エリスロポエチン遺伝子を用いた酸素応答性の遺伝子操作

課題番号 13671046

平成 13 年度～平成 15 年度科学研究費補助金

（基盤研究（C）(2)）研究成果報告書

平成 16 年 5 月

研究代表者　今川　重彦

（筑波大学臨床医学系）

ヒトエリスロポエチン(Epo)遺伝子発現は、低酸素環境下では Epo 遺伝子 3'エンハンサー領域の TACGTGCT 配列に hypoxia-inducible factor 1 (HIF-1)が結合して Epo 遺伝子発現を促進している。一方、通常大気中では Epo 遺伝子 5' プロモーター領域の AGATAA 配列に GATA が結合して Epo 遺伝子発現を負に制御している。このように、Epo 遺伝子発現は HIF-1 と GATA の両転写因子の均衡により調整されている。

この事実をふまえて以下の研究成果を得た。

1.

Epoは腎臓で産生されている。このため腎不全では尿毒素が増加・蓄積しEpo産生部位を障害するためにEpoが低下し、腎性貧血が発症するとされていた。しかし、腎不全に伴う腎性貧血患者でも、一過性の低酸素状態ではEpo産生能が保たれていることが確認されている。そこで我々は、Epo産生部位の臓器障害だけではなく、HIF-1とGATAの不均衡による腎性貧血発症機構を解析した。腎不全で著増する N^G-monomethyl L-argimine (L-NMMA)に着目し、低酸素でEpoを産生するHep3B細胞の実験系を用いた。この結果、NOS拮抗阻害薬であるL-NMMAによりNO・cGMP産生は阻害され、GATA結合活性及びGATA mRNA発現レベルが亢進することによりEpo遺伝子発現が抑制されて腎性貧血が発症することを認めた（Blood 96: 1716-1722, 2000)。また、この系にL-arginineを前処理すると、L-NMMAによるNO・cGMP産生低下は改善し、GATA結合活性及びGATAmRNA発現レベルは抑制されることでEpo遺伝子発現が改善され、腎性貧血が改善されることを認めた（Kidney Int 61: 396-404, 2002)。

N^G-monomethyl-L-arginine inhibits erythropoietin gene expression by stimulating GATA-2

Takahisa Tarumoto, Shigehiko Imagawa, Ken Ohmine, Tadashi Nagai, Masato Higuchi, Nobuo Imai, Norio Suzuki, Masayuki Yamamoto, and Keiya Ozawa

N^G-monomethyl-L-arginine (L-NMMA) has been reported to be elevated in uremic patients. Based on the hypothesis that the pathogenesis of the anemia of renal disease might be due to the perturbation of transcription factors of the erythropoietin (*Epo*) gene by L-NMMA, the present study was designed to investigate the effect of L-NMMA on *Epo* gene expression through the GATA transcription factor. L-NMMA caused decreased levels of NO, cyclic guanosine monophosphate (cGMP), and Epo protein in Hep3B cells. L-NAME (analogue of L-NMMA) also inhibited Epo production in anemic mice. Transfection of the Epo promoter-luciferase gene into Hep3B cells revealed that L-NMMA inhibited the Epo promoter activity. However, L-NMMA did not inhibit the Epo promoter activity when mutated Epo promoter (GATA to TATA) was transfected, and L-NMMA did not affect the enhancer activity. Electrophoretic mobility shift assays demonstrated the stimulation of GATA binding activity by L-NMMA. However, L-NMMA had no effect on the binding activity of hepatic nuclear factor-4, COUP-TF1, hypoxia-inducing factor-1, or NF-κB. Furthermore, cGMP inhibited the L-NMMA–induced GATA binding activity. L-NMMA also increased GATA-2 messenger RNA expression. These results demonstrate that L-NMMA suppresses *Epo* gene expression by up-regulation of the GATA transcription factor and support the hypothesis that L-NMMA is one of the candidate substances that underlie the pathogenesis of renal anemia. (Blood. 2000;96:1716-1722)

Introduction

In humans and mammals, erythropoiesis is regulated by the 30.4-kd glycoprotein hormone erythropoietin (Epo).[1] *Epo* gene expression is regulated by hypoxia through an oxygen sensor.[1] The major sites of Epo production are the liver in the fetus[2] and the kidney in the adult.[3] Peritubular capillary interstitial cells are thought to be the major site of production of Epo in the kidney.[4] The cause of the anemia of renal disease is believed to be damage to this site of the Epo production by renal failure.[5] In this regard, however, it is interesting to note that some patients with the anemia of renal disease still have the ability to produce Epo in response to acute blood loss and hypoxia.[6] On the other hand, other patients with renal failure do not have anemia.[7] These observations suggest that chronic perturbation of oxygen sensing or signal transduction or both underlie the pathogenesis of the anemia of renal disease rather than damage at the site of Epo production. Recently, N^G-monomethyl-L-arginine (L-NMMA) was reported to be undetectable in nonuremic subjects, whereas the concentration of L-NMMA was markedly elevated in uremic patients.[8] Based on this observation, we hypothesized that this substance may be a candidate uremic toxin responsible for renal anemia. However, the precise function of L-NMMA in mediating expression of the *Epo* gene remains to be elucidated. Because L-NMMA functions as an inhibitor of nitric oxide synthase (NOS),[9] it is expected to suppress the production of nitric oxide (NO) and cyclic guanosine 3′, 5′-monophosphate (cGMP). We have found that GATA transcription factors bind to a GATA site in the *Epo* gene promoter and negatively regulate the gene expression in Hep3B cells (Figure 1A).[10] In this study, we demonstrate that L-NMMA suppresses *Epo* gene expression by up-regulation of the GATA transcription factor.

Materials and methods

Cell culture and RNA preparation

The Hep3B cell line was obtained from the American Type Tissue Culture Collection (Rockville, MD). This cells were cultured in Dulbecco modified Eagle medium (Life Technologies, Gaithersburg, MD), supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL), and 10% heat-inactivated fetal bovine serum (FBS; Hyclone, Logan, UT) in 10-cm dishes. Cells were maintained in a humidified 5% CO_2/95% air incubator at 37°C. The cells were grown under conditions of hypoxia (1% oxygen) or normoxia as previously described.[11] These cells were stimulated where appropriate by the addition of L-NMMA to the culture medium. After incubation under hypoxic/normoxic or L-NMMA–stimulated/unstimulated conditions or both, the cells were harvested and cellular extracts were prepared. Total cellular RNA was also prepared by conventional methods.[12]

From the Department of Hematology, Jichi Medical School, Minamikawachi-machi, Tochigi-ken, Japan; Division of Hematology, Institute of Clinical Medicine, University of Tsukuba, Tennoudai, Tsukuba, Ibaraki, Japan; Center for Tsukuba Advanced Research Alliance and Institute of Basic Medical Sciences, University of Tsukuba, Tennoudai, Tsukuba, Ibaraki, Japan; Chugai Pharmaceutical Co, Ltd, Tokyo, Japan.

Submitted November 22, 1999; accepted April 25, 2000.

Supported by grants-in-aid for scientific research from the Ministry of Education, Science and Culture of Japan, and the Chugai Foundation, Tokyo, Japan.

Reprints: Shigehiko Imagawa, Division of Hematology, Institute of Clinical Medicine, University of Tsukuba, Tsukuba, Ibaraki 305-8575, Japan; e-mail: simagawa@md.tsukuba.ac.jp.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 U.S.C. section 1734.

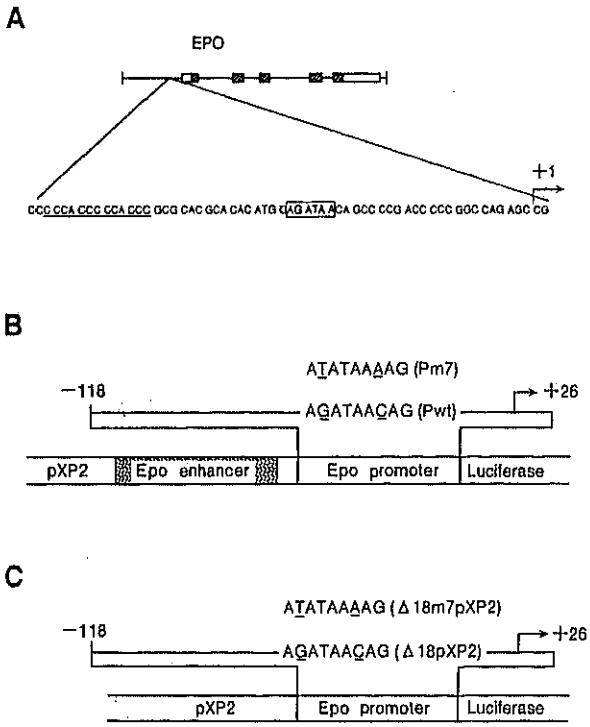


Figure 1. Constructs used in this study. (A) The GATA-binding site in the Epo 5′ promoter region (boxed) and repeated CACCC elements (underlined). (B) Diagrams of the reporter construct, the wild-type (Pwt) and the mutated GATA (Pm7) used in this study. The pEPLuc reporter construct is shown in the center. Shown above is the 144-bp insert from the Epo promoter. The mutation is indicated by underlining. The Epo enhancer contains an HIF-1 binding site. (C) Diagrams of the reporter constructs, Δ18pXP2, Δ18m7pXP2, used in this study. The mutation is indicated by underlining.

Transfection

Electroporation of the plasmids into Hep3B cells was performed as previously described.[10] A total of 3 to 10 × 10^6 cells were resuspended in 1 mL of 20 mmol/L HEPES buffer (pH 7.05) with 137 mmol/L NaCl, 5 mmol/L KCl, 0.7 mmol/L Na_2HPO_4, 6 mmol/L dextrose, 20 μg of vector DNA (circular DNA), and 500 μg carrier salmon sperm DNA, and then electroporated at a voltage of 250 V and a capacitance of 960 μF (Bio-Rad, Hercules, CA). The RSVCAT (Chloramphenicol acetyltransferase; 10 μg) plasmid or β-galactosidase was co-transfected as an internal standard for all transfection reactions.[10] The time constant of the shock was approximately 12 to 14 ms.

DNA binding assay

Nuclear extracts were prepared as previously described.[13] Protein concentrations were determined by assay (Bio-Rad) using bovine serum albumin (BSA) as a standard. Sense-strand oligonucleotide (wild-type: CATGCAGATAA CAGCCCCGAC) was end-labeled with T4 polynucleotide kinase (Toyobo, Tokyo, Japan) and annealed to a 4-fold excess of the unlabeled antisense oligonucleotide. Two nanograms of labeled probe was used in each binding reaction. The binding buffer consisted of 10 mmol/L Tris HCl (pH 7.5), 1 mmol/L EDTA, 4% Ficoll, 1 mmol/L dithiothreitol, and 75 mmol/L KCl. An equimolar mixture of poly[d(I-C)] and poly-[d(A-T)] (25 ng; Sigma, St Louis, MO) was used as a nonspecific competitor. The reaction mixtures (25 μL) were incubated for 15 minutes at 4°C and then electrophoresed on 5% nondenaturing polyacrylamide gels in 0.25 × TBE buffer (22 mmol/L Tris borate, 22 mmol/L boric acid, 0.5 mmol/L EDTA) at room temperature at 150 V for 1.5 hours as previously described.[13] Gels were vacuum dried and then autoradiography was performed using intensifying screens at −80°C for 24 hours. Monoclonal antibodies to hGATA-1, -2, and -3 were prepared as previously described.[14]

Plasmid vectors

We used the reporter plasmid pEPLuc described by Blanchard and coworkers[15] as a basic plasmid construct, in which both the 126-bp 3′ Epo enhancer (120 to 245-bp 3′ of the poly(A) addition site) and the 144-bp minimal Epo promoter (from −118 to +26 relative to the transcription initiation site) were placed upstream of the firefly luciferase (*Luc*) gene in pXP2,[16] resulting in Pwt[17] or V2-Ewt-Pwt-pXP2[17] (Figure 1B). This enhancer contained hypoxia-inducible factor 1 (HIF-1) binding site and steroid receptor response element (SRRE). In the mutant construct, the GATA sequence in the Epo promoter was mutated to TATA (AGATAACAG to ATATAAAAG). This mutant construct is called Pm7[17] or V3-Ewt-Pm7-pXP2[17] (Figure 1B). The 144-bp minimal Epo promoter (from −118 to +26 relative to the transcription initiation site) was placed upstream of the *Luc* gene in pXP2, resulting in Δ18pXP2 (Figure 1C). In the mutant construct, the GATA sequence in the Epo promoter was mutated to TATA (AGATAACAG to ATATAAAAG). This mutant construct is called Δ18m7pXP2 (Figure 1C). Construction of the hGATA-2 expression plasmid has been previously described.[14]

Northern blot analysis

Probes were labeled with [α-^{32}P]deoxycytidine triphosphate by random priming and used in RNA blot hybridization.[18] Formaldehyde gels for RNA electrophoresis were prepared as described.[18] RNA blot hybridization was performed using 25 μg of total RNA from Hep3B cells. The filter was hybridized to probe of hGATA-2 complementary DNA (cDNA). The same filter was stripped and rehybridized to probe consisting of the Epo cDNA, and a probe for ribosomal RNA to determine the level of RNA in each lane. Autoradiography was performed at −80°C and quantitated by densitometric scanning.

Anemic mice

The BDF1 mice were injected intraperitoneally with 0.2 mL of 10 mg N^G-nitro-L-arginine methyl ester (L-NAME)/mL phosphate-buffered saline (PBS) or 0.2 mL of PBS. Blood samples (0.3 mL) were obtained from the orbital vein at 0, 12, and 24 hours after injection of L-NAME. Epo levels in the serum were determined by radioimmunosorbent assay (RIA).

Other assays

Transfected Hep3B cells were washed with PBS and lysed in 10-cm dishes with 800 μL of cell lysis buffer (PicaGene, Toyo Ink, Tokyo). Luc activity in 20 μL of the cell extract was determined by Autolumat luminometer (Berthorude, Tokyo, Japan) for 10 seconds. Each measurement of relative light units was corrected by subtraction of the background and standardized to the RSVCAT or β-galactosidase internal transfection control activity. Hypoxic inducibility was defined as the ratio of the corrected relative light units of the hypoxic (1% O_2) dish to those of the normoxic (21% O_2) dish. CAT activity was determined as described by Neumann and colleagues.[19] NO was detected by the 2,3-diaminonaphthalene method,[20] cGMP was measured by enzyme immunosorbent assay (EIA),[21] and α-fetoprotein (AFP) was measured by RIA.

Results

Inhibition of Epo protein by L-NMMA

We first confirmed that L-NMMA was not cytotoxic at concentrations of up to 10^{-2} mol/L for Hep3B cells by the trypan blue dye exclusion method and the methyl-thiazol-diphenyl-tetrazolium (MTT) method (data not shown). Similarly, Fisher and coworkers reported that L-NMMA concentrations of up to 10^{-3} mol/L were not cytotoxic for Hep3B cells.[22] We then examined the effect of L-NMMA on the production of Epo protein in Hep3B cells. Incubation for 24 hours with 10^{-2} mol/L L-NMMA under hypoxic conditions showed an 80% inhibition of Epo, whereas 10^{-5}

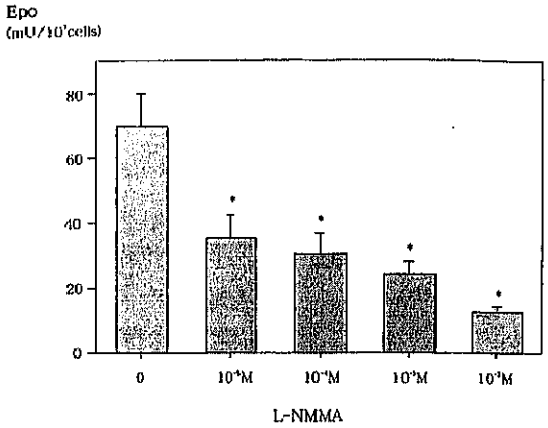


Figure 2. Effect of L-NMMA on Epo protein from Hep3B cells stimulated by hypoxia. Hep3B cells were incubated with different concentrations of L-NMMA under hypoxic conditions (1% O_2) for 24 h. Epo protein was measured by RIA. Four separate experiments were performed (n = 4). Error bars represent 1 SD. $^*P < .01$.

mol/L, 10^{-4} mol/L, and 10^{-3} mol/L L-NMMA each showed a 60% inhibition of Epo (Figure 2). To make sure that this inhibition of Epo protein by L-NMMA was specific, AFP was measured by RIA. Up to 10^{-2} mol/L L-NMMA did not inhibit the level of AFP (data not shown). These results suggest that L-NMMA specifically inhibited the production of Epo protein in Hep3B cells.

Inhibition of NO and cGMP by L-NMMA

L-NMMA is known to be an NOS inhibitor, and, therefore, a decrease in NO from cells incubated with L-NMMA was expected. To this end, Hep3B cells were incubated with different concentrations of L-NMMA. Hypoxia induced the secretion of NO, but the addition of L-NMMA inhibited this induction (Figure 3). Because NO stimulates guanylate cyclase (GC) to produce cGMP,[23] a decrease in cGMP from cells incubated with L-NMMA was also expected. Hypoxic Hep3B cells were incubated with different concentrations of L-NMMA. As shown in Figure 4, L-NMMA inhibited the secretion of cGMP from the cells.

Inhibition of serum Epo by L-NAME from L-NAME–injected mice

L-NAME was examined using an in vivo mouse assay, because L-NMMA is reported to be catabolyzed by N^G-dimethylarginine dimethylaminohydrolase (DDHA) in the intact kidney[24,25]; however, L-NAME is not catabolyzed by this enzyme.[26] Hecker and

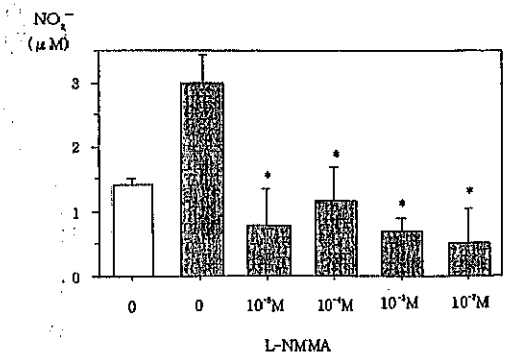


Figure 3. Effect of L-NMMA on NO from Hep3B cells. Hep3B cells were incubated under normoxic (21% O_2, □) or hypoxic (1% O_2, ■) conditions for 4 hours in the presence or absence of L-NMMA. NO was measured by the 2,3-diaminonaphthalene method. Three separate experiments were performed (n = 3). Error bars represent 1 SD. $^*P < .01$.

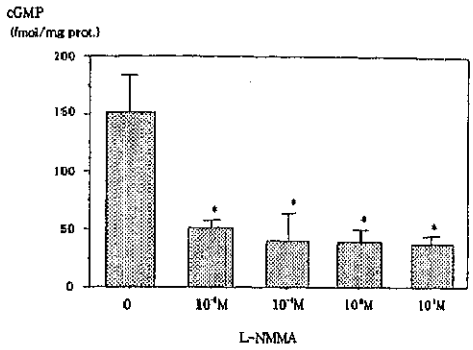


Figure 4. Effect of L-NMMA on cGMP from Hep3B cells stimulated by hypoxia. Hep3B cells were incubated with different concentrations of L-NMMA under hypoxia (1% O_2) for 2 hours. cGMP was measured by EIA. Two separate experiments were performed (n = 2). Error bars represent 1 SD. $^*P < .01$.

associates observed that L-NMMA was rapidly hydrolyzed to L-citrulline and lost the inhibitory effect in endothelial cells.[27] Furthermore, L-NMMA is continuously released into body fluids during the in vivo breakdown of the proteins and is assumed to be readily excreted in urine without reincorporation into proteins or further degradation in intact animals.[24] To identify the effect of L-NAME on Epo production in vivo, BDF1 mice were injected intraperitoneally with 0.2 mL of 10 mg L-NAME/mL PBS or 0.2 mL of PBS as a control. Blood samples (0.3 mL) were obtained from the orbital vein immediately after (0 hours) and at 12 and 24 hours after the injection of L-NAME (Figure 5). The serum Epo from the control (dashed line) increased to 66.4 mU/mL at 12 hours and to 276.8 mU/mL at 24 hours after the injection. The serum Epo from the mice injected with L-NAME (straight line) was 28.5 mU/mL at 12 hours and 98.8 mU/mL at 24 hours after the injection. These values were significantly lower than those of the control (Figure 5). These in vivo results are comparable with those obtained from the in vitro incubation of Hep3B cells (Figure 2).

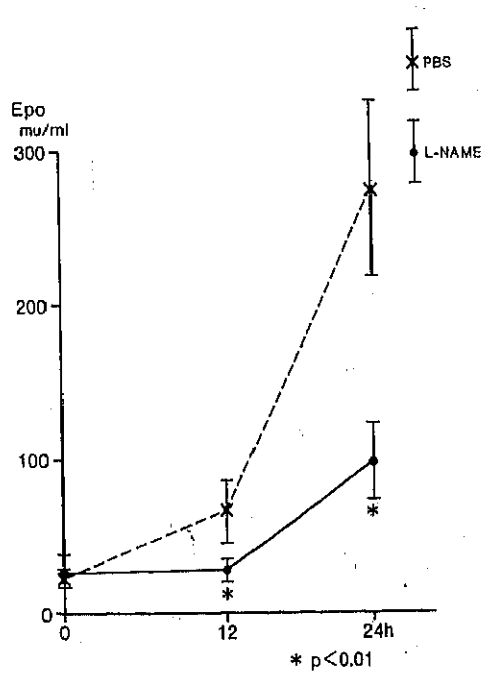


Figure 5. Effect of L-NAME on serum Epo from anemic mice. Five BDF1 mice were injected intraperitoneally with 0.2 mL of 10 mg L-NAME/mL PBS or 0.2 mL of PBS. Blood samples (0.3 mL) were taken from the orbital vein at 0, 12, and 24 hours after injection of L-NAME. Serum Epo levels were determined by RIA. Error bars represent 1 SD. $^*P < .01$.

Table 1. Effect of L-NMMA on the induction of the wild-type and mutated GATA motifs of the Epo promoter/enhancer with *Luc* reporter constructs in Hep3B cells

		Experiment 1		Experiment 2		Experiment 3		
	% O_2	RLU/CAT	Fold Induction	RLU/CAT	Fold Induction	RLU/CAT	Fold Induction	Fold Induction (mean ± 1 SD)
Pwt	21	56.6	×50.6	75.8	×63.4	54.3	×53.5	×55.8 ± 5.5
Pwt	1	2866.4		4807.9		2902.8		
Pwt + L-NMMA	21	42.6	×38.0	72.9	×41.0	46.1	×20.1	×33.0 ± 9.2
Pwt + L-NMMA	1	1619.8		2985.7		927.0		
Pwt + hGATA-2	21	50.7	×24.8	80.4	×23.4	49.4	×16.2	×21.5 ± 3.8
Pwt + hGATA-2	1	1257.1		1877.8		799.8		
Pwt + hGATA-2 + L-NMMA	21	57.7	×19.1	96.3	×11.7	45.6	×11.9	×14.2 ± 3.4
Pwt + hGATA-2 + L-NMMA	1	1101.6		1130.2		544.2		
Pm7	21	125.1	×75.9	61.0	×114.5	99.7	×100.2	×96.9 ± 15.9
Pm7	1	9498.1		6986.5		9988.6		
Pm7 + L-NMMA	21	113.6	×74.9	73.7	×114.2	62.8	×122.0	×103.7 ± 20.6
Pm7 + L-NMMA	1	8504.7		8413.8		7660.6		
Pm7 + hGATA-2	21	118.1	×138.8	112.0	×171.2	61.0	×132.0	×147.3 ± 17.1
Pm7 + hGATA-2	1	16386.4		19175.9		8050.9		
Pm7 + hGATA-2 + L-NMMA	21	182.7	×124.6	111.5	×135.8	126.1	×110.0	×123.5 ±10.6
Pm7 + hGATA-2 + L-NMMA	1	22766.1		15145.5		13870.4		

Wild-type (Pwt) and mutated (Pm7) GATA motifs. Hypoxic induction of the *Luc* gene expression is represented as a hypoxia/normoxia ratio. Fold induction indicates this hypoxia/normoxia ratio. Three separate experiments (duplicate sample) were performed (n = 3).

Inhibition of Epo promoter activity by L-NMMA

Expression of the *Epo* gene has been shown to be induced by hypoxia.[1] To elucidate the molecular mechanisms underlying the hypoxic induction of the *Epo* gene, plasmids containing both the promoter and enhancer of the *Epo* gene were used in a transient transfection assay into Hep3B cells.[10] One GATA and 2 CACCC motifs are in the promoter region, and one HIF-1 and one hepatic nuclear factor 4 (HNF-4) binding site are in the enhancer (Figure 1A). Both the promoter and enhancer were inserted upstream of the *Luc* gene to give rise to Pwt and Pm7 plasmids (Figure 1B). The latter contains a mutation in the promoter GATA element. We transfected Pwt and Pm7 into Hep3B cells and incubated the cells in the presence or absence of L-NMMA under 21% (normoxia) or 1% (hypoxia) oxygen for 24 hours. The hypoxic induction of *Luc* gene expression is represented as a hypoxia/normoxia ratio, as previously described.[10] Hypoxic induction from Pwt was 55.8 ± 5.5 fold higher than that from normoxic Pwt (mean ± 1 SD, n = 3) (Table 1 and Figure 6). Interestingly, the addition of L-NMMA inhibited the hypoxic induction of the *Luc* reporter gene expression from Pwt with hypoxia/normoxia ratio of only 33.0 ± 9.2 fold, 59.1% of that from Pwt incubated without L-NMMA (Table 1 and Figure 6). These results indicate that the hypoxic induction of the *Epo* gene expression is suppressed by L-NMMA through the *Epo* gene regulatory regions.

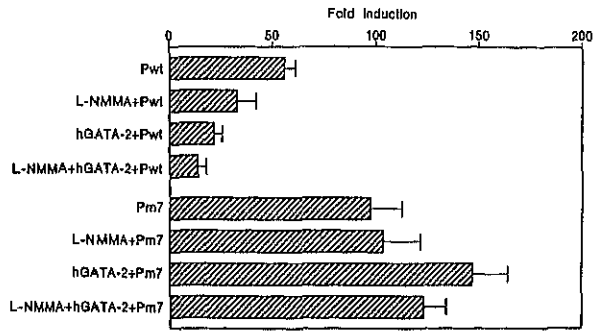


Figure 6. Effect of L-NMMA on the induction of the wild-type and mutated GATA motifs of the Epo promoter/enhancer with Luc reporter constructs in Hep3B cells. Wild-type (Pwt) and mutated (Pm7) GATA motifs. Hypoxic induction of *Luc* gene expression is represented as a hypoxia/normoxia ratio. Fold induction indicates this hypoxia/normoxia ratio. Three separate experiments (duplicate sample) were performed (n = 3). Error bars represent 1 SD.

We previously found that the GATA element in the promoter plays an important role in limiting the hypoxic induction of the *Epo* gene.[10] We therefore examined the contribution of the GATA site to the L-NMMA suppression of hypoxic *Epo* gene induction. To this end, Hep3B cells were co-transfected with Pwt and an hGATA-2 expression vector. The expression of hGATA-2 resulted in the inhibition of the hypoxic induction of the *Epo* gene. The *Luc* reporter gene was induced in the presence of hGATA-2 by 21.5 ± 3.8 fold, 43.2% of that from Pwt incubated without hGATA-2 (Table 1 and Figure 6). Furthermore, the exposure of Hep3B cells co-transfected with Pwt and hGATA-2 expression vector to L-NMMA resulted in further suppression with hypoxic induction of the reporter gene of only 14.2 ± 3.4 fold, 25.4% of that from the cells incubated without L-NMMA and hGATA-2 (Table 1 and Figure 6). These results suggest that hGATA-2 acts as a repressor of the hypoxic induction of the *Epo* gene and that the GATA sequence in the promoter mediates L-NMMA suppression.

The GATA element in the promoter only contributes to L-NMMA suppression

The contribution of the GATA element was further tested by using a reporter plasmid, Pm7, which contains GATA site mutations. We previously found that alone this GATA mutation affects the basal level expression of Luc reporter activity.[10] As was the case for Pwt, Luc expression was also strongly induced following the exposure of the transfected cells to hypoxia, 96.9 ± 15.9 fold (Table 1 and Figure 6). However, L-NMMA failed to affect the GATA mutant Pm7 Luc activity with hypoxic induction of 103.7 ± 20.6 fold, 107.0% of that from Pm7 only (Table 1 and Figure 6). Transfection of Pm7 into Hep3B cells, which express hGATA-2, resulted in hypoxic induction of 147.3 ± 17.1 fold, 152.0% of that from Pm7 only (Table 1 and Figure 6). Furthermore, the exposure of the cells co-transfected with Pm7 and hGATA-2 expression vector to L-NMMA showed 123.5 ± 10.6 fold, and 127.5% of that from Pm7 only (Table 1 and Figure 6). These results suggest that L-NMMA inhibits *Epo* gene expression through the GATA site in the Epo promoter rather than through the enhancer activity. To clearly identify whether this inhibitory effect of L-NMMA on *Epo*

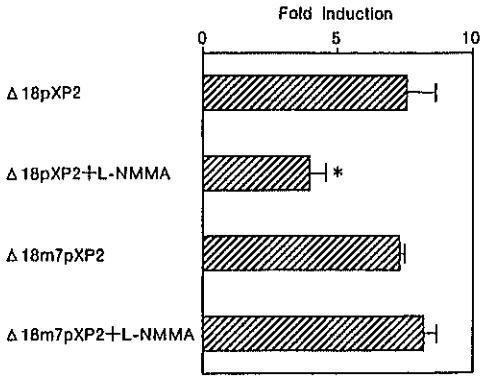


Figure 7. Effect of L-NMMA on the induction of the wild-type and mutated GATA motifs of the Epo promoter with Luc reporter constructs in Hep3B cells. Wild-type (Δ18pXP2) and mutated (Δ18m7pXP2) GATA motifs. Hypoxic induction of *Luc* gene expression is represented as a hypoxia/normoxia ratio. Fold induction indicates this hypoxia/normoxia ratio. Four separate experiments (duplicate sample) were performed (n = 4). Error bars represent 1 SD. $^*P < .01$.

gene expression was due to a GATA-2 or HIF-1 binding site or both, use of the construct that contained the Epo promoter only was advantageous. To this end, we then transfected Δ18pXP2 (wild type) or Δ18m7pXP2 (GATA site mutant) plasmids into Hep3B cells and incubated the cells both with or without additional L-NMMA under 21% or 1% oxygen for 24 hours (Figure 7). Hypoxic induction from Δ18pXP2 was 7.6 ± 1.0 fold higher than that from normoxic Δ18pXP2 (mean ± 1 SD, n = 4) (Figure 7). The addition of L-NMMA significantly inhibited the hypoxic induction of the *Luc* reporter gene expression from Δ18pXP2 with hypoxia/normoxia ratio of only 4.0 ± 0.6 fold, 52.6% of that from Δ18pXP2 incubated without L-NMMA (Figure 7). Hypoxic induction from Δ18m7pXP2 was 7.3 ± 0.2 fold higher that from normoxic Δ18m7pXP2. L-NMMA failed to affect the Δ18m7pXP2 Luc activity with hypoxic induction of 8.2 ± 0.5 fold, 112.3% of that from Δ18m7pXP2 only (Figure 7). These results clearly indicate that the inhibitory effect of L-NMMA on *Epo* gene expression was due to GATA-2, not HIF-1.

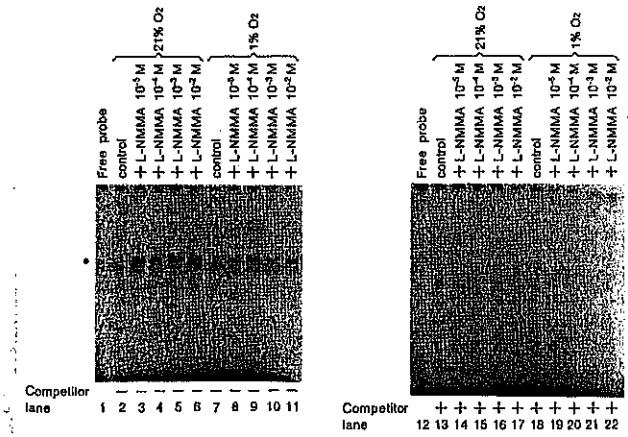


Figure 8. Effect of L-NMMA on the expression of GATA-2. EMSA was performed using 1.5 μg of protein from Hep3B cells under normoxia (lanes 2-6 and 13-17) and hypoxia (lanes 7-11 and 18-22), from Hep3B cells incubated with 10^{-5} mol/L L-NMMA (lanes 3, 8, 14, and 19), 10^{-4} mol/L L-NMMA (lanes 4, 9, 15, and 20), 10^{-3} mol/L L-NMMA (lanes 5, 10, 16, and 21), and 10^{-2} mol/L L-NMMA (lanes 6, 11, 17, and 22) for 1 hour. The dot at the left indicates the position of the GATA-2 transcription factor. A total of 25 ng (0.5 μL: 12.5-fold molar excess) of competitor DNA was added to each reaction mixture (lanes 13-22).

Enhancement of GATA-2 binding activity by L-NMMA

To examine whether L-NMMA treatment affects the binding activity of hGATA-2, nuclear extracts were prepared from the cells stimulated by L-NMMA for 1 hour under normoxic or hypoxic conditions, and electrophoretic mobility shift assays (EMSA) were performed with an oligonucleotide containing the wild-type GATA element (AGATAA) (Figure 8). L-NMMA induced the binding activity of GATA-2 (indicated by the circle) under normoxic and hypoxic conditions (lanes 3-6, 9-11). This binding activity was abolished by self-competitor (lanes 14-17, 20-22). To confirm that the band indicated by the circle was GATA-2, nuclear extracts were prepared from Hep3B cells under hypoxia with 10^{-4} mol/L L-NMMA for 1 hour, and incubated with 0.5 or 1.0 μL monoclonal antibodies of hGATA-1, 2, and 3 and then EMSA was performed under the same conditions as described in Figure 8. The control revealed a band of increased intensity (Figure 9, lane 2), and the addition of FCS further increased the intensity of this band, though the mechanism of this increase is unknown (Figure 9, lane 3). The addition of monoclonal antibodies of hGATA-1 and -3 resulted in bands of similar intensities (lanes 4, 5 and 8, 9); however, the band indicated by the circle disappeared with the addition of monoclonal hGATA-2 antibody (lanes 6 and 7). These results strongly suggest that the band indicated by the circle was a specific GATA-2 band.

To clarify the effect of cGMP on GATA-2 binding activity,

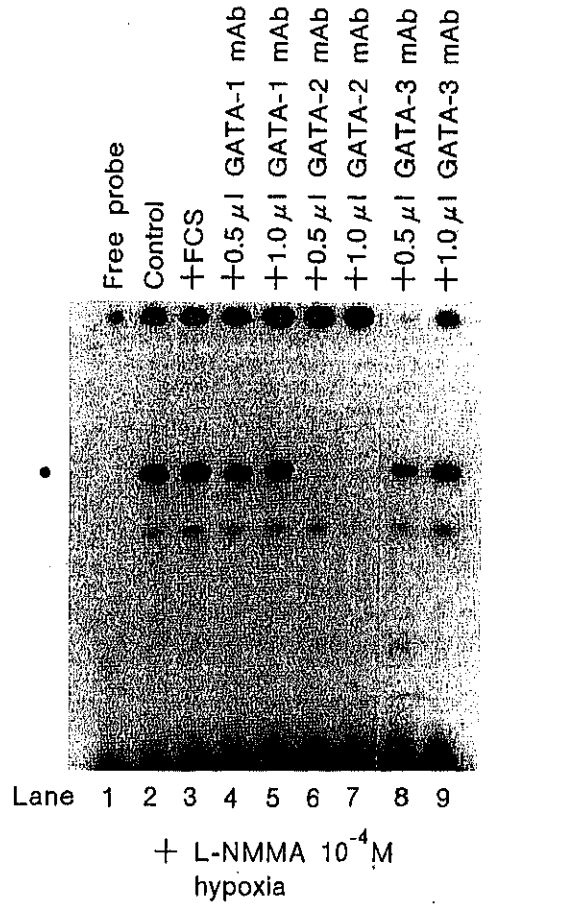


Figure 9. Effect of monoclonal antibodies on the expression of GATA. EMSA was performed using 1.5 μg of protein from Hep3B cells incubated with 10^{-4} mol/L L-NMMA under hypoxia (1% O_2) for 1 hour; 2 μL of FCS (lane 3) or 0.5 or 1.0 μL monoclonal antibodies of hGATA-1 (lanes 4 and 5), hGATA-2 (lanes 6 and 7), and hGATA-3 (lanes 8 and 9) were incubated with nuclear extracts overnight at 4°C and then EMSA was performed. The dot at the left indicates the position of the respective proteins.

nuclear extracts were prepared from Hep3B cells under hypoxia with the addition of 10^{-4} mol/L L-NMMA or cGMP, and EMSA was performed under the same conditions as described in Figure 8. The addition of cGMP inhibited the L-NMMA–induced binding activity of GATA-2 in a dose-dependent manner (data not shown). The effects of L-NMMA on the binding activity of HIF-1, HNF-4, chicken ovalbumin upstream promoter–transcription factor I (COUP-TF1), nuclear factor (NF)-κB were measured by EMSA. L-NMMA did not alter the binding activity of any of these transcription factors (data not shown).

GATA-2 messenger RNA expression was induced by L-NMMA

To examine whether GATA-2 and Epo messenger RNA (mRNA) expression levels were affected by the addition of L-NMMA, Northern blot analysis was performed. Northern blot analysis showed hypoxia-induced Epo mRNA expression; however, the addition of L-NMMA inhibited this induction of Epo mRNA (Figure 10A, middle panel). In contrast, hypoxia reduced GATA-2 mRNA expression, whereas L-NMMA induced the expression of GATA-2 mRNA (Figure 10A, upper panel, and B). The 28S used as a control revealed a constant level of mRNA expression from the cells incubated under normoxia or hypoxia and with or without L-NMMA (Figure 10A, lower panel).

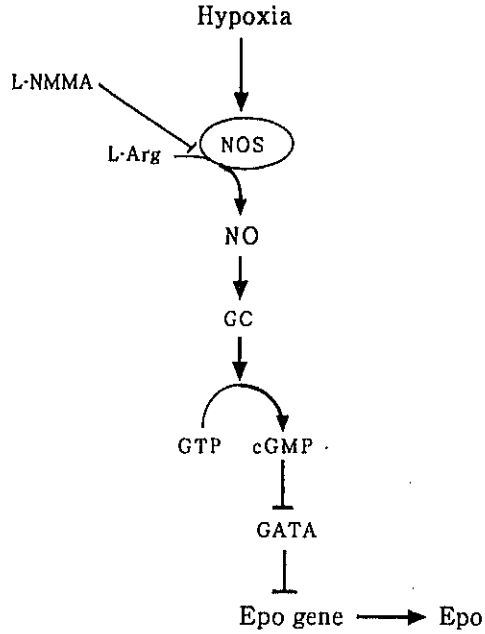


Figure 11. Hypothesis regarding the effect of L-NMMA on the expression of Epo.

Discussion

It was reported that L-NAME, also an NOS inhibitor, significantly blunted interferon-γ (IFN-γ) induction of Epo, nitrite, and cGMP in Hep3B cells.[22] Furthermore, L-NAME significantly decreased hypoxia-induced elevations of Epo mRNA and cGMP in an isolated perfused rat kidney system.[22] It has been suggested that NO plays an important role in the oxygen-sensing mechanism in Epo production.[22] However, the effect of an NOS inhibitor on the transcription factors of the *Epo* gene has not yet been clarified. NO is known to be an endothelium-derived relaxing factor[28] and has been found to play an important role in the physiology of the cardiovascular, immune, and nervous systems.[23,29] The biosynthesis of NO involves the oxidation of L-arginine by NOS.[30,31] NO stimulates a soluble GC, resulting in an increase in cGMP levels in cells.[23] The mRNA of nNOS is abundantly expressed in the kidney inner medulla and macula densa cells.[32] We hypothesized that NO produced by cNOS, which behaves as an intracellular or extracellular messenger, activates GC and, through stimulation of cGMP, consequently up-regulates *Epo* gene expression in peritubular cells. However, in chronic renal failure, we suspect that an NOS inhibitor such as L-NMMA suppresses *Epo* gene expression through inhibition of NO production. In the present study, we found that L-NMMA decreased the expression of NO and cGMP and increased the expression of GATA-2 mRNA and the level of GATA-2 binding activity, thereby inhibiting Epo promoter activity and causing a decrease in the expression of Epo protein (Figure 11).

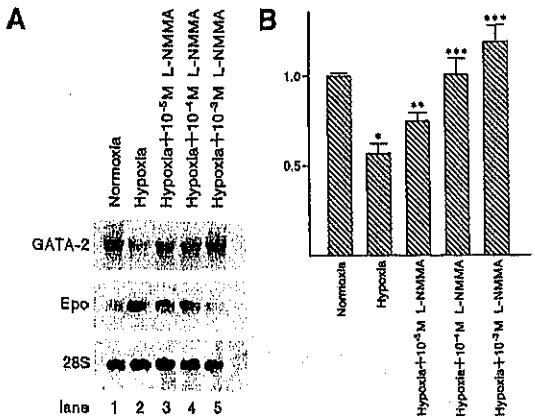


Figure 10. Effect of L-NMMA on the expression of GATA-2 and Epo mRNA. (A) Northern blot analysis was performed using 20 μg of RNA from Hep3B cells incubated under conditions of normoxia (21% O_2) (lane 1), conditions of hypoxia (1% O_2) (lane 2), hypoxia with 10^{-5} mol/L L-NMMA (lane 3), hypoxia with 10^{-4} mol/L L-NMMA (lane 4), and hypoxia with 10^{-3} mol/L L-NMMA (lane 5) for 8 hours. Upper, middle, and lower panels show GATA-2, Epo, and 28S mRNA, respectively. (B) Quantitative differences in GATA-2 mRNA levels were evaluated by molecular Imager FX. Abundance of GATA-2 mRNA is expressed as units of densitometry relative to normoxia. Three separate experiments were performed (n = 3). *Significance compared with normoxia, $P < .01$. **Significance compared with hypoxia, $P < .05$. ***Significance compared with hypoxia, $P < .01$.

The predialysis concentration of L-NMMA in patients with chronic renal failure is approximately 10^{-5} mol/L.[8] This concentration of L-NMMA significantly inhibited Epo protein production in Hep3B cells (Figure 2). However, a higher concentration of L-NAME in comparison to the serum concentration of L-NMMA in chronic renal failure was required for the inhibition of Epo production in vivo (Figure 5). There are 2 possible reasons for this. (1) A high local concentration of L-NMMA has a physiologic role in the kidney, liver, and pancreas. An inappropriately high concentration of L-NMMA due to renal failure may affect Epo production in peritubular cells despite its low serum concentration. (2) Not only L-NMMA but also asymmetric dimethylarginine (ADMA), which is also increased in patients with chronic renal failure,[33] causes a dose-dependent inhibition of NOS.[34]

In the present study, the effect of L-NMMA on GATA was investigated in detail. The effects of L-NMMA on HIF-1, HNF-4, COUP-TF1, NF-κB were assessed by EMSA and Epo promoter assay. L-NMMA did not alter the binding activity of HIF-1, HNF-4, COUP-TF1, or NF-κB at all. Neither EMSA nor *Epo* reporter gene transfection experiments showed any effect of L-NMMA on Epo enhancer activity. L-NMMA did not affect Luc activity when GATA in the Epo promoter was mutated (Pm7).

Recently, Kimura and coworkers reported that L-NAME did not interfere with hypoxia-induced vascular endothelial growth factor (VEGF) promoter (HIF-1 binding site) activation.[35] This result is compatible with our data. These results strongly suggest that L-NMMA affects GATA-2 binding.

The effect of NO on VEGF expression via HIF-1 is controversial. Some recent reports show an inhibitory effect of NO on VEGF expression. Huang and colleagues[36] and Sogawa and associates[37] have demonstrated that sodium nitroprusside (SNP, NO donor) suppresses hypoxia-induced *VEGF* gene activation and HIF-1 binding activity. SNP inhibits the hypoxic induction of the *VEGF* gene in a dose-dependent manner, in contrast to the effects S-nitroso-N-acetyl-D, L-penicillamine (SNAP) and 3-(2-hydroxy-1-(1-methylethyl)-2-nitrosohydrazino)-1-propanamine (NOC5) (another NO donor) as shown by Kimura and colleagues.[35] This concentration is clearly due to the specific nature of SNP. As to the effect of NO on Epo, Fisher and coworkers reported that serum levels of Epo in hypoxic polycythemic mice were significantly increased after injections of 200 μg/kg SNP.[22] Furthermore, cGMP levels in hypoxic Hep3B cells were also elevated. SNP (10 and 100 μmol/L) and NO (2 μmol/L) increased cGMP levels in Hep3B cells.[22] These results are compatible with our data and strongly suggest that L-NMMA inhibits Epo production via the GATA transcription factor.

Further analysis of the GATA-2 expression level and NO and cGMP from patients with renal anemia is necessary to clarify the details of the pathogenesis of renal anemia.

Acknowledgments

We thank D. L. Galson for providing the Pwt, Pm7, Δ18pXP2, and Δ18m7pXP2 plasmids. We also thank M. Nakamura and A. Yamazaki for their expert technical assistance.

References

1. Bunn HF, Poyton RO. Oxygen sensing and molecular adaptation to hypoxia. Physiol Rev. 1996; 76:839-885.
2. Zanjani ED, Poster J, Burlington H, Mann LI, Wasserman LR. Liver as the primary site of erythropoietin formation in the fetus. J Lab Clin Med. 1977;89:640-644.
3. Jacobson LO, Goldwasser E, Fried W, Plzak L. Role of the kidney in erythropoiesis. Nature. 1957;179:633-634.
4. Lacombe C, Da Silva J-L, Bruneval P, et al. Peritubular cells are the site of erythropoietin synthesis in the murine hypoxic kidney. J Clin Invest.1988;81:620-623.
5. McGonigle RJS, Wallin JD, Shadduck RK, Fisher JW. Erythropoietin deficiency and erythropoiesis in renal insufficiency. Kidney Int. 1984;26:437-444.
6. Esbach JW, Adamson JW. Anemia of end-stage renal disease. Kidney Int. 1985;28:1-5.
7. Walle AJ, Wong GY, Clemons GK, Garcia JF, Niedermayer W. Erythropoietin-hematocrit feedback circuit in the anemia of end-stage renal disease. Kidney Int. 1987;31:1205-1209.
8. Ribeiro ACM, Roberts NB, Lane C, Yaqoob M, Ellory JC. Accumulation of the endogenous L-arginine analogue N^G-monomethyl-L arginine in human end-stage renal failure patients on regular haemodialysis. Exp Physiol. 1996;81:475-481.
9. Gray GA, Schott C, Julou-Schaeffer G, Fleming I, Paratt JR, Stoclet J. The effect of inhibitors of the L-arginine/nitric oxide pathway on endotoxin-induced loss of vascular responsiveness in anesthetized rats. Br J Pharmacol. 1991;103:1218-1224.
10. Imagawa S, Yamamoto M, Miura Y. Negative regulation of the erythropoietin gene expression by the GATA transcription factors. Blood.1997;89: 1430-1439.
11. Imagawa S, Goldberg MA, Doweiko J, Bunn HF. Regulatory elements of the erythropoieitin gene. Blood. 1991;77:278-285.
12. Gilman M. Current Protocols in Molecular Biology, vol. 1. New York: John Wiley & Sons; 1988: 4.1.4.
13. Yamamoto M, Ko LJ, Leonard MW, Beug H, Orkin SH, Engel JD. Activity and tissue-specific expression of the transcription factor NF-E1 multi gene family. Genes Dev. 1990;4:1650-1662.
14. Nagal T, Harigae H, Ishihara H, et al. Transcription factor GATA-2 is expressed in erythroid, early myeloid, and CD34+ human leukemia-derived cell lines. Blood. 1994;84:1074-1084.
15. Blanchard KL, Acquaviva AM, Galson DL, Bunn H. Hypoxic induction of the human erythropoietin gene: cooperation between the promoter and enhancer, each of which contains steroid receptor response elements. Mol Cell Biol. 1992;12:5373-5385.
16. Nordeen SK. Luciferase reporter gene vectors for analysis of promoters and enhancers. Biotechniques. 1988;6:454-457.
17. Galson DL, Tsuchiya T, Tendler DS, et al. The orphan receptor hepatic nuclear factor 4 functions as a transcriptional activator for tissue-specific and hypoxia-specific erythropoietin gene expression and is antagonized by EAR3/COUP-TF1. Mol Cell Biol. 1995;15:2135-2144.
18. Riddle RD, Yamamoto M, Engel JD. Expression of δ-aminolevulinate syntase in avian cells: separate genes encode erythroid specific and nonspecific isozymes. Proc Natl Acad Sci U S A. 1989; 86:792-796.
19. Neumann JR, Morency CA, Russian KO. A novel rapid assay for chloramphenicol acetyltransferase gene expression. Biotechniques. 1987;5: 444.
20. Misco TP, Schilling RJ, Salvemini D, Moore WM, Currie MG. A fluorometric assay for the measurement of nitrite in biological samples. Anal Biochem.1993;214:11-16.
21. Ueno M, Rondon I, Beckman B, et al. Increased secretion of erythropoietin in human renal carcinoma cells in response to hypoxia. Am J Physiol. 1990;259 (Cell Physiol. 28):C427–C431.
22. Ohigashi T, Brookins J, Fisher JW. Interaction of nitric oxide and cyclic guanosine 3', 5'-monophosphate in erythropoietin production. J Clin Invest. 1993;92:1587-1591.
23. Moncada S, Palmer RMJ, Higgs EA. Nitric oxide: physiology, pathology, and pharmacology. Pharmacol Rev. 1991;43:109-142.
24. Kimoto M, Whitley G St J, Tsuji H, Ogawa T. Detection of N^G, N^G-dimethylarginine dimethylaminohydrolase in human tissues using a monoclonal antibody. J Biochem.1995;117:237-238.
25. Kimoto M, Tsuji H, Ogawa T, Sasaoka K. Detection of N^G, N^G-dimethylarginine dimethylaminohydrolase in the nitric oxide-generating systems of rats using monoclonal antibody. Arch Biochem Biophys.1993;300:657-662.
26. Ogawa T, Kimoto M, Sasaoka K. Purification and properties of a new enzyme, N^G, N^G-dimethylarginine dimethylaminohydrolase, from rat kidney. J Biol Chem. 1989;264;10205-10209.
27. Hecker M, Mitchell JA, Harris HJ, Katsura M, Thiemermann C, Vane JR. Endothelial cells metabolize N^G-monomethyl-L-arginine to L-citrulline and subsequently to L-arginine. Biochem Biophys Res Commun. 1990;167:1037-1043.
28. Ignarro LJ. Endothelium-derived nitric oxide: actions and properties. FASEB J. 1989;3:31-36.
29. Palmer RM, Ferrige AG, Moncada S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nature Lond. 1987;327:524-526.
30. Forstermann U, Schmidt HHHW, Pollock JS, et al. Isoform of nitric oxide: characterization and purification from different cell types. Biochem Pharmacol. 1991;42:1849-1857.
31. Marletta MA, Yoon PS, Iyengar R, Leaf CD, Wishnok JS. Macrophage oxidation of L-arginine to nitrite and nitrate: nitric oxide is an intermediate. Biochemistry. 1988;27:8706-8711.
32. Ito S, Ren Y. Evidence for the role of nitric oxide in macula densa control of glomerular hemodynamics. J Clin Invest. 1993;92:1093-1098.
33. Vallance P, Leone A, Calver A, Collier J, Moncada S. Accumulation of an endogenous inhibitor of nitric oxide synthesis in chronic renal failure. Lancet. 1992;339:572-575.
34. Vallance P, Leone A, Calver A, Collier J, Moncada S. Endogenous dimethylarginine as an inhibitor of nitric oxide synthesis. J Cardiovasc Pharmacol. 1992;20(suppl 12):S60–S62.
35. Kimura H, Weisz A, Kurashima Y, et al. Hypoxia response element of the human vascular endothelial growth factor gene mediates transcriptional regulation by nitric oxide: control of hypoxia-inducible factor-1 activity by nitric oxide. Blood. 2000;95:189-197.
36. Huang LE, Gu J, Schau M, Bunn HF. Regulation of hypoxia inducible factor 1α is mediated by an O_2-dependent degradation domain via the ubiquitin-proteasome pathway. Proc Natl Acad Sci U S A. 1998;95.7987-7992.
37. Sogawa K, Numayama-Tsuruta K, Ema M, Abe M, Abe H, Fujii-Kuriyama Y. Inhibition of hypoxia-inducible factor 1 activity by nitric oxide donors in hypoxia. Proc Natl Acad Sci U S A.1998;95:7368-7373.

Kidney International, Vol. 61 (2002), pp. 396–404

L-arginine rescues decreased erythropoietin gene expression by stimulating GATA-2 with L-NMMA

SHIGEHIKO IMAGAWA, TAKAHISA TARUMOTO, NORIO SUZUKI, HARUMI Y. MUKAI, YUICHI HASEGAWA, MASATO HIGUCHI, TOMOHIRO NEICHI, KEIYA OZAWA, MASAYUKI YAMAMOTO, and TOSHIRO NAGASAWA

Division of Hematology, Institute of Clinical Medicine, University of Tsukuba, Tsukuba, Ibaraki; Department of Hematology, Jichi Medical School, Tochigi; Center for Tsukuba Advanced Research Alliance and Institute of Basic Medical Sciences, University of Tsukuba, Tsukuba, Ibaraki; and Chugai Pharmaceutical Co., Ltd., Tokyo, Japan

L-arginine rescues decreased erythropoietin gene expression by stimulating GATA-2 with L-NMMA.

Background. N^G-monomethyl-L-arginine (L-NMMA) decreases the expression of nitric oxide (NO) and cyclic guanosine monophosphate (cGMP) and increases the expression of GATA-2 mRNA and levels of GATA-2 binding activity, thereby inhibiting erythropoietin (Epo) promoter activity and causing a decrease in the expression of Epo protein. In the present study, we examined the effect of L-arginine on Epo gene expression in Hep3B cells and BDF1 mice.

Methods. Hep3B cells were incubated with and without different concentrations of L-NMMA and/or L-arginine. Anemic mice were injected with phosphate-buffered saline (PBS) or L-NAME and L-arginine.

Results. Incubation with L-NMMA under hypoxic conditions inhibited Epo expression, but this inhibition was recovered by the addition of L-arginine. Hypoxia induced the secretions of NO and cGMP, but the addition of L-NMMA inhibited these inductions, though these inhibitions of NO and cGMP by L-NMMA were recovered by the addition of L-arginine. Hep3B cells transfected with the Epo promoter/enhancer-luciferase gene had Epo promoter activity. This activity was inhibited by L-NMMA, but it could be recovered by the addition of L-arginine. L-NMMA induced the binding activity of GATA-2 under hypoxic conditions. This binding activity was inhibited by the addition of L-arginine. The addition of cGMP inhibited L-NMMA-induced GATA-2 binding activity in a dose-dependent manner. The results of an in vivo mouse assay revealed that L-NAME inhibited the expression of Epo, but this inhibition of Epo expression by L-NAME was rescued by pretreatment with L-arginine.

Conclusion. L-arginine rescues decreased erythropoietin gene expression by stimulating GATA-2 with N^G-monomethyl-L-arginine.

Key words: L-NAME, L-NMMA, anemia, hypoxia, signal transduction, oxygen sensing, uremic toxin.

Received for publication March 28, 2001
and in revised form September 13, 2001
Accepted for publication September 14, 2001

Peritubular capillary interstitial cells are thought to be the major site of the production of erythropoietin (Epo) in the kidney [1]. The cause of the anemia of renal disease is believed to be damage to this site of Epo production [2]. In this regard, however, it is interesting to note that there are some patients with the anemia of renal disease who still have the ability to produce Epo in response to acute blood loss and hypoxia [3]. On the other hand, there are patients with renal failure who do not have anemia [4]. These results suggest that chronic perturbation of oxygen sensing and/or signal transduction rather than damage at the site of Epo production [5] underlie the pathogenesis of the anemia. Recently, it has been reported that N^G-monomethyl-L-arginine (L-NMMA) was undetectable in non-uremic subjects, but markedly elevated in uremic subjects [6]. Based on this observation, we hypothesized that L-NMMA is a candidate uremic toxin responsible for the anemia of renal disease [5]. However, the function of L-NMMA in mediating Epo gene expression has not been elucidated. Since L-NMMA functions as an inhibitor of nitric oxide synthase (NOS) [7], it was expected that it would suppress the production of nitric oxide (NO) and cyclic guanosine monophosphate (cGMP). GATA transcription factors have been demonstrated to bind to the GATA element in the Epo promoter and negatively regulate Epo gene expression [8]. Our previous study found that L-NMMA decreased the expression of NO and cGMP and increased the expression of GATA-2 mRNA and levels of GATA-2 binding activity, thereby inhibiting the Epo promoter activity and causing a decrease in the expression level of Epo protein [5]. In the present study,

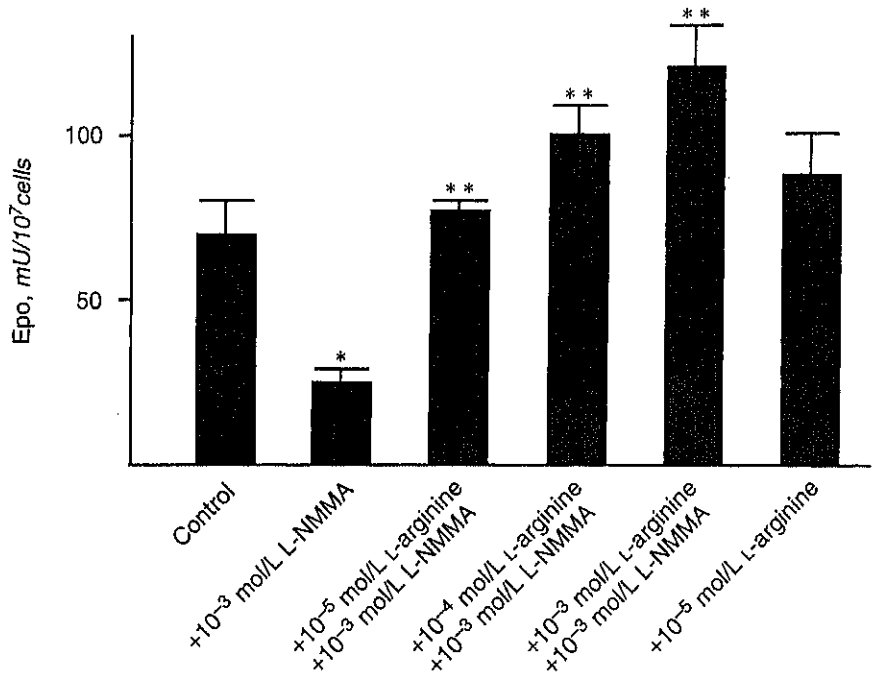


Fig. 1. Effect of L-NMMA and L-arginine on erythropoietin (Epo) protein from Hep3B cells stimulated by hypoxia. Hep3B cells were incubated with 10^{-3} mol/L L-NMMA and different concentrations of L-arginine under hypoxic conditions (1% O_2) for 24 hours. Epo protein was measured by RIA. Four separate experiments were performed ($N = 4$). Error bars represent one standard deviation. Significance values are: $*P < 0.01$ compared with control, and $**P < 0.01$ compared with 10^{-3} mol/L L-NMMA,.

we examined the effect of L-arginine on Epo gene expression in Hep3B cells and BDF1 mice to confirm our previous hypothesis.

METHODS

Cell culture and RNA preparation

The Hep3B cell line was obtained from the American Type Tissue Culture Collection (Rockville, MD, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies Inc., Gaithersburg, MD, USA), supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL) and 10% heat-inactivated fetal bovine serum (FBS; Hyclone, Logan, UT, USA), in 10 cm dishes. Cells were maintained in a humidified 5% CO_2/95% air incubator at 37°C. The cells were grown under conditions of hypoxia (1% oxygen) or normoxia as previously described [9]. These cells were stimulated where appropriate by the addition of L-NMMA to the culture medium. After incubation under hypoxic/normoxic and/or L-NMMA-stimulated/unstimulated conditions, the cells were harvested and cellular extracts were prepared. Total cellular RNA was also prepared by conventional methods [10].

Plasmid vectors

We used the reporter plasmid pEPLuc described by Blanchard et al [11] as a basic plasmid construct, in which both the 126-bp 3′ Epo enhancer [120 to 245-bp 3′ of the poly(A) addition site] and the 144-bp minimal Epo promoter (from −118 to +26 relative to the transcription initiation site) were placed upstream of the firefly luciferase (Luc) gene in pXP2 [12], resulting in Pwt [13] or V2-Ewt-Pwt-pXP2 [13]. This enhancer contained a hypoxia-inducible factor 1 (HIF-1) binding site and steroid receptor response element (SRRE).

Transfection

A total of 6 to 7 × 10^5 cells in six-well (10 cm^2 per well) tissue culture plates (Falcon) were washed with serum-free media. A mixture containing lipofectin (20 μg/ well; Life Technologies, Inc.), DNA constructs (2 μg/well) and β-galactosidase (1 μg/well) as an internal standard were co-transfected and the plates were incubated for 12 hours. The media was then changed to DMEM/FCS with or without L-NMMA or L-arginine and incubated for 24 hours under hypoxic/normoxic conditions.

DNA binding assay

Nuclear extracts were prepared as previously described [14]. Protein concentrations were determined by a Bio-Rad assay (Bio-Rad, Hertz, UK) using bovine serum albumin as a standard. Sense-strand oligonucleotide (wild type: CATGCAGATAACAGCCCCGAC) was end-labeled with T4 polynucleotide kinase (Toyobo, Tokyo, Japan) and annealed to a fourfold excess of the unlabeled antisense oligonucleotide. Two nanograms of labeled probe were used in each binding reaction. The binding buffer consisted of 10 mmol/L Tris HCl (pH 7.5), 1 mmol/L ethylenediaminetetraacetic acid (EDTA), 4% Ficoll, 1 mmol/L dithiothreitol and 75 mmol/L KCl. An equimolar mixture of poly[d(I-C)] and poly[d(A-T)] (25 ng; Sigma, St. Louis, MO, USA) was used as a nonspecific

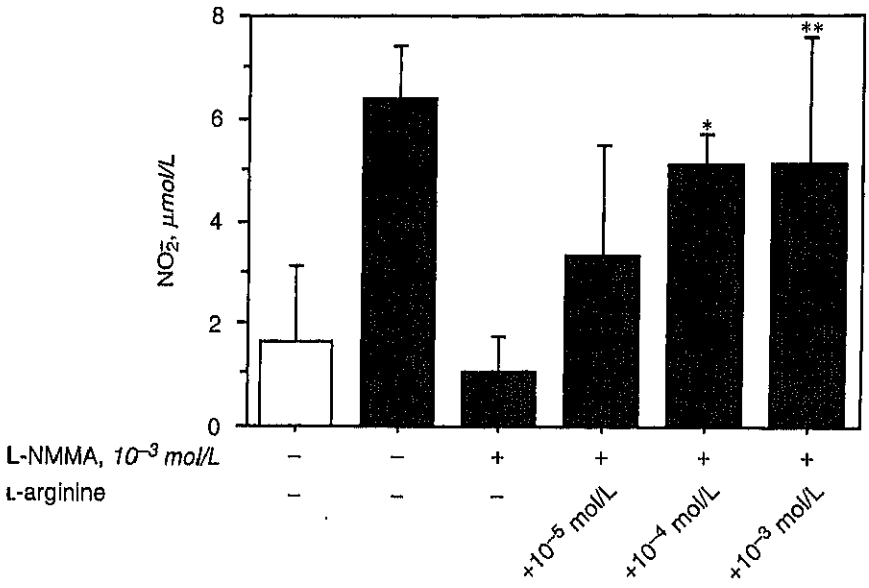


Fig. 2. Effect of L-NMMA and L-arginine on nitric oxide (NO) from Hep3B cells. Hep3B cells were incubated under normoxic (21% O_2) or hypoxic (1% O_2) conditions for 4 hours in the presence or absence of 10^{-3} mol/L L-NMMA and different concentrations of L-arginine. NO was measured by the 2,3-diaminonaphthalene method. Three separate experiments were performed ($N = 3$). Error bars represent one standard deviation. Significance values are: $*P < 0.005$ compared with 10^{-3} mol/L L-NMMA, and $**P<0.01$ compared with 10^{-3} mol/L L-NMMA. Symbols are: (□) normoxia, 21% O_2, (■) hypoxia, 1% O_2, $N = 3$.

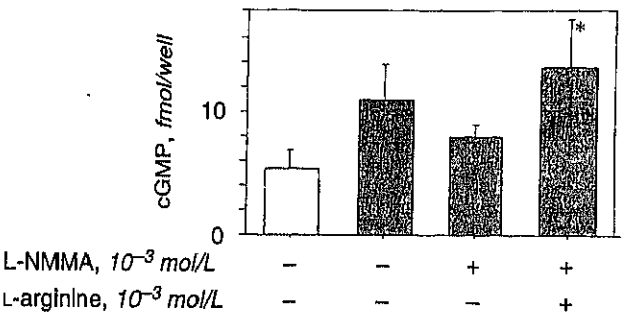


Fig. 3. Effect of L-NMMA and L-arginine on cGMP from Hep3B cells stimulated by hypoxia. Hep3B cells were incubated with 10^{-3} mol/L L-NMMA and 10^{-3} mol/L L-arginine under hypoxic (1% O_2) conditions for 2 hours. cGMP was measured by EIA. Three separate experiments were performed ($N = 3$). Error bars represent one standard deviation. Significance is $*P < 0.01$ compared with 10^{-3} mol/L L-NMMA. Symbols are: (□) normoxia, 21% O_2; (■) hypoxia, 1% O_2.

competitor. The reaction mixtures (25 μL) were incubated for 15 minutes at 4°C and then electrophoresed on 5% nondenaturing polyacrylamide gels in 0.25 × TBE buffer (22 mmol/L Tris borate, 22 mmol/L boric acid, 0.5 mmol/L EDTA) at room temperature at 150 V for 1.5 hours as previously described [14]. Gels were vacuum-dried and then autoradiography was performed using intensifying screens at −80°C for 24 hours.

Northern blot analysis

Probes were labeled with [α-^{32}P]deoxycytidine triphosphate by random priming and used in RNA blot hybridization [15]. Formaldehyde gels for RNA electrophoresis were prepared as described [15]. RNA blot hybridization was performed using 25 μg of total RNA from Hep3B cells. The filter was hybridized to a probe of hGATA-2

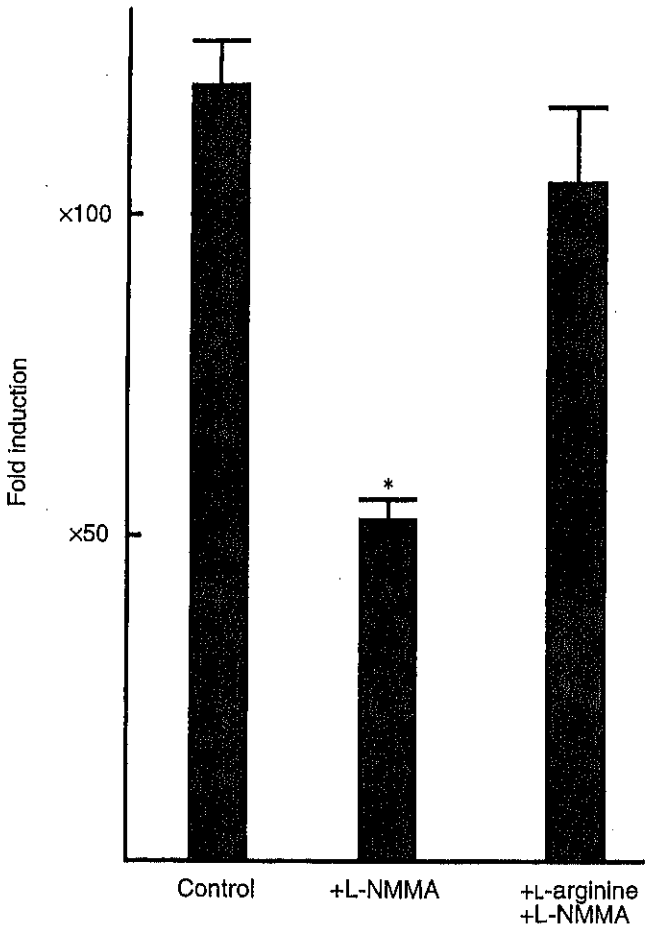


Fig. 4. Effect of L-NMMA and L-arginine on the induction of the Epo reporter constructs in Hep3B cells. Hypoxic induction of Luc gene expression is represented as a hypoxia/normoxia ratio. Fold induction indicates this hypoxia/normoxia ratio. Three separate experiments (duplicate samples) were performed ($N = 3$). Error bars represent one standard deviation. Significance compared with control, $*P < 0.01$.

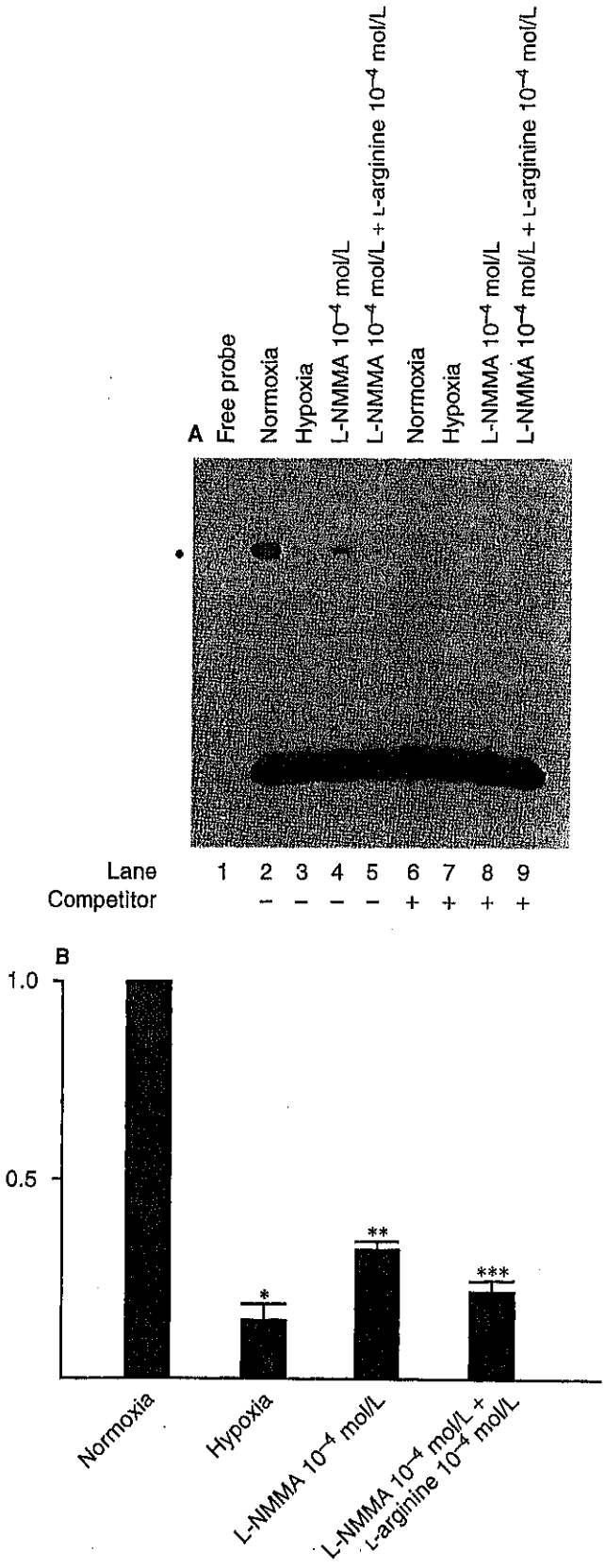


Fig. 5. Effect of L-NMMA and L-arginine on the expression of GATA-2. (*A*) EMSA was performed using 1.5 μg of protein from Hep3B cells under normoxia (lanes 2 and 6) and hypoxia (lanes 3–5 and 7–9), from Hep3B cells incubated with 10^{-4} mol/L L-NMMA (lanes 4 and 8), 10^{-4} mol/L L-NMMA and 10^{-4} mol/L L-arginine (lanes 5 and 9) for one hour. The dot at the left indicates the position of the GATA-2 transcription factor. A total of 25 ng (0.5 μL is a 12.5-fold molar excess) of competitor DNA was added to each reaction mixture (lanes 6–9). The autoradiograph is representative of three different experiments with similar results. (*B*) Densitometric analysis of the band indicated by the circle in panel A. Intensities are expressed relative to the control. Y-axis represents the intensity relative to that of the control. Three experiments from using different nuclear extracts were performed ($N = 3$). Error bars represent one standard deviation. Significances are: $*P < 0.005$ compared with normoxia; $**P < 0.005$ compared with hypoxia; $***P < 0.025$ compared with 10^{-4} mol/L L-NMMA.

cDNA. The same filter was stripped and rehybridized to a probe consisting of the ribosomal RNA to determine the level of RNA in each lane. Autoradiography was performed at −80°C.

Anemic mice

BDF1 mice were injected intra-peritoneally with 0.2 mL of 10 mg N^G-nitro-L-arginine methyl ester (L-NAME) /mL phosphate-buffered saline (PBS) or 0.2 mL of PBS as previously described [5], and 0.2 mL of 10 mg L-arginine added subsequently after the addition of L-NAME. Blood samples (0.3 mL) were obtained from the orbital vein at 0 and 24 hours after the injection of L-NAME. Epo levels in the serum were determined by radioimmunoassay (RIA). Hematocrits at the times 0 and 24 hours were 48% and 30%, respectively.

Other assays

Transfected Hep3B cells were washed with PBS and lysed in 10 cm^2 dishes with 400 μL of cell lysis buffer (Pica-Gene, Toyo Inc., Tokyo, Japan). Luc activity in 20 μL of the cell extract was determined by an Autolumat luminometer (Berthorude, Tokyo, Japan) for 10 seconds. Each measurement of relative light units was corrected by subtraction of the background and standardized to the β-galactosidase internal transfection control activity. Hypoxic inducibility was defined as the ratio of the corrected relative light units of the hypoxic (1% O_2) dish to those of the normoxic (21% O_2) dish. NO was detected by the 2,3 diaminonaphthalene method [16]. Intercellular cGMP was measured by enzyme immunoassay (EIA) [17].

RESULTS

Inhibition of Epo protein by L-NMMA was recovered by L-arginine

The effect of L-arginine was examined on the production of Epo protein, which was inhibited by L-NMMA in Hep3B cells. Incubation for 24 hours with 10^{-3} mol/L L-NMMA under hypoxic conditions resulted in 70% inhibition of Epo, but this inhibition was recovered by the addition of L-arginine (Fig. 1). While these results

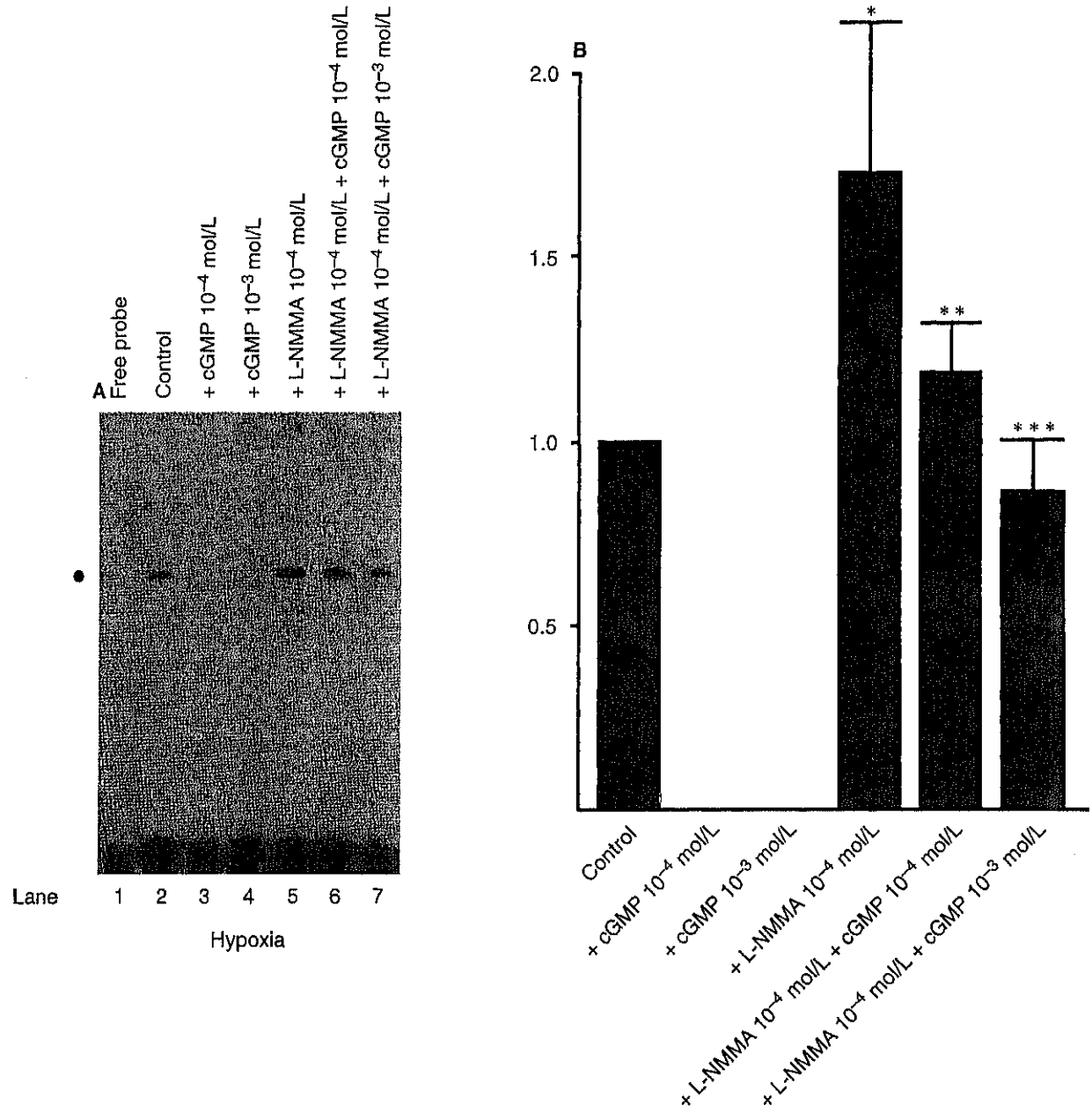


Fig. 6. Effect of cGMP on the expression of GATA. (*A*) EMSA was performed using 1.5 μg of protein from Hep3B cells incubated under hypoxia (1% O_2; lanes 2-7), with 10^{-4} mol/L cGMP (lane 3), 10^{-3} mol/L cGMP (lane 4), 10^{-4} mol/L + L-NMMA (lane 5), 10^{-4} mol/L + L-NMMA and 10^{-4} mol/L cGMP (lane 6), 10^{-4} mol/L L-NMMA and 10^{-3} mol/L cGMP (lane 7) for one hour. The autoradiograph is representative of three different experiments with similar results. (*B*) Densitometric analysis of the band indicated by the circle in panel A. Intensities are expressed relative to the control. Y-axis represents the intensity relative to that of the control. Three experiments from using different nuclear extracts were performed ($N = 3$). Error bars represent one standard deviation. Significance values are $^*P < 0.01$ compared with control, $^{**}P < 0.025$ compared with L-NMMA 10^{-4} mol/L; $^{***}P < 0.005$ compared with L-NMMA 10^{-4} mol/L.

suggest that L-NMMA specifically inhibited the production of Epo protein in Hep3B cells, this inhibition of Epo by L-NMMA was recovered by L-arginine.

Inhibition of NO and cGMP by L-NMMA was recovered by L-arginine

N^G-monomethyl-L-arginine is known to be a nitric oxide synthase (NOS) inhibitor, and therefore a decrease in NO from cells incubated with L-NMMA was expected. To this end, Hep3B cells were incubated with different concentrations of L-NMMA. Hypoxia induced the secretion of NO, but the addition of L-NMMA inhibited this induction. However, inhibition of NO by L-NMMA was reversed by the addition of 10^{-4} mol/L L-arginine (Fig. 2). Since NO stimulates guanylate cyclase (GC) to produce cGMP, a decrease in cGMP from cells incubated with

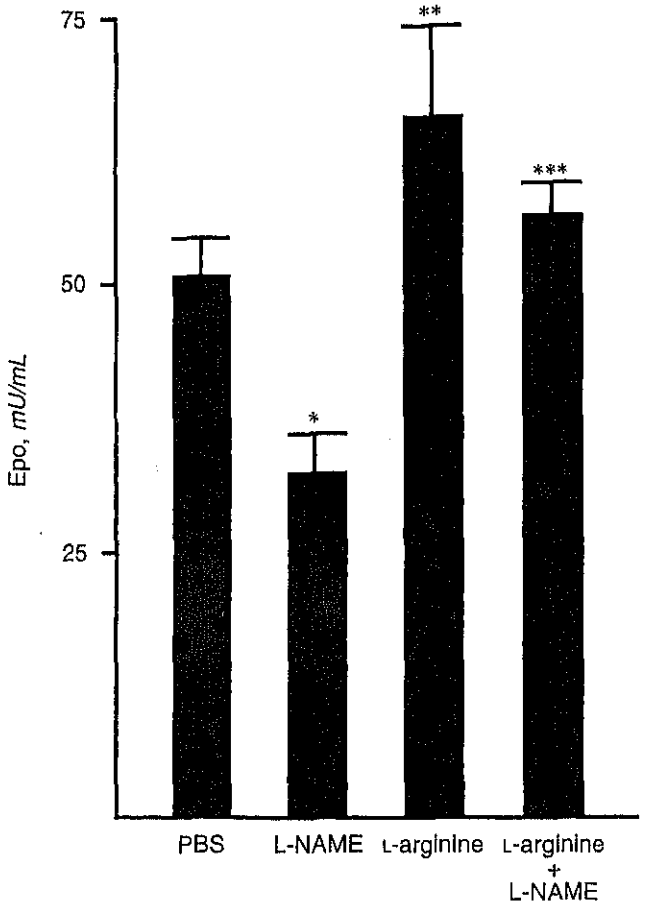


Fig. 7. Effect of L-NAME and L-arginine on serum Epo from anemic mice. BDF1 mice ($N = 5$) were injected intraperitoneally with 0.2 mL of 10 mg N^G-nitro-L-arginine methyl ester (L-NAME)/mL phosphate-buffered saline (PBS) or 0.2 mL of PBS as previously described, and 0.2 mL of 10 mg L-arginine after addition of L-NAME. Blood samples (0.3 mL) were obtained from the orbital vein at 0 and 12 hours after injection of L-NAME. Epo levels in the serum were determined by RIA. Significance values are: $*P < 0.01$ compared with PBS; $**P < 0.05$ compared with PBS; $***P < 0.005$ compared with L-NAME.

L-NMMA was also expected. To this end, hypoxic Hep3B cells were incubated with different concentrations of L-NMMA. As shown in Figure 3, hypoxia induced the secretion of cGMP, but the addition of 10^{-3} mol/L L-NMMA inhibited this induction. This inhibition of cGMP by L-NMMA was reversed by the addition of 10^{-3} mol/L L-arginine.

Inhibition of Epo promoter activity by L-NMMA was recovered by L-arginine

Pwt was transfected into Hep3B cells, and the cells incubated in the presence or absence of L-NMMA and/or L-arginine under 21% (normoxia) or 1% (hypoxia) oxygen for 24 hours. The hypoxic induction of Luc gene expression is represented as a hypoxia/normoxia ratio, as previously described [8]. Hypoxic expression from Pwt was 119.5 ± 6.7-fold higher than that from normoxic Pwt (mean ± 1 SD, $N = 3$; Fig. 4). The addition of L-NMMA inhibited the hypoxic induction of the Luc reporter gene expression from Pwt with a hypoxia/normoxia ratio of only 52.5 ± 2.7-fold, 43.9% of that from Pwt incubated without L-NMMA (Fig. 4). These results indicate that the hypoxic induction of the Epo gene expression is suppressed by L-NMMA through the Epo gene regulatory regions. However, this inhibition of Epo promoter activity by L-NMMA was reversed by the addition of L-arginine, with a hypoxia/normoxia ratio of 104.2 ± 11.1 in the presence of L-arginine and L-NMMA (Fig. 4).

Enhancement of GATA-2 binding activity by L-NMMA was inhibited by L-arginine

To examine whether L-arginine treatment affects the binding activity of hGATA-2, nuclear extracts were prepared from cells stimulated with L-NMMA for one hour under hypoxic conditions and an electrophoretic mobility shift assay (EMSA) was performed with an oligonucleotide containing the wild-type GATA element (AGATAA; Fig. 5). While normoxia induced the binding activity of GATA-2 (Fig. 5, lane 2), hypoxia reduced the GATA-2 binding activity (Fig. 5, lane 3). L-NMMA at 10^{-4} mol/L induced the binding activity of GATA-2 under hypoxic conditions (Fig. 5, lane 4), This enhanced binding activity by 10^{-4} mol/L L-NMMA was inhibited by the addition of 10^{-4} mol/L L-arginine (Fig. 5, lane 5).

cGMP inhibits GATA-2 binding activity

To clarify the effect of cGMP on GATA-2 binding activity, nuclear extracts were prepared from hypoxic Hep3B cells incubated in the absence or presence of 10^{-4} mol/L L-NMMA and/or cGMP, and EMSA was performed under the same conditions as described in Figure 5. L-NMMA at 10^{-4} mol/L induced GATA-2 binding activity (Fig. 6, lane 5), but the addition of cGMP inhibited hypoxia-induced or L-NMMA-induced GATA-2 binding activity in a dose-dependent manner (Fig. 6, lanes 2–4, 5–7).

Inhibition of serum Epo by L-NAME in vivo was recovered by L-arginine

L-NAME was examined using an in vivo mouse assay, since L-NMMA has been reported to be catabolized by N^G-dimethylarginine dimethylaminohydrolase (DDHA) in the intact kidney [18, 19]. However, L-NAME is not catabolized by this enzyme [20]. Hecker et al observed that L-NMMA was rapidly hydrolyzed to L-citrulline and lost its inhibitory effect in endothelial cells [21]. Furthermore, L-NMMA is continuously released into body fluids during the in vivo breakdown of proteins and was assumed to be readily excreted in urine without re-incorporation into proteins or further degradation in intact animals [18]. To identify the effect of L-NAME on Epo production in vivo, BDF1 mice were injected

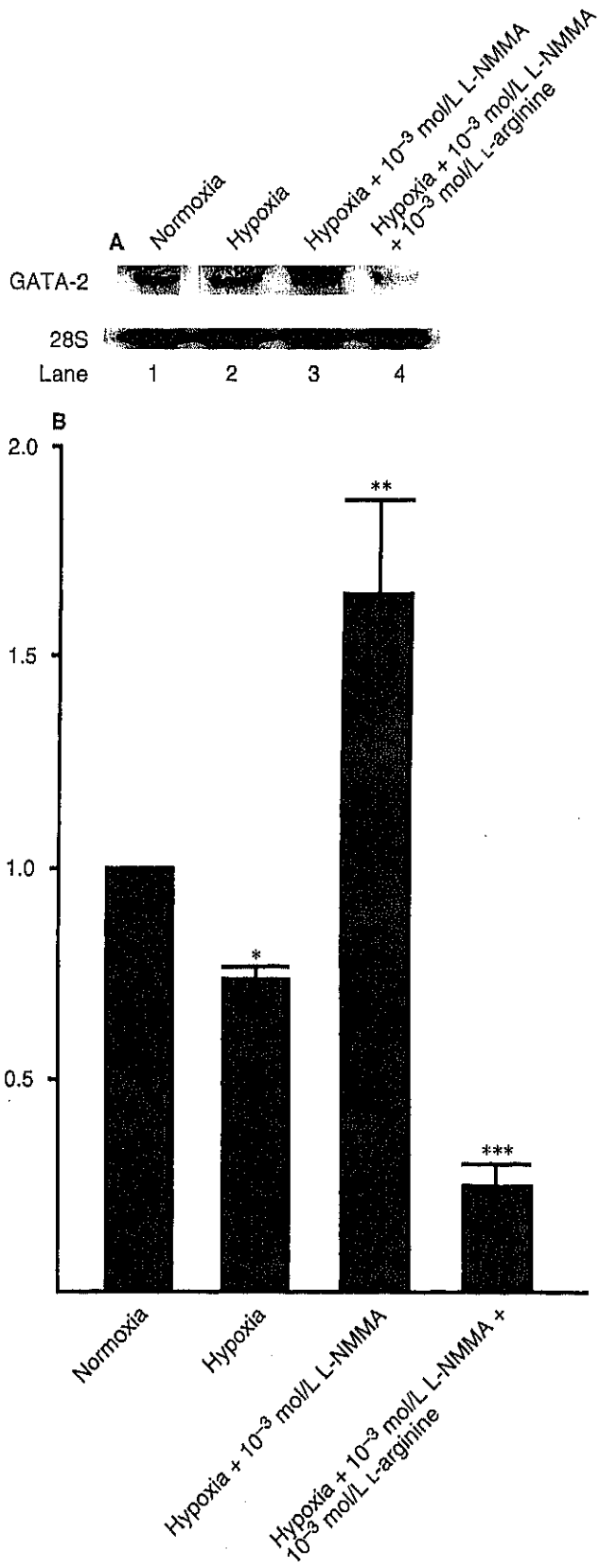


Fig. 8. Effect of L-NMMA on the expression of GATA-2 mRNA. (*A*) Northern blot analysis was performed using 25 μg of RNA from Hep3B cells incubated under normoxic conditions (21% O_2; lane 1), hypoxic conditions (1% O_2; lane 2), hypoxia with 10^{-3} mol/L L-NMMA (lane 3) and hypoxia with 10^{-3} mol/L L-NMMA and 10^{-3} mol/L L-arginine (lane 4) for eight hours. Upper and lower panels show GATA-2 and 28S mRNA, respectively. The autoradiograph is representative of three different experiments with similar results. (*B*) Densitometric analysis of the band of GATA-2 in panel A. Intensities are expressed relative to the control. Three experiments from using different nuclear extracts were performed ($N = 3$). Error bars represent one standard deviation. Significance values are: $*P < 0.005$ compared with normoxia; $**P < 0.005$ compared with hypoxia; $***P < 0.005$ compared with hypoxia + 10^{-3} mol/L L-NMMA.

intra-peritoneally with 0.2 mL of 5 mg L-NAME/mL PBS and/or 0.2 mL of 10 mg L-arginine or 0.2 mL of PBS as a control as previously described [5]. Blood samples (0.3 mL) were obtained from the orbital vein immediately after (0 hour) and at 12 hours after the injection of L-NAME (Fig. 7). Twelve hours after the injection, the serum Epo was 50.6 mU/mL in the control and 28.5 mU/mL in the L-NAME-injected mice. However, it was 65.1 mU/mL in the L-arginine-injected mice. The serum Epo from the pretreatment of L-arginine and subsequent L-NAME-injected mice was 56.0 mU/mL (Fig. 7). These results indicated that L-arginine rescued the decrease of Epo by L-NAME.

Enhancement of GATA-2 mRNA expression by L-NMMA was inhibited by L-arginine

To examine whether the level of GATA-2 expression was affected by the addition of L-NMMA, a Northern blot analysis was performed. The Northern blot analysis showed that hypoxia reduced GATA-2 mRNA expression (Fig. 8A, lane 2), while L-NMMA induced the expression of GATA-2 mRNA (Fig. 8A, lane 3). However, the addition of L-arginine inhibited this enhancement of GATA-2 mRNA by L-NMMA (Fig. 8A, lane 4). The 28S used as a control revealed a constant level of mRNA expression from the cells incubated under normoxia or hypoxia and with or without L-NMMA (Fig. 8A).

DISCUSSION

Nitric oxide-cGMP-G kinase is one of the pathways of hypoxia-induced signal transduction of Epo gene expression [22]. In chronic renal failure, we suspect that an inhibitor such as L-NMMA suppresses Epo gene expression through inhibition of NO production [5]. Our previous study found that L-NMMA decreased the expression of NO and cGMP and increased the expression of GATA-2 mRNA and the level of GATA-2 binding activity, thereby inhibiting Epo promoter activity and causing a decrease in the expression of Epo protein [5]. The effects of L-NMMA on HIF-1, hepatic nuclear factor 4 (HNF-4), chicken ovalbumin upstream promotor transcription factor 1 (COUP-TF1), nuclear factor-κB (NF-κB) were assessed by EMSA and an Epo promoter

assay. L-NMMA did not alter the binding activity of HIF-1, HNF-4, COUP-TF1, or NF-κB at all. Neither EMSA nor Epo reporter gene transfection experiments showed any effect of L-NMMA on Epo enhancer activity. L-NMMA did not affect Luc activity when GATA in the Epo promoter was mutated (Pm7) [5]. Recently, Kimura et al reported that L-NAME did not interfere with hypoxia-induced VEGF promoter (HIF-1 binding site) activation [23]. This result is compatible with our data. These results strongly suggest that L-NMMA affects GATA-2 binding.

The effect of NO on VEGF expression via HIF-1 is controversial. Some reports have shown an inhibitory effect of NO on VEGF and Epo expression. Huang et al [24] and Sogawa et al [25] have demonstrated that sodium nitroprusside (SNP, a NO donor) suppresses hypoxia-induced VEGF gene activation and HIF-1 binding activity. SNP inhibits the hypoxic induction of the VEGF gene in a dose-dependent manner, in contrast to the effects of S-nitroso-N-acetyl-D, L-penicillamine (SNAP) and 3-[hydroxy-1-(1-methylethyl)-2-nitrosohydrazino]-1-propanamine (NOC5) (other NO donors) shown by Kimura et al [23]. This difference may be due to the different types of NO donors. As Sandau et al reported that SNP releases cyanide prior to NO, it is far from being an ideal NO donor [26]. It is possible that cyanide's effects are as toxic. Those authors showed that a small NO concentration induced a faster but transient HIF-1α accumulation than higher doses of the same NO donor [26]. As to the effect of NO on Epo, Ohigashi et al reported that serum levels of Epo in hypoxic polycythemic mice were significantly increased after injections of 200 μg/kg SNP [22]. Furthermore, cGMP levels in hypoxic Hep3B cells also were elevated. SNP (10 and 100 μmol/L) and NO (2 μmol/L) increased cGMP levels in Hep3B cells [22]. These results are compatible with our data, and strongly suggest that L-NMMA inhibits Epo production via the GATA transcription factor.

Ohigashi et al reported that Rp-8-Bromo-cGMPs (a specific cGMP antagonist that binds only to the cGMP-binding sites of cGMP-dependent protein kinase) inhibited Epo mRNA expression in Hep3B cells exposed to hypoxia, which suggested that cGMP requires cGMP-dependent protein kinase to activate Epo gene expression [22]. However, the molecular mechanism of cGMP on GATA gene expression has not been analyzed yet in detail. We showed that cGMP inhibited the binding activity of GATA-2. The effect of cGMP on the expression level of GATA-2 mRNA and protein, especially the change of GATA-2 phosphorylation should be clarified.

The purpose of this study was examination of the effect of L-arginine on Epo gene expression in Hep3B cells and BDF1 mice to confirm our previous hypothesis. As a next step, further analysis of the GATA-2 expression level and NO, cGMP from patients with the anemia of renal disease is necessary to clarify the details of the pathogenesis of the anemia of renal disease.

ACKNOWLEDGMENTS

This work was supported by grants-in-aid for scientific research from the Ministry of Education, Science and Culture of Japan, Renal Anemia Foundation and the Chugai Foundation, Tokyo, Japan. We thank Hiromi Tanaka and Takako Hosoya for their expert technical assistance.

Reprint requests to Shigehiko Imagawa, M.D., Ph.D., Division of Hematology, Institute of Clinical Medicine, University of Tsukuba, Tsukuba, Ibaraki 305-8575, Japan.
E-mail: simagawa@md.tsukuba.ac.jp

REFERENCES

1. LACOMBE C, DA SILVA J-L, BRUNEVAL P, *et al*: Peritubular cells are the sites of erythropoietin synthesis in the murine hypoxic kidney. *J Clin Invest* 81:620–623, 1988
2. MCGONIGLE RJS, WALLIN JD, SHADDUCK RK, FISHER JW: Erythropoietin deficiency and erythropoiesis in renal insufficiency. *Kidney Int* 25:437–444, 1984
3. ESBACH JW, ADAMSON JW: Anemia of end-stage renal disease. *Kidney Int* 28:1–5, 1985
4. WALLE AJ, WONG GY, CLEMONS GK, *et al*: Erythropoietin-hematocrit feedback circuit in the anemia of end-stage renal disease. *Kidney Int* 31:1205–1209, 1987
5. TARUMOTO T, IMAGAWA S, OHMINE K, *et al*: N^G-monomethyl L-arginine inhibits erythropoietin gene expression by stimulating GATA-2. *Blood* 96:1716–1722, 2000
6. RIBEIRO ACM, ROBERTS NB, LANE C, *et al*: Accumulation of the endogenous L-arginine analogue N^G-monomethyl-L arginine in human end-stage renal failure patients on regular haemodialysis. *Exp Physiol* 81:475–481, 1996
7. GRAY GA, SCHOTT C, JULOU-SCHAEFFER G, *et al*: The effect of inhibitors of the L-arginine/nitric oxide pathway on endotoxin-induced loss of vascular responsiveness in anesthetized rats. *Br J Pharmacol* 103:1218–1224, 1991
8. IMAGAWA S, YAMAMOTO M, MIURA Y: Negative regulation of the erythropoietin gene expression by the GATA transcription factors. *Blood* 89:1430–1439, 1997
9. IMAGAWA S, GOLDBERG MA, DOWEIKO J, BUNN HF: Regulatory elements of the erythropoietin gene. *Blood* 77:278–285, 1991
10. GILMAN M: *Current Protocols in Molecular Biology*. New York, John Wiley and Sons, 1988, p 4.1.4
11. BLANCHARD KL, ACQUAVIVA AM, GALSON DL, BUNN H: Hypoxic induction of the human erythropoietin gene: Cooperation between the promoter and enhancer, each of which contains steroid receptor response elements. *Mol Cell Biol* 12:5373–5385, 1992
12. NORDEEN SK: Luciferase reporter gene vectors for analysis of promoters and enhancers. *BioTechniques* 6:454–457, 1988
13. GALSON DL, TSUCHIYA T, TENDLER DS, *et al*: The orphan receptor hepatic nuclear factor 4 functions as a transcriptional activator for tissue-specific and hypoxia-specific erythropoietin gene expression and is antagonized by EAR3/COUP-TF1. *Mol Cell Biol* 15:2135–2144, 1995
14. YAMAMOTO M, KO LJ, LEONARD MW, *et al*: Activity and tissue-specific expression of the transcription factor NF-E1 multi gene family. *Genes Dev* 4:1650–1662, 1990
15. RIDDLE RD, YAMAMOTO M, ENGEL JD: Expression of δ-aminolevulinate synthase in avian cells: Separate genes encode erythroid specific and non specific isozymes. *Proc Natl Acad Sci USA* 86:792–796, 1988
16. MISCO TP, SCHILLING RJ, SALVEMINI D, *et al*: A fluorometric assay for the measurement of nitrite in biological samples. *Anal Biochem* 214:11–16, 1993
17. UENO M, RONDON I, BECKMAN B, *et al*: Increased secretion of erythropoietin in human renal carcinoma cells in response to hypoxia. *Am J Physiol* 259(*Cell Physiol* 28):C427–C431, 1990

18. KIMOTO M WHITLEY G ST J, TSUJI H, OGAWA T: Detection of N^G,N^G-dimethylarginine dimethylaminohydrolase in human tissues using a monoclonal antibody. *J Biochem* 117:237–238, 1995
19. KIMOTO M, TSUJI H, OGAWA T, SASAOKA K: Detection of N^G, N^G-dimethylarginine dimethylaminohydrolase in the nitric oxide-generating systems of rats using monoclonal antibody. *Arch Biochem Biophys* 300:657–662, 1993
20. OGAWA T, KIMOTO M, SASAOKA K: Purification and properties of a new enzyme, N^G, N^G-dimethylarginine dimethylaminohydrolase, from rat kidney. *J Biol Chem* 264:10205–10209, 1989
21. HECKER M, MITCHELL JA, HARRIS HJ, *et al*: Endothelial cells metabolize N^G-monomethyl-L-arginine to L-citrulline and subsequently to L-arginine. *Biochem Biophys Res Commun* 167:1037–1043, 1990
22. OHIGASHI T, BROOKINS J, FISHER JW: Interaction of nitric oxide and cyclic guanosine 3′,5′-monophosphate in erythropoietin production. *J Clin Invest* 92:1587–1591, 1993
23. KIMURA H, WEISZ A, KURASHIMA Y, *et al*: Hypoxia response element of the human vascular endothelial growth factor gene mediates transcriptional regulation by nitric oxide: Control of hypoxia-inducible factor-1 activity by nitric oxide. *Blood* 95:189–197, 2000
24. HUANG LE, GU J, SCHAU M, BUNN HF: Regulation of hypoxia inducible factor 1α is mediated by an O_2-dependent degradation domain via the ubiquitin-proteasome pathway. *Proc Natl Acad Sci USA* 95:7987–7992, 1998
25. SOGAWA K, NUMAYAMA-TSURUTA K, EMA M, *et al*: Inhibition of hypoxia inducible factor 1 activity by nitric oxide donors in hypoxia. *Proc Natl Acad Sci USA* 95:7368–7373, 1998
26. SANDAU KB, FANDREY J, BRÜNE B: Accumulation of HIF-1α under the influence of nitric oxide. *Blood* 97:1009–1015, 2001

2.

H_2O_2 は細胞内を拡散するために intracellular messenger として働いている。そこで、H_2O_2 の Epo 遺伝子発現におよぼす効果を GATA と HIF-1 の両面から Hep3B 細胞系を用いて解析した。その結果、H_2O_2 は HIF-1 α鎖を不活性化するとともに、GATA 結合性を亢進させる両機序により Epo プロモーター活性が低下する機序を認めた (J Cell Physiol 186 : 260-267, 2001)。

JOURNAL OF CELLULAR PHYSIOLOGY 186:260–267 (2001)

Stimulation of GATA-2 as a Mechanism of Hydrogen Peroxide Suppression in Hypoxia-Induced Erythropoietin Gene Expression

MASAHIKO TABATA,[1] TAKAHISA TARUMOTO,[1] KEN OHMINE,[1] YUSUKE FURUKAWA,[2] KIYOHIKO HATAKE,[1] KEIYA OZAWA,[1] YUICHI HASEGAWA,[3] HARUMI MUKAI,[3] MASAYUKI YAMAMOTO,[4] AND SHIGEHIKO IMAGAWA[3*]

[1] *Department of Hematology, Jichi Medical School, Minamikawachi-Machi, Tochigi, Japan*
[2] *Division of Hematopoiesis, Institute of Hematology, Jichi Medical School, Minamikawachi-Machi, Tochigi, Japan*
[3] *Division of Hematology, Institute of Clinical Medicine, University of Tsukuba, Tsukuba, Ibaraki, Japan*
[4] *Center for Tsukuba Advanced Research Alliance and Institute of Basic Medical Sciences, University of Tsukuba, Tsukuba, Ibaraki, Japan*

Hydrogen peroxide (H_2O_2) has previously been shown to inhibit the DNA binding activity of hypoxia inducible factor-1 (HIF-1), the accumulation of HIF-1α protein and erythropoietin (Epo) gene expression. Epo gene expression has been previously shown to be down-regulated through a GATA binding site at its promoter region. In this study, the effect of H_2O_2 on Epo gene expression under hypoxic conditions through a GATA transcription factor was investigated. Hypoxic induction was found to be inhibited upon the addition of H_2O_2, and this effect could be reversed through the addition of catalase. Hypoxic induction was found to be suppressed by co-transfection with a human GATA-2 cDNA expression plasmid. Transfection of Hep3B cells with a reporter gene bearing a mutation at the promoter GATA binding site was found to be only mildly affected by the addition of H_2O_2. Electrophoretic gel mobility shift assays (EMSAs), using the Epo promoter GATA site as a probe and the GATA-2 protein extracted from Hep3B cells, showed that addition of H_2O_2 enhanced the binding of GATA-2 while addition of catalase inhibited this binding. From these results, we conclude that H_2O_2 increases the binding activity of GATA-2 in a specific manner, thereby suppressing the activity of the Epo promoter and thus inhibiting Epo gene expression. J. Cell. Physiol. 186:260–267, 2001. © 2001 Wiley-Liss, Inc.

Erythropoietin (Epo) is produced in the kidney (Jacobson et al., 1957) and fetal liver (Zanjani et al., 1977) in response to hypoxia, as well as in kidney in response to $CoCl_2$ administration (Goldwasser et al., 1958). In the human hepatoma cell lines Hep3B and $HepG_2$, both Epo mRNA and Epo protein are induced by hypoxia or $CoCl_2$ (Goldberg et al., 1987), making these cell lines useful for investigating the regulation of Epo gene expression. Considerable progress has been made in the identification and characterization of *cis*-acting enhancer elements contributing to the transcription of the Epo gene (Semenza et al., 1991a, 1991b; Semenza and Wang, 1992; Blanchard et al., 1992; Galson et al., 1995; Bunn and Poyton, 1996). However, the promoter of the Epo gene has not been well characterized. We previously have shown that CACCC elements at about −60 bp from the Epo CAP site are up-regulating sites for the Epo gene, whereas a GATA element located at −30 bp is a down-regulating site (Imagawa et al., 1997). In addition, the human GATA (hGATA)-1, 2, and 3 transcription factors have been found to specifically bind to this GATA site within the human Epo gene promoter and down-regulate Epo gene expression, especially the hGATA-2 transcription factor in Hep3B cells (Imagawa

Contract grant sponsor: The Ministry of Education, Science and Culture of Japan; Contract grant number: 09671130.

*Correspondence to: Shigehiko Imagawa, Division of Hematology, Institute of Clinical Medicine, University of Tsukuba, Tsukuba, Ibaraki 305-8575, Japan.
E-mail: simagawa@md.tsukuba.ac.jp

Received 30 May 2000; Accepted 25 September 2000

Published online in Wiley InterScience, 13 December 2000.

et al., 1997). Goldberg et al. (1988) have proposed that Hep3B cells have an oxygen-sensing mechanism in which hypoxia or $CoCl_2$ treatment results in a ligand-dependent conformational change in a heme protein, subsequently inducing Epo gene expression. It was further found that this heme protein may change its conformation depending on the ambient O_2 tension, suggesting that this conformational change induces Epo gene expression through some unknown intracellular signal (Goldberg et al., 1988). Studies on Epo-producing $HepG_2$ cell lines suggested the involvement of a b-type cytochrome in the oxygen-sensing process (Görlach et al., 1993). The presence of an NADPH oxidase-like heme protein in the oxygen-sensing system has been suggested by the observation that H_2O_2 is formed in these cells following exposure to oxygen. Using $HepG_2$ cells, Fandrey et al. (1994) found that H_2O_2 decreased Epo mRNA and Epo protein. Since H_2O_2 is able to freely diffuse within cells, it becomes one of the most likely candidates for an intracellular messenger molecule (Fandrey et al., 1994). And in this respect, H_2O_2-mediated inhibition of Epo gene expression has been reported to prevent (at least partly) the binding of hypoxia inducible factor-1 (HIF-1) to the HIF-1 binding site at the 3′-enhancer of the gene (Huang et al., 1996). Moreover, addition of H_2O_2 has been found to inhibit hypoxia-induced Epo mRNA and Epo protein production in Hep3B cells (Imagawa et al., 1996).

The present paper describes the effect of H_2O_2 on the Epo gene expression in Hep3B cells through the GATA site using transient transfection analysis. Hep3B cells were used because they are more responsive to hypoxia than $HepG_2$ cells (Goldberg et al., 1987). Epo promoter-luciferase and promoter/enhancer-luciferase constructs (both wild-type and a GATA site mutant promoter) were transfected into Hep3B cells and incubated with or without H_2O_2. We found that the H_2O_2 treatment of Hep3B cells resulted in an enhanced binding of the GATA-2 factor to its specific Epo gene promoter site. Therefore, H_2O_2 administration not only inhibits the HIF-1 binding activity, but also increases the binding of GATA-2 to a down-regulated site at the Epo promoter, thus providing another explanation to the mechanism by which H_2O_2 inhibits Epo gene transcription.

MATERIALS AND METHODS

Cell culturing and RNA preparations

The Hep3B cell line was obtained from the American Type Tissue Culture Collection (Rockville, MD). The cells were cultured in Dulbecco's modified Eagle's medium (Life Technologies Inc., Gaithersburg, MD), supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT) in 25 cm^2 tissue culture flasks. Cells were maintained in a humidified 5% CO_2/95% air incubator at 37°C and grown either under hypoxic (1% oxygen) or normoxic conditions, as previously described (Imagawa et al., 1997). The cells were stimulated upon addition of H_2O_2 (500 μM final concentration) or catalase (100 μg/ml) to the culture medium. After 24 h incubation under the hypoxic/normoxic and H_2O_2-stimulated/unstimulated conditions, the cells were harvested and cellular extracts prepared. Total cellular RNA was also prepared by conventional methods (Gilman, 1988). Fandrey et al. (1994) found that H_2O_2 concentrations up to 500 μM were not cytotoxic for $HepG_2$ cells, a hepatoma cell line that also expresses Epo. We subsequently showed that growth of Hep3B hepatoma cells also was not affected by the addition of 500 μM of H_2O_2 (Imagawa et al., 1996). Therefore, this concentration of H_2O_2 was used in the present study.

Transfection

Electroporation of the hGATA-2 expression vector into Hep3B cells was carried out as previously described (Imagawa et al., 1997). A RSVCAT (chloramphenicol acetyltransferase; 10 μg) plasmid was co-transfected as an internal standard in all transfection reactions (Imagawa et al., 1991).

Electrophoretic mobility shift assay

Nuclear extracts were prepared as described (Yamamoto et al., 1990). Protein concentrations were determined by a Bio-Rad assay (Bio-Rad, Japan) using bovine serum albumin as a standard. Sense strand oligonucleotides (wild type: CATGCAGATAACAGCCCCGAC, mutant oligo-1: CATGCAGCGAACAGCCCCGAC and mutant oligo-2: CATGCCGCGATCAGCCCCGAC) were end-labeled with T4 polynucleotide kinase (Toyobo, Tokyo) and annealed to a four-fold excess of the unlabeled antisense oligonucleotide. In each binding reaction, 2 ng of labeled probe was used. The binding buffer used contained 10 mM Tris–HCl (pH 7.5), 1 mM EDTA, 4% Ficoll, 1 mM dithiothreitol and 75 mM KCl. An equimolar mixture of poly[d(I-C)] and poly[d(A-T)] (Sigma, St. Louis, MO; 25 ng) was used as a nonspecific competitor. Reaction mixtures (25 μl) were incubated for 15 min at 4°C and subsequently electrophoresed on 5% non-denaturing polyacrylamide gels in 0.25 × TBE buffer (22 mM Tris–Borate, 22 mM boric acid, 0.5 mM EDTA) at room temperature at 150 V for 1.5 h as previously described (Yamamoto et al., 1990). After vacuum-drying, the gels were subjected to autoradiography using intensifying screens at −80°C for 24 h. A monoclonal antibody of hGATA-2 was prepared as described (Nagai et al., 1994).

Plasmid vectors

Four different kinds of pEPLuc plasmids (Pwt, Pm7, △18pXP2 and △18m7pXP2) were used. In Pwt, a 126 bp 3′ Epo enhancer (120–245 bp 3′ of the poly(A) addition site) and the 144 bp minimal Epo promoter (from −118 to +26 relative to the transcription initiation site) were placed upstream of the *Luc* gene in PXP2, or V2-Ewt-Pwt-PXP2 (Blanchard et al., 1992). In Pm7, the GATA sequence in the Epo promoter was mutated to TATA (i.e., AGATAACAG to ATATAAAAG). This mutant is also called V3-Ewt-Pm7-PXP2 (Blanchard et al., 1992). In △18pXP2, the 144 bp minimal Epo promoter (from −118 to +26 relative to the transcription initiation site) was placed upstream of the firefly luciferase (*Luc*) gene in PXP2 (Nordeen, 1988). △18m7pXP2 is identical to △18pXP2 except that the GATA sequence in the Epo promoter is mutated to TATA (i.e., AGATAACAG to ATATAAAAG). Construction of the hGATA-2 expression plasmid was described previously (Yamamoto et al., 1990).

Northern blot analysis

Probes were labeled with [α-^{32}P]deoxycytidine triphosphate by random priming and used in RNA blot hybridization (Riddle et al., 1989). Formaldehyde gels for RNA electrophoresis were prepared as described (Riddle et al., 1989). RNA blot hybridization was carried out using 25 μg total RNA from Hep3B cells, with filters hybridized with probe hGATA-2 cDNA. The same filter was stripped and re-hybridized to ribosomal RNA to determine the level of RNA in each lane.

Western blot analysis

Cells (1×10^7) were lysed directly in SDS/sample buffer (0.125 mol/liter Tris–HCl [pH 6.8], 10% glycerol, 2% SDS, 1% 2ME). Protein concentration of the samples was determined by the Protein Assay method (Nippon Bio Rad, Tokyo, Japan) and 9 μg of protein in these fractions was applied to a Laemmli gel system after supplementing with 0.0025% Bromphenol Blue. After electrophoretic separation, proteins were transferred to a polyvinylpyrrolidone difluoride (PVDF) membrane (Immobilon-P; Millipore, Bedford, MA). This membrane was blocked with Tris-buffered saline (TBS) containing 3% skim milk at 4°C overnight, and incubated with mouse anti-human GATA-2 antibody diluted with TBS containing 1% BSA. The membrane was then reacted with sheep anti-mouse IgG conjugated with horseradish peroxidase (Amersham). The position of immune complexes on the membrane was visualized using the ECL system (Amersham). The detailed method is described by Nagai et al. (1994).

Promoter assays

Transfected Hep3B cells rinsed with phosphate-buffered saline (PBS) were lysed in 10 cm dishes with 800 μl of cell lysis buffer (PicaGene, Toyo Ink, Tokyo). *Luc* activity was determined in 20 μl of the cell extract by an Autolumat luminometer (Berthorude) for 10 sec. Measurements expressed in relative light units (RLU), were corrected by subtracting the background and dividing the remainder by the RSVCAT internal transfection control activity. Hypoxic inducibility was defined as R (H/N), where H and N are the RLUs of the cells cultured under hypoxic (1% O_2) and normoxic (21% O_2) conditions, respectively. Chloramphenicol acetyl transferase (CAT) activity was determined as described by Neumann et al. (1987), and β-Gal assays were performed to normalize the transfection efficiency.

RESULTS

Inhibition of Epo promoter/enhancer activity by hydrogen peroxide

Transfection of Pwt into Hep3B cells and the cells incubated under normoxia and hypoxia showed 974 RLU/CAT U and 17697 RLU/ CAT U, respectively. Hypoxia induced an 18.2-fold increase in *Luc* reporter expression from Pwt (Fig. 1) as compared to normoxia (i.e., R (H/N) = 18.2). Addition of H_2O_2 reduced R (H/N) to just 9.5, thus significantly inhibiting the hypoxic induction of the *Luc* reporter gene expression from Pwt. The GATA site located at the Epo promoter plays an important role in the prevention of hypoxic induction of the Epo gene (Imagawa et al., 1997). For Hep3B cells

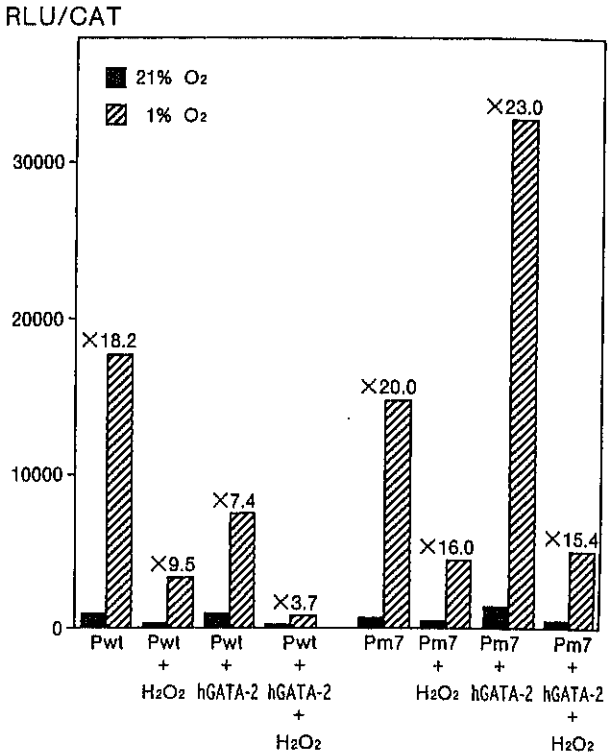


Fig. 1. Effect of H_2O_2 on the induction of the wild-type of the Epo promoter/enhancer with *Luc* reporter constructs in Hep3B cells. Numbers above the bars indicate the hypoxic induction, calculated as the ratio of induction under hypoxia/induction under normoxia.

that were co-transfected with Pwt and the hGATA-2 expression vector, R (H/N) was reduced to only 7.4, indicating that expression of hGATA-2 also inhibited the hypoxic induction of the Epo gene. Furthermore, exposure of these co-transfected cells to H_2O_2 resulted in an additional suppression of the hypoxic Epo gene response, i.e., R (H/N) was only 3.7. These results suggest that hGATA-2 acts as a suppressor of the hypoxic induction of the Epo gene, with the GATA sequence in the promoter mediating this H_2O_2 suppression.

The GATA site at the Epo promoter contributes to H_2O_2 suppression

Pm7-transfected cells (which bear a mutation in the Epo promoter GATA site), like Pwt-transfected cells, had a high R (H/N) value (20.0) (Fig. 1). However, the effect of H_2O_2 was not as significant in the Pm7-transfected cells as it was in the Pwt-transfected cells. H_2O_2 treatment reduced R (H/N) to only 16.0 (Fig. 1). Thus, the point mutation of the GATA binding site in the Epo gene promoter causes the plasmid to be less susceptible to the effects of H_2O_2 treatment, further supporting the idea that the GATA site plays a role in mediating the H_2O_2 suppression. While some basal level of H_2O_2 suppression in the Pm7-transfected cells exists, this residual suppression may be the result of the HIF-1 activity in the enhancer. Co-transfection of the hGATA-2 expression plasmid with Pm7 did not affect R (H/N), which remained at 23.0 (Fig. 1). This was expected because the promoter mutation completely inhibits GATA-

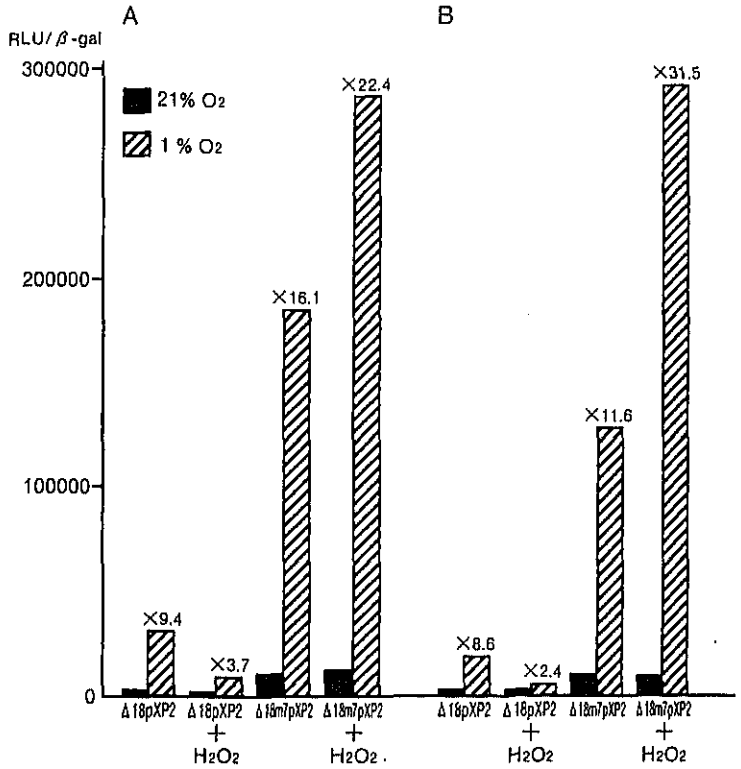


Fig. 2. Effect of H_2O_2 on the induction of the wild-type and mutated GATA site of the Epo promoter with *Luc* reporter constructs in Hep3B cells. Two different experiments were performed. Numbers above the bars are explained in the legend of Fig. 1.

2 binding. However, exposure of Hep3B cells co-transfected with Pm7 and hGATA-2 to H_2O_2 resulted in a R (H/N) of 15.4 (Fig. 1). This provides further evidence that the decrease in hypoxic induction may be due to the inhibition activity of HIF-1 only. These results thus directly demonstrate the contribution of the Epo promoter GATA site to the H_2O_2 hypoxic suppression response.

Inhibition of the Epo promoter activity by hydrogen peroxide

To confirm that the inhibitory effect of H_2O_2 on Epo gene expression was caused only by the GATA-2 or the HIF-1 binding site, a construct containing the Epo promoter only was used. For △18pXP2 cells, contain the intact Epo promoter, R (H/N) values in two replicate experiments were 9.4 and 8.6 (Fig. 2A,B). Addition of 500 μM H_2O_2 reduced R (H/N) to 3.7 and 2.4. Significantly, addition of H_2O_2 inhibited the hypoxic induction of the *Luc* reporter gene expression from △18pXP2. These results clearly indicate that the hypoxic induction of Epo reporter gene expression is suppressed upon H_2O_2 addition through the GATA site. For △18m7pXP2-transfected cells, in which the GATA site of the Epo promoter is mutated, R (H/N) was also high (16.1 and 11.6). No inhibitory effect of H_2O_2 was found in the △18m7pXP2-transfected cells, since R (H/N) remained high (22.4 and 31.5) in the presence of H_2O_2. These results strongly suggested that H_2O_2-mediated inhibition of the Epo gene is not only due to HIF-1, but also to GATA. Figure 2 shows that the GATA mutation is important in the basal expression of *Luc* and induction by hypoxia, since the GATA site works as a suppressive element, as previously reported (Imagawa et al., 1997). These data strongly support the view that GATA functions directly through this element.

Enhancement of GATA-2 binding activity by hydrogen peroxide

To determine whether the H_2O_2 treatment affected the binding activity of hGATA-2, Hep3B cells were stimulated by H_2O_2 under hypoxic conditions. Nuclear extracts prepared from the cells were used in EMSA with oligonucleotides containing the wild-type GATA site (AGATAA) (Fig. 3A, lanes 2–7). The circle indicates the position of the band corresponding specifically to the hGATA-2 probe complex (Fig. 3A, lanes 2–4, 8 and 9). When using the anti-GATA-2 monoclonal antibody, this band disappeared (Fig. 4, lane 3). This band also disappeared after addition of unlabeled wild-type oligonucleotide as a competitor (Fig. 3A, lanes 5–7). When unlabeled mutant oligonucleotides were added as competitors, this band was present (Fig. 3A, lane 8: mutant oligo-1: and lane 9: mutant oligo-2). However, the intensity of the band indicated by the circle was found to decrease in lane 9, with the intensity of the lower band concomitantly decreasing. This indicates that mutant oligo-2 does not compete with the band indicated by the circle. Furthermore, this band was not seen when labeled mutant oligonucleotides were used as

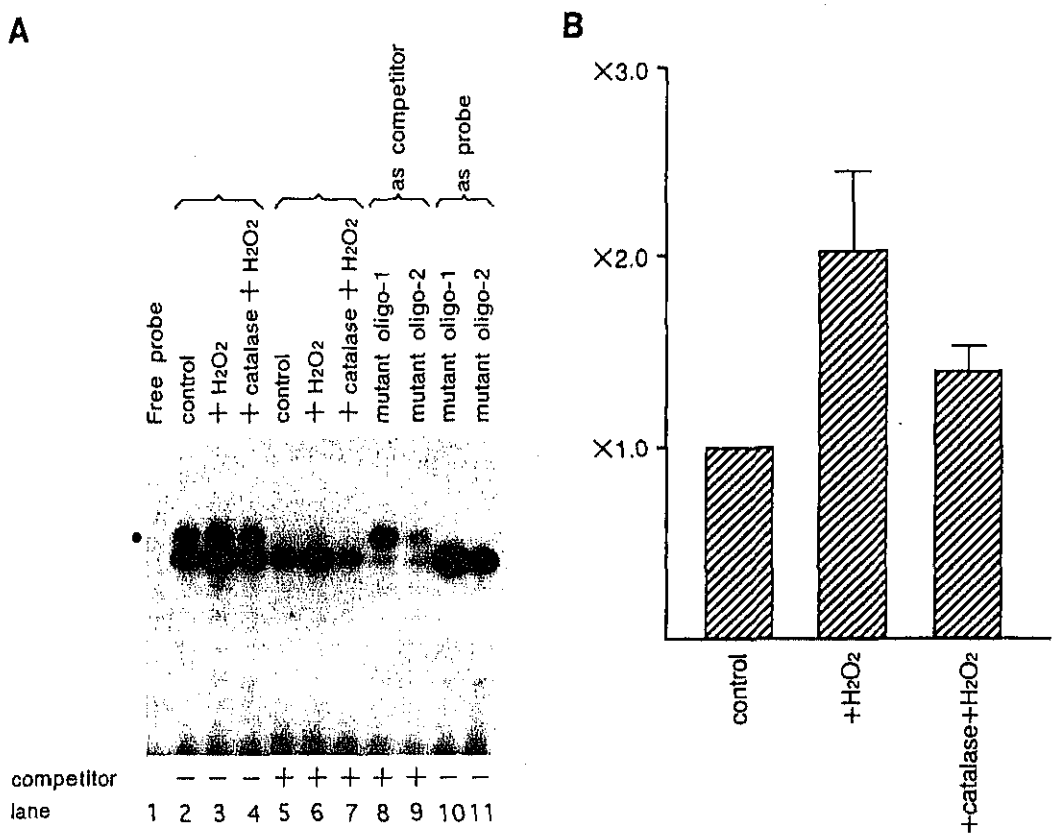


Fig. 3. Effect of H_2O_2 on the expression of hGATA-2 in Hep3B cells. A: EMSA using the Epo promoter GATA site as a probe, and the protein extracted from the cells. Each lane was loaded with 2.1 μg of protein from cell extracts. Cells were grown under hypoxia (lanes 2 and 5), with 500 μM H_2O_2 (lanes 3, 6) and with 100 μg/ml catalase and 500 μM H_2O_2 (lanes 4 and 7). The circle indicates the hGATA-2 transcription factor. A total of 25 ng (0.5 μl: 12.5-fold molar excess) of competitor DNA was added to each reaction mixture (lanes 5–7). B: Densitometric analysis of the band indicated by a circle in A. Intensities are expressed relative to the control. Y-axis represents the intensity relative to that of the control. Three experiments from using different nuclear extracts were performed (n = 3). Error bars represent the standard deviation.

probes (Fig. 3A, lane 10: mutant oligo-1 and lane 11: mutant oligo-2). These data indicate that the band is generated specifically through the binding of hGATA-2 to the Epo promoter GATA sequence probe, while the lower band is most likely the result of non-specific binding. Although the non-specific complex is not affected by self-competition, it is affected by two different competitors having mutations at the GATA site. hGATA-2 binding was enhanced after addition of H_2O_2 to the Hep3B cell culture (compare Fig. 3A, lanes 2 and 3), but it was inhibited upon the addition of catalase to the culture medium (Fig. 3A, lane 4). Three separate experiments from different nuclear extracts were carried out (data not shown). A densitometric analysis of this assay revealed that the addition of H_2O_2 increased the intensity of the hGATA-2 band by ≈2.0-fold (n = 3) (Fig. 3B) and this increase was reduced to a 1.4-fold increase (n = 3) upon the addition of catalase (Fig. 3B). This indicates that the enhancement of binding is a specific effect of H_2O_2. Although the intensity of the lower band also increases after addition of H_2O_2, this band does not disappear after addition of competitor and disappears after addition of mutant oligonucleotides as shown in Figure 3A, lanes 5–9. This result clearly indicates that the binding activity of GATA-2 is increased by H_2O_2. To determine whether the effect of H_2O_2 on the binding activity of GATA-2 is specific, EMSA was performed under the same conditions with an oligonucleotide containing an NF-κB element. As shown in Figure 5, the addition of H_2O_2 or catalase did not induce the binding activity of NF-κB. This result strongly suggests that the effect of H_2O_2 on the binding activity of GATA-2 is specific.

Effect of hydrogen peroxide on expression of GATA-2 mRNA and protein

The transcriptional or post-transcriptional effect of hydrogen peroxide on the GATA-2 binding activity was carried out by Northern blot analysis using 25 μg total RNA from Hep3B cells under normoxia (Fig. 6, lanes 1–3) and hypoxia (Fig. 6, lanes 4–6). Total RNAs were prepared from control Hep3B cells (lanes 1 and 4), Hep3B cells treated with 500 μM H_2O_2 (lanes 2 and 5) and Hep3B cells treated with 100 μg/ml catalase and 500 μM H_2O_2 (lanes 3 and 6). The band intensities in the Northern blot analysis with the hGATA-2 probe (Fig. 6A) as well as the band intensities of ribosomal RNA (an internal control) (Fig. 6B) were unaffected by the addition of H_2O_2 with or without catalase. Furthermore, a Western blot analysis was performed using 9.0 μg total

Fig. 4. EMSA showing the effect of an anti-GATA-2 monoclonal antibody. Hep3B cells were grown under hypoxia and 2.1 μg protein from a cell extract was applied to each lane. Nuclear extracts were incubated overnight at 4°C with 2 μl of FCS (lane 2) or anti-GATA-2 monoclonal antibody for knockout (lane 3). Circle shows GATA-2 transcription factor.

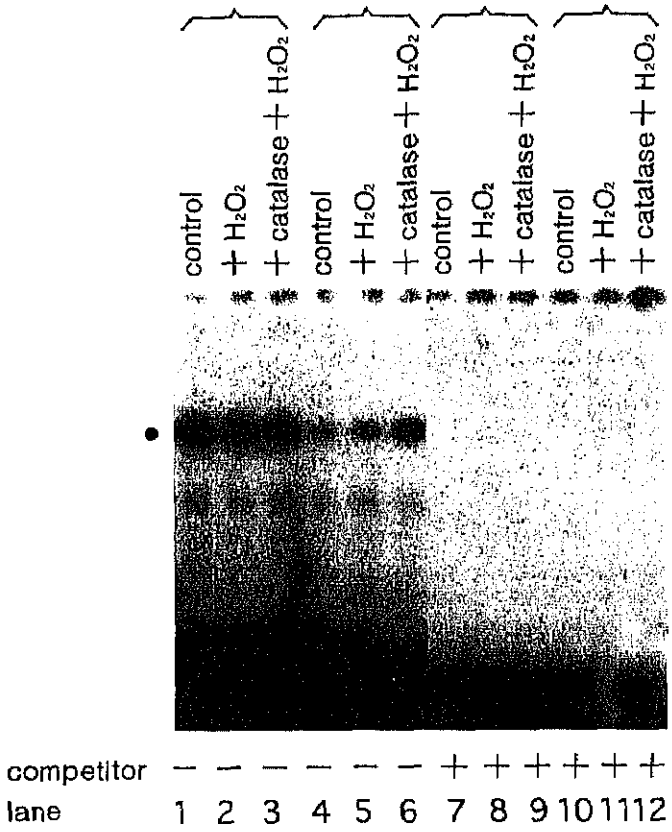


Fig. 5. EMSA showing effect of H_2O_2 on the expression of NF-κB in Hep3B cells. 2.1 μg protein from cell extracts was applied to each lane. Cells were grown under normoxia (lanes 1–3, 7–9), under hypoxia (lanes 4–6, 10–12), with 500 μM H_2O_2 (lanes 2, 5, 8 and 11) and with 100 μg/ml catalase and 500 μM H_2O_2 (lanes 3, 6, 9 and 12). Circle indicates NF-κB transcription factor. A total of 25 ng (0.5 μl: 12.5-fold molar excess) of competitor DNA was added to each reaction mixture (lanes 7–12).

protein from Hep3B cells under hypoxia, and with 500 μM H_2O_2 and/or 100 μg/ml catalase under hypoxia (Fig. 7). The intensities of the bands in the Western blot analysis with the GATA-2 antibody were also found to be unaffected by the addition of H_2O_2 and/or catalase (Fig. 7). These results indicate that the effect of increasing GATA-2 binding activity by hydrogen peroxide is due to post-transcriptional regulation.

DISCUSSION

Regulation of Epo gene expression is a complex process in which a change in intracellular oxygen tension (pO_2) is precisely transduced into a transcriptional response. It should be noted that H_2O_2 inhibits Epo gene expression by destabilizing HIF-1α, which binds to the 3′-enhancer of the Epo gene (Huang et al., 1996). This is intriguing, since H_2O_2 has been assumed to act as a signal inhibition molecule connecting oxygen sensitive proteins with transcription factor(s) regulating Epo gene expression (Fandrey et al., 1996). Indeed, Fandrey et al. (1994) showed that the addition of H_2O_2 inhibits Epo mRNA expression using competitive PCR, as well as Epo protein synthesis using $HepG_2$ cells. Subsequently, we reported that H_2O_2 inhibits Epo mRNA expression by competitive PCR and suggested that GATA-2 may have a role in this inhibition in Hep3B cells (Imagawa et al., 1996).

In the present study, H_2O_2 treatment was found to inhibit Epo promoter activity and increased the binding activity of GATA-2. As GATA-2 binds to the GATA site at the Epo promoter and downregulates Epo gene expression in Hep3B cells, this suppression of Epo gene expression by H_2O_2 is partly due to the enhanced GATA-2 binding activity. This effect may be ascribed to post-transcriptional regulation of GATA-2 by hydrogen peroxide, since the expression of GATA-2 mRNA and protein was unchanged upon the addition of hydrogen peroxide. Further analysis of the modulation of GATA-2, such as by phosphorylation (Towatari et al., 1995) upon hydrogen peroxide addition should be done to elucidate its mechanism.

The hypoxia-induced Epo promoter activity in Hep3B cells was reduced much more by treatment with H_2O_2 and GATA-2 rather than by either one of these treat-

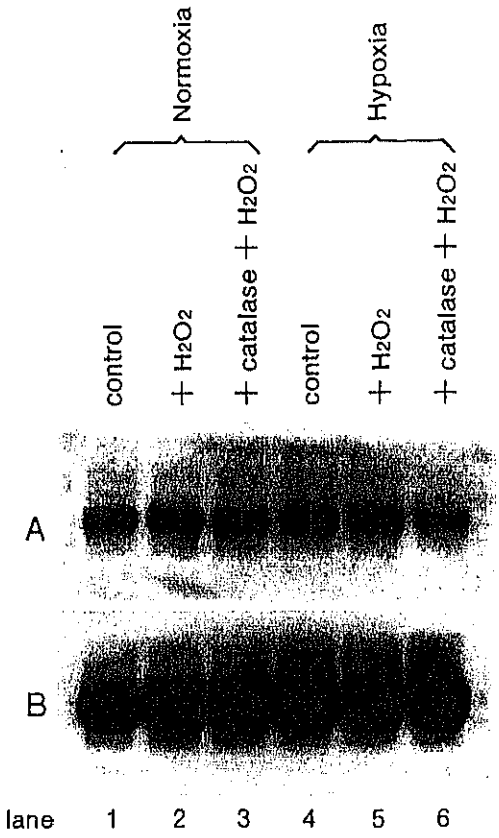


Fig. 6. Northern blot showing hGATA-2 mRNA expression in Hep3B cells. A: Cells grown under normoxia (lanes 1–3) and hypoxia (lanes 4–6). hGATA-2 cDNA was used as the probe. B: The same membrane stripped and re-hybridized to ribosomal RNA serving as an internal standard.

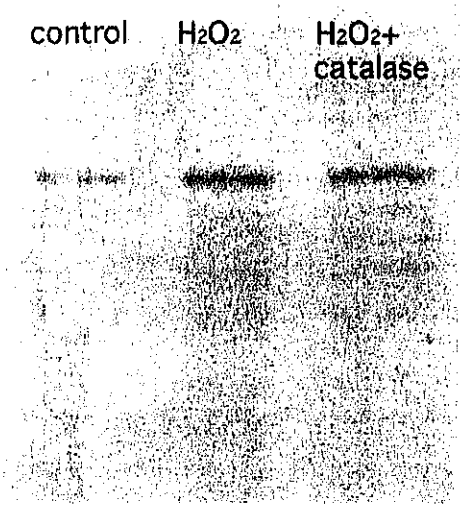


Fig. 7. Western bloting showing hGATA-2 protein expression in Hep3B cells. Cells were incubated under hypoxia (left), with 500 μM H_2O_2 under hypoxia (middle) and with 100 μg/ml catalase and 500 μM H_2O_2 under hypoxia (right). The circle indicates hGATA-2 protein.

ments alone. This suggests that the activity of transcription factors binding to the GATA site in the promoter and the HIF-1 site in the 3′-enhancer may be affected simultaneously by H_2O_2. Huang et al. (1998) identified an oxygen-dependent degradation (ODD) domain within HIF-1α that controls the degradation of HIF-1α by the ubiquitin-proteasome pathway. This degradation also acts as a type of post-transcriptional regulation (Huang et al., 1998). Therefore, H_2O_2 may stimulate the degradation of the ODD domain of HIF-1α as well as induce the binding of GATA-2, acting as a down regulator, and in cooperation with the destabilization of HIF-1α, it may result in a decreased expression of the Epo gene. Since the GATA family transcription factors are known to be upregulators (Orkin, 1990; Evans et al., 1990), the down-regulation of Epo gene expression by GATA-2 in Hep3B cells is intriguing. The binding activity of GATA-2 was especially enhanced by the addition of H_2O_2 to Hep3B cells. Further analysis of how H_2O_2 affects the binding of GATA-2 is needed to understand the H_2O_2-sensing process.

Hypoxia has been shown to induce an increase of vascular endothelial-derived growth factor (VEGF) mRNA expression in $HepG_2$ cells, and this increase is also suppressed by the addition of H_2O_2 (Huang et al., 1996). This H_2O_2 suppression of VEGF gene expression has been suggested to be mediated by HIF-1 (Huang et al., 1996). The exact mechanisms of H_2O_2 suppression of the expression of the Epo and VEGF genes at high pO_2, are not yet available. We speculate that similar mechanisms for Epo gene regulation and VEGF gene expression may exist. Indeed, GATA-2 has been shown to be an important regulator of gene expression in endothelial cells (Wilson et al., 1990). In addition, the HIF-1 transcription factor has been shown to be important in the hypoxic induction of Epo gene expression (Semenza et al., 1991a,b). The HIF-1 is known to affect the expression of the genes encoding VEGF (Levy et al., 1995), phospho-glycerokinase 1(PGK1) (Firth et al., 1994) and glucose transporter 1(Glut-1) (Ebert et al., 1995). HIF-1 is also activated in cells exposed to $CoCl_2$ or iron chelators (Semenza et al., 1991). These cell treatments enable HIF-1 to bind to a so-called consensus sequence (5′-TACGTGCT-3′) (Semenza et al., 1991). It has been suggested that HIF-1 interacts with another transcription factor HNF-4, in the hypoxic induction of Epo gene expression. HNF-4 is known to bind to steroid receptor response elements (SRRE) in the Epo gene 3′-enhancer as well as promoter regions. HNF-4 binds to the α-subunit of HIF-1 (Arany et al., 1996). This binding activates both α- and β-subunits of HIF-1 which causes HIF-1 to interact with the transcriptional activator p300, thus providing a mechanism to trigger the Epo mRNA transcription (Huang et al., 1997). The inhibition of Epo gene expression by H_2O_2 might originate from the suppression of p300 activity. In contrast, Epo gene expression is suppressed under the normoxic conditions. We assume that this is caused not only by suppression of the hypoxic-inducing mechanism, but also by the constitutive expression of GATA-2. Further analysis of switching and balancing up- and down-regulator activities is certainly necessary to better understand the oxygen-sensing mechanism of Epo gene expression.

ACKNOWLEDGMENTS

This study was supported by Grants-in-Aid from the Ministry of Education, Science and Culture of Japan and the Chugai Foundation. We would like to thank H.F. Bunn, J.D. Engel, J. Fandrey, W. Jelkmann, K. Igarashi and H. Pagel for helpful discussions and D.L. Galson for providing △18pXP2, △18m7pXP2, Pwt, and Pm7. We also thank M. Nakamura and A. Yamazaki for their expert technical assistance.

LITERATURE CITED

Arany Z, Huang LE, Eckner R, Bhattacharya S, Jiang C, Goldberg MA, Bunn HF, Livingston DM. 1996. An essential role for p300/CBP in the cellular response to hypoxia. Proc Natl Acad Sci USA 93:12969–12973.

Blanchard KL, Acquaviva AM, Galson DL, Bunn HF. 1992. Hypoxic induction of the human erythropoietin gene: Cooperation between the promoter and enhancer, each of which contains steroid receptor response elements. Mol Cell Biol 12:5373–5385.

Bunn HF, Poyton RO. 1996. Oxygen sensing and molecular adaptation to hypoxia. Physiol Rev 76:839–885.

Ebert BL, Firth JD, Ratcliffe PJ. 1995. Hypoxia and mitochondrial inhibitors regulate expression of glucose transporter-1 via distinct *cis*-acting sequences. J Biol Chem 270:29083–29089.

Evans T, Felsenfeld G, Reitman M. 1990. Control of globin gene transcription. Ann Rev Cell Biol 6:95–124.

Fandrey J, Frede S, Jelkmann W. 1994. Role of hydrogen peroxide in hypoxia-induced erythropoietin production. Biochem J 303:507–510.

Fandrey J, Frede S, Jelkmann W. 1996. Role of hydrogen peroxide in hypoxia-induced expression of the erythropoietin and the vascular endothelial growth factor genes. Mol Biol Hematopoiesis 5:523–530.

Firth JD, Ebert BL, Pugh CW, Ratcliffe PJ. 1994. Oxygen-regulated control elements in the phosphoglycerate kinase 1 and lactate dehydrogenase A genes: Similarities with the erythropoietin 3′ enhancer. Proc Natl Acad Sci USA 91:6496–6500.

Galson DL, Tsuchiya T, Tendler TS, Huang LE, Ren Y, Ogura T, Bunn HF. 1995. The orphan receptor hepatic nuclear factor 4 functions as a transcriptional activator for tissue-specific and hypoxia-specific erythropoietin gene expression and is antagonized by EAR3/COUP-TF-1. Mol Cell Biol 15:2135–2144.

Gilman M. 1988. Current Protocols in Molecular Biology, vol. 1. New York: Wiley. 4.1.4 p.

Goldberg MA, Glass GA, Cunningham JM, Bunn HF. 1987. The regulated expression of erythropoietin by two human hepatoma cell lines. Proc Natl Acad Sci USA 84:7972–7976.

Goldberg MA, Dunning SP, Bunn HF. 1988. Regulation of the erythropoietin gene: Evidence that the oxygen sensor is a heme protein. Science 242:1412–1415.

Goldwasser E, Jacobson LO, Fried W, Plzak LF. 1958. Studies on erythropoiesis. V. The effect of cobalt on the production of erythropoietin. Blood 13:55–60.

Görlach A, Holtermann G, Jelkmann W, Hancock JT, Jones SA, Jones OTG, Acker H. 1993. Photometric characteristics of heme proteins in erythropoietin-producing hepatoma cells ($HepG_2$). Biochem J 290:771–776.

Huang LE, Arany Z, Livingston DM, Bunn HF. 1996. Activation of hypoxia-inducible transcription factor depends primarily upon redox-sensitive stabilization of it's α subunit. J Biol Chem 271:32253–32259.

Huang LE, Ho V, Arany Z, Krainc D, Galson D, Tendler D, Livingston DM, Bunn HF. 1997. Erythropoietin gene regulation depends on heme-dependent oxygen sensing and assembly of interacting transcription factors. Kidney Int 51:548–552.

Huang LE, Gu J, Schau M, Bunn HF. 1998. Regulation of hypoxia-inducible factor 1α mediated by an O_2-dependent degradation domain via the ubiquitin-proteasome pathway. Proc Natl Acad Sci USA 95:7987–7992.

Imagawa S, Goldberg MA, Doweiko J, Bunn HF. 1991. Regulatory elements of the erythropoietin gene. Blood 77:278–285.

Imagawa S, Yamamoto M, Ueda M, Miura Y. 1996. Erythropoietin gene expression by hydrogen peroxide. Int J Hematol 64:189–195.

Imagawa S, Yamamoto M, Miura T. 1997. Negative regulation of the erythropoietin gene expression by the GATA transcription factors. Blood 89:1430–1439.

Jacobson LO, Goldwasser E, Fried W, Plzak L. 1957. Nature 179:633–634.

Levy AP, Levy NS, Wegner S, Goldberg MA. 1995. Transcriptional regulation of the rat vascular endothelial growth factor gene by hypoxia. J Biol Chem 270:13333–13340.

Nagai T, Harigae H, Ishihara H, Motohashi H, Minegishi N, Tsuchiya S, Hayashi N, Gu L, Andres B, Engel JD, Yamamoto M. 1994. Transcription factor GATA-2 is expressed in erythroid, early myeloid, and CD34+ human leukemia-derived cell lines. Blood 84:1074–1084.

Neumann JR, Morency CA, Russian KO. 1987. A novel rapid assay for chloramphenicol acetyltransferase gene expression. BioTechniques 5:444.

Nordeen SK. 1988. Luciferase reporter gene vectors for analysis of promoters and enhancers. BioTechniques 6:454–457.

Orkin SH. 1990. Globin gene regulation and switching. Cell 63:665–672.

Riddle RD, Yamamoto M, Engel JD. 1989. Expression of δ-aminolevulinate synthase in avian cells: Separate genes encode erythroid specific and non-specific isozymes. Proc Natl Acad Sci USA 86:792–796.

Semenza GL, Nejfelt MK, Chi SM, Antonarakis SE. 1991a. Hypoxia-inducible nuclear factors bind to an enhancer element located 3′ to the human erythropoeitin gene. Proc Natl Acad Sci USA 88:5680–5684.

Semenza G, Koury ST, Nejfelt MK, Gearhart JD, Antonarakis SE. 1991b. Cell-type specific and hypoxia-inducible expression of the human erythropoietin gene in transgenic mice. Proc Natl Acad Sci USA 88:8725–8729.

Semenza GL, Wang GL. 1992. A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. Mol Cell Biol 12:5447–5454.

Towatari M, May GE, Marais R, Perkins GR, Marshall CJ, Cowley S, Enver T. 1995. Regulation of GATA-2 phosphorylation by mitogen-activated protein kinase and interleukin-3. J Biol Chem 270:4101–4107.

Wilson DB, Dorfman DM, Orkin SH. 1990. A nonerythroid GATA-binding protein is required for function of the human preproendothelin-1 promoter in endothelial cells. Mol Cell Biol 10:4854–4862.

Yamamoto M, Ko LJ, Leonard MW, Beug H, Orkin SH, Engel JD. 1990. Activity and tissue-specific expression of the transcription factor NF-E1 multi gene family. Genes Dev 4:1650–1662.

Zanjani ED, Poster J, Burlington H, Mann LI, Wasserman LR. 1977. Liver as the primary site of erythropoietin formation in the fetus. J Lab Clin Med 89:640–644.

3.

イタイイタイ病に伴う貧血は、カドミウムによるEpo産生低下によって発症する。そこでカドミウムのEpo遺伝子発現におよぼす効果をGATAとHIF-1の両面からHep3B細胞系を用いて解析した。その結果、カドミウムはHIF-1α鎖を不活性化し、結合活性を低下させるが、GATAへの関与は認めなかった (Arch Toxicol 77 : 267-273, 2003)。

Arch Toxicol (2003) 77: 267–273
DOI 10.1007/s00204-003-0444-0

MOLECULAR TOXICOLOGY

Naoshi Obara · Shigehiko Imagawa
Yoko Nakano · Norio Suzuki
Masayuki Yamamoto · Toshiro Nagasawa

Suppression of erythropoietin gene expression by cadmium depends on inhibition of HIF-1, not stimulation of GATA-2

Received: 7 October 2002 / Accepted: 19 December 2002 / Published online: 7 March 2003

Abstract Long-term exposure of rats to cadmium (Cd) resulted in a marked suppression of erythropoietin (Epo) mRNA expression in the kidneys and the development of severe anemia. A recent report revealed that Cd inhibited hypoxia-inducible factor 1 (HIF-1) binding activity and Epo mRNA expression and protein production. However, Epo gene expression is also regulated by transcription factor GATA-2, which binds to the GATA binding site of the Epo promoter. To elucidate the mechanism of suppression of Epo by Cd, the effect of Cd on GATA-2 function was studied. Epo promoter/enhancer luciferase constructs, one with the wild-type promoter and another with a promoter with a mutant GATA site, were transfected into Hep3B zcells. No significant difference in Epo promoter activity in these two types of cells was observed in the presence of Cd. The binding activity of GATA-2 was not affected by Cd. This study showed that Cd inhibited HIF-1 binding activity and Epo promoter activity, and then suppressed Epo protein production. Inhibition of Epo gene expression by Cd depends on suppression of HIF-1 binding activity, not on alteration of GATA function.

Keywords Erythropoietin · Cadmium · GATA · Transcriptional regulation · Hypoxia-inducible factor 1α

Introduction

Itai-itai disease is a condition caused by long-term exposure of the inhabitants of Toyama prefecture, Japan, to cadmium intoxication. The characteristic clinical features of this disease include renal tubular dysfunction, osteomalacia, and anemia (Kasuya et al. 1992). A clinical report demonstrated that the anemia observed in patients with Itai-itai disease is caused primarily by the impaired production of erythropoietin (Epo) (Horiguchi et al. 1994). In addition, long-term exposure of rats to cadmium (Cd) resulted in the marked suppression of Epo mRNA expression in the kidneys, and the development of severe anemia (Horiguchi et al. 1996). Recently, Horiguchi et al. (2000) reported that Cd suppressed the binding activity of the hypoxia-inducible factor 1 (HIF-1) transcription factor, Epo mRNA expression and Epo protein production in a dose-dependent manner, with no apparent cell damage.

Epo gene expression is under the control of HIF-1 through an HIF-1 binding site in the Epo enhancer. HIF-1 is composed of a redox-sensitive HIF-1α subunit and a constitutively expressed HIF-1β subunit (Semenza and Wang 1992). Under normoxic conditions, HIF-1α is rapidly degraded, whereas under hypoxic conditions HIF-1α is stabilized and its transcription is stimulated (Huang et al. 1996, 1998). On the other hand, the Epo gene is negatively regulated under normoxic conditions by transcription factor GATA-2, which binds to the GATA site of the Epo promoter in Hep3B cells (Imagawa et al. 1997). Hep3B cells strongly express GATA-2 (Imagawa et al. 1997). Under normoxic conditions, Epo gene expression is not stimulated, because HIF-1α is degraded by prolyl hydroxylation (Ivan et al. 2001; Jaakkola et al. 2001), and Epo gene expression is suppressed by GATA. We have recently shown that N^G-monomethyl-L-arginine (L-NMMA), whose levels increase in chronic renal failure, inhibits production of nitric oxide (NO) and cyclic-GMP, stimulates GATA-2 binding activity and GATA-2 mRNA expression, and inhibits Epo promoter activity (Tarumoto et al. 2000; Imagawa et al. 2002). However, L-NMMA does not affect HIF-1. Hydrogen peroxide (H_2O_2) also inhibits Epo gene expression (Fandrey et al. 1994; Imagawa et al. 1996; Tabata et al. 2001). H_2O_2 treatment was found to

N. Obara · S. Imagawa (✉) · Y. Nakano · T. Nagasawa
Division of Hematology, Institute of Clinical Medicine, University of Tsukuba, 305-8575, Tsukuba, Ibaraki Japan
E-mail: simagawa@md.tsukuba.ac.jp
Tel.: +81-298-533124
Fax: +81-298-533124

N. Suzuki · M. Yamamoto
Center for Tsukuba Advanced Research Alliance and Institute of Basic Medical Sciences, University of Tsukuba, 305-8577, Tsukuba, Ibaraki Japan

inhibit Epo promoter activity and increased binding activity of GATA-2 (Imagawa et al. 1996; Tabata et al. 2001). As GATA-2 binds to the GATA site at the Epo promoter and downregulates Epo gene expression in Hep3B cells, this suppression of Epo gene expression by H_2O_2 is partly due to the enhanced GATA-2 binding activity (Imagawa et al. 1996; Tabata et al. 2001). The goal of the present study was to elucidate how Cd suppresses Epo, and especially how it affects GATA-2 function.

Materials and methods

Cell culture

An erythropoietin-producing hepatoma cell line (Hep3B) was obtained from the American Type Tissue Culture Collection (Rockville, MD, USA). Cells were incubated under both 21% (normoxia) and 1% (hypoxia) oxygen for 24 h as previously described (Imagawa et al. 1997). The hypoxic induction of the firefly luciferase (Luc) gene expression is represented as a hypoxia/normoxia ratio, as previously described (Imagawa et al. 1997).

Plasmid vectors

We used the reporter plasmid pEPLuc, described by Blanchard et al. (1992), as a basic plasmid construct in which both the 126-bp 3' Epo enhancer (120 to 245-bp 3' of the poly(A) addition site) and the 144-bp minimal Epo promoter (from −118 to +26 relative to the transcription initiation site) were placed upstream of Luc gene in pXP2 (Blanchard et al. 1992), resulting in Pwt or V2-Ewt-Pwt-pXP2 (Blanchard et al. 1992) (Fig. 1). This enhancer contained a hypoxia-inducible factor 1 (HIF-1) binding site and steroid receptor response element (SRRE). In Pm7, the GATA sequence in the Epo promoter was mutated to TATA (i.e., AGATAACAG to ATATAAAAG) (Imagawa et al. 1997). The 144-bp minimal Epo promoter were placed upstream the firefly Luc gene in pXP2, resulting in Δ18pXP2 (Fig. 1).

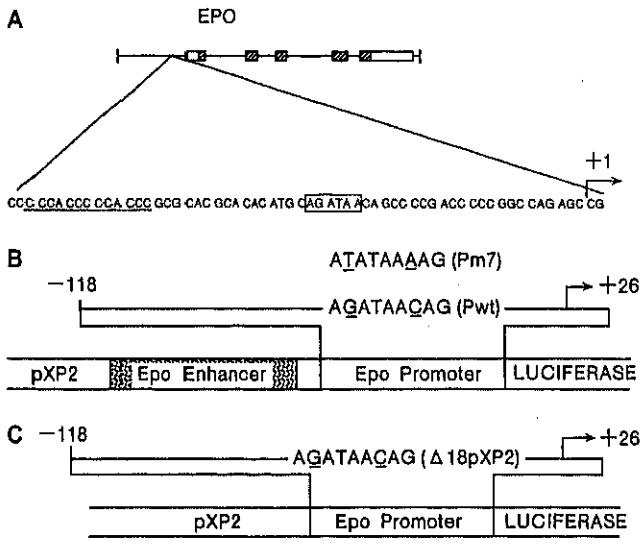


Fig. 1A–C Constructs used in this study. **A** The GATA-binding site in the erythropoietin (Epo) 5' promoter region (*boxed*) and repeated CACCC elements (*underlined*). **B** Diagrams of the reporter construct the wild-type (Pwt) and the mutated GATA (Pm7) used in this study. The pEPLuc reporter construct is shown in the center; shown above is the 144-bp insert from the Epo promoter. The mutation is indicated by *underlining*. The Epo enhancer contains an hypoxia-inducible factor 1 (HIF-1) binding site. **C** Diagram of the reporter construct, Δ18pXP2 used in this study

Transfection

A total of 7×10^5 cells in tissue culture plates (10 cm^2/well) (Falcon) were washed with serum-free media. A mixture containing lipofectin (Life Technologies, Inc., Gaithersberg, MD, USA; 20 μg/well), DNA constructs, and lac Z (1 μg/well) as an internal standard were co-transfected as previously described (Imagawa et al. 1997).

DNA binding assay

Nuclear extracts were prepared as previously described (Yamamoto et al. 1990). Protein concentrations were determined by Bio-Rad assay (Bio-Rad, Hercules, CA, USA) using bovine serum albumin as a standard. The sense-strand oligonucleotide (wild-type CATGCAGATAACAGCCCCGAC) was end-labeled with T4 polynucleotide kinase (Toyobo, Tokyo, Japan) and annealed to a four-fold excess of the unlabeled antisense oligonucleotide. Two nanogram of labeled probe was used in each binding reaction. The binding buffer consisted of 10 mM Tris HCl (pH 7.5), 1 mM EDTA, 4% Ficoll, 1 mM dithiothreitol and 75 mM KCl. An equimolar mixture of poly[d(I-C)] and poly[d(A-T)] (Sigma, St. Louis, MO, USA; 25 ng) was used as a nonspecific competitor. The reaction mixtures (25 μl) were incubated for 15 min at 4°C, and then electrophoresed on 5% nondenaturing polyacrylamide gels in 0.25× TBE buffer (22 mM Tris borate, 22 mM boric acid, 0.5 mM EDTA) at 150 V for 1.5 h at room temperature as previously described (Imagawa et al. 1997). Gels were vacuum-dried and then autoradiography was performed using intensifying screens at −80°C for 24 h.

Other assays

Luc activity in 20 μl of the cell extract was measured with an AutoLumat luminometer (Berthold, Bundoora, Australia) for 10 s. Each measurement of relative light units was corrected by subtraction of the background, and standardized to the β-galactosidase internal transfection control activity. Hypoxic inducibility was defined as the ratio of the corrected relative light units of the hypoxic (1% O_2) dish to those of the normoxic (21% O_2) dish as previously described (Tarumoto et al. 2000). Epo protein from Hep3B cells was measured by enzyme-linked immunosorbent assay.

Statistical analysis

Students' *t*-tests were used to assess the level of significant between treatment groups.

Results

Inhibition of Epo protein by Cd

We used the same dose (0.1–10 μM) of Cd in Hep3B cells as previously described (Horiguchi et al. 2000). Horiguchi and co-workers checked the viability of

Hep3B cells treated with Cd by measuring LDH activity released into medium to ascertain whether global Cd toxicity affected Epo production. Exposure of Hep3B cells to 0.1–10 μM Cd had no effect on cell viability (Horiguchi et al. 2000). We also checked Hep3B cell viability by trypan blue dye exclusion method. Exposure of Hep3B cells to 0.1–10 μM Cd had no effect on cell viability (data not shown). Cd dose-dependently inhibited the production of Epo protein in Hep3B cells (Fig. 2). After incubating Hep3B cells for 24 h under hypoxic conditions in the presence of 0, 0.5, 1.0 and 10.0 μM Cd, Epo concentrations were 315.1±71.9, 157.6±32.2, 111.9±14.9 and 76.3±15.1 mU Epo/mg protein, respectively (Fig. 2).

Inhibition of Epo promoter/enhancer activity by Cd

Hypoxic induction from Pwt was 54.6±17.7 times higher than that from normoxic Pwt (mean ±1 SD, $n=4$). Interestingly, Cd dose-dependently inhibited the hypoxic induction of Luc reporter gene expression from Pwt. Addition of 0.5, 1.0 and 10.0 μM Cd resulted in hypoxia/normoxia ratios of only 26.8±5.0, 16.2±3.8, and 3.6±1.3, respectively, which correspond to 49.1, 29.7, and 6.6% of the ratios obtained without Cd. These results indicate that the hypoxic induction of the Epo gene expression is suppressed by Cd through the Epo gene regulatory regions (Fig. 3).

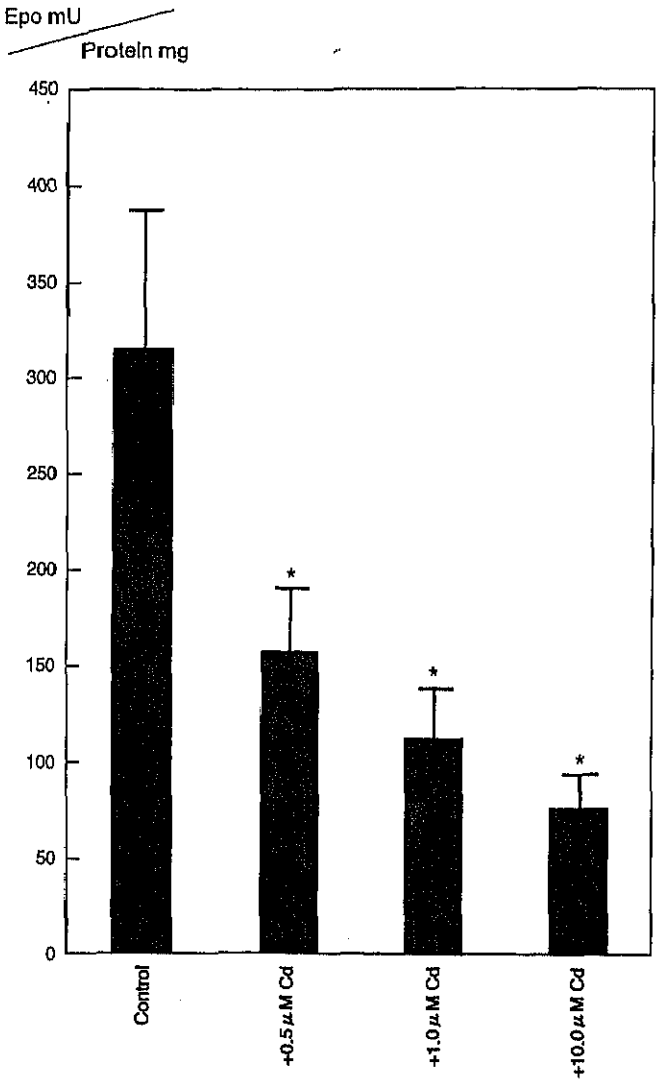


Fig. 2 Effect of cadmium on erythropoietin (Epo) protein from Hep3B cells stimulated by hypoxia. Hep3B cells were incubated with different concentrations of cadmium under hypoxic conditions (1% O_2) for 24 h. Epo protein was measured by enzyme-linked immunosorbent assay. Seven separate experiments were performed with triplicate samples ($n=7$). Error bars represent one standard deviation. *$P<0.005$, significance compared with control

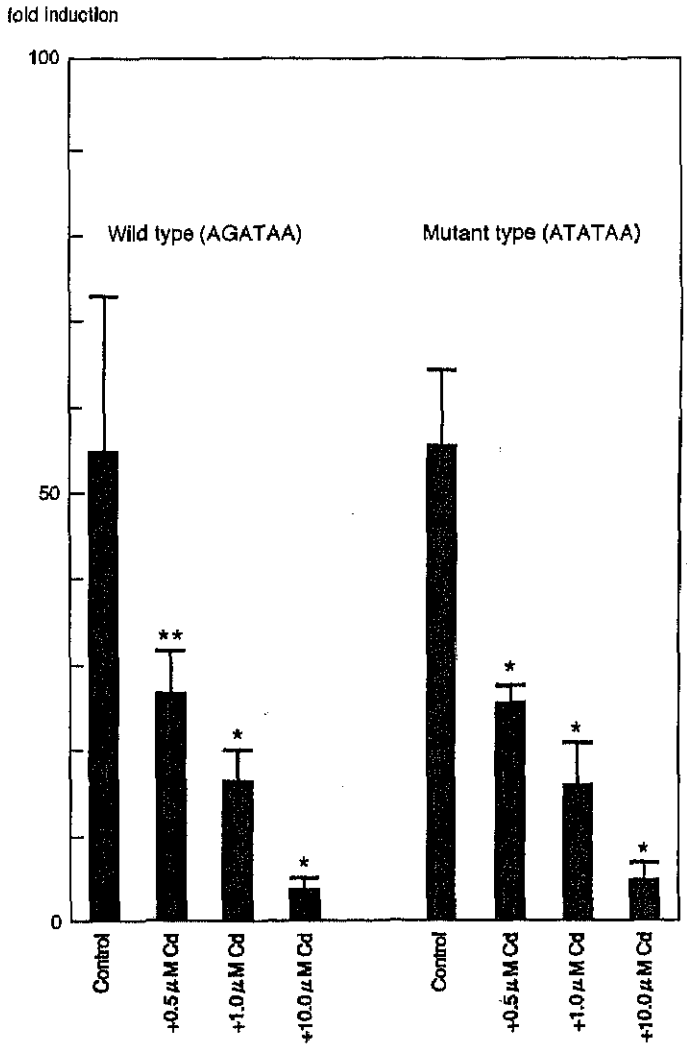


Fig. 3 Effect of cadmium on the induction of the erythropoietin (Epo) promoter/enhancer reporter constructs in Hep3B cells. Hypoxic induction of Luc gene expression is represented as a hypoxia/normoxia ratio. *Fold induction* indicates this hypoxia/normoxia ratio. Four separate experiments (triplicate samples) were performed ($n=4$). Error bars represent one standard deviation. *$P<0.005$, **$P<0.01$, significance compared with control

Hypoxia response element (HRE) in the enhancer only contributes to Cd suppression

The contribution of the GATA element was further tested by using a reporter plasmid, Pm7, which contains GATA site mutations. We previously found that the GATA mutation by itself affects the basal level expression of Luc reporter activity (Imagawa et al. 1997). As was the case with Pwt, Luc expression was also strongly induced (55.2 ± 7.8 fold) following exposure of the treated cells to hypoxia. Furthermore, Cd dose-dependently inhibited the hypoxic induction of Luc reporter gene expression from Pm7. Addition of 0.5, 1.0, and 10.0 μM Cd resulted in hypoxia/normoxia ratios of only 25.3 ± 1.8, 15.7 ± 5.0, and 4.5 ± 1.9, respectively, which correspond to 45.8, 28.4, and 8.2% of the ratios obtained without Cd (Fig. 3). These results suggest that the GATA element on the promoter does not contribute to the Cd suppression, and that HRE in the enhancer only contributes to Cd suppression. To elucidate whether GATA contributes to Cd suppression, Δ18pXP2 plasmid was transfected into Hep3B cells and incubated with and without Cd under a normoxic or hypoxic condition. Hypoxic induction from Δ18pXP2 was 7.8 ± 1.9 fold higher than that from normoxic Δ18pXP2 ($n = 4$). Addition of 0.5, 1.0 and 10.0 μM Cd resulted in hypoxia/normoxia ratios of 8.2 ± 2.6, 9.2 ± 0.4, and 6.8 ± 2.2, respectively (Fig. 4). Again these results strongly suggest that the GATA element on the promoter dose not contribute to the Cd suppression, and that HRE in the enhancer only contributes to Cd suppression.

Inhibition of H1F-1 binding activity by Cd

To determine whether Cd affects the binding activity of HIF-1, nuclear extracts were prepared from cells stimulated by Cd for 24 h under hypoxic conditions, and electrophoretic mobility shift assays (EMSAs) were performed with an oligonucleotide containing HRE. The addition of 1.0 and 10.0 μM Cd partially inhibited the binding activity of HIF under hypoxic conditions (Fig. 5A *right panel*, B), though the addition of 0.2 μM Cd increased the binding activity of HIF-1 by an unknown mechanism (Fig. 5A).

No effect of Cd on GATA binding activity

To examine whether the addition of Cd affects the binding activity of GATA-2, nuclear extracts were prepared from cells stimulated with Cd for 24 h under normoxic or hypoxic conditions, and EMSAs were performed with an oligonucleotide containing the GATA element. The addition of 0.2, 1.0 and 10.0 μM Cd did not affect the binding activity of GATA-2 under normoxic or hypoxic conditions (Fig. 6).

Discussion

Cd has been shown to generate intracellular reactive oxygen intermediates (ROIs) (Fariss 1991). Horiguchi et al. (2000) suggested that generation of ROIs underlies the ability of Cd to inhibit HIF-1 activation and Epo induction. However, the effect of Cd on GATA-2 function remains unknown. We have identified suppression of Epo gene expression by L-NMMA, which increased in the patients with chronic renal failure (Tarumoto et al. 2000; Imagawa et al. 2002), and by H_2O_2 (Tabata et al. 2001). L-NMMA (a NO synthase inhibitor) inhibits production of NO and cyclicGMP, and stimulates GATA-2 binding activity and mRNA expression, and then inhibits Epo promoter activity.

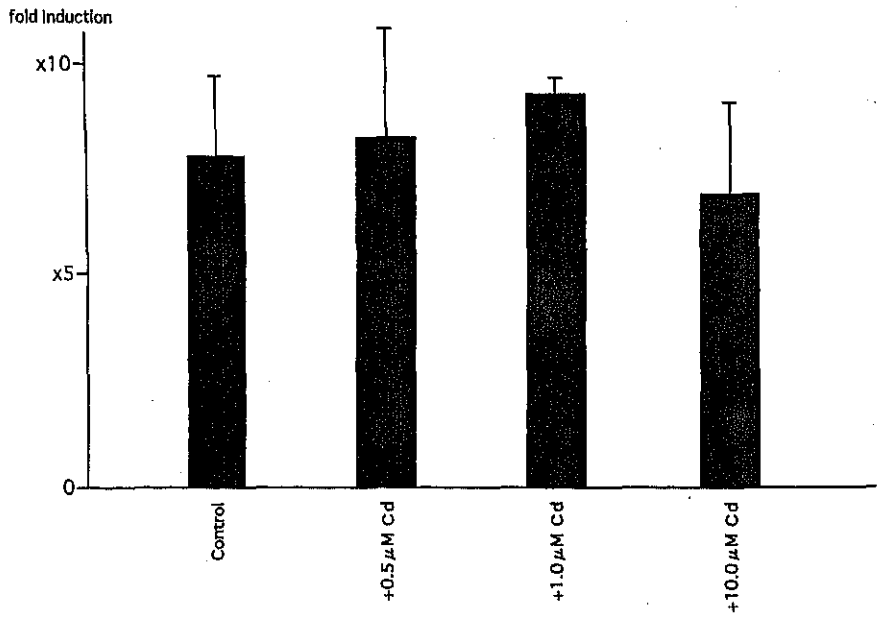


Fig. 4 Effect of cadmium on the induction of the erythropoietin (Epo) promoter constructs in Hep3B cells. Experimental condition were the same as described in Fig. 3

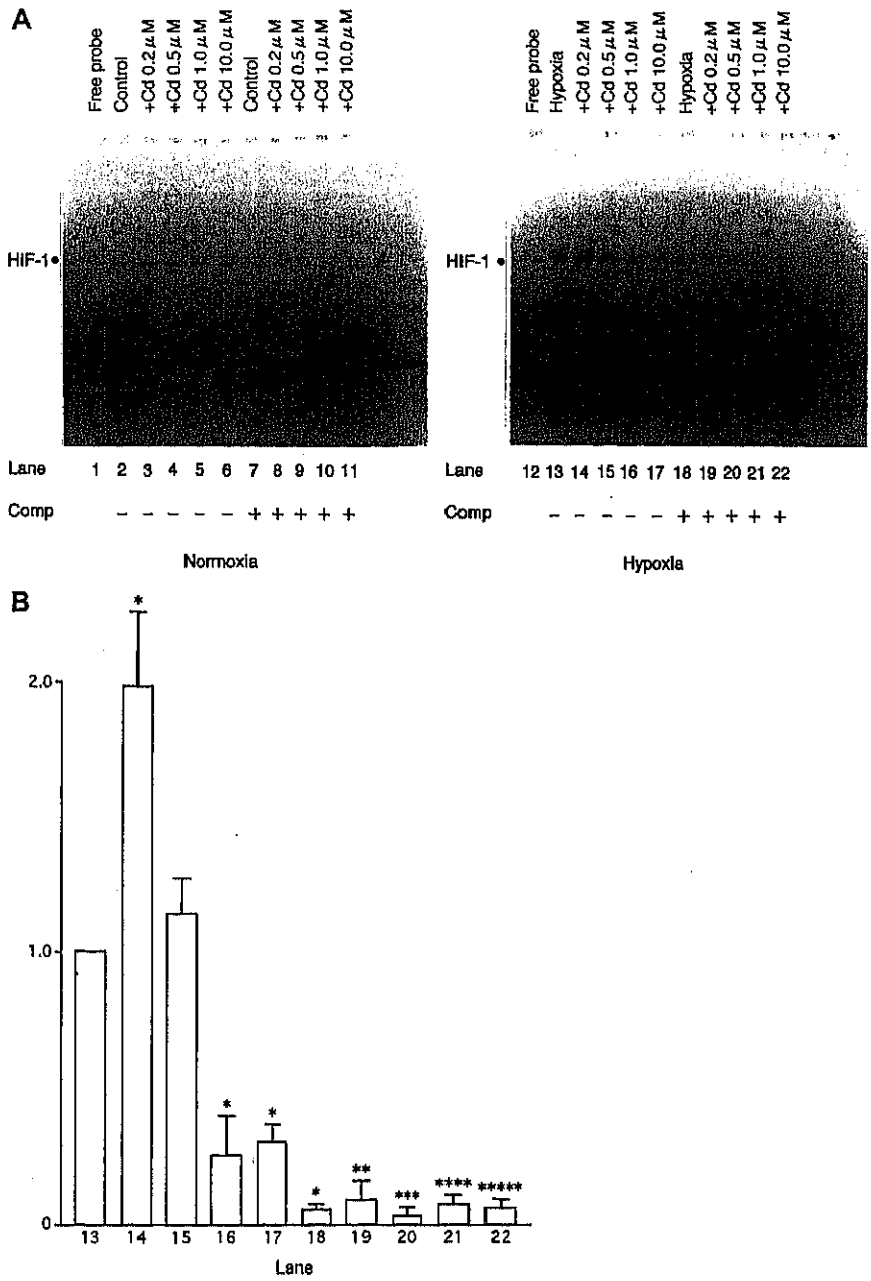


Fig. 5A,B Effect of cadmium on the expression of hypoxia-inducible factor 1 (HIF-1). A Electrophoretic mobility shift assay was performed using 1.5 μg protein from Hep3B cells under normoxia (lanes 2–11) and hypoxia (lanes 13–22), from Hep3B cells incubated with 0.2 μM cadmium (lanes 3, 8, 14 and 19), 0.5 μM cadmium (lanes 4, 9, 15 and 20), 1.0 μM cadmium (lanes 5, 10, 16 and 21) and 10.0 μM cadmium (lanes 6, 11, 17 and 22) for 24 h. The *dot* at the left indicates the position of HIF-1. A total of 25 ng (0.5 μl, 12.5-fold molar excess) of competitor DNA (*Comp*) was added to each reaction mixture (lanes 7–11 and 18–22). The autoradiograph is representative of three different experiments with similar results. B Densitometric analysis of the band indicated by the *circle* in A (lanes 13–22). Intensities are expressed relative to the control on the *Y*-axis. Three experiments using different nuclear extracts were performed ($n = 3$). Error bar represent one standard deviation. $^{*}P < 0.005$, significance compared with hypoxia Comp– (lane 13); $^{**}P < 0.005$, significance compared with Cd 0.2 μM Comp– (lane 14); $^{***}P < 0.005$, significance compared with Cd 0.5 μM Comp– (lane 15); $^{****}P < 0.005$, significance compared with Cd 1.0 μM Comp– (lane 16); $^{*****}P < 0.005$, significance compared with Cd 10.0 μM Comp– (lane 17)

However, L-NMMA does not affect HIF-1 (Fig. 7A) (Tarumoto et al. 2000; Imagawa et al. 2002). On the other hand, H_2O_2 inhibits Epo gene expression by both stimulating GATA-2 binding activity (Imagawa et al. 1996; Tabata et al. 2001) and by degrading HIF-1α (Fig. 7B) (Huang et al. 1996). In this study, we have shown that Cd inhibits Epo gene expression only by inhibiting HIF-1 binding activity, and not by stimulating GATA-2 binding activity (Fig. 7C). So far, we have identified the suppression of Epo gene expression by three different compounds: L-NMMA, H_2O_2 and Cd. The proposed mechanisms by which these compounds suppress Epo gene expression are shown in Fig. 7A,B and C, respectively. All three types of suppression were observed in the Hep3B cell line. To clarify the pathogenesis of the suppression of Epo gene expression, an in vivo system using mouse will be needed.

Other Epo inhibitors have been reported. Interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), and transforming growth factor-β (TGF-β) have been found to inhibit hypoxia-induced Epo production in vitro (Jelkmann et al. 1990; Faquin et al. 1992). These cytokines also inhibit erythroid progenitor cell proliferation, and thus play a major role in the pathogenesis of the anemia of chronic disease (Means and Krantz 1992). Jelkmann et al. (1992) reported that IL-1 and TNF-α reduce by approximately 50% the protein levels of Epo produced in HepG2 cells in response to hypoxia. Faquin et al. (1992) showed not only that IL-1 and TNF-α inhibit Epo production by up to 89%, but that TGF-β also has

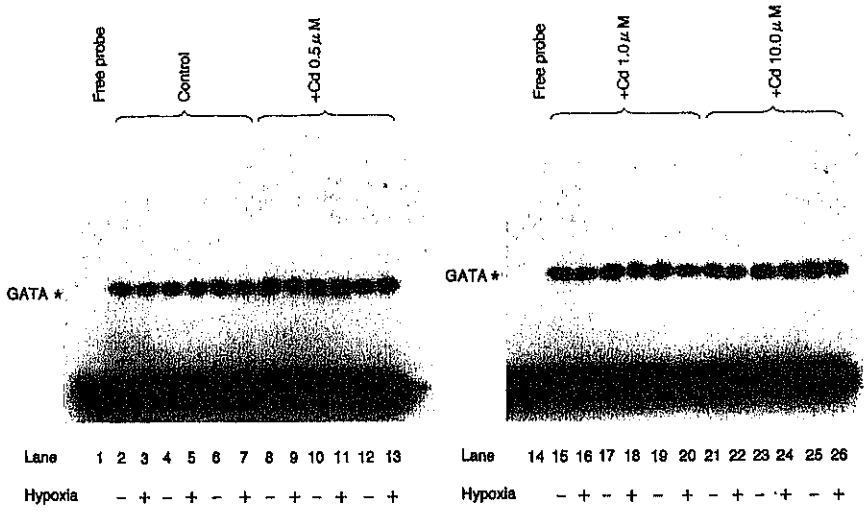


Fig. 6 Effect of cadmium on the expression of GATA. Electrophoretic mobility shift assay was performed using 1.5 μg protein from Hep3B cells under normoxia (lanes 2, 4, 6, 8, 10, 12, 15, 17, 19, 21, 23 and 25) and hypoxia (lanes 3, 5, 7, 9, 11, 13, 16, 18, 20, 22, 24 and 26), from Hep3B cells incubated with 0.5 μM cadmium (lanes 8–13), 1.0 μM cadmium (lanes 15–20), and 10.0 μM cadmium (lanes 21–26) for 24 h. The *asterisk* at the left indicates the position of GATA-2. Three experiments using different nuclear extracts were performed ($n=3$). The autoradiograph is representative of three different experiments with similar results

inhibitory effects that might differ from those of IL-1 and TNF-α. This effect appeared to occur at the level of the Epo mRNA (Faquin et al. 1992). Recently, La Ferla et al. (2002) showed that IL-1β and TNF-α increased GATA-2 and nuclear factor κB (NF-κB) binding activity and inhibited Epo promoter activity. Furthermore, functional inactivation of GATA-2 and NF-κB by oligodecoy techniques prevented the inhibition of Epo pro-

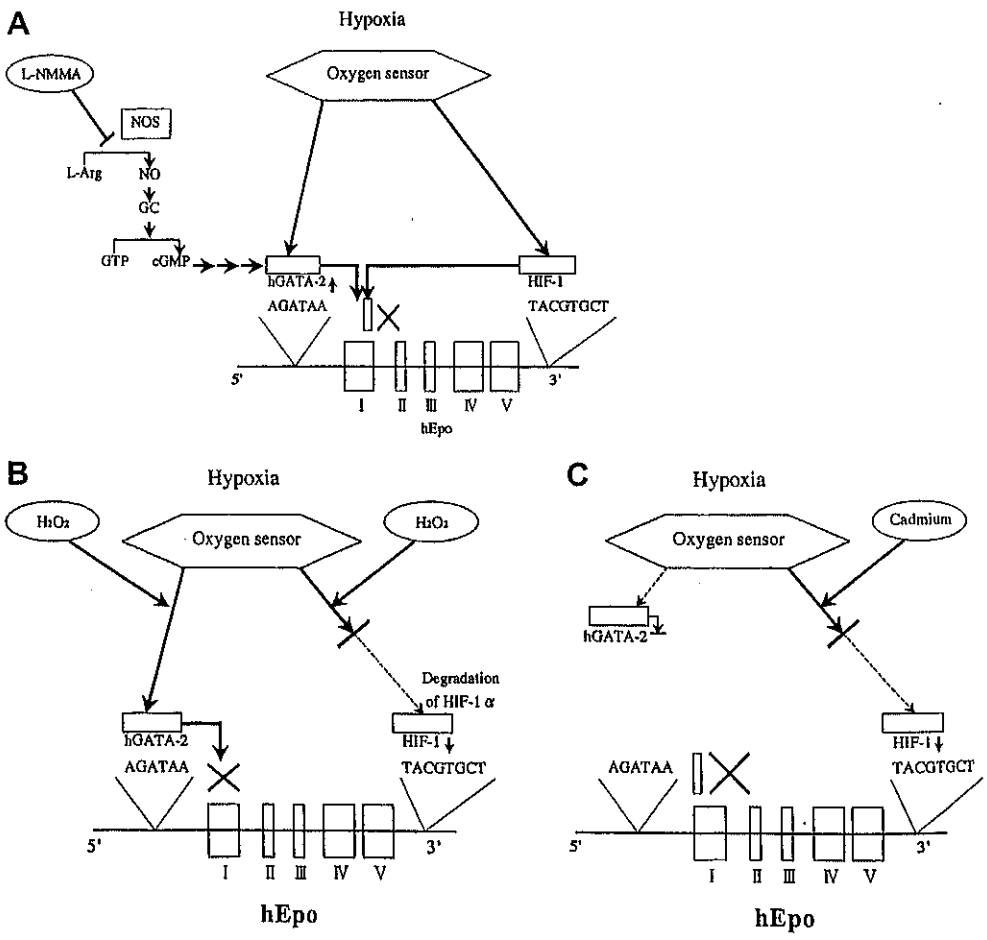


Fig. 7A–C Schema of inhibition of human erythropoietin (hEpo) gene expression by A N^G-monomethyl-L-arginine (L-NMMA), B H_2O_2, and C cadmium (*l-Arg* L-arginine, *NO* nitric oxide, *NOS* nitric oxide synthase, *GC* guanylate cyclase, *HIF-1* hypoxia-inducible factor 1)

duction by IL-Iβ and TNF-α. In HepG2 cells stably transfected with a dominant negative form of inhibitor IκBα, the activation of NF-κB was inhibited, which caused Epo mRNA levels and Epo protein secretion to increase. Both GATA-2 and NF-κB appear to be involved in the suppression of Epo gene expression by IL-Iβ and TNF-α in vitro, and may be responsible for the impaired Epo synthesis that is observed in inflammatory disease in vivo (La Ferla et al. 2002). To elucidate the mechanism by which Cd suppresses Epo expression, the effect of Cd on the function of NF-κB needs to be clarified.

Acknowledgements We thank Hiromi Tanaka and Ayako Hoshino for their expert technical assistance. This work was supported by Grants-in-aid for scientific research from the Ministry of Education, Science and Culture of Japan, and the Renal Anemia Foundation, Japan.

References

Blanchard KL, Acquaviva AM, Galson DL, Bunn HF (1992) Hypoxic induction of the human erythropoietin gene: cooperation between the promoter and enhancer, each of which contains steroid receptor response elements. Mol Cell Biol 12:5373–5385

Fandrey J, Frede S, Jelkmann W (1994) Role of hydrogen peroxide in hypoxic induced erythropoietin production. Biochem J 303:507–510

Faquin WC, Schneider IJ, Goldberg MA (1992) Effect of inflammatory cytokines on hypoxia-induced erythropoietin production. Blood 79:1987–1994

Fariss MW (1991) Cadmium toxicity: unique cytoprotective properties of alpha tocopheryl succinate in hepatocytes. Toxicology 69:63–77

Horiguchi H, Teranishi H, Niiya K, Aoshima K, Katoh T, Sakuragawa, N (1994) Hypoproduction of erythropoietin contributes to anemia in chronic cadmium intoxication: clinical study on Itai-Itai disease in Japan. Arch Toxicol 68:632–636

Horiguchi H, Sato M, Konno N, Fukushima M (1996) Long term cadmium exposure induces anemia in rats through hypoproduction of erythropoietin in the kidneys. Arch Toxicol 71:11–19

Horiguchi H, Kayama F, Oguma E, Willmore WG, Hradecky P, Bunn HF (2000) Cadmium and platinum suppression of erythropoietin production in cell culture: clinical implications. Blood 96:3743–3747

Huang LE, Arany Z, Livingston DM, Bunn HF (1996) Activation of hypoxia-inducible transcription factor depends primarily upon redox-sensitive stabilization of it's α–subunit. J Biol Chem 271:32253–32259

Huang LE, Gu J, Schau M, Bunn HF (1998) Regulation of hypoxia-inducible factor 1α is mediated by an O_2-dependgdent degradation domain via the ubiquitin-proteasome pathway. Proc Natl Acad Sci USA 95:7987–7992

Imagawa S, Yamamoto M, Ueda M, Miura Y (1996) Erythropoietin gene expression by hydrogen peroxide. Int J Hematol 64:184–195

Imagawa S, Yamamoto M, Miura Y (1997) Negative regulation of the erythropoietin gene expression by the GATA transcription factors. Blood 89:1430–1439

Imagawa S, Tarumoto T, Suzuki N, Mukai HY, Hasegawa Y, Higuchi M, Neichi T, Ozawa K, Yamamoto M, Nagasawa T (2002) L-Arginine rescues decreased erythropoietin gene expression by stimulating GATA-2 with L-NMMA. Kidney Int 61:396–404

Ivan M, Kondo K, Yang H, Kim W, Valiando J, Ohh M, Salic A, Asara JM, Lane WS, Kaelin WG Jr (2001) HIF-1α targeted for VHL-mediated destruction by proline hydroxylation: implications for O_2 sensing. Science 292:464–468

Jaakkola P, Mole DR, Tian YM, Wilson MI, Gielbert J, Gaskell SJ, Kriegsheim A, Hebestreit HF, Mukherji M, Schofield CJ, Maxwell PH, Pugh CW, Ratcliffe PJ (2001) Targeting of HIF-1α to the von Hippel-Lindau ubiquitylation complex by O_2-regulated prolyl hydroxylation. Science 292:468–472

Jelkmann W, Fandrey J, Wiedemann G (1990) Immunoreactive erythropoietin in the anemia of non-renal chronic disease. Biomed Biochim Acta 49:S265–S270

Jelkmann W, Pagel H, Wolff M, Fandrey J (1992) Monokines inhibiting erythropoietin production in human hepatoma cultures and in isolated perfused rat kidneys. Life Sci 50:301–308

Kasuya M, Teranishi H, Aoshima K, Katoh T, Horiguchi H, Morikawa Y, Nishijo M, Iwata K (1992) Water pollution by cadmium and the onset of Itai-itai disease. Water Sci Technol 25:145–156

La Ferla K, Reimann C, Jelkmann W, Hellwig-Burgel T (2002) Inhibition of erythropoietin gene expression signaling involves transcription factors GATA-2 and NF-κB. FASEB J 16:1811–1813

Means RT, Krantz SB (1992) Progress in understanding the pathogenesis of the anemia of chronic disease. Blood 80:1639–1647

Semenza GL, Wang GL (1992) A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. Mol Cell Biol 12:5447–5454

Tabata M, Tarumoto T, Ohmine K, Furukawa Y, Hatake K, Ozawa K, Hasegawa Y, Mukai H, Yamamoto M, Imagawa S (2001) Stimulation of GATA-2 as a mechanism of hydrogen peroxide suppression in hypoxia-induced erythropoietin gene expression. J Cell Physiol 186:260–267

Tarumoto T, Imagawa S, Ohmine K, Nagai T, Higuchi M, Imai N, Suzuki N, Yamamoto M, Ozawa K (2000) N^G-monomethyl-L-arginine inhibits erythropoietin gene expression by stimulating GATA-2. Blood 96:1716–1722

Yamamoto M, Ko LJ, Leonard MW, Beug H, Orkin SH, Engel JD (1990) Activity and tissue-specific expression of the transcription factor NF-E1 multi gene family. Genes Dev 4:1650–1662

4.

マウスEpo遺伝子のプロモーターは解明されていなかった。しかしヒトEpo遺伝子同様CAP部位より-30bp上流の TGATAA 配列に GATA が結合してマウス Epo 遺伝子発現を抑制していることを認めた (Int J Hematol 74 : 376-381, 2002)。

International Journal of
HEMATOLOGY

GATA Suppresses Erythropoietin Gene Expression through GATA Site in Mouse Erythropoietin Gene Promoter

Shigehiko Imagawa,[a] Norio Suzuki,[b] Ken Ohmine,[c] Naoshi Obara,[a] Harumi Y. Mukai,[a] Keiya Ozawa,[c] Masayuki Yamamoto,[b] Toshiro Nagasawa[a]

[a]*Division of Hematology, Institute of Clinical Medicine and*
[b]*Center for Tsukuba Advanced Research Alliance and Institute of Basic Medical Sciences, University of Tsukuba, Tsukuba, Ibaraki;* [c]*Department of Hematology, Jichi Medical School, Tochigi, Japan*

Received November 9, 2001; received in revised form January 15, 2002; accepted January 17, 2002

Abstract

The promoter and enhancer elements of the mouse erythropoietin (m*Epo*) gene, which have high homology with those of the human erythropoietin (h*Epo*) gene, were fused with *luciferase*. The construct was transfected into erythropoietin-producing hepatoma cell line (Hep3B) cells by lipofectin with lacZ as an internal standard. The wild type (TGATA) showed a 39.5-fold increase in induction by hypoxia. Mouse GATA-2 inhibited the hypoxic induction of the wild-type (m3), promoter-*luciferase* construct but not the hypoxic induction of the mutant (m4, 5) promoter-*luciferase* constructs. N^G-monomethyl L-arginine (L-NMMA) inhibited the hypoxic induction of the m3 promoter-luciferase construct, but this inhibition was recovered by L-arginine. H_2O_2 also inhibited the hypoxic induction of the m3 promoter-*luciferase* construct, but this inhibition was recovered by catalase. Gel shift assays performed on nuclear extracts of 293 cells overexpressing mGATA-1, -2, and -3 revealed that mGATA-1, -2, and -3 bind to the TGATA element of the mEpo promoter. These results indicate that mGATA binds to the TGATA site of the mEpo promoter and negatively regulates m*Epo* gene expression. Negative regulation of m*Epo* gene by GATA transcriptional factors is discussed. *Int J Hematol.* 2002;75:376-381.

Key words: Erythropoietin; GATA; Transcriptional regulation; L-NMMA; H_2O_2

1. Introduction

The human erythropoietin (h*Epo*) gene has been cloned [1] and is under the control of hypoxia-inducible factor-1 (HIF-1) through an HIF-1 binding site in its enhancer [2]. h*Epo* gene expression is negatively regulated by human GATA-2 (hGATA-2), which binds to the GATA site of the h*Epo* gene promoter in Hep3B cells [3]. Although most GATA transcription factors are known to be positive regulators [4], mGATA-2 and mGATA-3 were found to act as negative regulators of the expression of mouse peroxisome proliferator-activated receptor γ promoter [5], and hGATA-2 was shown to act as a negative regulator of h*Epo* gene expression [3]. Furthermore, we previously found that N^G-monomethyl L-arginine (L-NMMA) or H_2O_2 stimulates hGATA-2 binding activity and mRNA expression and then inhibits hEpo promoter activity [6,7]. Although the nucleotide sequence of the m*Epo* gene has high homology with that of the h*Epo* gene [8], the precise function of the m*Epo* gene promoter has not been identified. We made constructs containing the promoter and enhancer elements of the m*Epo*

Correspondence and reprint requests: Shigehiko Imagawa, MD, PhD, Division of Hematology, Institute of Clinical Medicine, University of Tsukuba, Tsukuba, Ibaraki 305-8575, Japan; phone and fax: 81-298-53-3124 (e-mail: simagawa@md.tsukuba.ac.jp).

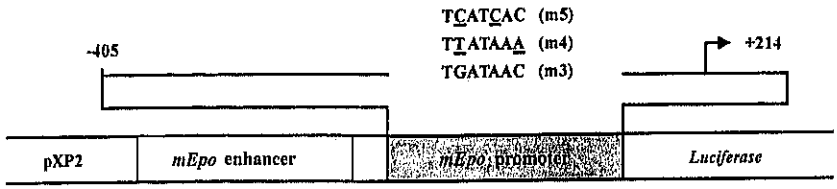


Figure 1. Diagrams of the reporter construct and the wild-type (m3) and the mutated GATA (m4 and m5) used in this study. The pEp*Luc* construct is shown in the center. Shown at top is the 619-bp insert from the Epo promoter. The mutation is indicated by underlining. m*Epo* indicates mouse erythropoietin.

gene, which has high homology with the h*Epo* gene, and *luciferase (Luc)* as a reporter gene, and then we performed transient transfection to Hep3B cells. Furthermore, nuclear extracts were prepared from 293 cells that overexpress mouse GATA-1, -2, and -3, and electrophoretic mobility shift assays (EMSAs) were performed with oligonucleotides containing the wild and mutant GATA elements.

Because the GATA site in the promoter and the HIF-1 binding site in the enhancer are liver-inducible elements [2], further analysis of kidney-inducible elements, using transgenic mice, should be performed to elucidate the complicated mechanism of *Epo* gene regulation. Therefore, the precise promoter function of the mouse *Epo* gene should be clarified by transfection analysis using cell lines.

2. Materials and Methods

2.1. Cell Culture

An erythropoietin-producing hepatoma cell line (Hep3B) was obtained from the American Type Tissue Culture Collection (Rockville, MD, USA). Cells were incubated under both 21% (normoxia) and 1% (hypoxia) oxygen for 24 hours as previously described [9]. The hypoxic induction of *Luc* gene expression is represented as a hypoxia/normoxia ratio as previously described [3].

2.2. Plasmid Vectors

A 129/SvJ mouse λ FIXII phage genomic DNA library (Stratagene, La Jolla, CA, USA) was screened with a probe corresponding to the promoter region of the human *Epo* gene. One phage clone (m*Epo*/FIXII) containing the mouse *Epo* coding region and 12 kb of the upstream flanking region was isolated. The *Xba*I-*Sac*I fragment, including the promoter region of the mouse *Epo* gene (–405 to +214 bp relative to the transcription initiation site), was excised from m*Epo*/FIXII and subcloned into the *Spe*I and *Sac*I sites of pBluescript II SK+ (Stratagene) plasmid vector (pmPWt). Three promoters were prepared, the wild-type promoter with a GATA element (m3, TGATAAC) and 2 mutant promoters (m4, TTATAAA, and m5, TCATCAC). m3 was mutated to m4 using the primer (5′-GCACACATGCTT ATAAAATC-3′) and to m5 using the primer (5′-GCACAC ATGCTCATCACATC-3′) by polymerase chain reaction (Figure 1) [10]. A 175-bp *Bgl*II-*Pvu*II fragment containing the 3′ enhancer region of the *Epo* gene [11] was excised from m*Epo*/FIXII and cloned into the *Bam*HI and *Eco*RV sites of pBluescript II SK+ (pmE). The reporter plasmid pEP*Luc* was used as a basic plasmid construct [12]. The human promoter and enhancer sequences in pE*PLuc* were replaced with those of the mouse *Epo* gene from pmPWt or its mutated counterparts using the *Hin*dIII and *Sac*I restriction sites (phEmP-*Luc*).

2.3. Transfection

A total of 7×10^5 cells in tissue culture plates (10 cm^2/well) (Falcon) were washed with serum-free media. A mixture containing lipofectin (20 μg/well) (Life Technologies, Rockville, MD, USA), DNA constructs, mGATA-2 (2 μg/well), and lacZ (1 μg/well) as an internal standard were cotransfected as previously described [6]. Ten micrograms of mouse GATA-1, -2, and -3, respectively, in the expression plasmids was transfected to 293 cells by lipofection (FuGene, Roche, Indianapolis, IN, USA) [4].

2.4. DNA Binding Assay

Nuclear extracts were prepared as previously described [4]. Protein concentrations were determined by BioRad protein assay (BioRad, Hercules, CA, USA) using bovine serum albumin as a standard. Sense-strand oligonucleotides (m3, GCA CAC ATG CTG ATA ACA TCC CCG ACC CCC; m4, GCA CAC ATG CTT ATA AAA TCC CCG ACC CCC; m5, GCA CAC ATG CTC ATC ACA TCC CCG ACC CCC) were end-labeled with T4 polynucleotide kinase (Toyobo, Tokyo, Japan) and annealed to a 4-fold excess of the unlabeled antisense oligonucleotides. Two nanograms of labeled probe (γ-^{32}P-ATP) was used in each binding reaction. The binding buffer consisted of 10 mM Tris HCl (pH 7.5), 1 mM EDTA, 4% Ficoll, 1 mM dithiothreitol, and 75 mM KCl. An equimolar mixture of poly[d(I-C)] and poly[d(A-T)] (Sigma, St. Louis, MO, USA; 25 ng) was used as a nonspecific competitor. The reaction mixtures (25 μL) were incubated for 15 minutes at 4°C and then electrophoresed on 5% nondenaturing polyacrylamide gels in 0.25 × TBE buffer (22 mM Tris Borate, 22 mM boric acid, 0.5 mM EDTA) at room temperature at 150 V for 1.5 hours as previously described [4]. Gels were vacuum-dried, and then autoradiography was performed using intensifying screens at –70°C for 2 hours. Either 0.5 μL or 1.0 μL of

mouse GATA-1, -2, and -3 monoclonal antibodies (Santa Cruz, Santa Cruz, CA, USA) were incubated with the mGATA-1, -2, and -3 transfected nuclear extracts for 20 minutes at 4°C, respectively.

2.5. Other Assays

Luc activity in 20 μL of the cell extract was determined by Autolumat luminometer (Berthold, Bundoora, Australia) for 10 seconds. Each measurement of relative light units was corrected by subtraction of the background and standardized to the β-galactosidase internal transfection control activity. Hypoxic inducibility was defined as the ratio of corrected relative light units of the hypoxic (1% O_2) dish to those of the normoxic (21% O_2) dish as previously described [6]. L-NMMA, L-arginine, H_2O_2 and catalase (all from Sigma) were used at 10^{-3} M, 10^{-3} M, 500 μM, and 100 μg/mL, respectively.

3. Results

3.1. Epo Promoter Activity Is Inhibited by mGATA-2

When the m3 promoter-*luciferase* construct was transfected into Hep3B cells, hypoxic induction of the m3 promoter-*luciferase* construct was 39.5 ± 9.7–fold higher than that of the normoxic m3 promoter-*luciferase* construct (mean ± 1 SD, n = 4) (Figure 2). Addition of mGATA-2 reduced the hypoxia/normoxia ratio to 11.7 ± 2.3, a 70.4% reduction (Figure 2). These results indicate that the hypoxic induction of *Epo* gene expression is suppressed by mGATA-2 through the *Epo* gene regulatory regions. When the mutant promoter constructs m4 and m5 were transfected into Hep3B cells, the hypoxia/normoxia ratios were 44.2 ± 8.4 and 39.2 ± 7.5, respectively. In the presence of mGATA-2, these ratios were unchanged. These results strongly indicate that m*Epo* gene expression, like h*Epo* gene expression, is negatively regulated by the binding of mGATA-2 through the GATA sequence. Furthermore, the addition of mGATA-2 to the TATA mutant (m4) promoter-*luciferase* construct was more effective in stimulating mEpo promoter activity than was the addition of mGATA-2 to the CATC mutant (m5) promoter-*luciferase* construct.

3.2. mEpo Promoter Activity Is Inhibited by L-NMMA and Recovered by L-Arginine

When Hep3B cells transfected with the m3 promoter-*luciferase* construct were incubated in the absence and presence of L-NMMA, the hypoxia/normoxia ratios were 45.6 ± 0.9 (mean ± 1 SD, n = 4) and 24.8 ± 3.2, respectively, representing a 45.6% reduction in the presence of L-NMMA (Figure 3). However, in the presence of both L-arginine and L-NMMA, the hypoxia/normoxia ratio recovered to 49.9 ± 3.8 (Figure 3). When the m4 promoter-*luciferase* construct was transfected into Hep3B cells, hypoxic induction of the m4 promoter-*luciferase* construct was 49.7 ± 8.5–fold. In the presence of L-NMMA or of L-arginine and L-NMMA, hypoxic inductions were unchanged, at 62.8 ± 5.3 and 58.7 ± 4.3, respectively (Figure 3). The results in this study were similar to the previous report with human *Epo* [6].

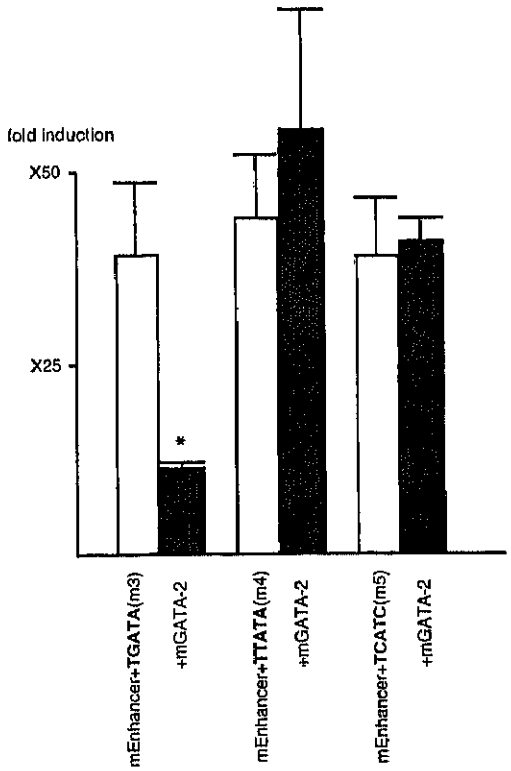


Figure 2. Mouse erythropoietin (mEpo) promoter activity and effect of mouse GATA-2. Wild-type and mutated mEpo constructs were transfected into erythropoietin-producing hepatoma cell line (Hep3B) cells by lipofectin. After incubation under hypoxia or normoxia with and without mGATA-2, luciferase and β-galactosidase were assayed. Four separate experiments (triplicate samples) were performed (n = 4). Error bars represent 1 standard deviation. *Significantly different from control, $P < .001$.

3.3. mEpo Promoter Activity Is Inhibited by H_2O_2 and Recovered by Catalase

When Hep3B cells transfected with the m3 promoter-*luciferase* construct were incubated in the presence of H_2O_2, the hypoxia/normoxia ratio decreased to 19.0 ± 4.7, a 51.2% reduction (Figure 4). However, in the presence of H_2O_2 and catalase, the ratio recovered to 37.7 ± 6.6 (Figure 4). When the m4 promoter-*luciferase* construct was transfected into Hep3B cells, the hypoxic induction of m4 promoter-*luciferase* construct was 45.8 ± 9.0–fold. However, the effect of H_2O_2 was not as significant in the m4-transfected cells as it was in the m3-transfected cells. The addition of H_2O_2 decreased the hypoxic induction to only 34.6 ± 1.7–fold. Some basal level of H_2O_2 suppression in the m4-transfected cells existed. Previous reports indicated that the suppression of *Epo* gene expression by H_2O_2 is caused by the inactivation of HIF-1α [13] and stimulation of GATA [7]. Because the GATA site

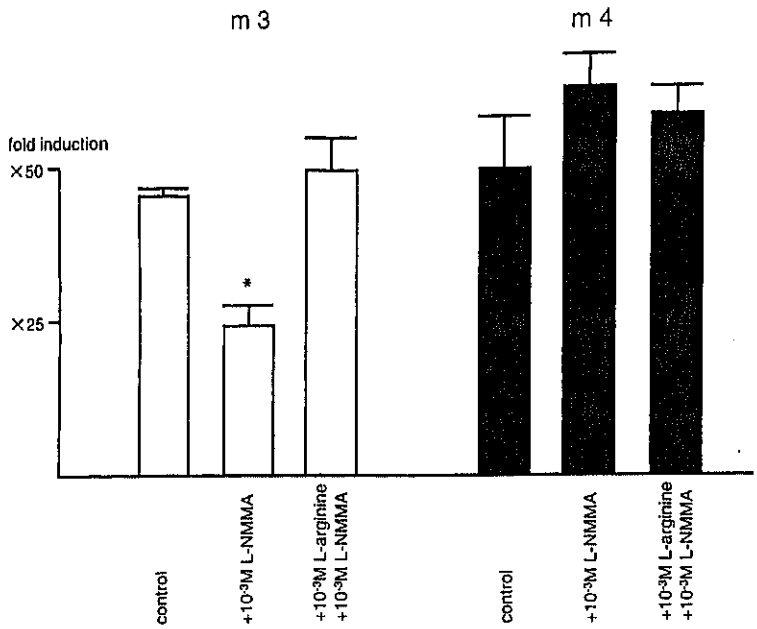


Figure 3. Effect of N^G-monomethyl L-arginine (L-NMMA) on mouse erythropoietin (mEpo) promoter activity. Experimental conditions were the same as those described in Figure 2. *Significantly different from control, $P < .001$.

was mutated to TATA in the m4 construct, the suppression by H_2O_2 in m4-transfected cells is caused by the inactivation of HIF-1α in the enhancer. However, in the presence of H_2O_2 and catalase, the ratio recovered to 46.0 ± 5.6-fold (Figure 4). These results indicate that H_2O_2 inhibits mEpo promoter activity through the GATA sequence. The results in this study were similar to the previous report with human *Epo* [7].

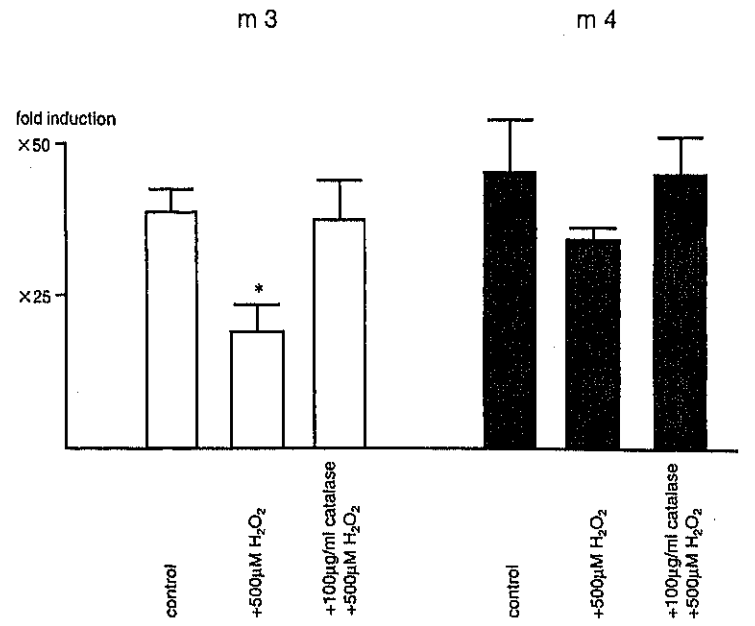


Figure 4. Effect of H_2O_2 on mouse erythropoietin (mEpo) promoter activity. Experimental conditions were the same as those described in Figure 2. *Significantly different from control, $P < .001$.

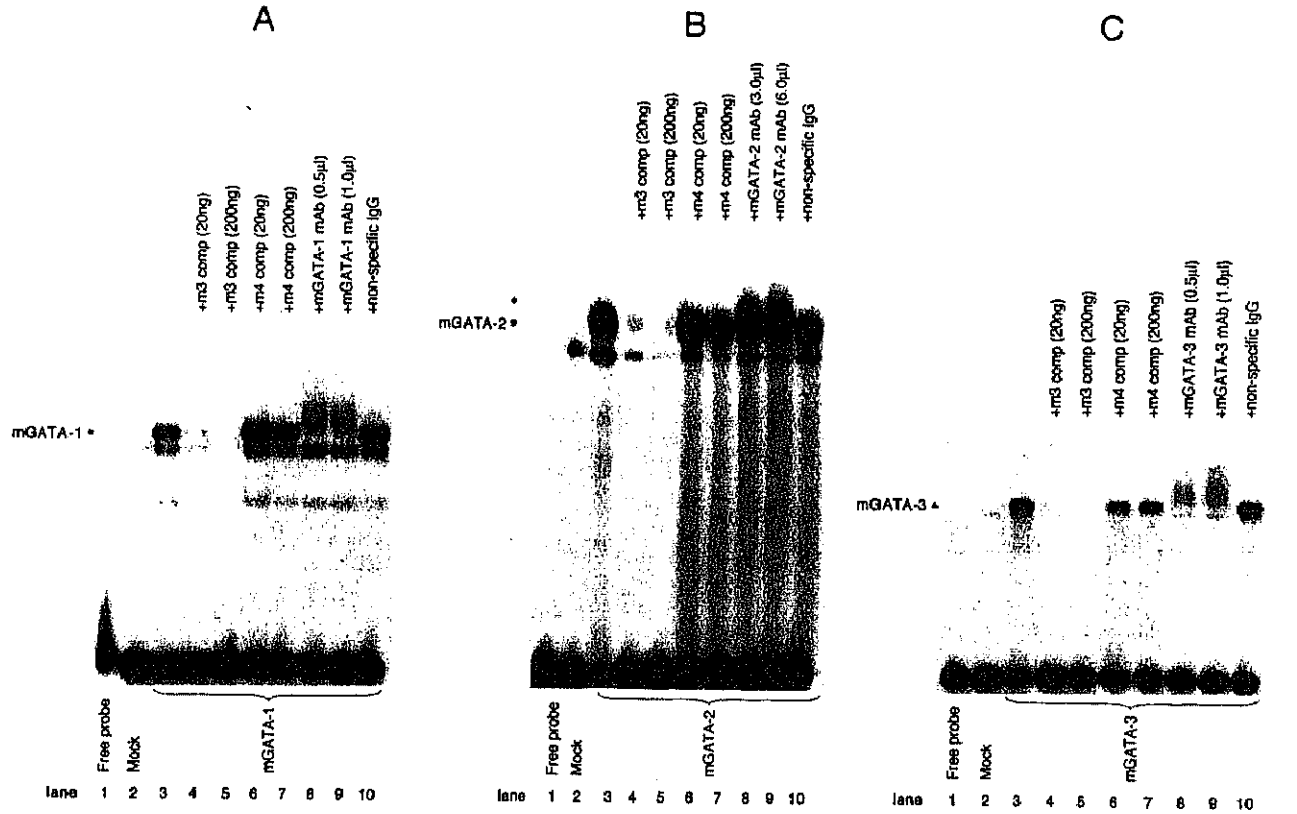


Figure 5. Electrophoretic mobility shift assays (EMSAs) with mouse GATA-1, -2, and -3 transfected nuclear extracts. Nuclear extracts were prepared from 293 cells that overexpress mouse GATA-1 (A), -2 (B), and -3 (C). EMSA was performed using 1.0 μg of protein from mock (lanes 2) and mGATA-1 (A), -2 (B), and -3 (C) overexpressing 293 cells (lanes 3-10) with an oligonucleotide containing the wild-type GATA element (m3). Twenty nanograms (10-fold molar excess) or 200 ng (100-fold molar excess) of competitor DNA containing the wild-type GATA element (m3) or mutant-type GATA element (m4) and 1.0 μL of nonspecific immunoglobulin (Ig)G was added to each reaction mixture (lanes 4-5, 6-7, 10). Either 0.5 μL or 1.0 μL of mouse GATA-1 (A) and -3 (C) monoclonal antibodies and 3.0 μL or 6.0 μL of mouse GATA-2 (B) monoclonal antibody (Santa Cruz, Santa Cruz, CA, USA) were incubated with the mouse GATA-1 (A), -2 (B), and -3 (C) transfected nuclear extracts for 20 minutes at 4°C (lanes 8-9). The circle, square, and triangle at left indicate the positions of the GATA-1 (A), -2 (B), and -3 (C) transcription factor, respectively. The asterisk at left indicates the position of the supershift band of GATA-2 (B).

3.4. Mouse GATA-1, -2, and -3 Bind to the TGATA Sequence

To determine whether mouse GATA-1, -2, and -3 bind to the TGATA sequence, nuclear extracts were prepared from 293 cells that overexpress mouse GATA-1, -2, and -3, and an EMSA was performed with an oligonucleotide containing the wild-type GATA element (m3) (Figure 5). Mouse GATA-1, -2, and -3 bound to this GATA element (Figure 5, lanes 3 and 10: positions of bands are indicated by circle, square, and triangle at left). These bands disappeared with the addition of an oligonucleotide containing the wild-type GATA element (m3) (Figure 5, lanes 4-5). However, these bands did not disappear with the addition of an oligonucleotide containing the mutant-type GATA element (m4) (Figure 5, lanes 6-7). To examine the specificity of these retarded bands, the mGATA-1, -2, and -3 transfected nuclear extracts were incubated with the mGATA-1, -2, and -3 monoclonal antibodies, respectively, for 20 minutes at 4°C. These retarded bands disappeared with the addition of mGATA-1 and -3 monoclonal antibodies, respectively (Figures 5A and 5C, lanes 8-9). The retarded band was supershifted with the addition of the mouse GATA-2 monoclonal antibody, as indicated by the asterisk (Figure 5B, lanes 8-9). These results strongly indicate that mouse GATA-1, -2, and -3 each specifically bind to the TGATA sequence.

4. Discussion

The preceding results show that mouse GATA-1, -2, and -3 each bind to the TGATA sequence of the mEpo promoter and thereby negatively regulate m*Epo* gene expression. The negative regulation of m*Epo* gene expression by mGATA-2 is similar to that observed in h*Epo* gene regulation. Hep3B cells are well-known human hepatoma cells that produce Epo constitutively [14]. Epo expression in these cells is also stimulated by hypoxia [14]. Furthermore, we found that Hep3B cells express GATA-2 [3]. Therefore,

in this study, the effect of mGATA-2 was evaluated by transient transfection.

Aird et al reported that TF-IID also binds to the TGATA element of the mEpo promoter and positively regulates m*Epo* gene expression [15]. These researchers demonstrated that mGATA-1 competes with basal factors to bind to the m*Epo* gene, and the initiation of in vitro transcription from a GATA box-containing promoter might be inhibited by GATA protein [15]. They showed that mGATA-2 also inhibits the initiation of transcription from rat platelet factor 4 (PF4) (AGATA sequence) in vitro and suggested that a more widely distributed GATA protein such as GATA-2 might be responsible for transcriptional repression of the mEpo gene [14]. We previously reported [3] that basic hEpo expression by the TATA mutant was higher than that of the wild type and that hGATA-2 up-regulated expression of the h*Epo* gene in the TATA mutant. Mouse GATA-2, like human GATA-2, up-regulates mEpo expression in the TATA mutant (m4) but not in the CATC mutant (m5). We speculate that mouse TF-IID also binds to the TGATA sequence and positively regulates m*Epo* gene expression and that mGATA-2 stimulates the cofactor of TF-IID.

GATA transcription factors are known to act as positive regulators of gene expression for most genes [4]. To act as a positive or negative regulator, GATA may assemble different combinations of cofactors depending on the gene and cell type [16]. Some proteins act as negative regulators through steric interference of preinitiation complex formation and may constitute a general mechanism of negative gene regulation [17-19]. Such proteins include those containing the engrailed homeodomain, Drosophila P-element transposase, and BPV-1 E2-transactivating proteins. In each case, the effect is mediated by specific transcription factors that have a dual function depending on the location of their cognate site relative to the TBP-binding site. In upstream promoter regions, these factors function as transcriptional activators, whereas in the vicinity of the TATA box, they inhibit the initiation of transcription by interfering with the assembly of basal factor complexes. This mechanism of transcriptional repression and negative regulation may have biologic importance.

As for human *Epo* gene regulation, L-NMMA and H_2O_2 inhibited the hypoxic induction of the wild-type promoter-*luciferase* construct, but these inhibitions were recovered by L-arginine and catalase, respectively. These results suggest that the mouse *Epo* gene is positively regulated by HIF-1 [2] in the enhancer and negatively regulated by GATA in the promoter. The destruction of this balance may be related to the failure of Epo production. Because this study indicates that the GATA site of the mouse and human *Epo* gene promoter negatively regulates the *Epo* gene, it is possible that GATA transcription factors might be the key regulators of the Epo production site that moves from the fetal liver to the adult kidney.

Acknowledgments

This work was supported by grants-in-aid for scientific research from the Ministry of Education, Science and Culture of Japan, Renal Anemia Foundation, and the Chugai Foundation, Tokyo, Japan. We thank Hiromi Tanaka for her expert technical assistance.

References

1. Jacobs K, Shoemaker C, Rudersdorf R, et al. Isolation and characterization of genomic and cDNA clones of human erythropoietin. *Nature.* 1985;313:806-810.
2. Semenza GL, Wang GL. A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. *Mol Cell Biol.* 1992;12:5447-5454.
3. Imagawa S, Yamamoto M, Miura Y. Negative regulation of the erythropoietin gene expression by the GATA transcription factors. *Blood.* 1997;89:1430-1439.
4. Yamamoto M, Ko LJ, Leonard MW, Beug H, Orkin SH, Engel JD. Activity and tissue-specific expression of the transcription factor NF-E1 multi gene family. *Genes Dev.* 1990;4:1650-1662.
5. Tong Q, Dalgin G, Xu H, et al. Function of GATA transcription factors in preadipocyte-adipocyte transition. *Science.* 2000;290: 134-138.
6. Tarumoto T, Imagawa S, Ohmine K, et al. N^G-monomethyl L-arginine inhibits erythropoietin gene expression by stimulating GATA-2. *Blood.* 2000;96:1716-1722.
7. Tabata M, Tarumoto T, Ohmine K, et al. Stimulation of GATA-2 as a mechanism of hydrogen peroxide suppression in hypoxia-induced erythropoietin gene expression. *J Cell Physiol.* 2001;186: 260-267.
8. Shoemaker CB, Mitsock LD. Murine erythropoietin gene: cloning, expression, and human gene homology. *Mol Cell Biol.* 1986;6: 849-858.
9. Imagawa S, Goldberg MA, Doweiko J, Bunn HF. Regulatory elements of the erythropoietin gene. *Blood.* 1991;77:278-285.
10. Ito W, Ishiguro H, Kurosawa Y. A general method for introducing a series of mutations into cloned DNA using the polymerase chain reaction. *Gene.* 1991;102:67-70.
11. Pugh CW, Tan CC, Jones RW, Ratcliffe PJ. Functional analysis of an oxygen-regulated transcriptional enhancer lying 3′ to the mouse erythropoietin gene. *Proc Natl Acad Sci U S A.* 1991;88: 10553-10557.
12. Blanchard KL, Acquaviva AM, Galson DL, Bunn HF. Hypoxic induction of the human erythropoietin gene: cooperation between the promoter and enhancer, each of which contains steroid receptor response elements. *Mol Cell Biol.* 1992;12:5373-5385.
13. Huang LE, Arany Z, Livingstone DM, Bunn HF. Activation of hypoxia-inducible transcription factor depends primarily upon redox-sensitive stabilization of its α-subunit. *J Biol Chem.* 1996; 271:32253-32259.
14. Goldberg MA, Glass A, Cunningham JM, Bunn HF. The regulated expression of erythropoietin by two human hepatoma cell lines. *Proc Natl Acad Sci U S A.* 1987;84:7972-7976.
15. Aird WC, Parvin JD, Sharp PA, Rosenberg RD. The interaction of GATA binding proteins and basal transcription factors with GATA box containing core promoters. *J Biol Chem.* 1994;269: 883-889.
16. Blobel GA, Nakajima T, Eckner R, Montminy M, Orkin SH. CREB-binding protein cooperates with transcription factor GATA-1 and is required for erythroid differentiation. *Proc Natl Acad Sci U S A.* 1998;95:2061-2066.
17. Ohkuma Y, Horikoshi M, Roeder RG, Desplan C. Engrailed, a homeodomain protein, can repress in vitro transcription by competition with the TATA box-binding protein transcription factor IID. *Proc Natl Acad Sci U S A.* 1990;87:2289-2293.
18. Dostatni N, Lambert PS, Sousa R, Ham J, Howley PM, Yaniv M. The functional BPV-1 E2 trans-activating protein can act as a repressor by preventing formation of the initiation complex. *Genes Dev.* 1991;5:1657-1671.
19. Kaufman PD, Rio DC. Drosophilia P-element transposase is a transcriptional repressor in vitro. *Proc Natl Acad Sci U S A.* 1991; 88:2613-2617.

5.

睡眠時無呼吸症候群(OSAHS)では、一過性不連続な低酸素刺激が加わる。この一過性低酸素刺激による生体内のEpoおよびvascular endothelial growth factor (VEGF) の反応を、OSAHS重症106例を用いて解析した結果、重症化するにつれ、Hbが増加しさらにVEGF は約5倍増加したがEpoの増加は1.6倍と低く、一過性の低酸素刺激による反応様式の相違を認めた (Blood 98 : 1255-1257, 2001, Blood 99 : 393-394, 2002)。さらに、この症例を用い、VEGFの増加がIL-6あるいはTNF-αの増加によるものかどうかを解析したが、IL-6あるいはTNF-αには変化を認めなかった (Respination 71 : 24-29, 2004) 。

Levels of vascular endothelial growth factor are elevated in patients with obstructive sleep apnea–hypopnea syndrome

Shigehiko Imagawa, Yuji Yamaguchi, Masato Higuchi, Tomohiro Neichi, Yuichi Hasegawa, Harumi Y. Mukai, Norio Suzuki, Masayuki Yamamoto, and Toshiro Nagasawa

To better understand how humans adapt to hypoxia, the levels of hemoglobin (Hb), serum erythropoietin (Epo), and vascular endothelial growth factor (VEGF) were measured in 106 patients with severe obstructive sleep apnea-hypopnea syndrome. The results indicated that temporal hypoxic stimulation increases Hb. Furthermore, a minor increase in Epo and a substantial increase in VEGF were found. The induction in patients with severe sleep apnea was greater than that reported in other types of hypoxia. (Blood. 2001;98: 1255-1257)

Introduction

Remarkable progress has been made in understanding the molecular basis of oxygen sensing and transcriptional regulation of physiologically relevant genes, including those encoding erythropoietin (Epo) and vascular endothelial growth factor (VEGF).[1] Induction of these genes confers multiple responses for maintenance of oxygen hemostasis. At the transcriptional level, these genes are all under the control of hypoxia-inducible factor-1 (HIF-1).[2] There is an HIF-1 binding site in the enhancer of the *Epo* gene[2] and in the promoter of the *VEGF* gene.[3] Both of these genes are induced by hypoxia in vivo and in vitro by means of a common oxygen and signaling pathway.[1] HIF-1 is a widely expressed heterodimeric protein composed of HIF-1α and aryl hydrocarbon nuclear translocator (ARNT) subunits, both of which belong to the rapidly growing PAS family of basic helix-loop-helix (bHLH) transcription factors.[4] At the messenger RNA (mRNA) level, both *HIF-1* and *ARNT* genes are constitutively expressed and not significantly up-regulated by hypoxia. Whereas changes in oxygen tension do not affect ARNT protein abundance, hypoxia markedly increases the levels of HIF-1α protein.[5] The oxygen-dependent degradation (ODD) of HIF-1α is mediated by an internal 200-residue ODD domain via the ubiquitin-proteasome pathway.[6] Despite these findings in vitro, very little is known about the steps underlying the activation of HIF-1 through the oxygen sensor by hypoxia in humans. Plasma Epo increases exponentially with the degree of hypoxia in humans.[7] High altitude stimulates Epo production in humans.[8] Obstructive sleep apnea is a recognized cause of sleep-associated hypoxemia.[9] Nocturnal oxygenation correlates with daytime awake arterial oxygen saturation, but it cannot be accurately predicted from awake measurements of oxygenation in patients with obstructive sleep apnea or chronic obstructive pulmonary disease.[10] Intermittent nocturnal hypoxia in patients with obstructive sleep apnea was not accompanied by elevated serum Epo or erythrocytosis.[11] However, the number of the subjects in this study was small (n = 26) and did not include severely affected patients. In the present study, the responses of VEGF and Epo to temporal hypoxic stimulation were assayed in patients with severe obstructive sleep apnea-hypopnea syndrome (OSAHS).

Study design

We measured the levels of hemoglobin (Hb), serum VEGF, and Epo in patients with severe OSAHS (n = 106) and compared them with the levels in controls (n = 45). Individuals with anemia (Hb < 12.0 g/dL), renal or liver disease, and coronary artery disease were excluded. Assays of serum VEGF and Epo were performed by enzyme-linked immunosorbent assay. Serum samples were obtained from the patients when they first came to the clinic. The patients, all of whom had severe OSAHS, were divided into 5 groups according to the apnea-hypopnea index (AHI; 30-49, 50-69, 70-89, 90-109, and > 110) and the controls had an AHI of less than 5, as shown in Table 1.

Results and discussion

With increases in the AHI, PaO_2 significantly decreased from 89.8% ± 9.4% (in the control group) to 78.2% ± 5.1% (in the AHI > 110 group) (Figure 1). In contrast to PaO_2, Hb significantly increased from 14.5 ± 1.4 g/dL (control) to 17.2 ± 0.3 g/dL (AHI > 110 group) (Table 1). Serum VEGF levels significantly ($P < .005$) increased from 150 ± 111 (control) to 755 ± 182 pg/mL (AHI > 110 group), 5 times higher than the control level

From the Division of Hematology, Institute of Clinical Medicine, University of Tsukuba, Tsukuba, Ibaraki, Japan; Center for Sleep Respiratory Disorder at Fukuoka, Fukuoka, Japan; Chugai Pharmaceutical Co, Ltd, Tokyo, Japan; and Center for Tsukuba Advanced Research Alliance and Institute of Basic Medical Sciences, University of Tsukuba, Tsukuba, Ibaraki, Japan.

Submitted January 22, 2001; accepted April 9, 2001.

Supported by grants-in-aid for scientific research from the Ministry of Education, Science and Culture of Japan, Renal Anemia Foundation, and the Chugai Foundation, Tokyo, Japan.

Reprints: Shigehiko Imagawa, Division of Hematology, Institute of Clinical Medicine, University of Tsukuba, Tsukuba, Ibaraki 305-8575, Japan; e-mail: simagawa@md.tsukuba.ac.jp.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 U.S.C. section 1734.

Table 1. Levels of Hb, serum Epo, and VEGF in patients with severe OSAHS and controls

AHI	Hb (g/dL)	Epo (mU/mL)	VEGF (pg/mL)	n
<5	14.5 ± 1.4	10 ± 5	150 ± 111	45
30-49	15.6 ± 1.1*	17 ± 20†	250 ± 202*	41
50-69	15.3 ± 1.2*	13 ± 7†	582 ± 415*	37
70-89	15.5 ± 1.2*	16 ± 14†	547 ± 517*	22
90-109	15.7 ± 1.3†	14 ± 10	450 ± 250*	6
>110	17.2 ± 0.3†	14 ± 5	755 ± 182*	4

Numbers represent mean ± 1 SD.

AHI indicates apnea-hypopnea index; Hb, hemoglobin; Epo, erythropoietin; VEGF, vascular endothelial growth factor; OSAHS, obstructive sleep apnea-hypopnea syndrome.

*$P < .005$ compared to AHI < 5.

†$P < .025$ compared to AHI < 5.

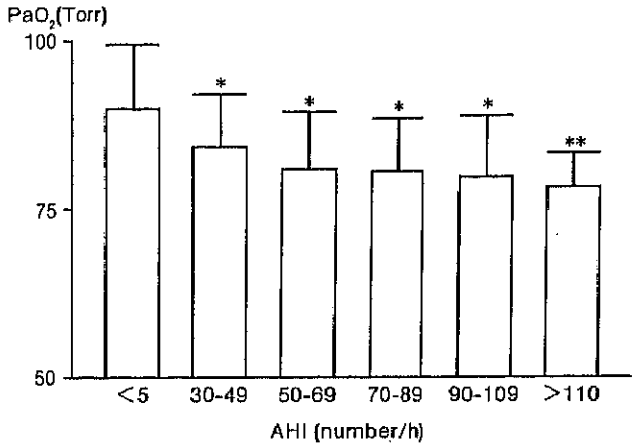


Figure 1. The level of PaO_2 in patients with severe OSAHS and controls. * Indicates $P < .005$; **, $P < .025$.

(Table 1). The serum Epo level in the control group was 10 ± 5 mU/mL (Table 1). Compared with the control level, Epo levels in the AHI 30 to 49, 50 to 69, and 70 to 89 groups were increased to 17, 13, and 16 mU/mL, respectively ($P < .025$), 1.6 times higher than the control level ($P < .025$) (Table 1). However, the levels in the AHI 90 to 109 and AHI greater than 110 groups were not increased ($P > .05$) (Table 1). Furthermore, there were no significant relationships between Epo and Hb, between VEGF and Hb, or between Epo and VEGF (data not shown).

Moore-Gillon and Cameron demonstrated that 2 hours of hypoxia (12% oxygen) per day leads to a rise in red cell mass in rats and that there is a dose-response relationship between the duration of hypoxia and red cell mass.[12] Other workers have shown that 1 hour of hypoxia (10% oxygen) per day leads to a rise in hematocrit in rats.[13] However, despite substantial nocturnal hypoxemia in some patients in the former study, there was no significant effect on serum Epo, and no significant change occurred when nocturnal hypoxemia was corrected by nasal continuous positive airway pressure.[11] Also, no patient had a serum Epo level more than 48 mU/mL, which was the upper limit of the normal range for the assay system used.[11] Thus, intermittent nocturnal hypoxemia in the patients was not accompanied by significantly elevated serum Epo levels. This finding conflicted with those of Cahan and associates,[14] who demonstrated that serum Epo levels in patients with obstructive sleep apnea were approximately 2-fold higher than those in normal subjects. Daytime hypoxemia appears to be an important determinant of serum Epo and red cell mass[15] in patients with chronic lung disease, but nocturnal hypoxemia does not appear to exert an appreciable independent influence on erythrocyte production.[16]

We found an increase in Hb, a minor increase in Epo, and a substantial increase in VEGF in the patients with OSAHS. The 1.6-fold increase in Epo in our study was compatible with that in a previous report.[14] This result indicates that a small increase in Epo allows for a corresponding increase in red cell mass. The resultant enhanced delivery of oxygen to tissues then dampens the hypoxic signal, thereby shutting off further stimulus for *Epo* gene transcription. This represents the closing of a negative feedback loop.

As to the response of VEGF by hypoxia, Gunga and colleagues reported reduced VEGF concentrations immediately after an ultramarathon run at high altitude.[17] Asano and coworkers measured a transient decrease of serum VEGF 10 days after the beginning of altitude training at 1886 m, followed by an increase, reaching maximum values on day 19.[18] Schobersberger and associates reported that VEGF in a group of runners was significantly elevated after they ran the Swiss Alpine Marathon of Devos (distance 67 km, altitude difference 2300 m) and further increased 2.4-fold until day 5 after exposure. Epo was also increased after exercise but reached a maximum 2 hours after the run (2-fold increase) and decreased thereafter.[19] They concluded that the increase of VEGF was due to both the stimulation of hypoxia and exercise. Especially after exercise, the tissue damage that occurred as a result of running increased the levels of cytokines such as interleukin 6 (IL-6) and tumor necrosis factor-α (TNF-α) which, in turn, may have stimulated the production of VEGF mRNA.[20] It is possible, though unlikely, that changes in IL-6 and TNF-α in patients with OSAHS contribute to the observed increase in VEGF and Epo.

References

1. Bunn HF, Poyton RO. Oxygen sensing and molecular adaptation to hypoxia. Physiol Rev. 1996; 76:839-885.
2. Semenza GL, Wang GL. A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. Mol Cell Biol. 1992;12:5447-5454.
3. Forsythe JA, Jiang B-H, Lyer NV, et al. Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. Mol Cell Biol. 1996;16:4604-4613.
4. Kallio PJ, Pongratz I, Gradin K, McGuire J, Poellinger L. Activation of hypoxia-inducible factor 1 α: post-transcriptional regulation and conformational change by recruitment of the Arnt transcription factor. Proc Natl Acad Sci U S A. 1997;5667-5672.
5. Huang LE, Arany Z, Livingston DM, Bunn HF. Activation of hypoxia-inducible factor depends primarily upon redox sensitive stabilization of its α subunit. J Biol Chem. 1996;271:32253-32259.
6. Huang LE, Gu J, Schau M, Bunn HF. Regulation of hypoxia-inducible factor 1 α is mediated by an O_2-dependent degradation domain via the ubiquitin-proteasome pathway. Proc Natl Acad Sci U S A. 1998;95:7987-7992.
7. Jelkmann W. Erythropoietin: structure, control of production, and function. Physiol Rev. 1992;72: 449-489.
8. Abbrecht PH, Littell JK. Plasma erythropoietin in men and mice during acclimatization to different altitudes. J Appl Physiol. 1972;32:54-58.
9. Guilleminault C, Tilkian A, Dement WC. The sleep apnea syndrome. Annu Rev Med. 1976;27:465-484.
10. McKeon JL, Murree-Allen K, Saunders NA. Prediction of oxygenation during sleep in patients with chronic obstructive lung disease. Thorax. 1988;43:312-317.
11. McKeon JL, Saunders NA, Murree-Allen K, et al. Urinary uric acid creatinine ratio, serum erythropoietin, and blood 2,3-diphosphoglycerate in patients with obstructive sleep apnea. Am Rev Respir Dis. 1990;142:8-13.

12. Moore-Gillon JC, Cameron IR. Right ventricular hypertrophy and polycythemia in rats after intermittent exposure to hypoxia. Clin Sci. 1985;69: 595-599.
13. Naitte EE, Doble EA. Threshold of intermittent hypoxia-induced right ventricular hypertropy in the rat. Respir Physiol. 1984;56:253-259.
14. Cahan C, Decker M, Robinowitz G, Washington L, Arnold J, Strohl KP. Hormonal and biochemical markers associated with sleep apnea [abstract]. Am Rev Respir Dis. 1989;139:A81.
15. Wedzicha JA, Cotes PM, Empey DW, Newland AC, Royston JP, Tam RC. Serum immunoreactive erythropoietin in hypoxic lung disease with and without polycythemia. Clin Sci. 1985;69:413-422.
16. Stradling JR, Lane DJ. Nocturnal hypoxemia in chronic obstructive pulmonary disease. Clin Sci. 1983;64:213-222.
17. Gunga HC, Kirsch K, Rocker L, et al. Vascular endothelial growth factor in exercising humans under different environmental conditions. Eur J Appl Physiol. 1999;79:484-490.
18. Asano M, Kaneoka K, Nomura T, et al. Increase in serum vascular endothelial growth factor levels during altitude training. Acta Physiol Scand. 1998; 162:455-459.
19. Schobersberger W, Hobisch-Hangen P, Fries D, et al. Increase in immune activation, vascular endothelial growth factor and erythropoietin after an ultramarathon run at moderate altitude. Immunobiology. 2000;201:611-620.
20. Minchenko A, Bauer T, Salceda S, Caro J. Hypoxia stimulation of vascular endothelial growth factor expression in vivo and in vitro. Lab Invest. 1994;71:374-379.

Response:

Elevation of vascular endothelial growth factor in patients with obstructive sleep apnea–hypopnea syndrome is not due to increased platelet counts

Gunsilius et al raise the possibility that the elevated vascular endothelial growth factor (VEGF) levels that we saw in our patients with obstructive sleep apnea–hypopnea syndrome (OSAHS) may be due to increased platelet counts that in turn lead to higher production of VEGF during clotting. Because we did not report platelet counts of our patients, this was a reasonable supposition. However, we can report additional data that argue against this hypothesis (Table 1). First, the platelet counts in our patients were normal, and second, there was no correlation between platelet count and apnea-hypopnea index (AHI). On the other hand, as reported in our paper,[1] VEGF levels increased significantly with increasing AHI.

Gunsilius et al wondered whether we observed edema, weight gain, or cardiopulmonary problems in our patients, which would be expected to result from chronic elevated VEGF levels. We did see edema, weight gain, and systemic hypertension in some of the patients with severe OSAHS, but there was no clear relationship between the levels of VEGF and these symptoms. However OSAHS is a temporary problem that occurs during only a portion of the day,[2] and half-lives of VEGF and erythropoietin (Epo) are less than 6 hours. Therefore, a lack of correlation between VEGF levels and these symptoms is not surprising. Still, cardiac arrhythmia and conduction disturbances[3] and pulmonary hypertension[4] have been reported in patients with sleep apnea, but in both cases these conditions were not chronic but occurred on a daily cycle.

Table 1. Levels of VEGF and platelet counts with severe OSAHS and controls

AHI	Serum VEGF (pg/mL)	Platelet count ($\times 10^4/\mu L$)	n
Less than 5 (control)	150 ± 111	24.8 ± 5.0	45
30-49	250 ± 202*	25.3 ± 7.2	41
50-69	582 ± 415*	24.4 ± 6.1	37
70-89	547 ± 517*	24.0 ± 4.3	22
90-109	450 ± 250*	25.9 ± 10.1	6
Greater than 110	755 ± 182*	28.0 ± 5.7	4

*$P < .005$ compared to AHI < 5.

The simple explanation for the elevated VEGF levels in patients with OSAHS is that it is induced by hypoxia. However, induction of VEGF may be a complex process that may involve other factors such as interleukin-6 and tumor necrosis factor α.[5] Further studies of these latter factors are needed to clarify the response of VEGF.

Shigehiko Imagawa, Yuji Yamaguchi, Masato Higuchi, Tomohiro Nakchi, Yuichi Hasegawa, Harumi Y Mukai, Norio Suzuki, Masayuki Yamamoto, and Toshiro Nagasawa.

Correspondence: Shigehiko Imagawa, Division of Hematology, Institute of Clinical Medicine, University of Tsukuba, Tsukuba, Ibaraki, Japan 305-8575; e-mail: simagawa@md.tsukuba.ac.jp

References

1. Imagawa S, Yamaguchi Y, Higuchi M, et al. Levels of vascular endothelial growth factor are elevated in patients with obstructive sleep apnea-hypopnea syndrome. Blood. 2001;98:1255-1257.
2. Eckardt K-U, Boutellier U, Kurtz A, Schopen M, Koller EA, Bauer C. Rate of erythropoietin formation in humans in response to acute hypobaric hypoxia. J Appl Physiol. 1989;66:1785-1788.
3. Guilleminault C, Connolly SJ, Winkle RA, et al. Cardiac arrhythmia and conduction disturbances during sleep in 400 patients with sleep apnea syndrome. Am J Cardiol. 1983;52:490-494.
4. Weitzenblum E, Krieger J, Apprill M, et al. Daytime pulmonary hypertension in patients with obstructive sleep apnea syndrome. Am Rev Respir Dis. 1988;138: 345-349.
5. Minchenko A, Bauer T, Salceda S, Caro J. Hypoxia stimulation of vascular endothelial growth factor expression in vivo and in vitro. Lab Invest. 1994;71:374-379.

Clinical Investigations

Respiration

Respiration 2004;71:24–29
DOI: 10.1159/000075645

Received: November 28, 2002
Accepted after revision: June 16, 2003

Interleukin-6 and Tumor Necrosis Factor-α in Patients with Obstructive Sleep Apnea-Hypopnea Syndrome

S. Imagawa[a] Y. Yamaguchi[b] K. Ogawa[b] N. Obara[a] N. Suzuki[c]
M. Yamamoto[c] T. Nagasawa[a]

[a]Division of Hematology, Institute of Clinical Medicine, University of Tsukuba, Ibaraki, [b]Center for Sleep Respiratory Disorder at Fukuoka, Fukuoka, and [c]Center for Tsukuba Advanced Research Alliance and Institute of Basic Medical Sciences, University of Tsukuba, Ibaraki, Japan

Key Words
Body mass index · Interleukin-6 · Sleep apnea · Tumor necrosis factor-α · Vascular endothelial growth factor

Abstract
Background: In previous studies, significantly elevated levels of vascular endothelial growth factor (VEGF) have been reported in patients with severe obstructive sleep apnea-hypopnea syndrome (OSAHS). On the other hand, plasma tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) have been significantly higher in mild sleep apneics than in normal controls. However, this study included a small number of patients and milder cases of OSAHS. ***Objectives and Methods:*** To assess the involvement of IL-6 and TNF-α in VEGF increases in patients with severe OSAHS, serum levels of IL-6 and TNF-α were determined in patients with severe OSAHS (n = 110) and compared to those of controls (n = 45) using an enzyme-linked immunosorbent assay. ***Results:*** No significant increase in IL-6 or TNF-α was detected in the present study cohort. However, the body mass index was significantly correlated with the severity of the apnea-hypopnea index. ***Conclusions:*** These data suggest that the elevation in VEGF is not directly related to IL-6 or TNF-α levels. However, the question of whether VEGF is the cause or the result of OSAHS remains to be determined. Further studies are needed to clarify the role of IL-6 and TNF-α in the pathogenesis of OSAHS, in which obesity should be entered as an independent factor.

Introduction

In a previous study, the responses of vascular endothelial growth factor (VEGF) and erythropoietin (EPO) to temporal hypoxic stimulation were assayed in 106 patients with severe obstructive sleep apnea-hypopnea syndrome (OSAHS), since both of the genes are regulated by hypoxia [1]. With increasing apnea-hypopnea index (AHI), PaO_2 significantly decreased from 89.8 ± 9.4 (in the control group) to 78.2 ± 5.1 Torr (in the AHI ≥ 110 group) [1]. In contrast to PaO_2, hemoglobin (Hb) significantly increased from 14.5 ± 1.5 (control) to 17.2 ± 0.3 g/dl (AHI ≥ 110 group). Serum VEGF levels signifi-

KARGER
Fax + 41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

0025–7931/04/0711–0024$21.00/0

Accessible online at:
www.karger.com/res

S. Imagawa
Division of Hematology
Institute of Clinical Medicine
University of Tsukuba, Ibaraki 305-8575 (Japan)
Tel./Fax +81 298 53 3124, E-Mail simagawa@md.tsukuba.ac.jp

cantly increased from 150 ± 111 (control) to 755 ± 182 pg/ml (AHI ≥110 group), 5 times higher than the control level [1]. The serum EPO level in the control group was 10 ± 5 mU/ml. EPO levels increased to 1.6 times the control level [1]. In patients with severe sleep apnea, VEGF production was increased compared to patients with other types of hypoxia [1]. There were no significant relationships between EPO and Hb, between VEGF and Hb, or between EPO and VEGF [1]. Gozal et al. [2] recently reported that circulating VEGF levels are also frequently elevated in patients with obstructive sleep apnea and suggested that milder VEGF plays a role in the regulation of tissue oxygen delivery. Although hypobaric hypoxia exposure during high-altitude climbing was not significantly associated with VEGF levels in randomly collected serum samples in humans [3], circulating levels of VEGF were elevated in the serum of experimentally induced hypoxia of a rodent model [4]. Interestingly, patients undergoing a surgical procedure will also temporarily increase circulating VEGF levels, suggesting that multiple pathophysiological pathways such as inflammatory processes and stress may underlie the upregulation of VEGF expression [5, 6]. Furthermore, Vgontzas et al. [7, 8] reported that plasma TNF-α and IL-6 were significantly higher in patients with the sleep apnea syndrome than in normal controls. However, the investigators examined milder patients and small numbers (AHI 63.7 ± 10.3; n = 12, AHI 48.7 ± 5.6; n = 14), and did not examine patients with severe OSAHS. It is possible that changes in IL-6 and TNF-α contribute to the observed increase in VEGF in our patients with severe OSAHS. Therefore, we hypothesized that increases in the cytokines IL-6 and TNF-α may induce the expression of VEGF in our patients with severe OSAHS.

Materials and Methods

Study Design

We measured serum IL-6 and TNF-α levels in patients with severe OSAHS (n = 110) and compared them with the levels obtained in controls (n = 45). Individuals with anemia (Hb <12.0 g/dl), renal or liver disease, and coronary arterial disease were excluded. Serum IL-6 and TNF-α were assayed by ELISA. When patients first came to the clinic, serum samples were obtained in the sitting position in the morning. The study cohort, consisting of patients with severe OSAHS, was divided into five patient groups according to the AHI (30–49, 50–69, 70–89, 90–109 and ≥110) and the control group, who had an AHI <5. Furthermore, patients with severe OSAHS were divided into six groups according to their BMI (<20, 20 ≤ BMI <25, 25 ≤ BMI <30, 30 ≤ BMI <35, 35 ≤ BMI <40, 40 kg/m^2 ≤ BMI). All patients gave written informed consent to participate in the study, and the study was reviewed and approved by a recognized ethics review committee of the Center for Sleep Respiratory Disorder. The study was performed in accordance with the Declaration of Helsinki.

Measurements of Serum IL-6 and TNF-α Concentrations

Assays of serum IL-6 and TNF-α were performed using an ELISA kit (R&D Systems, Minneapolis, Minn., USA). The minimum detectable doses of IL-6 and TNF-α are typically <0.70 and <10.0 pg/ml, respectively, in our hands. Intra-assay precision and inter-assay coefficients of variation of IL-6 are 4.2 and 6.4% and of TNF-α 5.2 and 7.4%.

Analysis

Student's t test was used to assess the level of significance.

Results

To elucidate the hypoxic levels of the patients, the relationship between desaturation and AHI was investigated. As shown in figure 1, desaturation was well correlated with the severity of AHI in these patients (fig. 1). At each BMI, desaturation was well correlated with AHI (BMI <20: r = 0.41, 20 ≤ BMI <25: r = 0.47, 25 ≤ BMI <30: r = 0.61, 30 ≤ BMI <35: r = 0.52, 35 ≤ BMI <40: r = 0.73, 40 ≤ BMI: r = 0.64). Serum IL-6 was 24.4 pg/ml in controls. Although serum IL-6 levels were 38.5 and 34.5 pg/ml in the group of AHI 70–89 and AHI 90–109, respectively, in the patients with severe OSAHS they were not significantly different from the controls (table 1, fig. 2). On the other hand, serum TNF-α level was 25.0 pg/ml in the controls (table 2). In the group of AHI 90–109, and that ≥110, TNF-α was increased (30.7 and 39.8 pg/ml, respectively) compared to the controls, but the differences were not significant (table 2, fig. 3). Vgontzas et al. [7] reported that plasma TNF-α and IL-6 were significantly higher in mild sleep apneics than in normal controls. Furthermore, they reported a strong positive correlation between IL-6 and BMI [8]. In the present study, the BMI of the controls was 22.9 kg/m^2 and was significantly correlated with the severity of AHI (table 3, fig. 4). BMI was weakly correlated with IL-6 levels in the group of AHI <5: r = 0.134: p < 0.01, AHI 30–49: r = 0.130: p < 0.01, AHI 50–69: r = 0.082: p < 0.01, and AHI 90–109: r = 0.368: p < 0.05, respectively. BMI was also weakly correlated with TNF-α levels in AHI <5: r = 0.078: p < 0.01, AHI 30–49: r = 0.334: p < 0.01, AHI 50–69: r = 0.097: p < 0.01, AHI 70–89, r = 0.115: p < 0.05, and AHI ≥ 110: r = 0.584: p < 0.01, respectively (table 4).

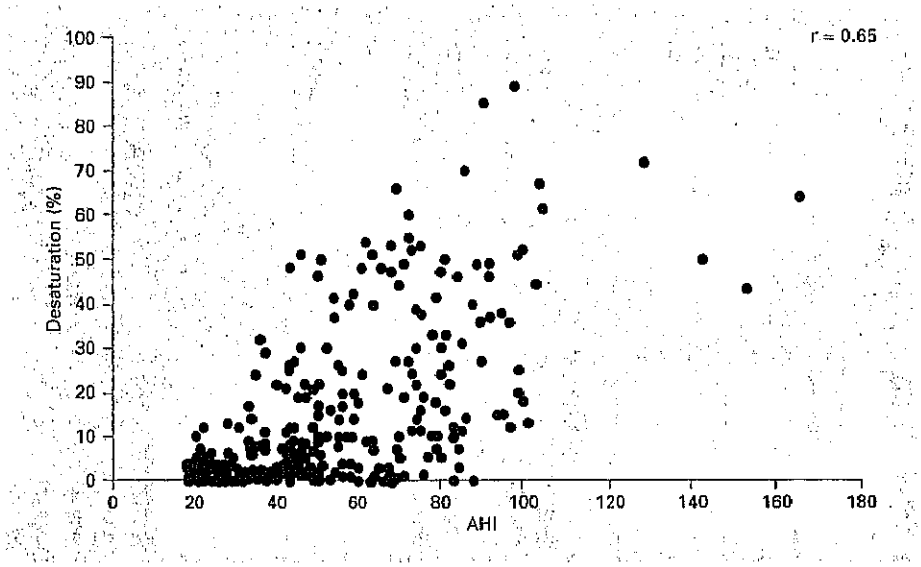


Fig. 1. Relationship between desaturation and AHI.

Table 1. Serum IL-6 concentrations in the patients with severe OSAHS

AHI	IL-6 pg/ml	Sample No.
<5	24.4±77.0	46
30–49	19.3±56.3	45
50–69	6.4±18.2	36
70–89	38.5±121.0	24
90–109	34.5±55.4	7
≥110	30.1±42.1	5

Table 2. Serum TNF-α concentrations in the patients with severe OSAHS

AHI	TNF-α pg/ml	Sample No.
<5	25.0±26.4	47
30–49	21.7±22.7	45
50–69	26.7±37.5	38
70–89	28.6±27.9	24
90–109	30.7±21.1	6
≥110	39.8±37.9	5

Table 3. BMI in the patients with severe OSAHS

AHI	BMI kg/m^2	Sample No.
<5	22.9±2.9	46
30–49	26.7±3.8	45
50–69	26.4±5.4	37
70–89	28.5±3.6	24
90–109	32.1±6.6	8
≥110	36.3±3.0	5

Table 4. Relationships between BMI and IL-6 or TNF-α

AHI	BMI:IL-6		BMI:TNF-α	
<5	y = 3.64x – 59.33	r = 0.134*	y = 0.71x + 6.47	r = 0.078*
30–49	y = – 2.12x + 7.92	r = 0.130*	y = 2.58x – 45.34	r = 0.334*
50–69	y = 0.38x + 16.72	r = 0.082*	y = 0.93x + 52.63	r = 0.097*
70–89	y = 0.79x + 17.53	r = 0.024	y = 0.79x + 17.53	r = 0.115**
90–109	y = – 2.91x + 128.24	r = 0.368**	y = 0.95x – 0.95	r = 0.321
≥110	y = 1.04x + 2.11	r = 0.081	y = –8.32x + 331.74	r = 0.584*

* $p < 0.01$, ** $p < 0.05$.

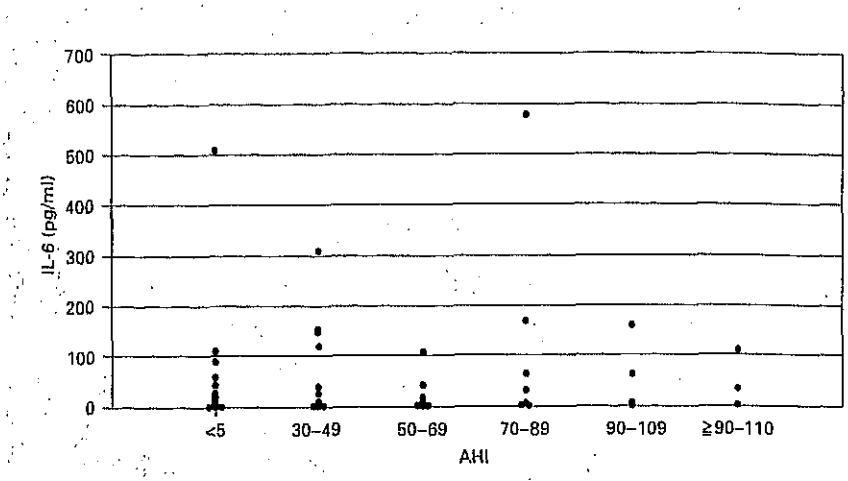


Fig. 2. Relationship between IL-6 and AHI.

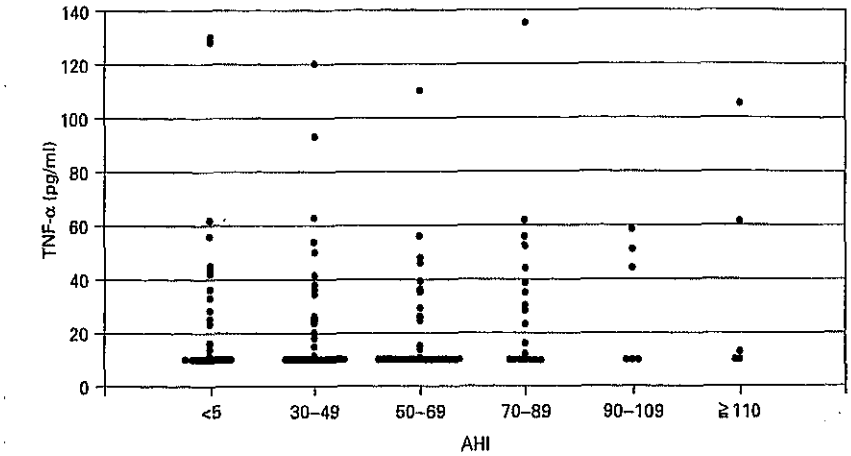


Fig. 3. Relationship between TNF-α and AHI.

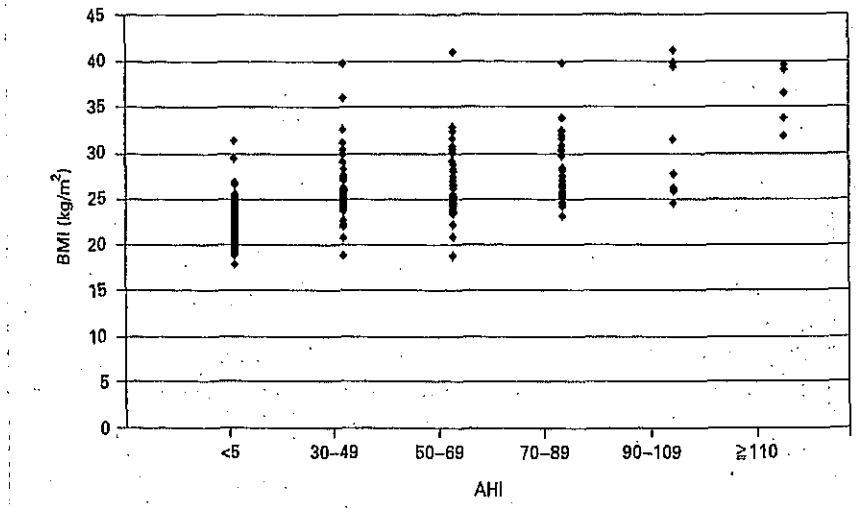


Fig. 4. Relationship between BMI and AHI.

Discussion

Based on available information on IL-6 in human diseases, the serum IL-6 concentration seems to be a good indicator of activation of the inflammatory cascade and a predictor of subsequent organ dysfunction [9]. Respiratory problems and hypoventilation during sleep are common among obese patients [10]. Inflammatory cytokines seem to be involved in the regulation of sleep in animals and humans [11, 12]. Vgontzas et al. [7, 8] concluded that TNF-α and IL-6 may mediate somnogenic activity and fatigue associated with excessive daytime somnolence in obese patients with sleep apnea. They demonstrate that serum IL-6 concentrations were positively correlated with the level of obesity assessed by BMI. In contrast, in a study by Roytblat et al. [13], IL-6 levels were comparable in obese patients and patients with obstructive sleep apnea syndrome. In their study, IL-6 levels were 34-fold higher in the patients with obesity-hypoventilation than in the controls, and 8-fold higher than in the patients with the obstructive sleep apnea syndrome. However, serum IL-6 levels were increased in all obese subjects [13]. The relationship between BMI and AHI found in the present study clearly shows that severe sleep apnea is associated with obesity. To elucidate the role of IL-6 and TNF-α in the pathogenesis of OSAHS, obesity should be entered as an independent factor into this study.

TNF-α correlates strongly with lipolysis, and this cytokine causes marked insulin resistance [14–16] and stimulates leptin secretion [17–19]. Circulating concentrations of leptin are not only proportional to the total body fat but also to the degree of insulin resistance [20]. Chronic leptin administration has been associated with sympathetic system activation and elevation of blood pressure [21], suggesting that it might play a role in the pathogenic expression of sleep apnea [8]. In fact, Vgontzas et al. [8] reported that sleep apneic men had higher plasma concentrations of the adipose tissue-derived hormone leptin than non-apneic obese men. To elucidate the changes in VEGF more precisely, leptin should be measured in patients with severe OSAHS.

VEGF is an endothelial-cell-specific mitogen that increases oxygen delivery to peripheral tissues by stimulating angiogenesis and improving tissue capillary density [22]. Increased VEGF protein expression occurs following hypoxia by enhanced transcriptional regulation of the VEGF gene through binding of the hypoxia-inducible factor-1 (HIF-1) to a hypoxic response element in the 5′ flanking region of the VEGF gene promoter [23]. Recently, elevated serum VEGF concentrations have been reported in several conditions that are not inherently associated with the typical intermittent hypoxia seen in OSAHS. In a cohort of patients with cystic fibrosis, elevated VEGF levels decreased after antibiotic therapy associated with clinical improvement in inflammatory markers [24]. Similarly, increased VEGF levels occur in several oncological conditions including brain, breast, and colorectal cancers, and have been proposed as clinically applicable prognostic markers [25–29]. In the present study, we found no significant elevation in the levels of IL-6 or TNF-α in the patients with severe OSAHS. Our data suggest that the elevation in VEGF is not directly related to the levels of IL-6 or TNF-α. One could envision that VEGF expression could be altered by confounding factors to make the correlations between VEGF and TNF-α/IL-6 more tenuous than in healthy subjects, animals, or cell cultures.

Whether the increment in VEGF in the patients with severe OSAHS was the cause or the result of OSAHS remains unclear. Further detailed studies on OSAHS will be needed.

Acknowledgments

We thank Hiromi Tanaka and Ayako Hoshino for their expert technical assistance. This work was supported by grants-in-aid for scientific research from the Ministry of Education, Science and Culture of Japan.

References

1 Imagawa S, Yamaguchi Y, Higuchi M, Neichi T, Hasegawa Y, Mukai HY, Suzuki N, Yamamoto M, Nagasawa T: Levels of vascular endothelial growth factor are elevated in patients with obstructive sleep apnea-hypopnea syndrome. Blood 2001;98:1255–1257.

2 Gozal D, Lipton AJ, Jones KL: Circulating vascular endothelial growth factor levels in patients with obstructive sleep apnea. Sleep 2002; 25:59–65.

3 Pazlicek V, Marti HH, Grad S, Gibbs JS, Kol C, Wenger RH, Gassman M, Kohl J, Maly FE, Oelz O, Koller EA, Schirlo C: Effects of hypobaric hypoxia on vascular endothelial growth factor and the acute phase response in subjects who are susceptible to high-altitude pulmonary oedema. Eur J Appl Physiol 2000;81:497–503.

4 Christou H, Yoshida A, Arthur V, Morita T, Kourenbanas S: Increased vascular endothelial growth factor production in the lungs of rats with hypoxia-induced pulmonary hypertension. Am J Respir Cell Mol Biol 1993;18:768–776.

5 Bondestam J, Salven P, Jaankela-Saari H, Ikonen T, Lepantalo M, Mattila S, Joensuu H: Major surgery increases serum levels of vascular endothelial growth factor only temporarily. Am J Surg 2000;179:57–59.

6 Richard DE, Berra E, Ponyssegur J: Nonhypoxic pathway mediates the induction of hypoxia-inducible factor 1α in vascular smooth muscle cells. J Biol Chem 2000;275:26765–26771.

7 Vgontzas AN, Papanicolaou DA, Bixlen EO, Kales A, Tyson K, Chrousos GP: Elevation of plasma cytokines in disorders of excessive daytime sleepiness: Role of sleep disturbance and obesity. J Clin Endocrinol Metab 1997;82: 1313–1316.

8 Vgontzas AN, Papanicolaou DA, Bixler EO, Hopper K, Lotsikas A, Lin HM, Kales A, Chrousos GP: Sleep apnea and daytime sleepiness and fatigue: Relation to visceral obesity, insulin resistance, and hypercytokinemia. J Clin Endocrinol Metab 2000;85:1151–1158.

9 Bifel WL, Moore EE, Moore FA, Peteson VM: Interleukin-6 in the injured patient. Ann Surg 1996;224:647–664.

10 Fox GL, Whalley DG, Began OR: Anesthesia for the morbidly obese: Experience with 110 patients. Br J Anaesth 1981;83:811–816.

11 Kaspäs L, Hong L, Cady AB, Opp MR, Postlethwaite AE, Seyer JM, Krueger JM: Somnogenic, pyrogenic, and anorectic activities of tumor necrosis factor-alpha and TNF-alpha fragments. Am J Physiol 1992;263:R708–R715.

12 Opp MR, Kaspas L, Toth LA: Cytokine involvement in regulation of sleep. Proc Soc Exp Biol Med 1992;201:16–27.

13 Roytblat L, Rachinsky M, Fisher A, Greemberg L, Shapira Y, Douvdevani A, Gelman S: Raised interleukin-6 levels in obese patients. Obes Res 2000;8:673–675.

14 Hotamisligil GS, Shargill NS, Spiegelman BM: Adipose expression of tumor necrosis factor-α: Direct role in obesity-linked insulin resistance. Science 1993;259:87–91.

15 Orban Z, Remaley AT, Sampson M, Trajanoski Z, Chrousos GP: The differential effect of food intake and β-adrenergic stimulation on adipose-derived hormones and cytokines in man. J Clin Endocrinol Metab 1999;84:2126–2133.

16 Beisel WR: Herman Award Lecture: Infection-induced malnutrition – From cholera to cytokines. Am J Clin Nutr 1995;62:813–819.

17 Sarraf P, Frederich RC, Turner EM, Ma G, Jaskowiak NT, Rivet DJ III, Flier JS, Lowell BB, Fraker DL, Alexander.HR: Multiple cytokines and acute inflammation raise mouse leptin levels: Potential role in inflammatory anorexia. J Exp Med 1997;185:171–175.

18 Mantzoros CS, Moschos S, Avramopoulos I, Kaklamani V, Liolios A, Doulgerakis DE, Griveas I, Katsilambros N, Flier JS: Leptin concentrations in relation to body mass index and the tumor necrosis factor-α system in humans. J Clin Endocrinol Metab 1997;82:3408–3413.

19 Zumbach MS, Wolfgang M, Boehme J, Wahl P, Stremmel W, Ziegler R, Nawroth PP: Tumor necrosis factor increases serum leptin levels in humans. J Clin Endocrinol Metab 1997;82: 4080–4082.

20 de Courten M, Zimmet P, Hodge A, Collins V, Nicolson M, Staten M, Dowse G, Alberti KG: Hyperleptinaemia: The missing link in the metabolic syndrome? Diabet Med 1997;14: 200–208.

21 Shek EW, Brands MW, Hall JE: Chronic leptin infusion increases arterial pressure. Hypertension 1998;31:409–414.

22 Gunga HC, Kirsch K, Rocker L, Behn C, Koralewski E, Davila EH, Estrade MI, Johannes B, Wittels P, Jelkmann W: Vascular endothelial growth factor in exercising humans under different environmental conditions. Eur J Appl Physiol 1999;79:484–490.

23 Marti HH, Risau W: Systemic hypoxia changes the organ specific distribution of vascular endothelial growth factor and its receptors. Proc Natl Acad Sci USA 1998;95:15809–15814.

24 McColley SA, Stellmach V, Boas SR, Jain M, Grawford SE: Serum vascular endothelial growth factor is elevated in cystic fibrosis and decreases with treatment of acute pulmonary exacerbation. Am J Respir Crit Care Med 2000;161:1877–1880.

25 Los M, Aarsman CJ, Terpstra L, Wittebol-Post D, Lips CJ, Blijham GH, Voest EE: Elevated ocular levels of vascular endothelial growth factor in patients with von Hippel-Lindau disease. Ann Oncol 1997;10:1015–1022.

26 Takano S, Yochii Y, Kondo S, Suzuki H, Maruno T, Shirai S, Nose T: Concentration of vascular endothelial growth factor in the serum and tumor tissue of brain tissue patients. Cancer Res 1996;56:2185–2190.

27 Speirs V, Atkin ST: Production of VEGF and expression of the VEGF receptors Flt-1 and KDR in primary cultures of epithelial and stromal cells derived from breast tumors. Br J Cancer 1999;80:898–903.

28 Fujisaki K, Mitsuyama K, Toyonaga A, Matsuo K, Tanikawa K: Circulating vascular endothelial growth factors in patients with colorectal cancer. Am J Gastroenterol 1998;93:269–252.

29 Hyodo I, Doi T, Endo H, Hosokawa Y, Nishikawa Y, Tanimizu M, Jinno K, Kotani Y: Clinical significance of plasma vascular endothelial growth factor in gastrointestinal cancer. Eur J Cancer 1998;34:2041–2045.

6.

慢性貧血は、IL-Iβあるいは TNF-αが増加するために Epo 産生が低下し発症する。最近 Jelkmann らは、IL-Iβあるいは TFN-αが GATA および NF-κB 結合活性を亢進させるために Epo プロモーター活性を低下させる機序を解明した。そこで GATA 特異的阻害薬である K-7174 を Hep3B 細胞系に添加して解析した。この結果、IL-Iβあるいは TNF-αにより GATA 結合活性が増加し Epo プロモーター活性が低下して Hep3B 細胞の産生する Epo は低下する。しかし、K-7174 を前処理すると IL-βあるいは TNF-αによる GATA 結合活性亢進が抑制されるために Epo プロモーター活性が上昇して Epo 蛋白が増加することを認めた。また、マウスに IL-Iβあるいは TNF-αを腹腔内注射すると Epo 産生が低下し、網状赤血球・Hb が低下した。しかし K-7174 を腹腔内注射すると IL-Iβあるいは TNF-αによる Epo の産生低下は解除され、網状赤血球・Hb が増加し、慢性貧血が改善することを認めた (FASEB J 17: 1742-1744, 2003)。

A GATA-specific inhibitor (K-7174) rescues anemia induced by IL-1β, TNF-α, or L-NMMA[1]

SHIGEHIKO IMAGAWA,*,[2] YOKO NAKANO,* NAOSHI OBARA,* NORIO SUZUKI,† TAKESHI DOI,‡ TATSUHIKO KODAMA,§ TOSHIRO NAGASAWA,* AND MASAYUKI YAMAMOTO†

*Division of Hematology, Institute of Clinical Medicine, University of Tsukuba, Tsukuba, Ibaraki 305-8575, Japan; †Center for Tsukuba Advanced Research Alliance and Institute of Basic Medical Sciences, University of Tsukuba, Tsukuba, Ibaraki 305-8577, Japan; ‡Kowa Research Institute, Kowa Co., Ltd., Tokyo 103-8433, Japan; and §Laboratory for Systems Biology and Medicine Research Center for Advanced Science and Technology, University of Tokyo, Tokyo 153-8904, Japan

SPECIFIC AIM

One common pathogenesis of anemia of chronic diseases (ACD) and with renal disease appears to be stimulation of GATA binding activity by interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), or N^G-monomethyl L-arginine (L-NMMA), which inhibits erythropoietin (Epo) promoter activity. We investigated the ability of K-7174 (a GATA-specific inhibitor) to improve Epo production after inhibition by IL-1β, TNF-α, or L-NMMA treatment in Hep3B cells in vitro and in an in vivo mouse assay.

PRINCIPAL FINDINGS

1. Epo protein production, which is inhibited by IL-1β or TNF-α, is rescued by K-7174

Hypoxia (1% O_2) induced Epo protein production from aliquots of 3×10^6 Hep3B cells over 24 h. However, 15 U/mL IL-1β and 220 U/mL TNF-α each inhibited Epo protein production, and addition of 10–20 μM K-7174 rescued these inhibitions in a dose-dependent manner (**Fig. 1**).

2. Epo promoter activity, which is inhibited by IL-1β, TNF-α, or L-NMMA, is rescued with K-7174

Hep3B cells were transiently transfected with a construct spanning regions of the human Epo enhancer containing hypoxia-inducible factor 1 (HIF-1) and a promoter containing a GATA element (Pwt: wild-type). Hypoxic induction of Epo promoter activity from Pwt was 33.7-fold higher than that from normoxic Pwt. Addition of 15 U/mL IL-1β, 220 U/mL TNF-α, or 10^{-3} M L-NMMA inhibited induction from Pwt with lowered hypoxic/normoxic ratios of 19.9-, 20.7-, or 19.8-fold, respectively. However, the addition of 10 μM K-7174 increased induction from hypoxic Pwt to 40.3-fold higher than that from normoxic Pwt. Furthermore, the combination of 10 μM K-7174 with + IL-1β, + TNF-α, or + L-NMMA rescued hypoxic inductions from Pwt, with increased hypoxia/normoxia ratios of 43.0-, 49.6-, or 35.6-fold, respectively. These results indicate that K-7174 rescues the suppression of *Epo* gene expression by IL-1β, TNF-α, or L-NMMA through the *Epo* gene regulatory regions.

3. The binding activity of GATA, which is enhanced by IL-1β, TNF-α, or L-NMMA, is inhibited with K-7174

Hep3B cells showed strong GATA DNA binding by an electrophoretic mobility shift assay (EMSA). The addition of K-7174 decreased the intensity of this band dose dependently. Stimulation with 15 U/mL IL-1β, 220 U/mL TNF-α, or 10^{-3} M L-NMMA for 24 h led to an increase in GATA binding. These increments of GATA binding activity induced by IL-1β, TNF-α, or L-NMMA were inhibited by the addition of 10 μM K-7174. Thus, IL-1β, TNF-α, and L-NMMA all enhance GATA binding activity, which in turn inhibits Epo promoter activity and protein production.

4. K-7174 increases Epo production by mice in vivo and rescues the inhibition of hemoglobin (Hb) concentrations and reticulocyte counts by IL-1β or TNF-α

To elucidate the effects of K-7174, IL-1β, and TNF-α on Epo production, Hb concentrations, and reticulocyte counts in an in vivo mouse assay, blood samples (0.3 mL) were obtained from the orbital vein of ICR mice at 0, 12, 24, and 36 h. Compared with PBS control, intraperitoneal injection of IL-1β decreased Epo production, Hb concentrations, and reticulocyte counts

[1] To read the full text of this article, go to http://www.fasebj.org/cgi/doi/10.1096/fj.02-1134fje; doi: 10.1096/fj.02-1134fje

[2] Correspondence: Division of Hematology, Institute of Clinical Medicine, University of Tsukuba, Tsukuba, Ibaraki 305-8575, Japan. E-mail: simigawa@md.tsukuba.ac.jp

0892-6638/03/0017-1742 © FASEB

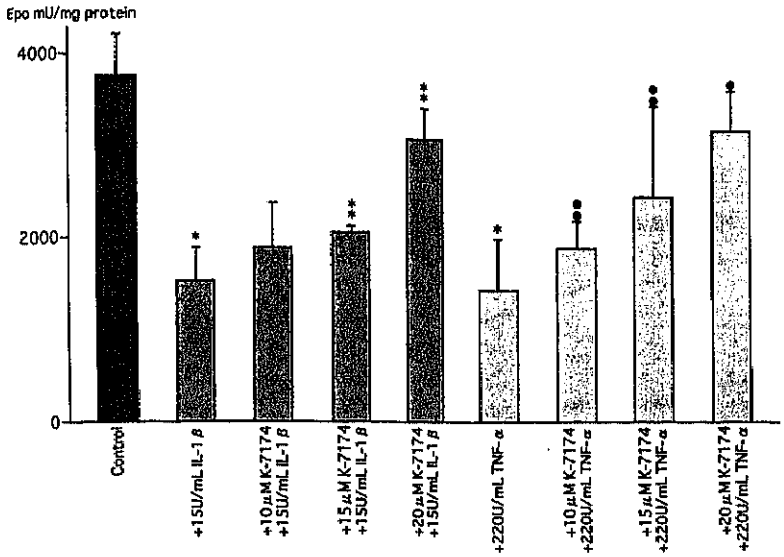


Figure 1. Effects of different doses of K-7174 on the inhibition of Epo protein production from Hep3B cells by IL-1β and TNF-α. Aliquots of 3×10^6 Hep3B cells were incubated with 15 U/mL rhIL-1β, 220 U/mL rhTNF-α, or 10, 15, 20 μM K-7174 under hypoxic conditions (1% O_2) for 24 h. The expression level of Epo protein was measured with RIA. Separate experiments were performed (n=8). Error bars represent 1 standard deviation. *Significance compared with control, $P < 0.005$. **Significance compared with 15 U/mL IL-1β, $P < 0.005$. *Significance compared with 220 U/mL TNF-α, $P < 0.005$. **Significance compared with 220 U/mL TNF-α, $P < 0.025$.

on days 3, 6, and 10 (**Fig. 2**). Injection of TNF-α also inhibited Epo production, Hb concentrations, and reticulocyte counts on the same days (Fig. 2). Compared with these results, injection of K-7174 significantly increased Epo production, Hb concentrations, and reticulocyte counts on the same days, while injection of K-7174 significantly rescued the inhibitions of Epo production, Hb concentrations, and reticulocyte counts by IL-1β or TNF-α (Fig. 2). Thus, K-7174 treatment increased Epo production, Hb concentrations, and reticulocyte counts in vivo.

CONCLUSIONS AND SIGNIFICANCE

By in vitro cell assay, both 15 U/mL IL-1β and 220 U/mL TNF-α, inhibited Epo protein production, and this was rescued by 10 μM K-7174. The three treatments (15 U/mL IL-1β, 220 U/mL TNF-α, and 10^{-3} M L-NMMA) each inhibited Epo promoter activity and the addition of K-7174 reversed this. The EMSA showed that addition of K-7174 decreased GATA binding activity, which in turn was increased with the addition of IL-1β, TNF-α, or L-NMMA. Intraperitoneal injections of IL-1β or TNF-α into mice decreased Hb concentrations and reticulocyte counts. However, the addition of K-7174 reversed this anemia produced by IL-1β or TNF-α. K-7174 was developed as a low molecular weight anti-inflammatory drug. It is known to be a specific inhibitor of GATA. Intraperitoneal injection of 30 mg/kg K-7174 into ICR mice produced no significant change in the weight of the spleen or the numbers of white blood cells and platelets (data not shown). A dose of 300 mg/kg for rats (10-fold higher than the dose used in this study) revealed no adverse effects on the liver, kidney, or on hematopoiesis (data not shown). Furthermore, K-7174 decreases the production of circulating glutamic-oxaloacetic transaminase (GOT) and

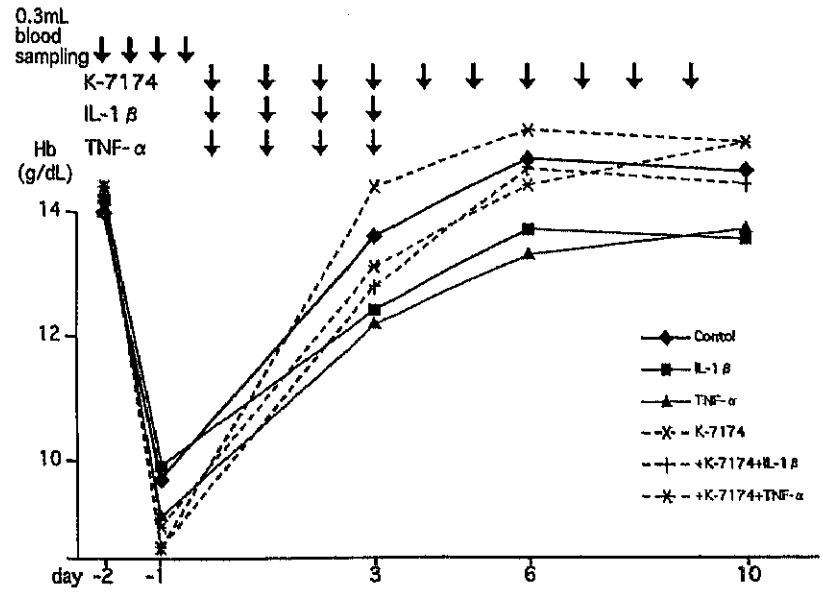


Figure 2. Effects of K-7174 on the decrease of hemoglobin (Hb) concentrations from ICR mice induced by IL-1β or TNF-α. ICR mice were injected intraperitoneally with 0.1 mL of PBS (control) on days 0–9, 1.67×10^4 U rmIL-β on days 0–3, 3.33×10^5 U rmTNF-α on days 0–3, or 0.1 mL (30 mg/kg) K-7174 on days 0–9 (n=6). Blood samples (0.3 mL) were taken from the orbital vein at 0, 12, 24, 36 h and on days 3, 6, and 10.

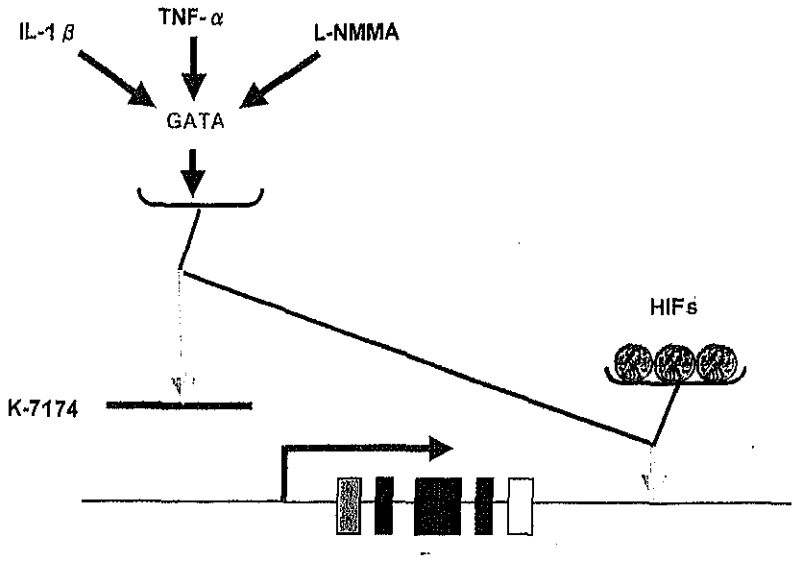


Figure 3. Schematic drawing of the erythropoietin gene expression and inhibition by *IL-1β*, TNF-α, or L-NMMA through the GATA site of the Epo promoter. K-7174 rescues the suppression of *Epo* gene expression by IL-1β, TNF-α, or L-NMMA.

lactate dehydrogenase (LDH), which are increased by the injection of IL-1β and TNF-α into ICR mice (data not shown). Thus, the anemia induced by IL-1β or TNF-α can be prevented by simultaneous administration of K-7174 (Fig. 3). This study raises the possibility of using K-7174 as a novel drug for treating ACD and for anemia associated with renal disease. We are presently investigating the effects of K-7174 using DBA/2FG-pcy mice as a model of anemia associated with renal disease. FJ

The FASEB Journal express article 10.1096/fj.02-1134fje. Published online July 18, 2003.

A GATA-specific inhibitor (K-7174) rescues anemia induced by IL-1β, TNF-α, or L-NMMA

Shigehiko Imagawa,* Yoko Nakano,* Naoshi Obara,* Norio Suzuki,† Takeshi Doi,‡ Tatsuhiko Kodama,§ Toshiro Nagasawa,* and Masayuki Yamamoto†

*Division of Hematology, Institute of Clinical Medicine, University of Tsukuba, Tsukuba, Ibaraki 305-8575; †Center for Tsukuba Advanced Research Alliance and Institute of Basic Medical Sciences, University of Tsukuba, Tsukuba, Ibaraki 305-8577; ‡Kowa Research Institute, Kowa Co., Ltd., Tokyo 103-8433; and §Laboratory for Systems Biology and Medicine Research Center for Advanced Science and Technology, University of Tokyo, Tokyo 153-8904, Japan

Corresponding author: Shigehiko Imagawa, Division of Hematology, Institute of Clinical Medicine, University of Tsukuba, Tsukuba, Ibaraki 305-8575, Japan. E-mail: simagawa@md.tsukuba.ac.jp

ABSTRACT

Interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), or N^G-monomethyl-L-arginine (L-NMMA) are increased in patients with chronic disease-related anemia. They increase the binding activity of GATA and inhibit erythropoietin (Epo) promoter activity. In this study, we examined the ability of K-7174 (a GATA-specific inhibitor) to improve Epo production when inhibited by treatment with IL-1β, TNF-α, or L-NMMA. Epo protein production and promoter activity were induced in Hep3B cells with 1% O_2. However, 15 U/ml IL-1β, 220 U/ml TNF-α, or 10^{-3} M L-NMMA inhibited Epo protein production and promoter activity, respectively. Addition of 10 μM K-7174 rescued these inhibitions of Epo protein production and promoter activity induced by IL-1β, TNF-α, or L-NMMA, respectively. Electrophoretic mobility shift assays revealed that addition of K-7174 decreased GATA binding activity, which was increased with the addition of IL-1β, TNF-α, or L-NMMA. Furthermore, intraperitoneal injection of mice with IL-1β or TNF-α decreased the hemoglobin concentrations and reticulocyte counts. However, the addition of K-7174 reversed these effects. These results raise the possibility of using K-7174 as therapy to treat anemia.

Key words: interleukin-1β • N^G-monomethyl-L-arginine • tumor necrosis factor-α • erythropoietin

Anemia commonly occurs in patients with chronic diseases such as malignancies, chronic inflammatory states, or infections. Such anemia of chronic diseases (ACD) results in part from inadequate red cell production (1). The mechanisms responsible for ACD are unclear. However, the condition is generally associated with the production of cytokines by activated macrophages that in turn suppress erythropoietin (Epo) production. The pathogenesis of this process is obscure, although it may result from a multifactorial process that includes abnormal iron reutilization (1, 2), inappropriately low serum Epo levels for the degree of anemia in the individual patient, and a decreased bone marrow response to the Epo that is present. Steady-state serum Epo levels are frequently decreased relative to the degree of anemia in patients with a

variety of infectious, inflammatory, and malignant disorders, including acquired immunodeficiency syndrome (AIDS), rheumatoid arthritis (RA), ulcerative colitis, and cancer (3–7). Furthermore, the administration of recombinant human Epo may partially or even completely correct anemia in those patients with chronic diseases in whom the Epo response is blunted (8–13), strongly suggesting that a relative lack of endogenous Epo contributes to the pathogenesis of these anemias.

The disorders associated with ACD are characterized by the production of certain inflammatory cytokines, primarily macrophage derived, including interleukin-1α (IL-1α), IL-1β, IL-6, transforming growth factor-β (TGF-β), and tumor necrosis factor-α (TNF-α; refs 14–19). In patients with chronic inflammatory diseases, cellular or humoral factors, including TNF and IL-1, may cause suppression of the bone marrow response to Epo (20–24). There may be a role for cytokines in the impaired Epo response associated with RA. Faquin et al. (25) reported that IL-1 (α or β), TNF-α, and TGF-β inhibited production of Epo from the hepatoma cell line Hep3B. This effect appeared to occur at the level of Epo mRNA production. Jelkmann et al. (26), using the HepG2 cell line, reported similar results for IL-1 and TNF-α but noted no inhibition by TGF-β. In addition, they reported that IL-1β inhibits Epo production in isolated serum-free perfused kidneys.

Recently, La Ferla et al. (27) reported the molecular mechanisms of *Epo* gene inhibition by IL-1β or TNF-α in patients with ACD. They reported that treatment with IL-1β or TNF-α increased DNA binding activities of the transcription factors GATA-2 and NF-κB and inhibited Epo promoter activity and Epo protein production (27). Thus both GATA-2 and NF-κB seem to be involved in the suppression of *Epo* gene expression by IL-1β and TNF-α in vitro and may be responsible for the impaired Epo synthesis in inflammatory diseases in vivo (27). On the other hand, N^G-monomethyl-L-arginine (L-NMMA), a nitric oxide synthase (NOS) inhibitor, is increased in patients with chronic renal failure (28). We found that L-NMMA inhibits NO•cGMP production, increases the binding activity of GATA and mRNA expression, and inhibits Epo promoter activity (29). Therefore, one common pathogenesis of ACD and anemia with renal disease appears to be via the stimulation of GATA binding activity by IL-1β, TNF-α, or L-NMMA. K-7174 (a GATA-specific inhibitor), which acts through a mechanism independent of NF-κB activity, suppresses the binding activity of GATA proteins (30). In this study, we examined the ability of K-7174 to improve Epo production, which had been inhibited by IL-1β, TNF-α, or L-NMMA treatment in Hep3B cells in vitro and in an in vivo mouse assay.

MATERIALS AND METHODS

Cell culture

An Epo-producing hepatoma cell line (Hep3B) was obtained from the American Type Tissue Culture Collection (Rockville, MD). Cells were incubated under both 21% (normoxia) and 1% (hypoxia) oxygen for 24 h as described previously (21). Aliquots of 3×10^6 Hep3B cells were incubated with 15 U/ml recombinant human IL-1β (rhIL-1β; Roche Pharmaceuticals), 220 U/ml recombinant human TNF-α (rhTNF-α; Roche), or 10, 15, or 20 μM K-7174 (Kowa, Tokyo, Japan) (Fig. 1; ref 30) under hypoxic conditions (1% O_2) for 24 h. The hypoxic induction of the firefly luciferase (*Luc*) gene expression is represented here as a hypoxia/normoxia ratio, as described previously (31, 32).

Plasmid vectors

We used the reporter plasmid pEPLuc described by Blanchard et al. (33) as a basic plasmid construct. In this, both the 126-bp 3′ Epo enhancer and the 144-bp minimal Epo promoter were placed upstream of the *Luc* gene in pXP2 (34). The Epo enhancer corresponds to nt 120 to 245 on the 3′ side of the poly(A) addition site, and the Epo promoter corresponds to nt -118 to +26 relative to the transcription initiation site. This resulting plasmid is referred to as Pwt (35). This enhancer contained a hypoxia-inducible factor 1 (HIF-1) binding site and a steroid receptor response element (SRRE).

Transfection

Aliquots of 8×10^5 cells in tissue culture plates (10 cm^2/well: Falcon) were washed with serum-free media. A mixture containing lipofectin (Life Technologies, Inc., 20 μg/well), DNA constructs, and a LacZ expression plasmid (1 μg/well) as an internal standard was cotransfected as described previously (32). After transfection, cells were incubated with 15 U/ml rhIL-1β, 220 U/ml rhTNF-α, 10^{-3} M L-NMMA (Calbiochem), or 10 μM K-7174 (Kowa) under normoxic or hypoxic conditions for 24 h.

DNA binding assay

Nuclear extracts were prepared as described previously (36). Protein concentrations were estimated by Bio-Rad protein assay (Bio-Rad, Hertz, UK) using bovine serum albumin as a standard. A sense-strand oligonucleotide (wild-type: CATGCAGATAACAGCCCCGAC) was end-labeled with T4 polynucleotide kinase (Toyobo, Tokyo, Japan) and annealed to a fourfold excess of the unlabeled antisense oligonucleotide. Two-nanogram aliquots of the labeled probe were used in each binding reaction. The binding buffer consisted of 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 4% Ficoll, 1 mM dithiothreitol, and 75 mM KCl. An equimolar mixture of poly[d(I-C)] and poly[d(A-T)] (Sigma, St. Louis, MO; 25 ng) was used as a nonspecific competitor. The reaction mixtures (25 μl) were incubated for 15 min at 4°C and then electrophoresed on 5% nondenaturing polyacrylamide gels in 0.25 × TBE buffer (22 mM Tris borate, 22 mM boric acid, and 0.5 mM EDTA) at room temperature at 150 V for 1.5 h as described previously (36). Gels were vacuum dried, and autoradiography was performed using intensifying screens at –80°C for 24 h.

In vivo mice assay

ICR mice were purchased from Clea Japan (Tokyo) and used as healthy controls. They were housed in autoclaved metal cages and were given a standard diet (CM; Oriental Yeast, Tokyo, Japan) and tap water ad libitum in an air-conditioned room. Mice were intraperitoneally administered with recombinant mouse IL-1β (rmIL-1β; Roche), recombinant mouse TNF-α (rmTNF-α; Roche), or K-7174 (Kowa). Blood samples (0.3 ml) were obtained from the orbital vein and used for hematological analysis. Epo was measured with RIA. Thirty-six ICR mice were divided into six groups of six (A-F) to examine the effects of K-7174 on rescuing the inhibition of hemoglobin (Hb), reticulocytes, and Epo by IL-1β or TNF-α. The injection schedule for the six groups is shown in Table 1. Blood samples were obtained from the orbital vein on days 3, 6, and 10. To elucidate the effects of K-7174, IL-1β, and TNF-α on Epo production in vivo, blood samples (0.3 ml) were obtained from the orbital vein at 0, 12, 24, and 36 h. Group A was injected

with 100 μl PBS on days 0-9. Group B was injected with 1.67×10^4 U IL-1β on days 0-3. Group C was injected with 3.33×10^5 U TNF-α on days 0-3. Group D was injected with 30 mg/kg K-7174 on days 0-9. Group E was injected with 1.67×10^4 U IL-1β on days 0-3 and 30 mg/kg K-7174 on days 0-9. Group F was injected with 3.33×10^5 U TNF-α on days 0-3 and 30 mg/kg K-7174 on days 0-9.

Hematological blood parameters

Blood was collected from the retro-orbital plexus into EDTA-coated microcapillaries. Hb concentration was determined in the colorimetric way (total Hb, Sigma Diagnostics, St. Louis, MO). Reticulocyte counts were done on smears of blood that had been stained with methylene blue according to standard procedures. At least 1000 red blood cells were counted in each determination.

Other assays

Luc activity in 20 μl aliquots of the cell extract was determined using an Autolumat luminometer (Berthorude, Tokyo, Japan) for 10 s. Each measurement of relative light units was corrected by subtraction of the background and standardized to the β-galactosidase internal transfection control activity. Hypoxic inducibility was defined as the ratio of the corrected relative light units of the hypoxic (1% O_2) dish to those of the normoxic (21% O_2) dish as described previously (32). Concentrations of Epo from Hep3B cells or mice were measured by RIA as described above.

Statistical analysis

Welch tests were used to assess the level of significance between treatment groups.

RESULTS

Epo protein production, which is inhibited by IL-1β or TNF-α, is rescued with K-7174

Hypoxia-induced Epo protein production from the Hep3B cells resulted in an activity of 3753 ± 462 mU/mg protein (Fig. 2). In the presence of 15 U/ml IL-1β, the increase in Epo protein production by hypoxia was only 1514 ± 365 mU/mg protein. In the presence of 220 U/ml TNF-α, the increase in Epo protein by hypoxia was only 1422 ± 560 mU/mg protein. Addition of 10 μM K-7174 rescued the inhibition of Epo protein by IL-1β to 1878 ± 485 mU/mg protein and that of Epo protein by TNF-α to 1874 ± 297 mU/mg protein (Fig. 2). To elucidate the dose-dependent effect of K-7174 on production of Epo on Hep3B cells, different doses of K-7174 were added to the culture. Addition of 15 or 20 μM K-7174 rescued the inhibition of Epo protein production by IL-1β to 2044 ± 70 and 3065 ± 336 mU/mg protein and that of Epo protein production by TNF-α to 2442 ± 985 and 3165 ± 425 mU/mg protein, respectively (Fig. 2). These results indicate that K-7174 affects Epo production by Hep3B cells in a dose-dependent manner.

Epo promoter activity, which is inhibited by IL-1β, TNF-α, or L-NMMA, is rescued with K-7174

We previously reported that L-NMMA was not cytotoxic at concentrations of up to 10^{-2} M for Hep3B cell by the trypan blue dye exclusion method and methyl-thiazol-diphenyl-tetrazolium (MTT) method (29). Hypoxic induction of Epo promoter activity from Pwt was 33.7-fold (±4.8:

n=6) higher than that from normoxic Pwt (Fig. 3). Interestingly, the addition of 15 U/ml IL-1β, 220 U/ml TNF-α, or 10^{-3} M L-NMMA inhibited the hypoxic induction of Luc reporter gene expression from Pwt with a hypoxia/normoxia ratio of only 19.9 ± 6.5-, 20.7 ± 4.1-, or 19.8 ± 6.4-fold. The inhibitions of induction in the presence of IL-1β, TNF-α, or L-NMMA were 59.1, 61.4, or 58.8% of the induction from Pwt without IL-1β, TNF-α, or L-NMMA (Fig. 3). These results indicate that the hypoxic induction of the *Epo* gene expression is suppressed by IL-1β, TNF-α, or L-NMMA through the *Epo* gene regulatory regions.

However, the addition of 10 μM K-7174 induced hypoxic induction from Pwt to 40.3 ± 5.9-fold higher than that from normoxic Pwt (Fig. 3). Furthermore, the addition of 10 μM K-7174 to the +IL-1β, +TNF-α, or +L-NMMA rescued hypoxic inductions of Luc reporter gene expression from Pwt with hypoxia/normoxia ratios of 43.0 ± 10.1-, 49.6 ± 17.3-, and 35.6 ± 2.9-fold, respectively. The inductions with K-7174 combined with IL-1β, TNF-α, or L-NMMA were 127.6, 147.2, and 105.6% greater, respectively, than those from Pwt induced without IL-1β, TNF-α, or L-NMMA (Fig. 3). These results strongly indicate that K-7174 rescues the suppression of *Epo* gene expression by IL-1β, TNF-α, or L-NMMA through the *Epo* gene regulatory regions.

To elucidate the effect of K-7174 via the GATA site in the Epo promoter region, Pm7 (mutated: AGATAA→ATATAA) was transfected into Hep3B cells and incubated with or without IL-1β, TNF-α, L-NMMA, or K-7174 under 1% O_2 or 21% O_2. Hypoxic induction from Pm7 was 52.2 ± 7.8 (n=4)-fold higher than that from normoxic Pm7. In contrast to Pwt, the addition of 15 U/ml IL-1β, 220 U/ml TNF, or 10^{-3} M L-NMMA did not inhibit the hypoxic induction of Luc reporter gene expression from Pm7, with hypoxia/normoxia ratios of 55.7 ± 8.8-, 56.2 ± 14.8-, or 57.6 ± 17.7-fold, respectively. The addition of 10 μM K-7174 to control, +IL-1β, +TNF-α, or +L-NMMA did not induce hypoxic inductions of Luc reporter gene expression from Pm7 with a hypoxia/normoxia ratio of 61.8 ± 12.8-, 64.1 ± 12.8-, 68.4 ± 12.4-, or 59.3 ± 12.6-fold, respectively. These results indicate that K-7174 rescues the suppression of *Epo* gene expression by IL-1β, TNF-α, or L-NMMA through the GATA site of the Epo promoter and suggest the possibility of that K-7174 might be effective as a drug for treating anemia.

The binding activity of GATA is inhibited with K-7174

An electrophoretic mobility shift assay (EMSA) revealed the specific binding activity of GATA (Fig. 4, left panel, closed circle). The addition of K-7174 decreased the intensity of this band dose dependently (Fig. 4). Thus K-7174 inhibits GATA binding activity dose dependently. The addition of 15 U/ml IL-1β or 220 U/ml TNF-α enhanced binding activity of GATA (Fig. 5, lanes 3, 4). These increments of GATA binding activity by IL-1β or TNF-α were inhibited by the addition of 10 μM K-7174 (Fig. 5, lanes 6, 7). These results suggest that IL-1β or TNF-α enhance GATA binding activity, thereby inhibiting Epo promoter activity and protein production, while K-7174 reduces GATA binding activity and stimulates the Epo promoter activity and protein production.

The addition of 10^{-3} M L-NMMA enhanced the binding activity of GATA (Fig. 6, lane 3). The increment of GATA binding activity by 10^{-3} M L-NMMA was inhibited by the addition of 10 μM K-7174 (Fig. 6, lane 4). These results suggest that L-NMMA also enhances GATA binding activity and inhibits the Epo promoter activity and protein production, whereas K-7174 reduces GATA binding activity in such conditions, stimulating the Epo promoter activity and protein production. However, the addition of K-7174 did not affect HIF-1 binding activity (data not shown). The above results prompted us to investigate the effect of K-7174 in a mouse in vivo assay.

K-7174 rescues the decrease of Hb by IL-1β or TNF-α

To elucidate the effects of K-7174, IL-1β, and TNF-α on Epo production in vivo, blood samples (0.3 ml) were obtained from orbital vein at 0, 12, 24, and 36 h. Thirty-six ICR mice were divided into six groups (A-F: Fig. 7A and B). The injection schedule is shown in Table 1 (n=6: Fig. 7A and B). The Hb concentration of the control group was 14.0 ± 0.8 g/dl on day –2, 9.7 ± 0.8 g/dl on day –1, 13.6 ± 0.7 g/dl on day 3, 14.8 ± 0.7 g/dl on day 6, and 14.6 ± 0.6 g/dl on day 10 (Fig. 7A and B). Injection of IL-1β decreased the Hbs on days 3, 6, and 10 (Fig. 7). Injection of TNF-α also decreased the Hbs on these days (Fig. 7A and B). However, injection of K-7174 significantly increased the Hbs. Furthermore, K-7174 rescued the decrease of Hbs by IL-1β and TNF-α, respectively (Fig. 7A and B).

K-7174 increases the reticulocyte counts of mice

The reticulocyte counts (in permillage of total red cells) of mice in the above experiments were measured to elucidate the effect of K-7174 on reticulocyte count by mice in vivo. The reticulocyte counts were 53.4 ± 15.4‰ on day –2. The PBS control produced 247.2 ± 26.7‰ on day 3, 167.6 ± 25.8‰ on day 6, and 108.8 ± 9.4‰ on day 10, respectively. Compared with the controls, injection of IL-Iβ decreased the reticulocyte counts on days 3, 6, and 10 (Fig. 8). For the same days, injection of TNF-α also inhibited the reticulocyte counts (Fig. 8), whereas injection of K-7174 significantly increased it (Fig. 8) and injection of K-7174 significantly rescued the inhibition of the reticulocytes production by IL-Iβ or TNF-α (Fig. 8).

K-7174 increases the Epo production of mice

The serum Epo levels of mice in the above experiments were assayed by RIA to elucidate the effect of K-7174 on Epo production by mice in vivo. Serum Epo levels were 10.5 ± 5.4 mU/ml on day –2, and at day –1 the level had increased to 108.7 ± 45.9 mU/ml (Fig. 9). The PBS control produced 17.8 ± 2.2 mU/ml on day 3, 12.8 ± 0.7 mU/ml on day 6, and 13.6 ± 0.8 mU/ml on day 10, respectively. Compared with the controls, injection of IL-1β decreased Epo production on days 3, 6, and 10 (Fig. 9). For the same days, injection of TNF-α also inhibited Epo production (Fig. 9), whereas injection of K-7174 significantly increased it (Fig. 9) and injection of K-7174 significantly rescued the inhibition of Epo production by IL-1β or TNF-α (Fig. 9). These results clearly show that K-7174 increased Epo production, reticulocyte counts, and Hb concentrations.

DISCUSSION

We have shown here that K-7174 has the ability to rescue the inhibition of Epo promoter activity and Epo protein production by various cytokines. By in vitro cell assay, 15 U/ml IL-1β and 220 U/ml TNF-α inhibited Epo protein production. Addition of 10 μM K-7174 rescued these inhibitions of Epo protein production. The three treatments, 15 U/ml IL-1β, 220 U/ml TNF-α, and 10^{-3} M L-NMMA, each inhibited Epo promoter activity, but the addition of K-7174 reversed this. The EMSA revealed that addition of K-7174 decreased GATA binding activity, which in turn was increased with the addition of IL-1β, TNF-α, or L-NMMA. Intraperitoneal injections of IL-1β or TNF-α into mice decreased Epo production, reticulocyte counts, and Hb concentrations. However, the administration of K-7174 rescued Epo production, reticulocyte counts, and Hb concentration induced by IL-Iβ or TNF-α from these inhibitions. The main reason of this mechanism might be dependent on the stimulation of Epo production by K-7174. This increment of Epo by K-7174

stimulates proliferation of the erythroid progenitor cells, releasing reticulocytes from bone marrow and spleen to peripheral blood (37). On the other hand, down-regulation of *GATA-1* gene expression is needed for the terminal maturation of erythroid cells (38 and unpublished data). Therefore, direct inhibition of the DNA binding activity of GATA-1 by K-7174 might induce to release the reticulocytes into peripheral blood rapidly.

K-7174 was developed as a low molecular weight anti-inflammatory drug and is known to be a specific inhibitor of GATA (30). Umetani et al. (30) reported that K-7174 inhibited the expression of vascular cell adhesion molecule-1 (VCAM-1) induced by either TNF-α or IL-1β, without affecting the induction of intracellular adhesion molecule-1 or E-selectin. K-7174 had no effect on the stability of VCAM-1 mRNA (30). EMSA revealed that its inhibitory effect on VCAM-1 induction was mediated by an effect on the binding to the GATA motifs in the *VCAM-1* gene promoter region (30). K-7174 does not influence binding to any of the following binding motifs: octamer binding protein, AP-1, SP-1, ets, NF-κB, or interferon regulatory factor (30). The inhibitory effect of K-7174 on GATA seems to be via inhibition of GATA acetylation or through the stimulation of GATA de-acetylation (30, 39). Inhibition of acetylation of transcription factors induces inhibition of DNA binding (40, 41). Sano et al. (40) reported that acetylation of c-Myb by CREB binding protein (CBP) increases the trans-activating capacity of c-Myb by enhancing its association with CBP. The p53 tumor suppressor is a transcriptional factor whose activity is modulated by protein stability and posttranslational modifications including acetylation. Luo et al. (41) showed that the de-acetylation of p53 strongly represses p53-dependent transcriptional activation.

GATA proteins are types of zinc finger transcription factors that recognize the consensus (A/T) GATA (A/G) sequence or related sequences (42, 43). GATA proteins are also essential for the differentiation and function of hematopoietic cells (44). Intraperitoneal injection of 30 mg/kg K-7174 into ICR mice produced no significant change in the weight of the spleen or the numbers of white blood cells and platelets (data not shown). A dose of 300 mg/kg for rats (10 times higher than the dose that was used in this study) revealed no adverse effects on the liver, kidney, or hematopoiesis (data not shown). Furthermore, K-7174 decreases the production of circulating glutamic-oxaloacetic transaminase (GOT) and lactate dehydrogenase (LDH), which are increased by the injection of IL-1β and TNF-α into ICR mice (data not shown). These results demonstrate that the anemia induced by IL-1β or TNF-α can be prevented by the simultaneous administration of K-7174.

Papadaki et al. (45) recently found that patients with RA exhibit a low frequency and increased apoptosis of bone marrow erythroid progenitors and precursor cells because of increased local production of TNF-α. They reported that blocking the activity of TNF-α with a specific chimeric human/mouse monoclonal antibody improves the ACD seen in patients with RA and the beneficial effect of the treatment is mediated by down-regulating the TNF-α-induced apoptotic mechanisms in bone marrow (45). However, deprivation of Epo induces apoptosis of immature erythroid colony-forming cells through down-regulation of the Bcl-X (L) anti-apoptotic protein (46). Thus ACD is not only caused by increased TNF-α but also by IL-1β. Thus TNF-α blockade may not be a suitable therapy for patients with ACD caused by cancer.

This study raises the possibility of using K-7174 as a novel drug for treating ACD and for anemia associated with renal disease. We are presently investigating the effect of K-7174 using DBA/2FG-pcy mice as a model of anemia associated with renal disease.

ACKNOWLEDGMENTS

We thank Wolfgang Jelkmann for helpful discussions and suggestions. We also thank H. Tanaka and A. Hoshino for expert technical assistance. This work was supported by grants-in-aid for scientific research from the Ministry of Education, Science and Culture of Japan, and the Kowa Foundation, Tokyo, Japan.

REFERENCES

1. Douglas, S. W., and Adamson, J. W. (1975) The anemia of chronic disorders: studies of marrow regulation and iron metabolism. *Blood* **45,** 55–65

2. Zarrabi, M. H., Lysik, R., DiStefano, J., and Zucker, S. (1977) The anemia of chronic disorders: studies of iron reutilization in the anemia of experimental malignancy and chronic inflammation. *Br. J. Haematol.* **35,** 647–658

3. Hochberg, M. C., Arnold, C. M., Hogans, B. B., and Spivak, J. L. (1988) Serum immunoreactive erythropoietin in rheumatoid arthritis: impaired response to anemia. *Arthritis Rheum.* **31,** 1318–1321

4. Miller, C. B., Jones, R. J., Piantadosi, S., Abeloff, M. D., and Spivak, J. L. (1990) Decreased erythropoietin response in patients with the anemia of cancer. *N. Engl. J. Med.* **322,** 1689–1692

5. Baer, A. N., Dessypris, E. N., Goldwasser, E., and Krantz, S. B. (1987) Blunted erythropoietin response to anemia in rheumatoid arthritis. *Br. J. Haematol.* **66,** 559–564

6. Johannsen, H., Jelkmann, W., Wiedemann, G., Otte, M., and Wagner, T. (1989) Erythropoietin/haemoglobin relationship in leukaemia and ulcerative colitis. *Eur. J. Haematol.* **43,** 201–206

7. Spivak, J. L., Barnes, D. C., Fuchs, E., and Quinn, T. C. (1989) Serum immunoreactive erythropoietin in HIV-infected patients. *JAMA* **261,** 3104–3107

8. Pincus, T., Olsen, N. J., Russell, I. J., Wolfe, F., Harris, E. R., Schnitzer, T. J., Boccagno, J. A., and Krantz, S. B. (1990) Multicenter study of recombinant human erythropoietin in correction of anemia in rheumatoid arthritis. *Am. J. Med.* **89,** 161–168

9. Means, R. T., Olsen, N. J., Krantz, S. B., Dessypris, E. N., Graber, S. E., Stone, W. J., O'Neil, V. L., and Pincus, T. (1989) Treatment of the anemia of rheumatoid arthritis with recombinant human erythropoietin: clinical and in vitro studies. *Arthr. Rheum.* **32,** 638–642

10. Eschbach, J. W., Egrie, J. C., Downing, M. R., Browne, J. K., and Adamson, J. W. (1987) Correction of the anemia of end-stage renal disease with recombinant human erythropoietin: results of a combined Phase I and II clinical trial. *N. Engl. J. Med.* **316,** 73–78

11. Ludwig, H., Elke, F., Kotsmann, H., Hocker, P., Gisslinger, H., and Barnas, U. (1990) Erythropoietin treatment of anemia associated with multiple myeloma. *N. Engl. J. Med.* **322,** 1693–1699

12. Fischl, M., Galpin, J. E., Levine, J. D., Groopman, J. E., Henry, D. H., Kennedy, P., Miles, S., Robbins, W., Starrett, B., Zalusky, R., et al. (1990) Recombinant human erythropoietin for patients with AIDS treated with zidovudine. *N. Engl. J. Med.* **322,** 1488–1493

13. Rudnick, S., Abels, R., and Danna, R. (1990) Correction of chronic anemia in cancer patients by r-HuEpo (EPREX). *Proc. Am. Soc. Clin. Oncol.* **9,** 182

14. Lee, G. R. (1983) The anemia of chronic disease. *Semin. Hematol.* **20,** 61–80

15. Dinarello, C. A. (1989) Interleukin-1 and its related cytokines. *Cytokines* **1,** 105

16. Bendtzen, K. (1988) Interleukin 1, interleukin 6 and tumor necrosis facor in infection, inflammation and immunity. *Immunol. Lett.* **19,** 183–191

17. Dowton, S. B., and Colten, H. R. (1988) Acute phase reactants in inflammation and infection. *Semin. Hematol.* **25,** 84–90

18. Levine, B., Kalman, J., Mayer, L., Fillit, H. M., and Padker, M. (1990) Elevated circulating levels of tumor necrosis factor in severe chronic heart failure. *N. Engl. J. Med.* **323,** 236–241

19. Border, W. A., Okuda, S., Languino, L. R., Sporn, M. B., and Ruoslahti, E. (1990) Suppression of experimental glomerulonephritis by antiserum against transforming growth factor β1. *Nature* **346,** 371–374

20. Johnson, C. S., Cook, C. A., and Furmanski, P. (1990) In vivo suppression of erythropoiesis by tumor necrosis factor-α (TNF-α): reversal with exogenous erythropoietin (Epo). *Exp. Hematol.* **18,** 109–113

21. Schooley, J. C., Kullgren, B., and Allison, A. C. (1987) Inhibition by interleukin-1 of the action of erythropoietin on erythroid precursors and its possible role in the pathgenesis of hypoplastic anaemias. *Br. J. Haematol.* **67,** 11–17

22. Ulich, T. R., Castillo, J., and Yin, S. (1990) Tumor necrosis factor exerts dose-dependent effects on erythropoiesis and myelopoiesis in vivo. *Exp. Hematol.* **18,** 311–315

23. Clinbon, U., Bonewald, L., Caro, J., and Roodman, G. D. (1990) Erythropoietin fails to reverse the anemia in mice continuously exposed to tumor necrosis factor-alpha in vivo. *Exp. Hematol.* **18,** 438–441

24. Means, R. T., Dessypris, E. N., and Krantz, S. B. (1990) Inhibition of human colony-forming-unit erythroid by tumor necrosis factor requires accessory cells. *J. Clin. Invest.* **86,** 538–541

25. Faquin, W. C., Schneider, T. J., and Goldberg, M. A. (1992) Effect of inflammatory cytokines on hypoxia-induced erythropoietin production. *Blood* **79,** 1987–1994

26. Jelkmann, W., Pagel, H., Wolff, M., and Fandrey, J. (1991) Monokines inhibiting erythropoietin production in human hepatoma cultures and in isolated perfused rat kidneys. *Life Sci.* **50,** 301–308

27. La Ferla, K., Reimann, C., Jelkmann, W., and Hellwig-Bürgel, T. (2002) Inhibition of erythropoietin gene expression signaling involves transcription factors GATA-2 and NF-κB. *FASEB J.* **16,** 1811–1813

28. Ribero, A. C. M., Roberts, N. B., Lane, C., Yaqoob, M., and Ellory, J. C. (1996) Accumulation of the endogenous L-arginine analogue N^G-monomethyl L-arginine in human end-stage renal failure patients on regular haemodialysis. *Exp. Physiol.* **81,** 475–481

29. Tarumoto, T., Imagawa, S., Ohmine, K., Nagai, T., Higuchi, M., Imai, N., Suzuki, N., Yamamoto, M., and Ozawa, K. (2000) N^G-monomethyl L-arginine inhibits erythropoietin gene expression by stimulating GATA-2. *Blood* **96,** 1716–1722

30. Umetani, M., Nakao, H., Doi, T., Iwasaki, A., Ohtaka, M., Nagoya, T., Mataki, C., Hamakubo, T., and Kodama, T. (2000) A novel cell adhesion inhibitor, K-7174, reduces the endothelial VCAM-1 induction by inflammatory cytokines, acting through the regulation of GATA. *Biochem. Biophys. Res. Commun.* **272,** 370–374

31. Imagawa, S., Goldberg, M. A., Doweiko, J., and Bunn, H. F. (1991) Regulatory elements of the erythropoietin gene. *Blood* **77,** 278–285

32. Imagawa, S., Yamamoto, M., and Miura, Y. (1997) Negative regulation of the erythropoietin gene expression by the GATA transcription factors. *Blood* **89,** 1430–1439

33. Blanchard, K. L., Acquaviva, A. M., Galson, D. L., and Bunn, H. (1992) Hypoxic induction of the human erythropoietin gene: cooperation between the promoter and enhancer, each of which contains steroid receptor response elements. *Mol. Cell. Biol.* **12,** 5373–5385

34. Nordeen, S. K. (1989) Luciferase reporter gene vectors for analysis of promoters and enhancers. *Biol. Techniques* **6,** 454–458

35. Galson, D. L., Tsuchiya, T., Tendler, D. S., Huang, L. E., Ren, Y., Ogura, T., and Bunn, H. F. (1995) The orphan receptor hepatic nuclear factor 4 functions as a transcriptional activator for tissue-specific and hypoxia-specific erythropoietin gene expression and is antagonized by EAR3/COUP-TF1. *Mol. Cell. Biol.* **1,** 2135–2144

36. Yamamoto, M., Ko, L. J., Leonard, M. W., Beng, H., Orkin, S. H., and Engel, J. D. (1990) Activity and tissue-specific expression of the transcription factor NF-E1 multi gene family. *Genes Dev.* **4,** 1650–1662

37. Reiff, R. H., Nutter, J. Y., Donohue, D. N., and Finch, C. A. (1958) The relative number of marrow reticulocytes. *Am. J. Clin. Pathol.* **30,** 199–203

38. Whyatt, D., Lindeboom, F., Karis, A., Ferreira, R., Milot, E., Hendriks, R.,de Bruijn, M., Langeveld, A., Gribnau, J., Grosveld, F., and Philipsen, S. (2000) An intrinsic but cell-nonautonomous defect in GATA-1-overexpressing mouse erythroid cells. *Nature* **406,** 519-524

39. Boyes, J., Byfield, P., Nakatani, Y., and Ogryzko, V. (1998) Regulation of activity of the transcription factor GATA-1 by acetylation. *Nature* **396,** 594-598

40. Sano, Y., and Ishii, S. (2001) Increased affinity of c-Myb for CREB-binding protein (CBP) after CBP-induced acetylation. *J. Biol. Chem.* **276,** 3674–3682

41. Luo, J., Su, F., Chen, D., Shilloh, A., and Gu, W. (2000) Deacetylation of P53 modulate its effect on cell growth and apoptosis. *Nature* **408,** 377–381

42. Merika, M., and Orkin, S. H. (1993) DNA-binding specificity of GATA family transcription factors. *Mol. Cell. Biol.* **13,** 3999–4010

43. Ko, L. J., and Engel, J. D. (1993) DNA binding specificities of the GATA transcription factor family. *Mol. Cell. Biol.* **13,** 4011–4022

44. Shivdasarni, R. A., and Orkin, S. H. (1996) The transcriptional control of hematopoiesis. *Blood* **87,** 4025–4039

45. Papadaki, H. A., Kritikos, H. D., Valatas, V., Boumpas, D. T., and Eliopoulos, G. D. (2002) Anemia of chronic disease in rheumatoid arthritis is associated with increased apoptosis of bone marrow erythroid cells: improvement following anti-tumor factor-α antibody therapy. *Blood* **100,** 474–482

46. Silva, M., Grillot, D., Benito, A., Richard, C., Nunez, G., and Fernandez-Luna, J. L. (1996) Erythropoietin can promote erythroid progenitor survival by repressing apoptosis through Bcl-XL and Bcl-2. *Blood* **88,** 1576–1582

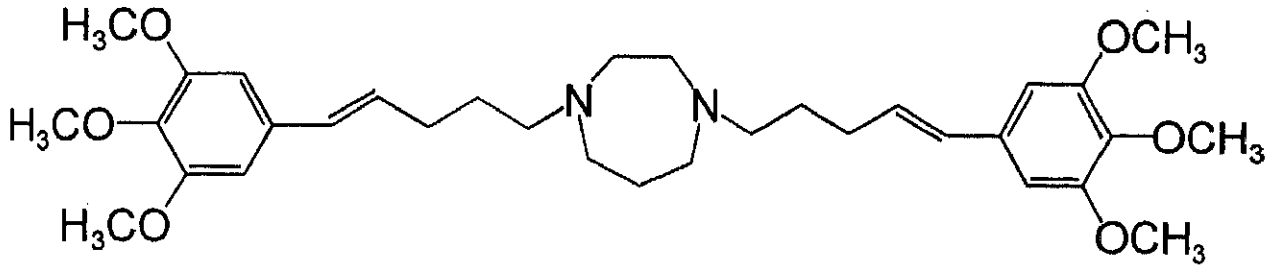
Received November 26, 2002; accepted May 19, 2003.

Table 1

Injection schedule of groups of mice used to examine the effects on K-7174 on rescuing the inhibitions by IL-1β or TNF-α

Purpose (Group Size)	Group	Injection		Day									
		Item	Amount	0	1	2	3	4	5	6	7	8	9
Effects on hemo-globin and Epo (n=6)	A	PBS	100 μl	x	x	x	x	x	x	x	x	x	x
	B	IL-1β	1.67×10^4 U	x	x	x	x						
	C	TNF-α	3.33×10^5 U	x	x	x	x						
	D	K-7174	30 mg/kg	x	x	x	x	x	x	x	x	x	x
	E	IL-1β	1.67×10^4 U	x	x	x	x						
		K-7174	30 mg/kg	x	x	x	x	x	x	x	x	x	x
	F	TNF-α	3.33×10^5 U	x	x	x	x						
		K-7174	30 mg/kg	x	x	x	x	x	x	x	x	x	x

Fig. 1



K-7174

Figure 1. Chemical structure of K-7174.

Fig. 2

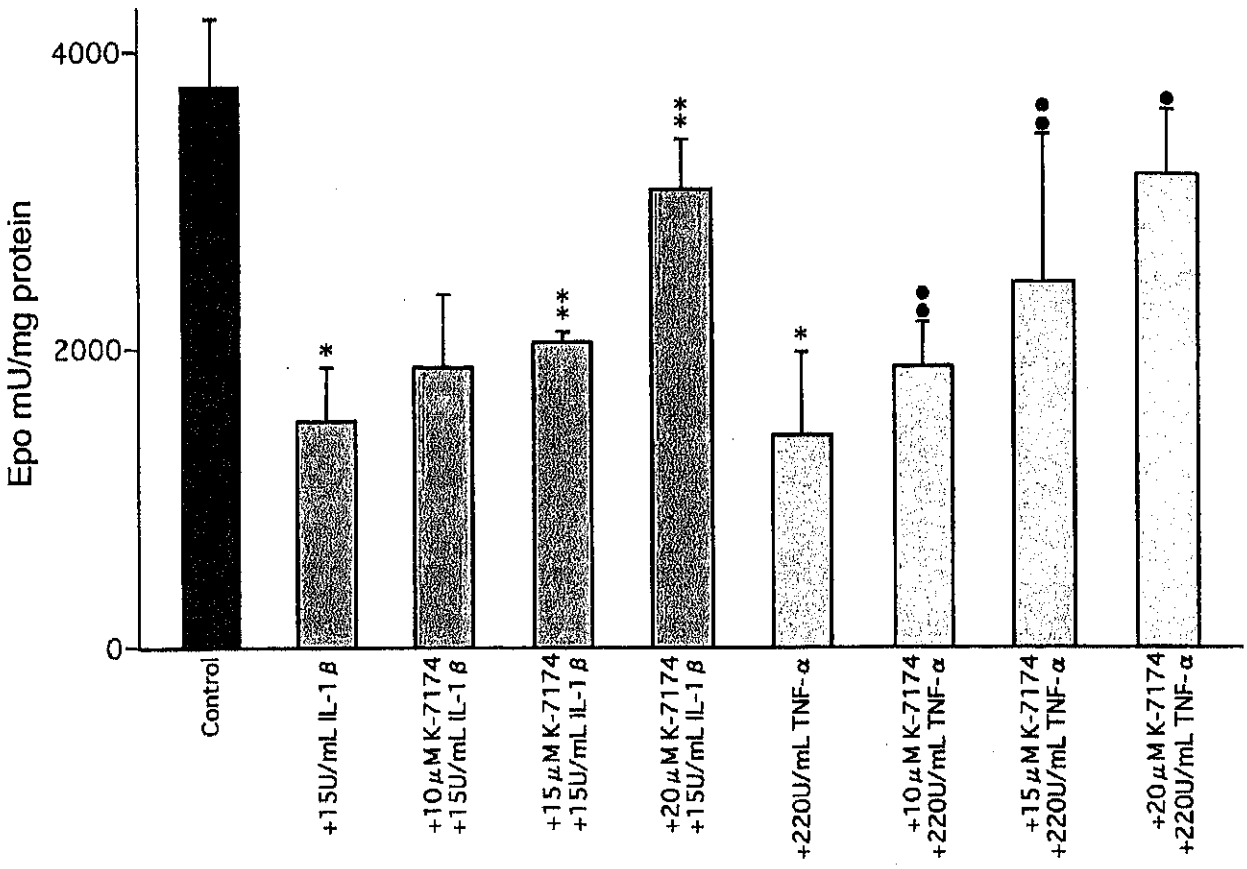


Figure 2. Effects of different doses of K-7174 on the inhibition of Epo protein production from Hep3B cells by IL-1β or TNF-α. Aliquots of 3×10^6 Hep3B cells were incubated with 15 U/ml rhIL-1β, 220 U/ml rhTNF-α, or 10, 15, or 20 μM K-7174 under hypoxic conditions (1% O_2) for 24 h. The expression level of Epo protein was measured with RIA. Separate experiments were performed (n=8). Error bars are 1SD. *Significance compared with control, $P < 0.005$. **Significance compared with 15 U/ml IL-1β, $P < 0.005$. •Significance compared with 220 U/ml TNF-α, $P < 0.005$. ••Significance compared with 220 U/ml TNF-α, $P < 0.025$.

Fig. 3

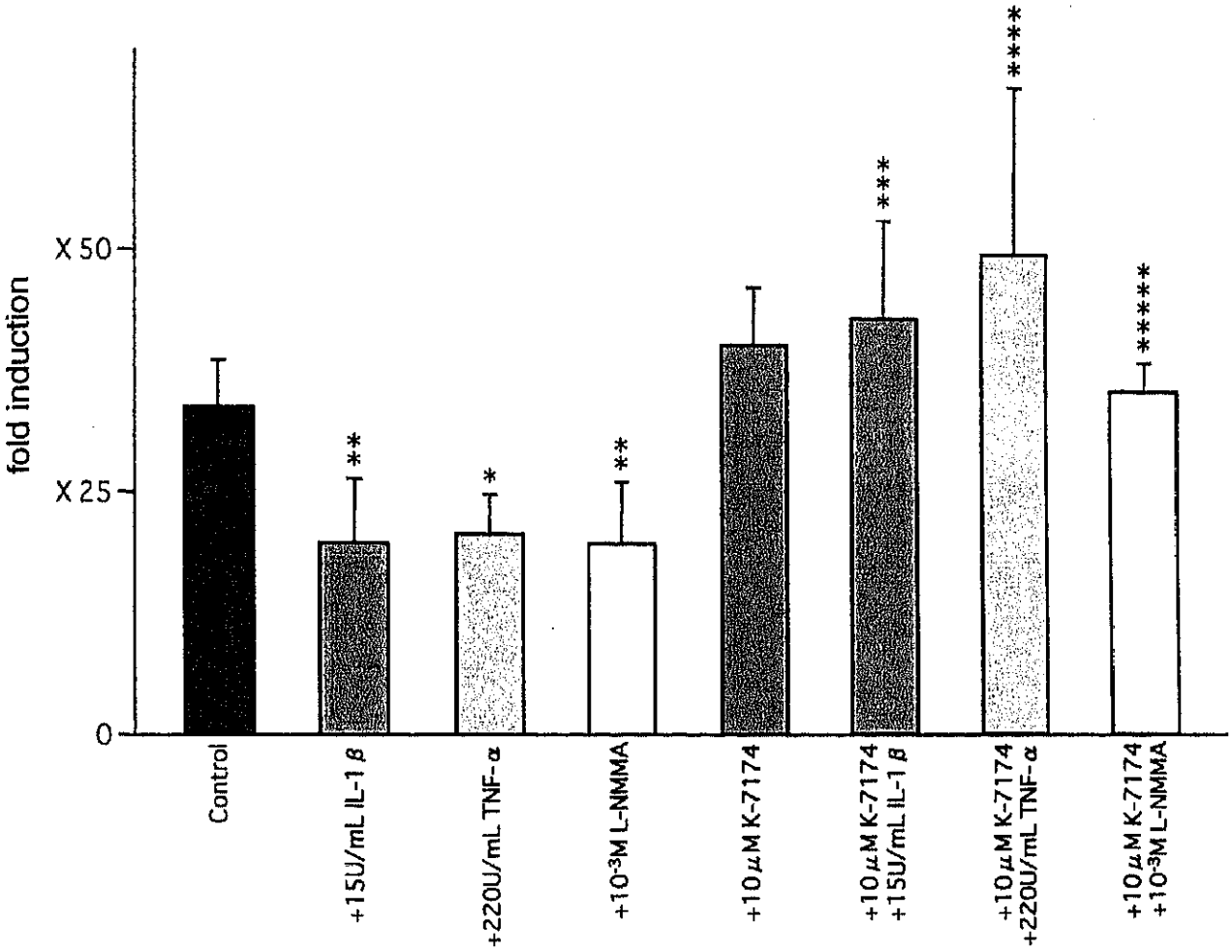


Figure 3. Effects of K-7174 on the inhibition of induction of the wild-type (Pwt) Epo promoter/enhancer with Luc reporter construct in Hep3B cells by IL-1β, TNF-α, or L-NMMA. Wild-type (Pwt) Epo promoter/enhancer with Luc reporter construct was transfected into 8×10^5 Hep3B cells and incubated with 15 U/ml rhIL-1β, 220 U/ml rhTNF-α, 10^{-3} M L-NMMA, or 10 μM K-7174 under normoxic (21% O_2) or hypoxic conditions (1% O_2) for 24 h. Hypoxic induction of Luc gene expression is represented here as a hypoxia/normoxia ratio. The fold induction reported indicates this hypoxia/normoxia ratio. Six separate experiments (quadruple samples) were performed (n=6). Error bars are 1SD. *Significance compared with control, $P < 0.005$. **Significance compared with control, $P < 0.01$. ***Significance compared with IL-1β, $P < 0.005$.

Fig. 4

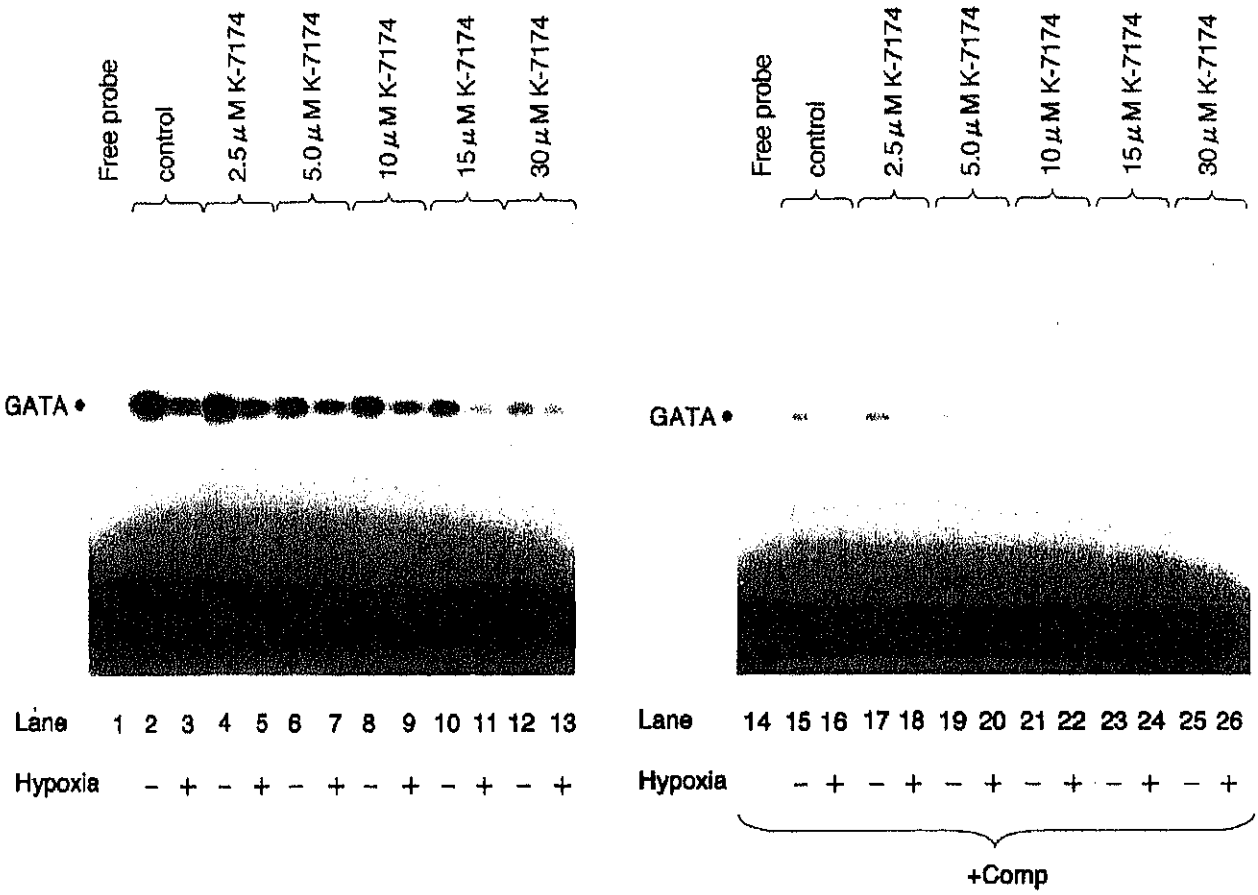


Figure 4. Effects of different doses of K-7174 on the expression of GATA. Electrophoretic mobility shift assay (EMSA) was performed using 1 μg of protein from Hep3B cells under normoxia (lanes 2, 4, 6, 8, 10, 12, 15, 17, 19, 21, 23, and 25) and hypoxia (lanes 3, 5, 7, 9, 11, 13, 16, 18, 20, 22, 24, and 26) and from Hep3B cells incubated with 2.5 μM K-7174 (lanes 4, 5, 17, and 18), 5 μM K-7174 (lanes 6, 7, 19, and 20), 10 μM K-7174 (lanes 8, 9, 21, and 22), 15 μM K-7174 (lanes 10, 11, 23, and 24), and 30 μM K-7174 (lanes 12, 13, 25, and 26) for 24 h. The dot at the left is the position of the GATA transcription factor. A total of 25 ng (0.5 μl: 12.5-fold molar excess) of competitor DNA was added to each reaction mixture (lanes 15–26).

Fig. 5

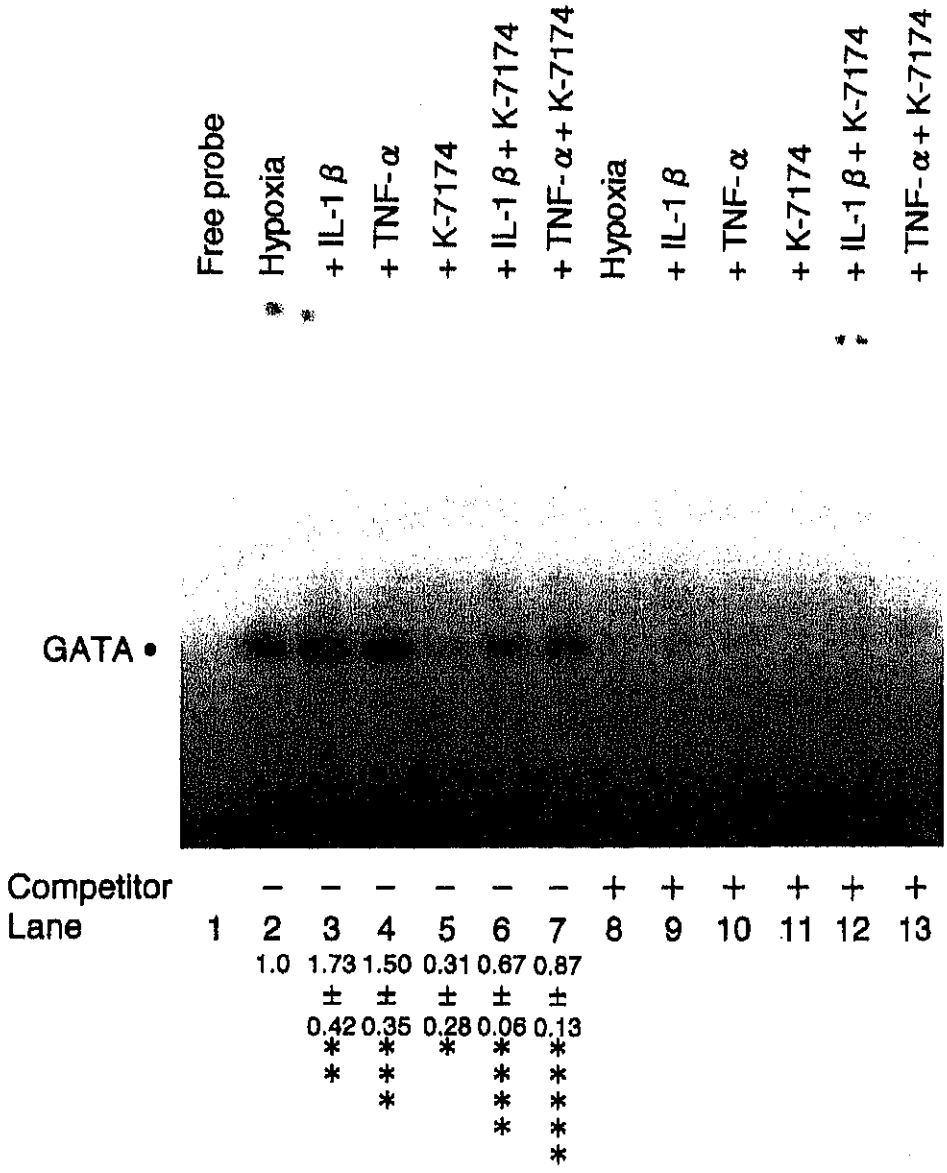


Figure 5. Effects of K-7174 on the enhanced expression of GATA by IL-1β or TNF-α. EMSA was performed using 1 μg of protein from Hep3B cells under hypoxia (lanes 2-13) and incubated with 15 U/ml rhIL-1β (lanes 3 and 9), 220 U/ml rhTNF-α (lanes 4 and 10), 10 μM K-7174 (lanes 5 and 11), 15 U/ml rhIL-1β plus 10 μM K-7174 (lanes 6 and 12), and 220 U/ml rhTNF-α plus 10 μM K-7174 (lanes 7 and 13) for 24 h. The dot at the left is the position of the GATA transcription factor. A total of 25 ng (0.5 μl: 12.5-fold molar excess) of competitor DNA was added to each reaction mixture (lanes 8–13). Numbers below the bands are means ± 1SD by the densitometric analysis of the band indicated by the circle in panel. Five experiments using different nuclear extracts were performed (n=5). *Significance compared with hypoxia, $P < 0.01$. **Significance compared with hypoxia, $P < 0.025$. ***Significance compared with hypoxia, $P < 0.05$. ****Significance compared with IL-Iβ, $P < 0.01$. *****Significance compared with TNF-α, $P < 0.025$.

Fig. 6

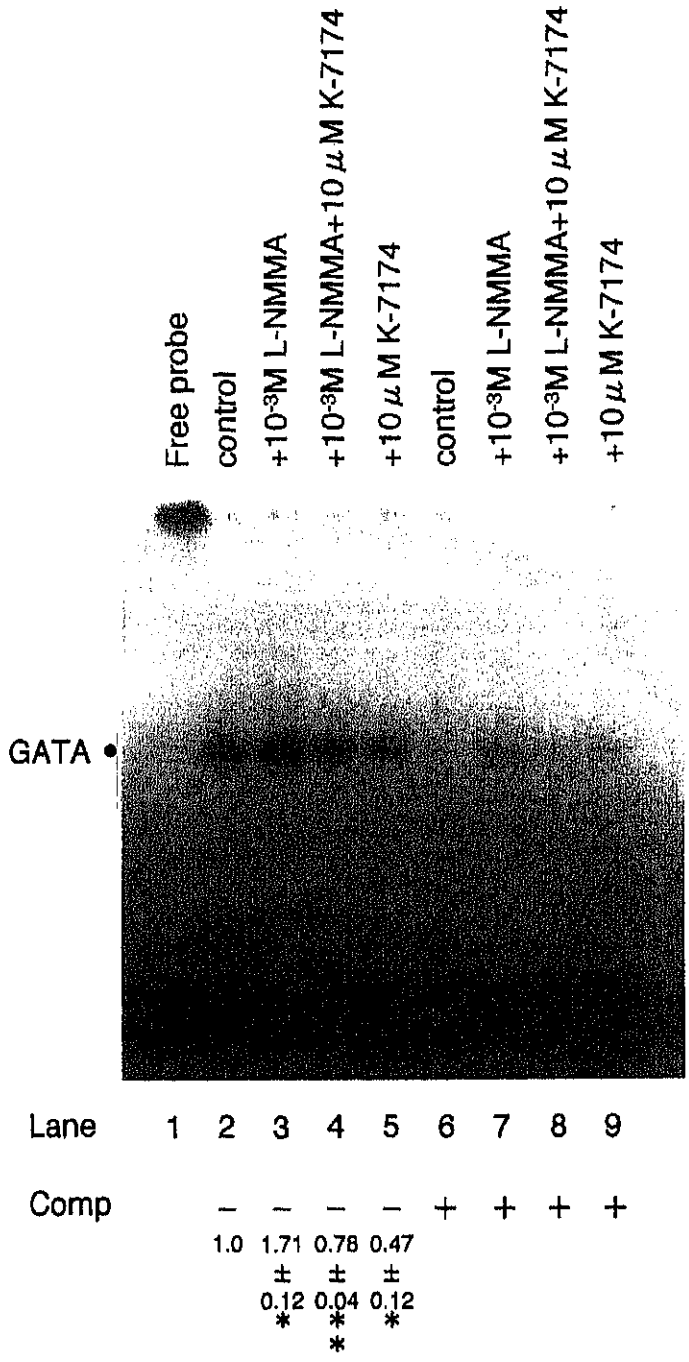


Figure 6. Effects of K-7174 on the enhanced expression of GATA induced by L-NMMA. EMSA was performed using 1 μg of protein from Hep3B cells under normoxia (21% O_2) (lanes 2–9) and incubated with 10^{-3} M L-NMMA (lanes 3 and 7), 10^{-3} M L-NMMA plus 10 μM K-7174 (lanes 4 and 8), and 10 μM K-7174 (lanes 5 and 9), for 24 h. The dot at the left is the position of the GATA transcription factor. A total of 25 ng (0.5 μl: 12.5-fold molar excess) of competitor DNA was added to each reaction mixture (lanes 6–9). Numbers below the bands are means ± 1SD by the densitometric analysis of the band indicated by the circle in panel. Five experiments using different nuclear extracts were performed (n=5). *Significance compared with hypoxia, $P < 0.005$. **Significance compared with L-NMMA, $P < 0.005$.

Fig. 7

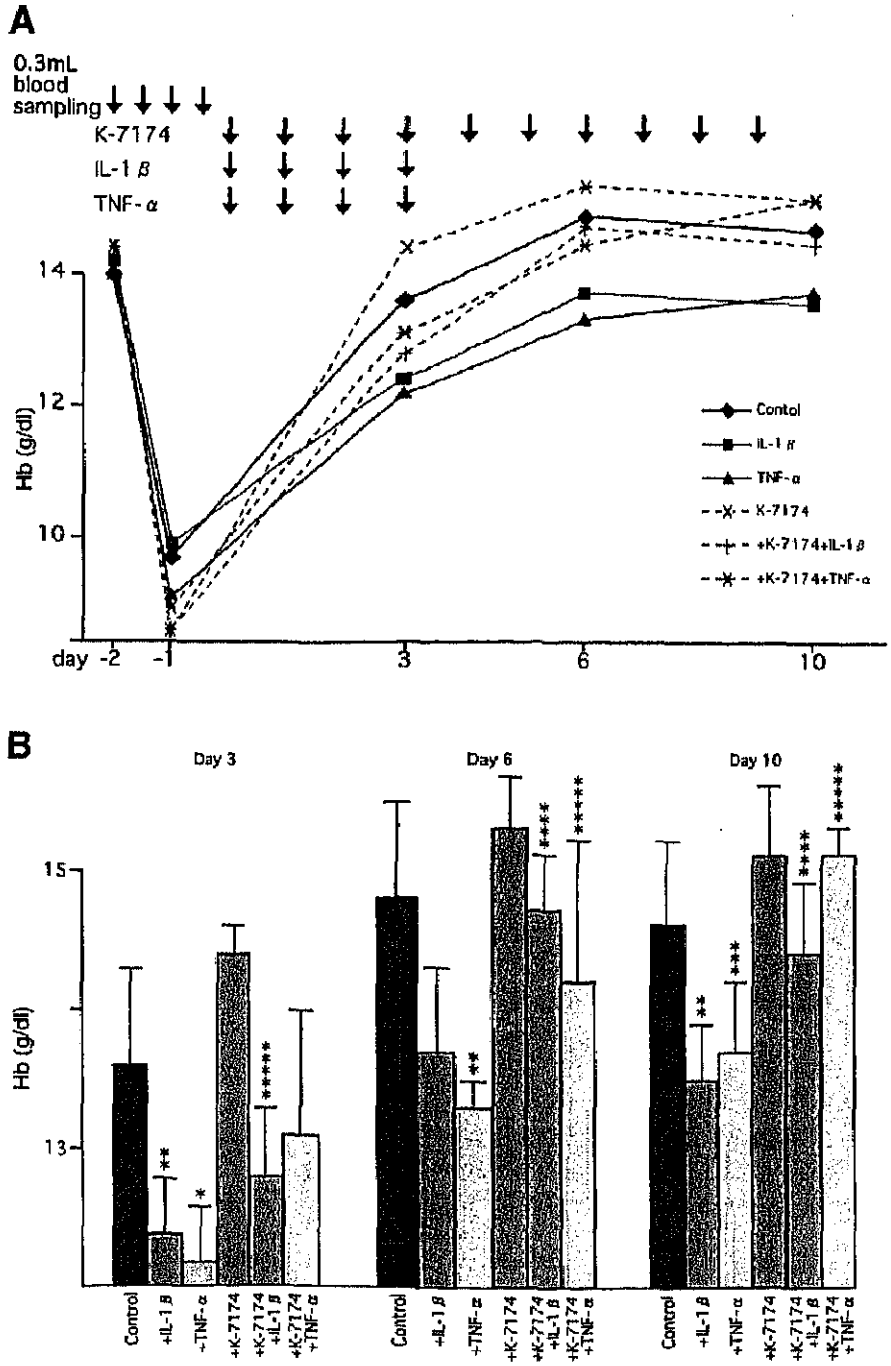


Figure 7. Effects of K-7174 on the decrease of hemoglobin (Hb) concentrations from ICR mice induced by IL-1β or TNF-α. A) ICR mice were injected intraperitoneally with 0.1 ml of PBS (control) on days 0–9, 1.67×10^4 U rmIL-β on days 0–3, 3.33×10^5 U rmTNF-α on days 0–3, and 0.1 ml of 30 mg/kg K-7174 on days 0–9 (n=6). Blood samples (0.3 ml) were taken from the orbital vein at 0, 12, 24, 36 h, and days 3, 6, and 10. B) *Significance compared with control, $P < 0.005$. **Significance compared with control, $P < 0.01$. ***Significance compared with control, $P < 0.05$. ****Significance compared with IL-Iβ, $P < 0.01$. *****Significance compared with IL-Iβ, $P < 0.05$. ******Significance compared with TNF-α, $P < 0.005$.

Fig. 8

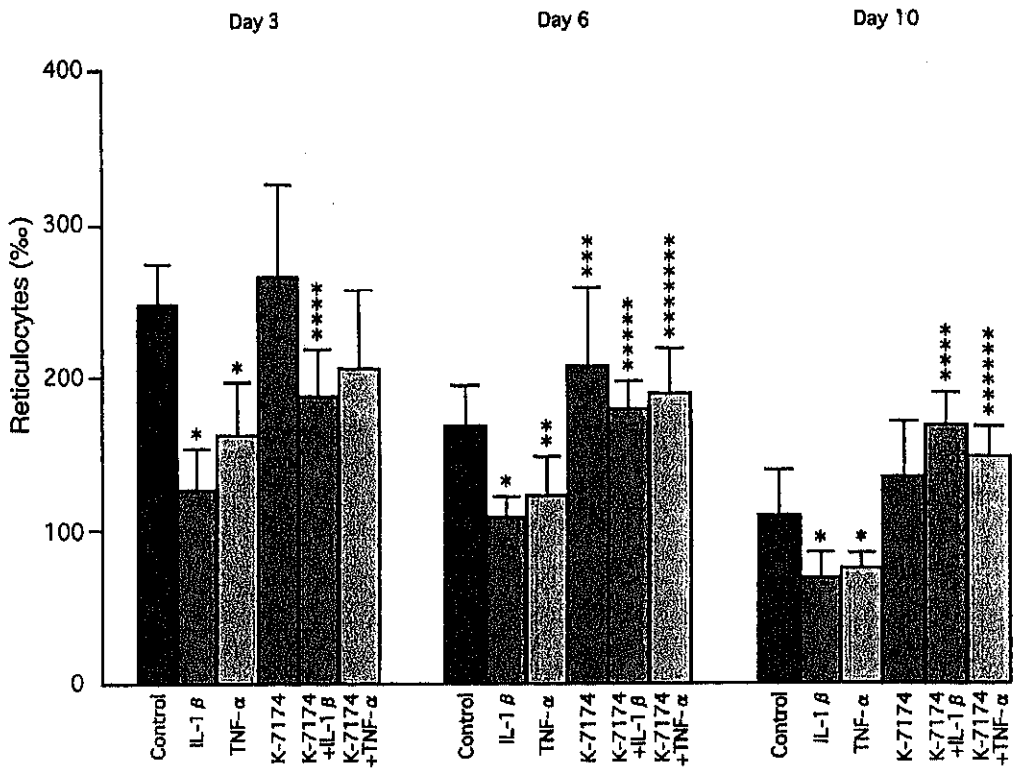


Figure 8. Effects of K-7174 on the reticulocyte counts from ICR mice induced by IL-Iβ or TNF-α. Experimental conditions were the same as described in the **Fig. 7** legend. *Significance compared with control, $P < 0.005$. **Significance compared with control, $P < 0.025$. ***Significance compared with control, $P < 0.05$. ****Significance compared with IL-1β, $P < 0.005$. *****Significance compared with IL-1β, $P < 0.01$. ******Significance compared with TNF-α, $P < 0.005$. *******Significance compared with TNF-α, $P < 0.05$.

Fig. 9

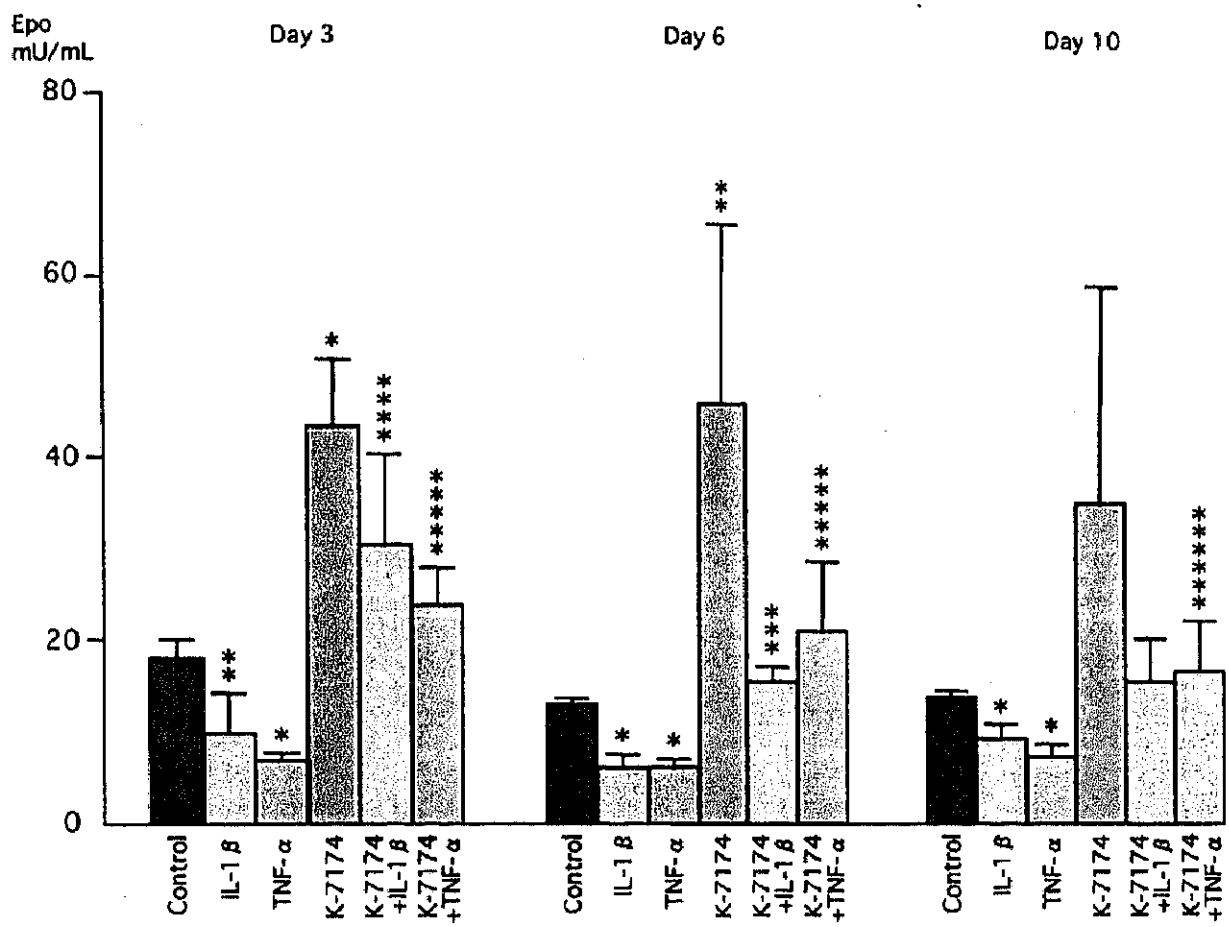


Figure 9. Effects of K-7174 on the decrease of serum concentrations of Epo in ICR mice induced by IL-1β or TNF-α. Experimental conditions were the same as described in the **Fig. 7** legend. Serum Epo levels were determined by RIA. *Significance compared with control, $P < 0.005$. **Significance compared with control, $P < 0.025$. ***Significance compared with IL-1β, $P < 0.005$. ****Significance compared with IL-1β, $P < 0.025$. *****Significance compared with TNF-α, $P < 0.005$. ******Significance compared with TNF-α, $P < 0.025$.

7.

他

Identification and characterization of 2 types of erythroid progenitors that express GATA-1 at distinct levels

Norio Suzuki, Naruyoshi Suwabe, Osamu Ohneda, Naoshi Obara, Shigehiko Imagawa, Xiaoqing Pan, Hozumi Motohashi, and Masayuki Yamamoto

Transcription factor GATA-1 is essential for the development of the erythroid lineage. To ascertain whether strict control of GATA-1 expression level is necessary for achieving proper erythropoiesis, we established transgenic mouse lines expressing green fluorescent protein (GFP) under the control of the *GATA-1* gene hematopoietic regulatory domain. We examined the *GATA-1* expression level by exploiting the transgenic mice and found 2 GFP-positive hematopoietic progenitor fractions in the bone marrow. One is the GFP^{high} fraction containing mainly CFU-E and proerythroblasts, which coexpress transferrin receptor, while the other is the GFP^{low}/transferrin receptor–negative fraction containing BFU-E. Since the intensity of green fluorescence correlates well with the expression level of GATA-1, these results indicate that GATA-1 is highly expressed in erythroid colony-forming unit (CFU-E) but low in erythroid burst-forming unit (BFU-E), suggesting that the incremental expression of GATA-1 is required for the formation of erythroid progenitors. We also examined GFP-positive fractions in the transgenic mouse spleen and fetal liver and identified fractions containing BFU-E and CFU-E, respectively. This study also presents an efficient method for enriching the CFU-E and BFU-E from mouse hematopoietic tissues. (Blood. 2003;102:3575-3583)

Introduction

Pluripotent hematopoietic progenitors are committed to and differentiated along the erythroid lineage by the control of various cytokines, growth factors, and signals from the microenvironment.[1] In hematopoietic progenitors, these signals are transduced to transcription factors in the nucleus, and the progenitor cells differentiate and mature into erythrocytes by changing their gene expression profiles.[2] GATA-1 is a zinc-finger transcription factor, which plays a central role in erythropoiesis. GATA-1 binds to the GATA factor-binding motifs (T/A)GATA(A/G) that have been identified in the regulatory sequences of many genes expressed in erythroid and megakaryocytic cells.[3-5] Expression of GATA-1 is restricted to erythroid, megakaryocytic, eosinophilic, basophilic, and mast cells within the hematopoietic system.[6] The Sertoli cells in the testis also express GATA-1.[7]

Mutations in the *GATA-1* gene cause defects in erythropoiesis, platelet formation, mast cell maturation, and eosinophil development.[8-14] GATA-1–null mutant embryonic stem (ES) cells could not differentiate into mature erythrocytes due to the arrest of erythroid maturation at the proerythroblast stage.[15,16] The arrest provoked rapid apoptosis of the proerythroblasts.[17] We previously established *GATA-1* gene knockdown ES cells, in which the GATA-1 expression level was reduced to approximately 5% of that in wild-type ES cells.[10,18] Proerythroblast-like cells derived from the GATA-1 knockdown ES cells have an ability to proliferate vigorously, but a GATA-1 level of 5% cannot sustain the gene expression required for maturation of proerythroblasts.[18] McDevitt et al also demonstrated that a 4- to 5-fold decrease in GATA-1 expression caused defects in erythroid cell maturation.[19] These results suggest the presence of a threshold GATA-1 expression level at the proerythroblast stage, which is required to sustain erythroid differentiation.

We previously reported that an 8.0-kb genomic fragment of the *GATA-1* gene, *G1-HRD* (*GATA-1-hematopoietic regulatory domain*),[20,21] is sufficient for recapitulation of the endogenous GATA-1 expression profile; transgenic GATA-1 cDNA expression under the control of *G1-HRD* fully rescued the GATA-1 germ line mutant mouse from lethal dyserythropoiesis.[22] Surprisingly, GATA-2 and GATA-3 under *G1-HRD* regulation also could rescue the GATA-1 knockdown mutant mice from embryonic lethality.[22] Thus, the transcriptional regulation of the timing, place, and abundance of *GATA-1* gene expression appears to be more important for GATA-1 function than the domain structure and functional specificity of GATA-1. In support of this contention, the overexpression of GATA-1 in erythroid cells caused a defect in erythroid terminal maturation.[23] However, since the expression profile of GATA-1 during erythroid differentiation has not been examined in detail, a solid conclusion for the relationship between GATA-1 expression and its functional contribution to erythropoiesis has not yet been formed.

Thus, we set about examining the expression profile of GATA-1 during erythropoiesis through the preparation and analysis of transgenic mouse lines expressing green fluorescent protein (*GFP*) transgene under the control of *G1-HRD* (*G1-HRD-GFP* transgene).

From the Center for Tsukuba Advanced Research Alliance (TARA), Institutes of Basic Medical Sciences, Institutes of Clinical Medicine, and Exploratory Research for Advanced Technology (ERATO) Environmental Response Project, University of Tsukuba, Japan.

Submitted April 14, 2003; accepted July 21, 2003. Prepublished online as *Blood* First Edition Paper, July 31, 2003; DOI 10.1182/blood-2003-04-1154.

Supported by grants from the Ministry of Education, Science, Sports and Culture, JST-ERATO, Japan Society for the Promotion of Science-the Research for the Future Program (JSPS-RFTF), Center for Renewable Energy and Sustainable Technology, the Naito Foundation, and Program for Promotion of Basic Research Activities for Innovative Biosciences. N. Suzuki is a Japan Health Science Foundation postdoctoral fellow.

Reprints: Masayuki Yamamoto, Center for TARA, University of Tsukuba, 1-1-1 Tennoudai, Tsukuba 305-8577, Japan; e-mail: masi@tara.tsukuba.ac.jp.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 U.S.C. section 1734.

We found that GFP expression in these transgenic mice substantially, if not completely, recapitulated the endogenous expression profile of GATA-1. This reliable *G1-HRD-GFP* expression made it possible to separate the bone marrow cells into 6 consecutive erythroid populations. By means of the purification approach using the GFP (or GATA-1) expression level, 2 classic erythroid progenitors, colony-forming unit-erythroid and burst forming unit-erythroid (CFU-E and BFU-E, respectively) were delineated into discrete subfractions separable by a flow cytometer. The results revealed that the GFP^{high} cell fraction contains mainly CFU-E and proerythroblasts, whereas the GFP^{low} cell fraction contains BFU-E, demonstrating that GATA-1 is expressed at the highest level in CFU-E and proerythroblasts among erythroid differentiation.

In addition, taking advantage of the ability to mark the erythroid progenitors with high efficiency by green fluorescence, we developed a novel method for the real-time detection of CFU-E. As an example of such an approach, we show here detection of the CFU-E in the livers of erythropoietin receptor (EpoR)–deficient mutant embryos, although the conventional colony assay has never detected the presence of CFU-E in the EpoR-null mutant embryos.[24] Thus, our novel approach provides an important clue for the analysis of hematopoietic disorders.

Materials and methods

Mice

The *G1-HRD* (IE3.9int) vector has been described previously.[20] In constructing the *G1-HRD-GFP* transgene, *G1-HRD* was ligated to GFP cDNA. We established 3 lines of *G1-HRD-GFP* transgenic mice, and the highest GFP-expressing line was mainly used in this study. For screening the transgenic mice, the tail DNA was extracted and polymerase chain reaction (PCR) was performed using a pair of primers, GFPs4 5′-CTGAAGTTCATCTGCACCACC-3′) and GFPas4 5′-GAAGTTGTACTCCAGCTTGTGC-3′). To induce anemia, 6-week-old mice were injected with 1.2 mg phenylhydrazine (Sigma, St Louis) intraperitoneally on day 1 and day 2. On day 5, the spleen and bone marrow were analyzed. The EpoR-deficient mice[25] (Jackson Laboratories, Bar Harbor, ME) were mated with *G1-HRD-GFP* mice.

Cell sorting

Sorting and analysis of cells were performed using FACSVantage (Becton Dickinson, San Jose, CA). Mononucleated cell suspensions from the bone marrow, spleen, and embryonic day 12.5 (E12.5) fetal liver were prepared and incubated with biotinylated monoclonal antibodies recognizing Mac-1, Gr.1, Ter119, B220, CD4, and CD8. Hematopoietic lineage marker–negative (Lin^-) cells were enriched by magnetic separation using streptavidine-conjugated magnetic beads (Polyscience, Warrington, PA) and then stained with PE (phycoerythrin)–conjugated anti-CD71 and APC (allophycocyanin)–conjugated anti–c-Kit antibodies. All antibodies were obtained from BD Pharmingen (San Diego, CA).

Staining of intracellular GATA-1

Cells were fixed on ice for 1 hour with 0.25% paraformaldehyde. After washing, the cells were permeabilized by 3 cycles of freezing and thawing. After one overnight reaction with rat anti–GATA-1 monoclonal antibody (N6),[7] the cells were stained with PE-conjugated anti–rat IgG antibody (BD Pharmingen) and analyzed by FACSCalibur (Becton Dickinson).

Colony assay

Sorted cells were cultured in 1 mL of 0.8% methylcellulose medium containing 30% fetal bovine serum (FBS). For detection of CFU-E, medium was supplemented with 4 U/mL Epo. For detection of BFU-E, medium was supplemented with Epo (4 U/mL) and stem cell factor (SCF) (100 ng/mL). SCF, interleukin-3 (IL-3), IL-6 (100 ng/mL each), and Epo (4 U/mL) were added for the formation of the myeloid colonies. Colonies were counted after 3 days (CFU-E) or 7 days (BFU-E and myeloid colonies) of culturing. To distinguish erythroid colonies, colonies were stained with benzidine before counting. All supplemented cytokines were provided by Kirin Brewery (Takasaki, Japan). These assays were performed in triplicate and repeated more than 3 times, and the results are shown with standard deviations.

Reverse transcription–polymerase chain reaction (RT-PCR)

RNA was purified from cells using RNeasy (Qiagen, Basel, Switzerland) and reverse transcribed by SenscriptRT (Qiagen) and random hexamers. PCR was performed for the detection of GATA-1 (pair of primers: 5′-ACTCGTCATACCACTAAGGT-3′ and 5′-AGTGTCTGTAGGCCTCAGCT-3′), GATA-2 5′-ACACACCACCCGATACCCACCTAT-3′ and 5′-GCCTAGCCCATGGCAGTCACCATGCT-3′), c-mpl 5′-AGCTGCTGTCCACAGAAACC-3′ and 5′-GTCATTTCGTGACTTGAAGTGGC-3′), EpoR 5′-GATTCTGGAGTGCCTGGTCTGAGCC-3′ and 5′-GGTGTCGACCTTCAATGGGAACAC-3′), IL-7R 5′-CAAAGTCCGATCCATTCCCCATAAC-3′ and 5′-GTTTTCTTATGATCGGGGAGACTAGG-3′). Hypoxanthine phosphoribosyltransferase (HPRT) was used as an internal control.[26] GATA-1 and -2 mRNA levels were also measured quantitatively using primer pairs 5′-CAGAACCGGCCTCTCATCC-3′ and 5′-TAGTGCATTGGGTGCCTGC-3′ for GATA-1; 5′-GAATGGACAGAACCGGCC-3′ and 5′-AGGTGGTGGTTGTCGTCTGA-3′ for GATA-2 and a VIC-labeled oligo-DNA probe 5′-CCCAAGAAGCGAATGATTGTCAGCAAA-3′ for GATA-1; 5′-AAGCGGAGGCTGTCTGCTGCCAG-3′ for GATA-2 by ABI PRISM 7700 (Perkin-Elmer, Foster City, CA). Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as internal standard (primer pair, 5′-GAAGGTGAAGGTCGGAGTC-3′ and 5′-GAAGATGGTGATGGGATTTC-3′; JOE-labeled probe, 5′-CAAGCTTCCCGTTCTCAGCC-3′).

Measurement of the intracellular-free calcium

Bone marrow cells were incubated with 3 μM Indo-1-AM (Molecular Probes, Eugene, OR) for 30 minutes at 37°C then stained on ice with PE-conjugated anti–c-Kit or anti-Ter119 antibodies. Cells were incubated with ionomycin (3 μg/mL, Calbiochem, Darmstadt, Germany) or 10 U/mL Epo at 37°C for 30 minutes. The ratio of florescence intensity (405 nm/480 nm) was measured by BD-LSR (Becton Dickinson).[27]

Results

GFP and GATA-1 expression in the hematopoietic progenitor fraction from *G1-HRD-GFP* transgenic mouse

Several lines of evidence have demonstrated that *G1-HRD*, the *GATA-1* gene hematopoietic regulatory domain, markedly recapitulates the endogenous expression profile of GATA-1 in the erythroid lineage.[3,20] In order to identify hematopoietic cells expressing GATA-1, we established 3 transgenic mouse lines that express *GFP* reporter gene under the control of *G1-HRD*. We first analyzed the GFP expression profile in the transgenic mice using the fluorescent microscope. The expression of GFP was observed specifically in hematopoietic tissues, including yolk sac, fetal liver, spleen, and bone marrow (data not shown), showing very good agreement with our previous analyses.[20,22,26,28] Microscopic analysis also detected the expression of GFP in peripheral enucleated red blood cells and platelets (data not shown), perhaps due to the stable nature of GFP.

We then analyzed the expression of GFP by fluorescence activated cell sorting (FACS). Within the mononucleated cell (MNC) fraction from bone marrow, most Ter119-positive erythroid cells (data not shown) and 12% of lineage marker–negative (Lin^-) cells expressed GFP (Figure 1A). Since the $Lin^-/c\text{-}Kit^+$ fraction

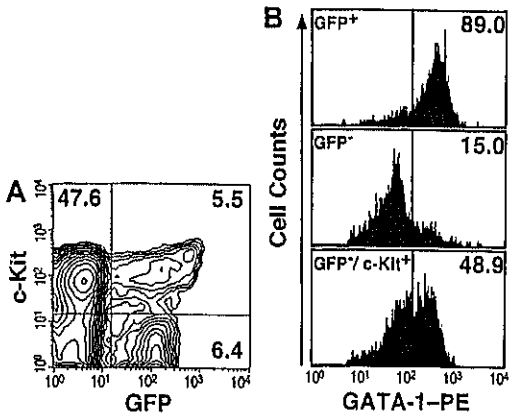


Figure 1. Expression analyses of GFP and GATA-1 in hematopoietic progenitors. (A) The Lin⁻ fraction from the bone marrow of the *G1-HRD-GFP* transgenic mouse was stained with APC-conjugated anti–c-Kit antibody and analyzed by FACS. The percentage of cells in each quadrangle is shown. (B) Immunostaining of intracellular GATA-1 in the sorted cells. Almost all GFP⁺ cells in the Lin⁻ fraction were GATA-1 positive (top panel). Most GFP⁻ cells in the Lin⁻ fraction were GATA-1–negative (middle). The Lin⁻/c-Kit⁺/GFP⁻ fraction contains approximately half of GATA-1–positive cells (bottom panel).

contains hematopoietic progenitors, we analyzed the expression of GFP and c-Kit in the Lin⁻ fraction from transgenic bone marrow. Approximately 10% of Lin⁻/c-Kit⁺ cells expressed GFP, whereas half of the GFP⁺ cells in the Lin⁻ fraction were c-Kit⁻ (Figure 1A). Morphological analyses revealed that the GFP⁺/Lin⁻/c-Kit⁺ fraction contains erythroblasts and many eosinophils with eosinophilic granules (data not shown). Interestingly, essential roles for GATA-1 in eosinophil development have been reported.[12,13]

To examine the specificity of the GFP expression, we compared the expression profile of GFP with that of GATA-1 protein. To this end, immunostaining of intracellular GATA-1 in FACS-sorted bone marrow cells was conducted. The result revealed that almost all GFP⁺ cells were positive for intracellular GATA-1 staining (Figure 1B, top panel), whereas only 15% of cells in the GFP⁻ fraction were GATA-1 positive (middle panel). Similarly, when Lin⁻/c-Kit⁺ cells were sorted into GFP⁺ and GFP⁻ fractions, almost all GFP⁺ cells were GATA-1 positive (data not shown). In contrast, approximately 50% of cells in the GFP⁻/Lin⁻/c-Kit⁺ fraction expressed endogenous GATA-1 (Figure 1B, bottom panel). One plausible explanation for this discrepancy is that the *G1-HRD* may not contain the sufficient regulatory sequence to recapitulate fully the *GATA-1* gene expression in the erythroid progenitors at immature stages of differentiation. As these data were reproducible in the other 2 *G1-HRD-GFP* transgenic mouse lines, the consistency of the GFP expression with that of the endogenous GATA-1 were demonstrated.

BFU-E and CFU-E are concentrated in the CD71⁻/GFP⁺ and CD71⁺/GFP⁺ fractions, respectively

To characterize the hematopoietic progenitors expressing GATA-1, we examined GFP expression in the Lin⁻/c-Kit⁺ fraction of *G1-HRD-GFP* transgenic mice. The bone marrow cells in the Lin⁻/c-Kit⁺ fraction were stained with PE-conjugated anti-CD71 antibody and analyzed by FACS (Figure 2A). CD71 is the transferrin receptor (TfR) and is known as a marker for proliferating hematopoietic cells.[29] The GFP-positive fraction was clearly divided into 2 fractions by the expression of CD71. The green fluorescent intensity of GFP⁺/CD71⁺ cells was higher than that of GFP⁺/CD71⁻ cells (Figure 2A). The mean fluorescent intensities of the GFP⁺/CD71⁺ and GFP⁺/CD71⁻ fractions were 360 and 125, respectively. It should be noted that the GFP⁺/CD71⁺ fraction is small (4.1% of Lin⁻/c-Kit⁺ cells) but contains solely the high level GFP-expressing cells (Figure 2A).

Based on these results, we speculate that the expression of the *G1-HRD-GFP* transgene can be used to separate the erythroid progenitor fraction from the fraction containing progenitors for the other hematopoietic lineages within the bone marrow cells. Especially, we hypothesized that the CD71 marker might separate the erythroid progenitors into 2 fractions in collaboration with high- and low-level GFP expression.

To test this hypothesis, we carried out a colony assay using cells in these fractions. The GFP⁺/CD71⁺ fraction contained CFU-E abundantly, but there were no other colony-forming cells (Figure 2B-D). The GFP⁺/CD71⁻ fraction also contained CFU-E, but the number was much less than that in the GFP⁺/CD71⁺ fraction

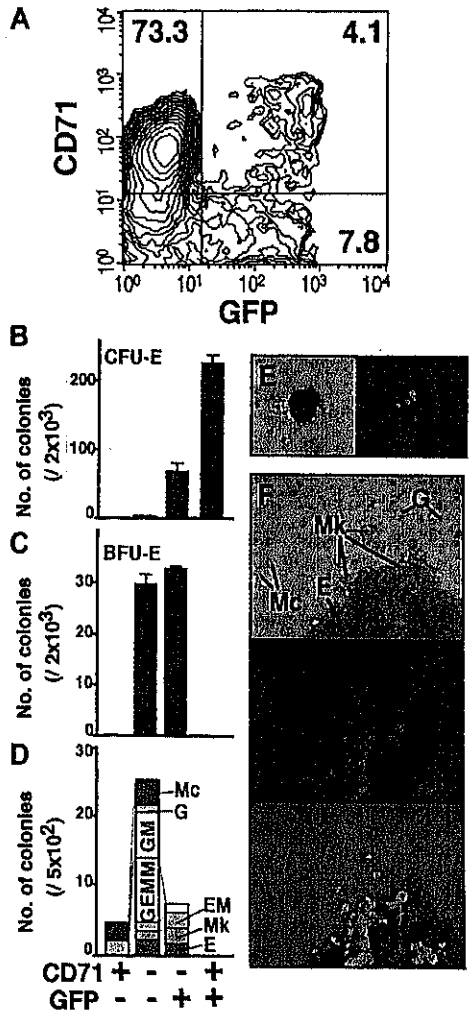


Figure 2. Colony assay of GFP⁺ cells in the bone marrow. (A) The Lin⁻/c-Kit⁺ fraction is subdivided into 4 fractions with the expression of GFP and CD71. The percentage of cells in each quadrangle is shown. Cells in each fraction were sorted and cultured with only Epo (B), Epo and SCF (C), or Epo, SCF IL-6, and IL-3 (D). Colonies were counted 3 days (B) or 7 days (C, D) after culturing. (E) A colony derived from CFU-E in the CD71⁺/GFP⁺ fraction was stained by benzidine (left panel, blue staining), and some of the cells in the colony were detected by GFP expression under the fluorescent microscope (right panel). (F) A mixed colony derived from a CD71⁻/GFP⁺ cell was observed by bright (top panel) and fluorescent (middle panel) microscopy. GFP expression was detected in red cells (erythrocytes [E]) and large cells (megakaryocytes [Mk]) but not in small white cells (granulocytes [G]) and large extended cells (macrophages [Mc]). The top and middle panels were merged in the bottom panel (F). Original magnifications, × 200 (E) and × 100 (F).

(Figure 2B). Importantly, we found that 1.5% of cells in this fraction formed BFU-E–derived colonies (Figure 2C). In addition, a few colonies were found to contain only megakaryocytes (Mk) or megakaryocytes and erythrocytes (EM, common precursor of megakaryocyte and erythrocyte) in the GFP$^+$/CD71$^-$ fraction. We also observed a few colonies containing granulocytes, erythrocytes, megakaryocytes, and macrophages (GEMM, mixed colony) in the GFP$^+$/CD71$^-$ fraction (Figure 2D). BFU-E also was included in the GFP$^-$/CD71$^-$ fraction, but this fraction contained many myeloid colony-forming cells including CFU-GM (granulocyte and macrophage) and CFU-GEMM. There were colony-forming unit-granulocyte and -macrophage in the GFP$^-$/CD71$^+$ fraction, but no other colonies were observed (Figure 2B-D).

The benzidine-positive (ie, hemoglobinized erythroid) cells in the CFU-E–derived colony from the GFP$^+$/CD71$^+$ fraction expressed various intensities of green fluorescence (Figure 2E). GFP expression also was detected in megakaryocytes and erythroid cells, but not in granulocytes and macrophages, in the mixed colonies derived from the GFP$^+$/CD71$^-$ fraction (Figure 2F). We also observed GFP-positive megakaryocytes and erythroid cells in the mixed colonies derived from the GFP$^-$/CD71$^-$ fraction (data not shown). These results suggest that the GFP$^-$/CD71$^-$ and GFP$^+$/CD71$^-$ fractions may contain progenitors differentiating into the cells in the GFP$^+$/CD71$^+$ fraction. GFP expression changes dynamically during erythroid and megakaryocytic cell differentiation. We noticed, through flow cytometry and microscopic analyses, that in all the GFP$^+$ cells in these colonies, green fluorescent intensity was weaker than in primary c-Kit$^+$ cells in the bone marrow (data not shown and Figure 2E-F). This observation further supports our contention that the expression level of GATA-1 in erythroid progenitors decreases along with progenitor cell differentiation.

Based on these results, we propose that Lin$^-$/c-Kit$^+$/GFP$^+$/CD71$^-$ cells in the bone marrow are an early erythroid progenitor (EEP) fraction containing BFU-E in abundance, whereas Lin$^-$/c-Kit$^+$/GFP$^+$/CD71$^+$ cells are a late erythroid progenitor (LEP) fraction containing abundantly CFU-E. Thus, we report here for the first time the efficient enrichment of CFU-E and BFU-E erythroid progenitors from normal bone marrow cells using flow cytometry.

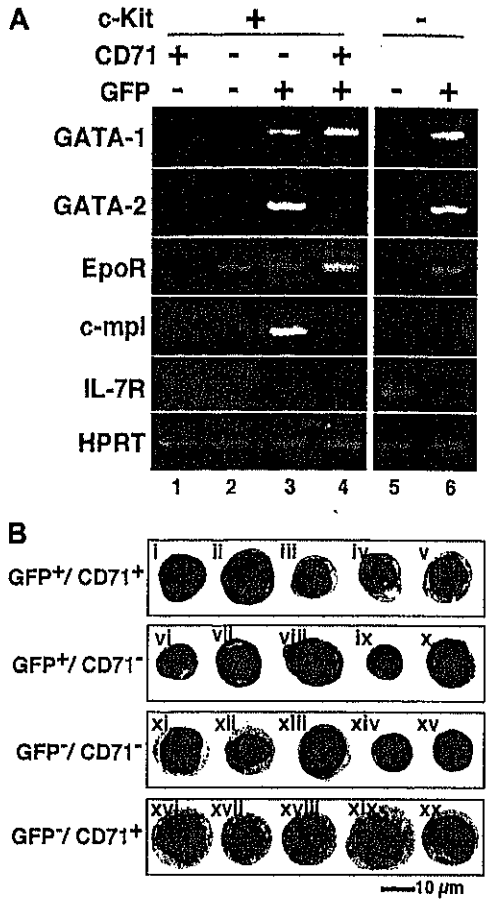


Figure 3. Gene expression and morphology of GFP$^+$ progenitor cells. (A) RNA was extracted from the sorted cells in the indicated fractions, and RT-PCR was performed to detect GATA-1, GATA-2, EpoR, c-mpl, and IL-7R expression. The bone marrow Lin$^-$/c-Kit$^+$ hematopoietic progenitor fraction was subdivided into 4 fractions (lanes 1-4). EEP and LEP fractions are represented in lanes 3 and 4, respectively. GFP$^-$ (lane 5) and GFP$^+$ (lane 6) cells in the Lin$^-$/c-Kit$^-$ fractions also are shown. (B) The morphology of the sorted cells are shown by Wright-Giemsa staining. Proerythroblast-like cells were detected in the LEP fraction (GFP$^+$/CD71$^+$; i,ii). It is likely that erythroid progenitors are i, ii, iii, iv, v, and vi in the LEP and EEP (GFP$^+$/CD71$^-$) fractions. All cells in the GFP$^-$/CD71$^+$ fraction contained azurophil granules (xvi-xx).

Gene expression and morphology of early and late erythroid progenitors

In order to characterize the erythroid progenitor fractions EEP and LEP, we sorted the mouse bone marrow cells of each fraction by FACS and defined their gene expression profiles through RT-PCR analysis. As expected, the expression of GATA-1 mRNA was detected predominantly in the GFP$^+$ fractions (Figure 3A, lanes 3, 4, and 6), and the level was higher in the LEP fraction (Lin$^-$/c-Kit$^+$/GFP$^+$/CD71$^+$) than that in the EEP fraction (Lin$^-$/c-Kit$^+$/GFP$^+$/CD71$^-$). Consistent with our antibody permeation analysis (Figure 1B), GATA-1 expression also was detected, albeit very weakly, in the c-Kit$^+$/GFP$^-$ fraction (lane 2; see "Change in GATA-1 expression level during erythroid differentiation").

We found that mRNAs for GATA-2 and c-mpl were highly expressed in the EEP fraction (Figure 3A, lane 3). The expression of these mRNAs could not be detected in the LEP fraction. The expression of GATA-1 and GATA-2 mRNAs also was detected in the Lin$^-$/c-Kit$^-$/GFP$^+$ fraction containing eosinophils and erythroblasts (lane 6). This agrees closely with our previous analysis showing that eosinophils express GATA-1 and GATA-2.[12] Importantly, erythropoietin receptor (EpoR) mRNA was expressed in the fractions expressing GATA-1, and this result shows excellent agreement with the notion that EpoR is expressed most abundantly at the CFU-E stage and is essential for the survival and differentiation of the CFU-E.[24]

Our data demonstrate that c-mpl is specifically expressed in the EEP fraction (lane 3), which contains 2 megakaryocyte progenitors, CFU-Mk and CFU-EM (Figure 2). Although we could not detect c-mpl expression in the Lin$^-$/c-Kit$^+$/GFP$^-$/CD71$^-$ fraction (lane 2), which contains hematopoietic stem cells (HSCs), there are many reports showing c-mpl expression in the HSC.[30] The Lin$^-$/c-Kit$^+$/GFP$^-$/CD71$^-$ fraction contains not only HSCs, but also progenitors for various other hematopoietic lineages. Therefore, c-mpl expression in the HSC might have been diluted so that we could not detect its expression. The expression of interleukin-7 receptor (IL-7R) was detected specifically in the Lin$^-$/c-Kit$^-$/GFP$^-$ fraction (Figure 3A, lane 5). Since IL-7R is a good marker for the lymphoid lineage,[30] this fraction seems to contain mainly lymphoid progenitors, which do not express GATA-1, GATA-2, EpoR, or c-mpl.

We then examined the morphology of the sorted cells in the c-Kit$^+$ fraction. In the LEP (GFP$^+$/CD71$^+$) fraction, there were many proerythroblast-like cells, which were stained deep purple in

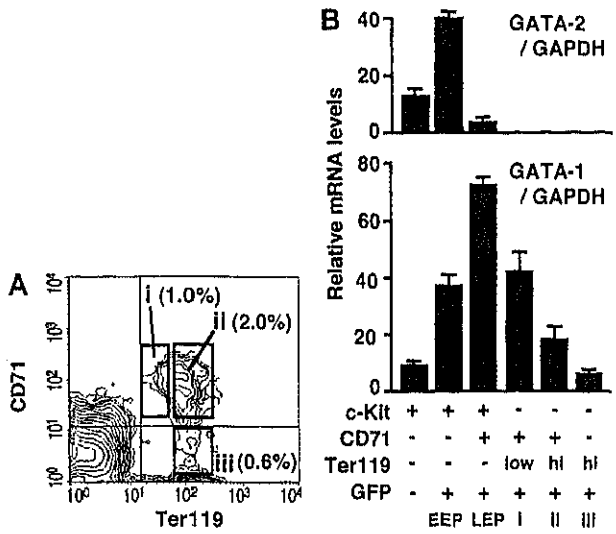


Figure 4. Quantitative analyses of GATA-1 and GATA-2 mRNAs during erythroid differentiation. (A) Bone marrow cells were analyzed for CD71 and Ter119 expression. Cells in open boxes (i-iii) were sorted and analyzed for GATA-1 expression level in B. The percentage of cells in each box is shown. (B) The relative GATA-1 and GATA-2 mRNA levels at different stages of erythroid cell development were measured by quantitative RT-PCR and normalized to the level of GAPDH mRNA. Erythroid differentiation is indicated by the x-axis from left to right.

the cytoplasm and deep red in the nuclei compared with other GFP$^+$ cells by Wright-Giemsa staining (Figure 3Bi-ii). The nuclei appeared to be loose and coarse. No basophilic or polychromatic erythroblasts were observed in the c-Kit$^+$ fraction (Figure 3B). The LEP fraction also contained cells with light purple–colored cytoplasm, which seem to be CFU-E, as judged by colony assay (iii-iv). These CFU-E–like cells also were observed in the EEP fraction (v). On the contrary, we could not delineate as to which cells correspond to BFU-E or megakaryocytic progenitors in the EEP (GFP$^+$/CD71$^-$) fraction. All cells in the GFP$^-$/CD71$^+$ fraction had azurophilic granules (xvi-xx), indicating that this fraction contains myeloid progenitors. Thus, the *G1-HRD-GFP* reporter transgene enabled us to divide the CD71$^+$ fraction into myeloid and erythroid lineages. Finally, showing excellent agreement with our assignment through colony assays, the GFP$^-$/CD71$^-$ fraction appears to contain multilineage progenitors (xiv-xv) and early myeloid cells (xi-xii).

Change in GATA-1 expression level during erythroid differentiation

In the previous sections, we identified and isolated c-Kit$^+$ progenitors corresponding to the 3 consecutive stages of erythroid differentiation from the bone marrow of *G1-HRD-GFP* transgenic mice. In this regard, one preceding report demonstrated that the expression level of CD71 decreases and that of Ter119 increases during late erythroblast maturation in the spleen.[31] According to the report, proerythroblast (CD71$^+$/Ter119^{low}), basophilic and polychromatic erythroblast (CD71$^+$/Ter119^{high}), and orthochromatic erythroblast (CD71$^-$/Ter119^{high}) can be separated from the spleen by flow cytometry.[31] We therefore examined CD71 and Ter119 expressions in the bone marrow of *G1-HRD-GFP* mice and found that bone marrow cells also contain these 3 erythroblast fractions (Figure 4A). The morphology of the bone marrow and spleen cells in these fractions was similar (data not shown), suggesting that bone marrow cells can also be separated into 3 late erythroid progenitors by means of CD71 and Ter119 antigens.

We envisage from these analyses that we could obtain erythroid lineage cells from 6 consecutive stages of differentiation. We examined the expression profiles of GATA-1 and GATA-2 during erythroid differentiation using sorted bone marrow cells by RT-PCR analysis. The results of the quantitative RT-PCR are shown as a graph (Figure 4B). The results clearly demonstrate that GATA-1 increases from EEP to LEP and decreases during maturation of erythroid progenitors. The LEP fraction (Lin$^-$/c-Kit$^+$/GFP$^+$/CD71$^+$) expresses GATA-1 at the highest level throughout the 6 stages of erythroid differentiation, based on the RT-PCR and green fluorescent intensity data. On the other hand, the expression level of GATA-2 is the highest in the EEP fraction and is decreased to undetectable level after the LEP stage of differentiation (Figure 4B). This is the first report demonstrating a fluctuation in the GATA-1 and -2 expression levels during in vivo erythropoiesis.

The number of GFP-positive erythroid progenitors increases in the anemic spleen

To clarify whether erythroid fractions similar to the EEP and LEP also exist in the spleen, we examined the expression of GFP in the spleen of the *G1-HRD-GFP* transgenic mice. To our expectation, we identified both EEP (Lin$^-$/c-Kit$^+$/GFP$^+$/CD71$^-$) and LEP (Lin$^-$/c-Kit$^+$/GFP$^+$/CD71$^+$) fractions in the spleen (Figure 5A).

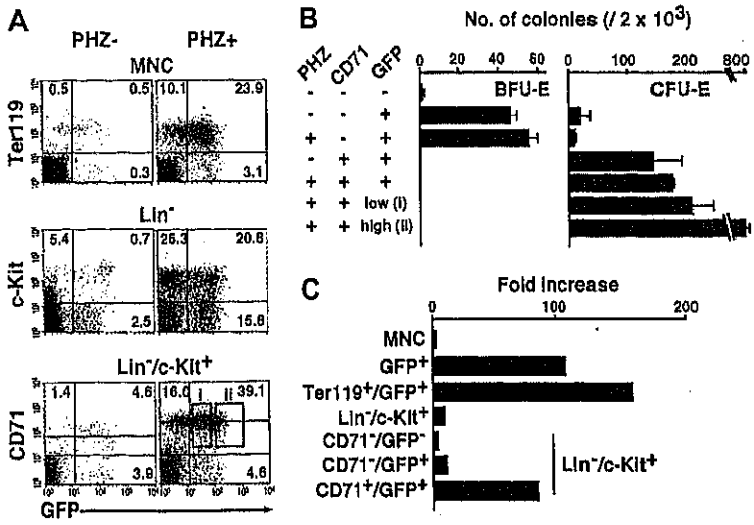


Figure 5. FACS analysis and colony assay of the normal and anemic spleen of *G1-HRD-GFP* transgenic mouse. (A) Spleen mononucleated cells (MNCs) were obtained from *G1-HRD-GFP* transgenic mice before (PHZ−, left) and after (PHZ+, right) injection of phenylhydrazine (PHZ). The MNC, Lin$^-$ fraction, and Lin$^-$/c-Kit$^+$ fraction were analyzed by FACS (top, middle, and bottom panels, respectively). The percentage in each quadrangle is shown. The dotted lines show the mean intensities of CD71 expression in CD71$^+$/GFP$^+$ fractions (bottom panels). (B) Results of colony assay in the normal and anemic spleens. Two thousand cells from the indicated fractions were analyzed. Fractions I and II are shown by open boxes in A. (C) The cell number of the erythroid fractions of the spleen were increased by PHZ injection. Fold increases between the PHZ-induced anemic spleens, and normal spleens are indicated in this graph. The results are shown with standard deviations.

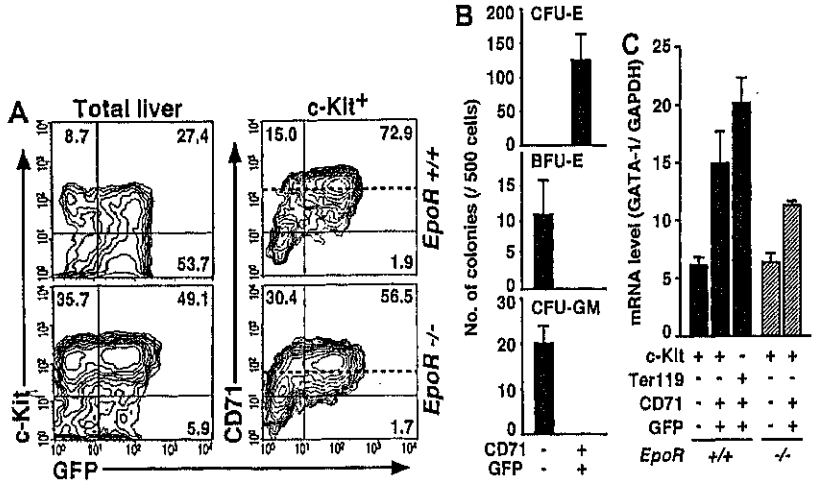


Figure 6. Analyses of normal and abnormal fetal erythropoiesis by the *G1-HRD-GFP* transgene. (A) Total (left) and c-Kit$^+$ (right) cells from wild-type (top) and EpoR-null (bottom) E12.5 fetal livers with *G1-HRD-GFP* transgene were analyzed by FACS. The percentage in each quadrangle is shown. The means of the CD71 expression levels in the CD71$^+$ fractions from each embryo are indicated by dotted lines. (B) Colony assay of sorted cells from *G1-HRD-GFP* transgenic fetal liver. (C) Result of the quantitative RT-PCR analysis of GATA-1 in *EpoR*$^{+/+}$*::G1-HRD-GFP*$^+$ (closed bars) and *EpoR*$^{-/-}$*::G1-HRD-GFP*$^+$ (hatched bars) fetal liver cell fractions. The GATA-1 mRNA levels were normalized to the level of GAPDH mRNA. The results are shown with standard deviations.

The EEP and LEP fractions consisted of 3.9% and 4.6% of Lin$^-$/c-Kit$^+$ cells, respectively. Colony assays revealed that the spleen EEP and LEP fractions contain mainly BFU-E and CFU-E, respectively (Figure 5B), as was the case for the bone marrow.

Upon induction of anemia with phenylhydrazine (PHZ), the number of GFP$^+$ cells in the spleen was markedly increased. Fold increases in various GFP$^+$ fractions in the PHZ-induced anemic spleens from normal mouse spleen are indicated in Figure 5C. The numbers of the erythroblast (Ter119$^+$/GFP$^+$) and LEP cells were increased approximately 100-fold. Importantly, we detected GFP^{high} and GFP^{low} fractions in the latter fraction of anemic spleen (Figure 5A, boxes i,ii), which were CD71$^+$. When we examined the colony-forming activity of these subfractions, we found, surprisingly, that more than 40% of cells in the box ii subfraction formed CFU-E–derived colonies (Figure 5B). To our knowledge, this is the most efficient strategy for isolating CFU-E.

Consistent with the previous observation that CFU-E increased in the anemic spleen by about 100-fold,[32] the total number of the cells in the LEP fraction increased by approximately 100-fold (Figure 5C). Since the number of CFU-E–derived colonies also increased (Figure 5B) concomitantly with an increase in the LEP fraction (Figure 5C), the frequency of CFU-E in the LEP fraction does not change much in the normal and anemic spleen. This observation suggests that anemic stimuli similarly increase not only the CFU-E, but also the rest of the cells in the LEP fraction.

The means of CD71-PE fluorescent intensities, which are shown by dotted lines in Figure 5A (bottom panels), were 100 and 300 in normal and anemic spleens, respectively, indicating that the expression of CD71 in each cell in the LEP fraction was increased in the anemic mice. These data are consistent with reports that *TfR* gene expression is induced by hypoxia and proliferation signals.[29,33] Intriguingly, while the TfR expression level was induced in the LEP fraction, it remained not to be expressed in the EEP fraction (Figure 5A). Taken together, there are both EEP and LEP fractions in the spleen, as is the case in the bone marrow, and the LEP cells are dramatically increased in the anemic condition.

GFP expression in normal and dyserythropoietic fetal livers

To clarify whether the *G1-HRD-GFP* transgene faithfully recapitulates the expression profile of the *GATA-1* gene in fetal liver, we examined the E12.5 fetal liver of the transgenic mouse. FACS analyses revealed that there were GFP$^+$ cells in the Ter119$^+$ erythroblasts (data not shown) and in the c-Kit$^+$ hematopoietic progenitors, as was the case for the mouse bone marrow and spleen (Figure 6A, upper left panel). To our surprise, however, c-Kit$^+$ fetal liver cells were divided into 2 main populations, GFP$^+$/CD71$^+$ and GFP$^-$/CD71$^-$ (Figure 6A, upper right panel). We could not find a GFP$^+$/CD71$^-$ cell fraction, which corresponds to the EEP fraction in the adult hematopoietic tissues.

Colony assay was then performed exploiting c-Kit$^+$/CD71$^-$/GFP$^-$ and c-Kit$^+$/CD71$^+$/GFP$^+$ cells from the fetal liver. Like the LEP fraction of the adult bone marrow, the c-Kit$^+$/CD71$^+$/GFP$^+$ fraction contained CFU-E (Figure 6B). On the other hand, BFU-E and CFU-GM cells resided in the GFP$^-$/CD71$^-$ fraction (Figure 6B).

We then examined the expression levels of GATA-1 mRNA in these fetal liver cell fractions by quantitative RT-PCR. The expression level of GATA-1 in the c-Kit$^+$/CD71$^-$/GFP$^-$ fraction (containing BFU-E) was less than 45% of that in the c-Kit$^+$/CD71$^+$/GFP$^+$ fraction (containing CFU-E, Figure 6C). These results indicate that *G1-HRD* is not active in BFU-E in the fetal liver, even though they expressed GATA-1 endogenously; in other words, *G1-HRD* is insufficient for gene expression in fetal liver BFU-E. We thus suggested that the fetal liver CD71$^+$/GFP$^+$ fraction is the counterpart of the LEP fraction in the adult hematopoietic tissues, and the counterpart of the EEP fraction must be contained in the CD71$^-$/GFP$^-$ fraction. Upon normalizing the GATA-1 mRNA levels in the fetal liver and bone marrow cell fractions with that of GAPDH, we found it interesting that the GATA-1 levels in all of the liver fractions were lower than those in the bone marrow EEP and LEP fractions (Figures 4B, 6C).

It has been reported that fetal liver cells from erythropoietin receptor (EpoR)–null mutant embryos form CFU-E–derived colonies when they are infected with the retrovirus vector expressing EpoR.[24] This observation suggests that there may be CFU-E in the embryonic liver of the EpoR-null mutant, but that the cells cannot be detected by colony assay without the retroviral complementation of EpoR. In order to ascertain the presence of CFU-E in the liver of EpoR-null mutant embryos, we examined *G1-HRD-GFP* expression. The *G1-HRD-GFP* transgene was introduced into *EpoR*$^{-/-}$ mice by breeding, and FACS analysis was performed exploiting the liver of *EpoR*$^{-/-}$*::G1-HRD-GFP*$^+$ embryos.

Our study shows good agreement with previous reports that there are only a residual number of Ter119$^+$ cells in the liver of EpoR-null mutant embryos.[26] In contrast, however, our study revealed a comparable number of c-Kit$^+$ cells ($7.0\sim9.0 \times 10^4$/liver) to that of wild-type (Figure 6A). Similar to the wild-type case, the c-Kit$^+$ cells were subdivided into CD71$^-$/GFP$^-$ and CD71$^+$/GFP$^+$ fractions (Figure 6A, right panels). The level of

GATA-1 mRNA and green fluorescent intensities in these fractions were comparable to that of wild-type embryos (Figure 6A,C). It is noteworthy that the mean intensity of CD71 expression in the $CD71^+/GFP^+$ fraction of the EpoR-null embryo was about 30% of that of wild type (55 and 190, respectively), perhaps reflecting the cellular proliferating activity (dotted lines in Figure 6A). Thus, our approach using the flow cytometer and *G1-HRD-GFP* transgene enabled the development of a new method for investigating the existence of CFU-E in various hematopoietic disorders.

Change in the intracellular calcium ion storage during erythroid maturation

Taking advantage of the progress achieved here in isolating erythroid progenitors from mouse bone marrow, we attempted to settle the controversy over the effect of Epo on the mobility of intracellular calcium ions in erythroid cells. When calcium ions are effused from the endoplasmic reticulum (ER) by various signals, the intracellular free calcium ion (IFC) plays a role in cellular signal transduction. Whereas there are many reports concerning the role of Ca^{2+} as a second messenger in Epo signal transduction in erythroid cells, it remains unclear whether Epo induces the increase of IFC in primary hematopoietic cells.[34]

We exploited erythroid progenitor fractions isolated from the bone marrow of a *G1-HRD-GFP* transgenic mouse and measured the concentration of IFC by indo-1 staining and flow cytometry in the presence or absence of Epo. Treatment of the cells with Epo for 5 to 30 minutes induced IFC in $c\text{-}Kit^-/GFP^+$ (3.3%) and $Ter119^+/GFP^+$ (4.4%) cells, but not in the other fractions (Figure 7). This suggests that, if calcium is involved in Epo signaling, its role is likely specific to $Ter119^+$ erythroblasts rather than to $c\text{-}Kit^+$ erythroid progenitors.

We then examined total cellular $[Ca^{2+}]$, since Ca^{2+} is also known to be an important factor for maintenance of the red cell membrane structure.[35] The calcium ionophore, ionomycin, forces the release of total Ca^{2+} from the ER, enabling us to measure the total cellular $[Ca^{2+}]$. We analyzed various cell fractions from the bone marrow by indo-1 staining and flow cytometry after exposure to ionomycin for 30 minutes. Most of the cells in the $Ter119^+$ erythroblast fraction retained a high concentration of Ca^{2+}, but the other cell fractions ($c\text{-}Kit^+$ or $Ter119^-$) contained less than 10% of cells retaining Ca^{2+} (Figure 7). These results demonstrate that Ca^{2+} is accumulated during the differentiation of erythroid progenitors into erythroblasts. Taken together, these data suggest that the intracellular calcium concentration plays important roles in the regulation of Epo signal transduction and maintenance of erythrocyte structure in erythroblasts but not in erythroid progenitors.

Discussion

This study unveils, for the first time, the dynamic changes in the expression level of GATA-1 during erythropoiesis. We have divided erythroid lineage cells into 6 consecutive stages of differentiation by means of *G1-HRD-GFP* transgene expression. Especially, we identified 2 new erythroid cell fractions, $Lin^-/c\text{-}Kit^+/CD71^+/GFP^{high}$ and $Lin^-/c\text{-}Kit^+/CD71^-/GFP^{low}$, in the mouse bone marrow and named them as LEP (late erythroid progenitor) and EEP (early erythroid progenitor) fractions, respectively. These fractions also exist in the spleen and embryonic liver. GFP expression made it possible to design an efficient enrichment procedure for these erythroid progenitors from hematopoietic tissues.

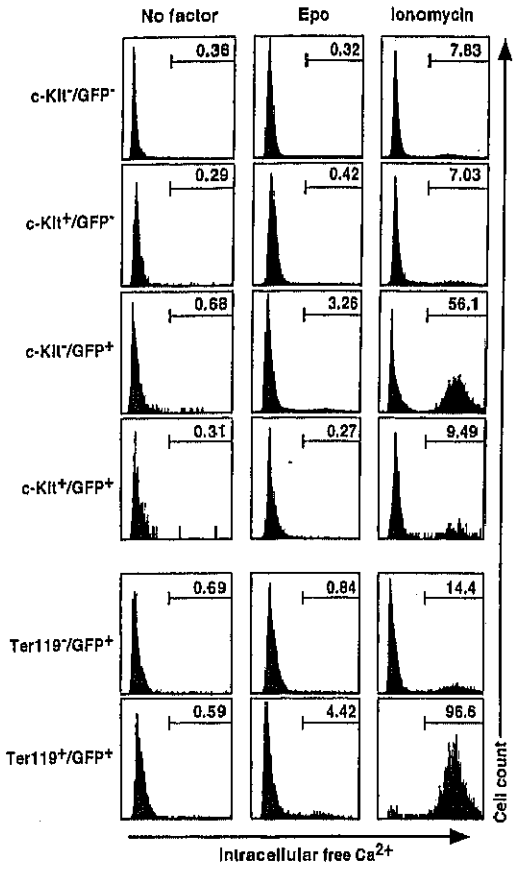


Figure 7. Measurement of intracellular free calcium concentration upon exposure to Epo and Ionomycin in the bone marrow cell fractions. Bone marrow cells from *G1-HRD-GFP* transgenic mice were analyzed for their intracellular free calcium concentration after supplementation of Epo (10 U/mL) or Ionomycin (3 μg/mL) by Indo-1 flow cytometry. The percentages of cells containing IFC in each fraction are indicated.

We exploited CD71/TfR antigen in this cell fractionation method, as the number of CFU-E–derived colonies was reported to increase upon supplementation of the culture medium with transferrin.[36] Interestingly, there was no change in the number of BFU-E by such treatment.[36] Through expression analysis of the transcription factor genes, we further revealed that EEP cells coexpress both GATA-1 and GATA-2, whereas LEP cells do not express GATA-2 but highly express GATA-1. This observation shows very good agreement with the previous observation that the GATA-2 expression was down-regulated but that of GATA-1 was up-regulated during erythroid cell differentiation.[37] Since GATA-2 overexpression inhibits erythroid differentiation,[38] this change in the expression of GATA factors seems to be important for normal erythropoiesis. The decisive moment at which this switching of GATA factors seems to occur is during the differentiation stages between EEP and LEP.

The dynamical change of GATA-1 expression level along with the erythroid differentiation is summarized in Figure 8. This GATA-1 expression profile provides a couple of important clues for understanding GATA-1 function in erythroid lineage cells. First, the highest expression of GATA-1 is observed in the LEP stage, which is before the accumulation of hemoglobin.[31] CD71/TfR is highly expressed at this stage and acts in the cellular uptake of the iron-transferrin complex. The LEP cells also appear to vigorously synthesize heme through GATA-1–mediated transactivation of heme biosynthesis enzyme genes, such as those coding for erythroid 5-aminolevulinate synthase (ALAS-E) and porphobilinogen deaminase. Consistent with this notion, mutation of the

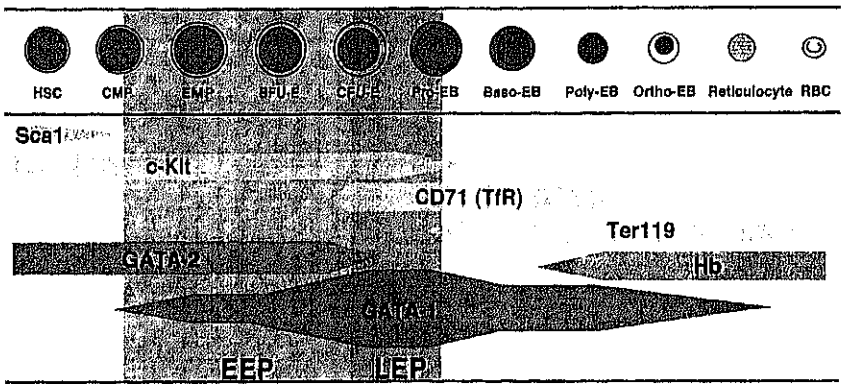


Figure 8. Summary of the GATA-1 expression profile during erythroid differentiation. The thickness of the bar indicates the level of gene expression. GATA-1 expression starts at the bipotential (erythrocyte and megakaryocyte) progenitor stage (EMP, erythroid/megakaryocytic precursors) and increases upon differentiation into the proerythroblast. Sca1, c-Kit, and GATA-2 were expressed in hematopoietic stem cells (HSCs) and common myeloid progenitors (CMPs), and their expressions decrease with the progression of differentiation.[30] When the expression level of GATA-1 decreases in Ter119$^+$ erythroblasts, hemoglobin (Hb) accumulates in the cells.[31] Pro-EB indicates proerythroblast; Baso-EB, basophilic erythroblast; Poly-EB, polychromatic erythroblast; Ortho-EB, orthochromatic erythroblast; RBC, red blood cell.

GATA-1 gene was shown to repress the expression of these heme biosynthetic enzymes.[18] Since the defect of heme biosynthesis by mutation of the *ALAS-E* gene causes decrease of the TfR expression,[39] we suggest that the high expression of GATA-1 that induces synthesis of heme is needed for the TfR expression in the LEP cells. Thus, abundant expression of GATA-1 must be required for the differentiation of erythroid progenitors at the LEP stage.

Secondly, we demonstrate here that the level of GATA-1 decreases after the erythroid progenitor stage (Figure 4). Since constitutive overexpression of GATA-1 in erythroblasts inhibits cell maturation,[23] this down-regulation of GATA-1 must be necessary for terminal maturation of the erythrocytes. However, the molecular basis for the GATA-1 reduction during terminal erythroid maturation is unclear at present. In this regard, it is interesting to note that caspase was reported to cause specific degradation of the GATA-1 protein in erythroblasts.[40] Decrease of GATA-1 protein may cause down-regulation of its transcription through inhibiting autoregulation.[41]

The use of *G1-HRD-GFP* transgenic mouse lines enables us to efficiently enrich erythroid progenitors from mouse hematopoietic tissues. Whereas Ter119 and glycophorin-A are important surface markers of differentiated erythroblasts and mature erythrocytes, these markers are not present in erythroid progenitors such as BFU-E and CFU-E.[42] In fact, there are no suitable surface markers/antibodies for the isolation of erythroid progenitors. This limitation has hampered our attempt to purify and characterize steady-state erythroid progenitors from normal mouse bone marrow, and therefore we usually enrich CFU-E from anemic spleen. This study makes it possible to purify erythroid progenitors from normal mouse hematopoietic tissues, and we are now able to assess the erythropoiesis occurring in various hematopoietic disorders. Furthermore, it should be emphasized that we could obtain 800 CFU-E–derived colonies from 2000 plated cells purified from anemic spleen. To our knowledge, this is the most efficient method to enrich erythroid progenitors.

We found that all CFU-E resides in the GFP$^+$ fraction of bone marrow, whereas more than half of the BFU-E resides in this fraction. This is consistent with the result that approximately half of Lin$^-$/c-Kit$^+$/GFP$^-$ cells express GATA-1 protein. The important observation here is that in the fetal liver, BFU-E do not express GFP at all, even though the BFU-E–containing fraction expresses endogenous GATA-1 mRNA. One plausible explanation for this discrepancy is that there may be different regulatory mechanisms for *GATA-1* gene transcription in the fetal liver and adult hematopoietic tissues. Additional regulatory elements might be required for *GATA-1* gene expression in early erythroid progenitors, as the *G1-HRD* appeared to be insufficient for recapitulating expression of the endogenous *GATA-1* gene in very immature hematopoietic progenitors.

Although the colony-forming assay is widely used to evaluate hematopoietic cell dysfunction, there is an inherent problem with this approach in that we have never assessed the progenitor cells when they lack the abilities to proliferate and differentiate, such as the hematopoietic progenitors in the EpoR, c-Myb, and Runx1 mutant mouse lines.[24,43,44] Importantly, we detected GFP$^+$ erythroid progenitors in the EpoR-null embryonic livers. Since the GFP$^+$ fraction contains CFU-E abundantly in the wild-type embryos, this result strongly argues that the EpoR-deficient embryos actually contain CFU-E, but the cells cannot differentiate further due to the lack of the Epo-EpoR signaling pathway. We therefore propose that the *G1-HRD-GFP* transgene is a useful means for the detection of CFU-E in the mutant animals with defective erythropoiesis.

There has been controversy over the mobility of intracellular free Ca^{2+} (IFC) in the erythroid progenitors and its relation to Epo.[34] One line of evidence indicates that the Ca^{2+} level is low in the erythroid progenitors, whereas another line of evidence shows that the Ca^{2+} level is high in the progenitors. To obtain insight into this issue, we measured the Ca^{2+} concentration in the erythroid progenitors purified by means of GFP expression. We found that while most of the Ter119$^+$ erythroblasts contain a high concentration of Ca^{2+} in the endoplasmic reticulum (ER), c-Kit$^+$/GFP$^+$ erythroid progenitors contain a low or undetectable level of Ca^{2+}. An Epo stimulus induces Ca^{2+} efflux from the ER to the cytoplasm only in mature erythroblasts and not in early erythroid progenitors. This result is consistent with our previous analysis[27] and clearly demonstrates that changes in the Ca^{2+} concentration do not affect Epo signaling in the progenitor stage. On the contrary, the biologic significance of Ca^{2+} modulation on Epo function in erythroblasts remains to be clarified.

Finally, we wish to present the usefulness of the *G1-HRD* for transgenic expression of various effector genes in erythroid and megakaryocytic cell lineages. We have established transgenic mouse lines expressing small Mafs, EpoR, Stat3, TR2/4, and GATA factors under the control of *G1-HRD*.[21,22,26,45,46] We recently showed that EpoR-null mutant mice can be rescued from dyserythropoiesis and embryonic lethality by introducing the *G1-HRD-EpoR* transgene,[26] as *G1-HRD* directs the expression of the effector gene in CFU-E, which require the expression of EpoR for survival and differentiation. We envisage that *G1-HRD* will be useful in therapy for dyserythropoiesis in the future.

Acknowledgments

We thank Drs Shigeko Nishimura, Toshiro Nagasawa, Satoru Takahashi, Fumiki Katsuoka, and Tania O'Connor for discussion and advice. We also thank Kirin Brewery for their generous supply of cytokines.

References

1. Zhu J, Emerson SG. Hematopoietic cytokines, transcription factors and lineage commitment. Oncogene. 2002;21:3295-3313.
2. Cantor AB, Orkin SH. Transcriptional regulation of erythropoiesis: an affair involving multiple partners. Oncogene. 2002;21:3368-3376.
3. Yamamoto M, Takahashi S, Onodera K, Muraosa Y, Engel JD. Upstream and downstream of erythroid transcription factor GATA-1. Genes Cells. 1997;2:107-115.
4. Shivdasani RA. Molecular and transcriptional regulation of megakaryocyte differentiation. Stem Cells. 2001;19:397-407.
5. Ohneda K, Yamamoto M. Roles of hematopoietic transcription factors GATA-1 and GATA-2 in the development of red blood cell lineage. Acta Haematol. 2002;108:237-245.
6. Weiss MJ, Orkin SH. GATA transcription factors: key regulators of hematopoiesis. Exp Hematol. 1995;23:99-107.
7. Ito E, Toki T, Ishihara H, et al. Erythroid transcription factor GATA-1 is abundantly transcribed in mouse testis. Nature. 1993;362:466-468.
8. Pevny L, Simon MC, Robertson E, et al. Erythroid differentiation in chimaeric mice blocked by a targeted mutation in the gene for transcription factor GATA-1. Nature. 1991;349:257-260.
9. Fujiwara Y, Browne CP, Cunniff K, Goff SC, Orkin SH. Arrested development of embryonic red cell precursors in mouse embryos lacking transcription factor GATA-1. Proc Natl Acad Sci U S A. 1996;93:12355-12358.
10. Takahashi S, Onodera K, Motohashi H, et al. Arrest in primitive erythroid cell development caused by promoter-specific disruption of the GATA-1 gene. J Biol Chem. 1997;272:12611-12615.
11. Harigae H, Takahashi S, Suwabe N, et al. Differential roles of GATA-1 and GATA-2 in growth and differentiation of mast cells. Genes Cells. 1998;3:39-50.
12. Hirasawa R, Shimizu R, Takahashi S, et al. Essential and instructive roles of GATA factors in eosinophil development. J Exp Med. 2002;195:1379-1386.
13. Yu C, Cantor AB, Yang H, et al. Targeted deletion of a high-affinity GATA-binding site in the GATA-1 promoter leads to selective loss of the eosinophil lineage in vivo. J Exp Med. 2002;195:1387-1395.
14. Migliaccio AR, Rana RA, Sanchez M, et al. GATA-1 as a regulator of mast cell differentiation revealed by the phenotype of the GATA-1 low mouse mutant. J Exp Med. 2003;197:281-296.
15. Blobel GA, Simon MC, Orkin SH. Rescue of GATA-1–deficient embryonic stem cells by heterologous GATA-binding proteins. Mol Cell Biol. 1995;15:626-633.
16. Pevny L, Lin CS, D'Agati V, et al. Development of hematopoietic cells lacking transcription factor GATA-1. Development. 1995;121:163-172.
17. Weiss MJ, Orkin SH. Transcription factor GATA-1 permits survival and maturation of erythroid precursors by preventing apoptosis. Proc Natl Acad Sci U S A. 1995;92:9623-9627.
18. Suwabe N, Takahashi S, Nakano T, Yamamoto M. GATA-1 regulates growth and differentiation of definitive erythroid lineage cells during in vitro ES cell differentiation. Blood. 1998;92:4108-4118.
19. McDevitt MA, Shivdasani RA, Fujiwara Y, et al. A "knockdown" mutation created by cis-element gene targeting reveals the dependence of erythroid cell maturation on the level of transcription factor GATA-1. Proc Natl Acad Sci U S A. 1997;94:6781-6785.
20. Onodera K, Takahashi S, Nishimura S, et al. GATA-1 transcription is controlled by distinct regulatory mechanisms during primitive and definitive erythropoiesis. Proc Natl Acad Sci U S A. 1997;94:4487-4492.
21. Motohashi H, Katsuoka F, Shavit JA, Engel JD, Yamamoto M. Positive or negative MARE-dependent transcriptional regulation is determined by the abundance of small Maf proteins. Cell. 2000;103:865-875.
22. Takahashi S, Shimizu R, Suwabe N, et al. GATA factor transgenes under GATA-1 locus control rescue germline GATA-1 mutant deficiencies. Blood. 2000;96:910-916.
23. Whyatt D, Lindeboom F, Karis A, et al. An intrinsic but cell-nonautonomous defect in GATA-1–overexpressing mouse erythroid cells. Nature. 2000;406:519-524.
24. Wu H, Liu X, Jaenisch R, Lodish HF. Generation of committed erythroid BFU-E and CFU-E progenitors does not require erythropoietin or the erythropoietin receptor. Cell. 1995;83:59-67.
25. Kieran MW, Perkins AC, Orkin SH, Zon LI. Thrombopoietin rescues in vitro erythroid colony formation from mouse embryos lacking the erythropoietin receptor. Proc Natl Acad Sci U S A. 1996;93:9126-9131.
26. Suzuki N, Ohneda O, Takahashi S, et al. Erythroid-specific expression of the erythropoietin receptor rescued its null mutant mice from lethality. Blood. 2002;100:2279-2288.
27. Imagawa S, Smith BR, Palmer-Crocker R, Bunn HF. The effect of recombinant erythropoietin on intracellular free calcium in erythropoietin-responsive cells. Blood. 1989;73:1452-1457.
28. Fujimoto T, Ogawa M, Minegishi N, et al. Stepwise divergence of primitive and definitive haematopoietic and endothelial cell lineages during embryonic stem cell differentiation. Genes Cells. 2001;6:1113-1127.
29. Sposi NM, Cianetti L, Tritarelli E, et al. Mechanisms of differential transferrin receptor expression in normal hematopoiesis. Eur J Biochem. 2000;267:6762-6774.
30. Akashi K, Traver D, Miyamoto T, Weissman IL. A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. Nature. 2000;404:193-197.
31. Socolovsky M, Nam H, Fleming MD, et al. Ineffective erythropoiesis in Stat5a(−/−)5b(−/−) mice due to decreased survival of early erythroblasts. Blood. 2001;98:3261-3273.
32. Vannucchi AM, Paoletti F, Linari S, et al. Identification and characterization of a bipotent (erythroid and megakaryocytic) cell precursor from the spleen of phenylhydrazine-treated mice. Blood. 2000;95:2559-2568.
33. Tacchini L, Bianchi L, Bernelli-Zazzera A, Cairo G. Transferrin receptor induction by hypoxia: HIF-1–mediated transcriptional activation and cell-specific post-transcriptional regulation. J Biol Chem. 1999;274:24142-24146.
34. Schaefer A, Magocsi M, Marquardt H. Signalling mechanisms in erythropoiesis: the enigmatic role of calcium. Cell Signal. 1997;9:483-495.
35. Bookchin RM, Lew VL. Sickle red cell dehydration: mechanisms and interventions. Curr Opin Hematol. 2002;9:107-110.
36. Rich IN, Sawatzki G, Kubanek B. Specific enhancement of mouse CFU-E by mouse transferrin. Br J Haematol. 1981;49:567-573.
37. Labbaye C, Valtieri M, Barberi T, et al. Differential expression and functional role of GATA-2, NF-E2, and GATA-1 in normal adult hematopoiesis. J Clin Invest. 1995;95:2346-2358.
38. Ikonomi P, Rivera CE, Riordan M, et al. Overexpression of GATA-2 inhibits erythroid and promotes megakaryocyte differentiation. Exp Hematol. 2000;28:1423-1431.
39. Nakajima O, Takahashi S, Harigae H, et al. Heme deficiency in erythroid lineage causes differentiation arrest and cytoplasmic iron overload. EMBO J. 1999;18:6282-6289.
40. De Maria R, Zeuner A, Eramo A, et al. Negative regulation of erythropoiesis by caspase-mediated cleavage of GATA-1. Nature. 1999;401:489-493.
41. Nishimura S, Takahashi S, Kuroha T, et al. A GATA box in the GATA-1 gene hematopoietic enhancer is a critical element in the network of GATA factors and sites that regulate this gene. Mol Cell Biol. 2000;20:713-723.
42. Kina T, Ikuta K, Takayama E, et al. The monoclonal antibody TER-119 recognizes a molecule associated with glycophorin A and specifically marks the late stages of murine erythroid lineage. Br J Haematol. 2000;109:280-287.
43. Mucenski ML, McLain K, Kier AB, et al. A functional c-myb gene is required for normal murine fetal hepatic hematopoiesis. Cell. 1991;65:677-689.
44. Okuda T, van Deursen J, Hiebert SW, Grosveld G, Downing JR. AML1, the target of multiple chromosomal translocations in human leukemia, is essential for normal fetal liver hematopoiesis. Cell. 1996;84:321-330.
45. Kirito K, Osawa M, Morita H, et al. A functional role of Stat3 in in vivo megakaryopoiesis. Blood. 2002;99:3220-3227.
46. Tanabe O, Katsuoka F, Campbell AD, et al. An embryonic/fetal beta-type globin gene repressor contains a nuclear receptor TR2/TR4 heterodimer. EMBO J. 2002;21:3434-3442.

Erythroid-specific expression of the erythropoietin receptor rescued its null mutant mice from lethality

Norio Suzuki, Osamu Ohneda, Satoru Takahashi, Masato Higuchi, Harumi Y. Mukai, Tatsutoshi Nakahata, Shigehiko Imagawa, and Masayuki Yamamoto

Erythropoietin (Epo) and its receptor (EpoR) are indispensable to erythropoiesis. Although roles besides angiogenesis, such as neuroprotection and heart development, have been reported for the Epo-EpoR system, the precise contribution of Epo-EpoR to these nonhematopoietic tissues requires clarification. Exploiting a *GATA-1* minigene cassette with hematopoietic regulatory domains, we established 2 lines of transgene-rescued EpoR-null mutant mice expressing EpoR exclusively in the hematopoietic lineage. Surprisingly, despite the lack of EpoR expression in nonhematopoietic tissues, these mice develop normally and are fertile. As such, we could exploit them for analyzing the roles of the Epo-EpoR system in adult hematopoiesis and in nonhematopoietic tissues. These rescued lines showed a differential level of EpoR expression in erythroid cells; one expressed approximately 40%, and the other expressed 120% of the wild-type EpoR level. A colony formation assay showed that erythroid progenitors in the 2 mutant lines exhibit distinct sensitivity to Epo. The circulating Epo level was much higher in the transgenic line with a lower EpoR expression. In response to induced anemia, the plasma Epo concentrations increased in both lines. Notably, the timing of the peak of plasma Epo concentration was delayed in both lines of rescued mice compared with wild type, suggesting that, in wild-type mice, nonhematopoietic EpoR contributes to the regulation of plasma Epo concentration. We thus conclude that nonhematopoietic expression of EpoR is dispensable to normal mouse development and that the expression level of EpoR regulates erythropoiesis by controlling the sensitivity of erythroid progenitors to Epo. (Blood. 2002;100:2279-2288)

Introduction

Erythropoietin (Epo) stimulates the proliferation and differentiation of erythroid progenitors.[1] Its receptor, EpoR, is a member of the type 1 cytokine receptor family characterized by a single transmembrane domain.[2] Recently, the importance of the Epo-EpoR system in primitive and definitive erythropoiesis was determined by generating lines of mutant mice lacking either the *Epo* or *EpoR* gene.[1,3,4] Both *Epo* and *EpoR* homozygous mutant mice died of severe anemia between embryonic day 13 (E13) and E15. Existence of the erythroid progenitors erythroid colony-forming unit (CFU-E) and erythroid burst-forming unit (BFU-E) in the livers of these mutants indicated that Epo-EpoR is not required for the commitment of hematopoietic progenitors to the erythroid lineage.[5,6] However, primitive erythropoiesis is partially impaired by the absence of the Epo-EpoR pathway, as shown by the smaller number of primitive erythrocytes observed in the yolk sac blood islands of homozygous mutant embryos compared with heterozygous mutant and wild-type embryos.[1,3,4] In contrast, contribution of the Epo-EpoR system is crucial during definitive erythropoiesis, namely for proliferation, survival, and differentiation of erythroid progenitors in the later stages of differentiation.[1,3,4]

Expression of EpoR is not restricted to the hematopoietic lineage, but it can be identified in various nonhematopoietic tissues. For example, EpoR is expressed in epicardium and pericardium.[7] In addition, Epo has been shown to promote the proliferation of endothelial cells, fetal liver stromal cells, and skeletal muscle satellite cells.[8-11] Epo can also assist in the recovery of neurons from injury.[12,13] This widespread expression profile of EpoR strongly suggests that a lack of Epo-EpoR may affect various biologic aspects. However, because germ-line *Epo-EpoR* mutant embryos die in utero, it is unknown whether the Epo-EpoR pathway is actually required for the development and activity of cells in nonhematopoietic tissues.

GATA-1 has been shown to regulate the expression of the Epo receptor[14] and most other erythroid genes. Transgenic expression of GATA-1 under the transcriptional control of the *GATA-1* locus hematopoietic regulatory domain (*GATA-1–HRD*) rescued GATA-1 knockdown mice from embryonic lethality by restoring GATA-1 expression in hematopoietic tissues.[15,16] Therefore, we exploited the *GATA-1–HRD* to rescue *EpoR* gene knockout mice from embryonic lethality by expressing the *EpoR* transgene specifically in erythroid cells.

The *GATA-1–HRD-EpoR* transgene recovered erythropoiesis in EpoR-null mice, rescuing the mutants from embryonic lethality to give fertile mice with a normal phenotype throughout their lives. We assume, therefore, that nonhematopoietic expression of EpoR is dispensable to normal mouse development. We generated 2 lines

From the Center for Tsukuba Advanced Research Alliance, Institutes of Basic Medical Sciences and Clinical Medicine, University of Tsukuba; Product Research Laboratory, Chugai Pharmaceutical, Tokyo; and Department of Pediatrics, Graduate School of Medicine, Kyoto University, Japan.

Submitted January 16, 2002; accepted March 15, 2002. Prepublished online as *Blood* First Edition Paper, May 31, 2002; DOI 10.1182/blood-2002-01-0124.

Supported by grants from the Ministry of Education, Science, Sports and Culture (M.Y.), JSPS-RFTF and CREST (M.Y.), and PROBRAIN (S.T.).

Reprints: Masayuki Yamamoto, Center for TARA, University of Tsukuba, 1-1-1 Tennoudai, Tsukuba 305-8577, Japan; e-mail: masi@tara.tsukuba.ac.jp.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 U.S.C. section 1734.

of EpoR-rescued mice and, compared with wild-type levels, one line expresses EpoR at 40%, whereas the other expresses EpoR at 120% in bone marrow cells. These mice do not suffer from polycythemia or anemia because the in vivo plasma Epo level is stringently regulated. Importantly, though, erythroid progenitors in the bone marrow of the low and high EpoR-expressing lines showed different sensitivity to Epo in colony-formation assays. Thus, the expression level of EpoR regulates erythropoiesis by controlling the sensitivity of erythroid progenitor cells to Epo.

Materials and methods

Mice and construction of EpoR transgene

EpoR-deficient (C57Bl/6) mice[4] were supplied by Jackson Laboratories (Bar Harbor, ME). EpoR cDNA was ligated to a genomic fragment containing *GATA-1–HRD*.[15] Transgene constructs were injected into fertilized eggs derived from BDF1 parents, and 6 independent lines of transgenic mice were obtained. Two lines of mice, Tg-A and Tg-B, were mated with $EpoR^{+/-}$ mice to establish the compound mutant mice $EpoR^{+/-}$::*HG1-EpoR*. $EpoR^{-/-}$::*HG1-EpoR* mice were obtained by crossing the former mice with $EpoR^{+/-}$ mice. Resultant mice and embryos were genotyped by polymerase chain reaction (PCR), Southern blot analysis, or both. Genomic tail DNA was prepared and digested with *Avr*II. Southern membranes were hybridized with a ^{32}P-labeled probe, as indicated in Figure 1A. PCR analysis with genomic DNA was also performed to amplify endogenous *EpoR* alleles (387 bp) and transgenes (303 bp). The PCR primer pair used was primer EpoR3S (5′-GGTGAGTCACGAAAGTCATG-3′) and primer EpoR4AS (5′-ACACGTCCACTTCATATCGG-3′), corresponding to the exon III and the exon IV sequence, respectively, of the *EpoR* gene. All mice were treated according to the regulations of the Standards for Human Care and Use of Laboratory Animals of the University of Tsukuba.

RNA and RT-PCR

After isolation from various mouse tissues using ISOGEN (Nippon-Gene, Osaka, Japan), total RNA was incubated with DNase I (RQ1; Promega, Madison, WI). Samples were reverse-transcribed by Super-Script II, and random hexamers (both from Gibco BRL, Rockville, MD) and the PCR reaction (40 cycles) were performed as follows: 40 seconds at 95°C, 40 seconds at 60°C, and 60 seconds at 72°C. The PCR primers used were primer-1, 5′-ACGAAACAGGGGCGCTGGAG-3′; primer-2, 5′-ACACGTCCACTTCATATCGG-3′; and primer-3, 5′-TCCTCTGCATCAACAAGCCC-3′ (Figure 1C). Hypoxanthine guanine phosphoribosyl transferase (HPRT) was used as an internal control; the sense primer was 5′-GCTGGTGAAAAGGACCTCT-3′, and the antisense primer was 5′-CACAGGACTAGAACACCTGC-3′. PCR products were electrophoresed on an agarose gel and were transferred to a nylon membrane (Zeta-Probe; BioRad, Hercules, CA) for hybridization with a ^{32}P-labeled probe. The expression of endogenous and transgenic EpoR was detected independently to avoid contamination of genomic DNA in such a highly sensitive detection method.

Histologic analysis and TUNEL assay

Embryos and tissues were fixed in 4% paraformaldehyde for 30 minutes and embedded in polyester wax (BDH Laboratory, Poole, England). Sections (5 μm) were incubated with rabbit anti-GFP antibody (diluted 1:1000; Molecular Probes, Eugene, OR), rat anti–PECAM-1 monoclonal antibody (1:500; BD PharMingen, San Diego, CA) and mouse anti–alpha sarcomeric muscle actin monoclonal antibody. Specific antibody binding was visualized using either horseradish peroxidase (HRP)–conjugated goat anti-mouse immunoglobulin that was polymerized by dextran (EnVision; DAKO, Carpinteria, CA) or HRP-conjugated anti-rabbit immunoglobulin (Biosource, Camarillo, CA) secondary antibody. TUNEL assay was performed using an in situ apoptosis detection kit (TAKARA, Osaka, Japan). Color detection was performed using diaminobenzidine (250 mg/mL), H_2O_2 (0.01%), and $NiCl_2$ (0.05%) as a chromogen. Kernechtrot solution was used for counterstaining in all sections.

Flow cytometry

FACS analysis was performed with the CellQuest program (Becton Dickinson, San Jose, CA). Single-cell suspensions from E12.5 livers were prepared and incubated with CD16/CD32 (2.246) antibody (1:200) on ice for 15 minutes. Subsequently, cells were stained with allophycocyanin (APC)-conjugated anti-Ter119, fluorescein isothiocyanate (FITC)–conjugated anti-CD41, phycoerythrin (PE)-conjugated anti-CD44, and PE-conjugated anti–c-Kit antibodies for 30 minutes. After the final wash, viable cells were selected by propidium iodide (PI) staining. APC-, FITC-, and

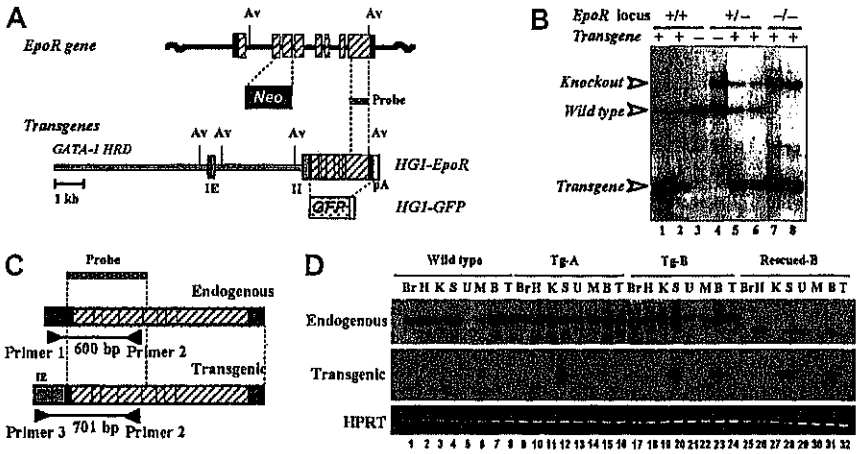


Figure 1. Establishment of rescued *EpoR* mutant mouse lines. (A) Structure of the wild-type and mutant *EpoR* locus[4] and design of the *HG1-EpoR* and *HG1-GFP* transgenes. The *GATA-1–HRD* minigene containing exons IE and II of the mouse *GATA-1* gene was ligated to either EpoR or GFP cDNA to give *HG1-EpoR* and *HG1-GFP*, respectively. The translated and untranslated exons of the *EpoR* gene are shown as solid and hatched boxes, respectively. *Neo*, pA, and Av represent the neomycin resistance gene cassette, polyadenylation signal, and *Avr*II sites, respectively. (B) Genotyping of transgenic and rescued mice by Southern blot analysis. Tail DNA samples digested with *Avr*II were hybridized to the *EpoR* probe shown in panel A. Tg-A (lane 1) and Tg-B (lane 2) mice contain 40 and 4 copies of the *EpoR* transgene, respectively. Note that the wild-type *EpoR* band (4.2 kb) is absent in lanes 7 and 8 (Tg-B rescued mice), and a knockout allele band is present in lanes 4 to 8. (C) Expected mRNA structures of endogenous and transgenic *EpoR*. Transgene-derived *EpoR* mRNA includes exons IE and II of the *GATA-1–HRD*, so that its amplicon can be distinguished from endogenous *EpoR*-derived mRNA using the primer sets shown. (D) Endogenous and transgenic EpoR mRNA expression determined by RT-PCR analysis. Samples of total RNA from various tissues of wild-type (lanes 1-8), Tg-A (lanes 9-16), Tg-B (lanes 17-24), and Rescued-B (lanes 25-32) mice were analyzed. PCR was performed using primer sets Primer 1 with 2 and Primer 3 with 2 to detect endogenous (600 bp) and transgenic (701 bp) EpoR transcripts, respectively. HPRT was used as an internal control. Br indicates brain; H, heart; K, kidney; S, spleen; U, uterus; M, muscle; B, bone marrow; and T, testis.

PE-conjugated rat IgG2b were used as isotype-matched controls. The antibodies were all obtained from BD PharMingen.

RT-PCR analysis with sorted bone marrow cells

Mononuclear cells from adult mouse bone marrow were prepared using Histopaque1082 (Sigma, St Louis, MO). Cell suspensions were stained with PI- and PE-conjugated streptavidin after incubation with biotin-conjugated anti-Ter119, anti-CD34 or anti–c-Kit antibodies. Fluorescence intensity of the cells was analyzed, and 5×10^4 green fluorescence protein $(GFP)^+$ or GFP^- cells in PI^- fractions were sorted. RNA was extracted by RNeasy (QIAGEN, Basel, Switzerland) and reverse-transcribed by Sensiscript (QIAGEN). To detect GATA-1 mRNA, these samples were rendered for PCR amplification (35 cycles) using the primer pair 5′-ACTCGTCATACCACTAAGGT-3′ and 5′-AGTGTCTGTAGGCCTCAGCT-3′.

Epo binding assay

The number of EpoR on the hematopoietic cell surfaces was measured, as described previously.[17] For each genotype, mononuclear cells were obtained from the bone marrow of 4 to 8 mice of 10 to 12 weeks of age.

Colony assays

Fetal liver cells (1×10^4) or bone marrow mononucleated cells (2×10^4) were cultured in 1 mL 0.8% methylcellulose medium containing 30% fetal bovine serum (FBS), 1% bovine serum albumin (BSA), 0.1 mM 2-mercaptoethanol and various concentrations of recombinant human Epo (Chugai Pharmaceutical, Tokyo, Japan). After 2 days (fetal liver) or 3 days (bone marrow) of culturing, cells were stained with benzidine, and positive colonies were counted. Bone marrow mononucleated cells (1×10^5) were cultured in the same medium supplemented with 100 ng/mL stem cell factor (SCF; R&D Systems, Minneapolis, MN) and various concentrations of Epo for 7 days, and benzidine-positive erythroid bursts were counted. These assays were performed in triplicate and repeated 3 times, and the results are shown with standard deviations.

Induction of anemia

Six-week-old mice were anesthetized with diethylether. After withdrawing 0.4 mL blood from the retro-orbital venous plexus using heparin-coated microtubes, an aliquot of the collected blood sample was used for determining the hematopoietic indices and leukocyte and platelet numbers using an automatic counter (Nihon-Kouden, Tokyo, Japan). Plasma was also isolated, and the Epo concentration was measured by radioimmunoassay.[17]

Results

Rescue of EpoR-null mutant mice from embryonic lethality by *GATA-1–HRD-EpoR* transgene

We generated 6 lines of transgenic mice expressing full-length EpoR under the control of *GATA-1–HRD* (*HG1-EpoR*) (Figure 1A). Genomic Southern blot analysis revealed that the Tg-A line (Figure 1B, lane 1) contains approximately 40 copies of the *HG1-EpoR* transgene, whereas the Tg-B line (lane 2) has only 4 copies. To monitor the expression of the *EpoR* transgene, line Tg-A was coinjected with a *GATA-1–HRD-GFP* expression construct (*HG1-GFP*, Figure 1A). In the anticipation that the expression level of the *EpoR* transgene will vary according to the transgene copy number, we selected lines Tg-A and Tg-B for use in a genetic rescue experiment of EpoR-deficient mice.

$EpoR^{+/-}$ mice possessing either Tg-A or Tg-B were crossed with $EpoR^{+/-}$ mice to yield $EpoR^{-/-}$::*Tg* mice. Resultant pups were genotyped by PCR, Southern blot analysis, or both, and, according to Mendelian inheritance, an expected number of mice were found to have an $EpoR^{-/-}$::*Tg-A* or $EpoR^{-/-}$::*Tg-B* genotype (Table 1). Remarkably, no $EpoR^{-/-}$ mice lacking the *EpoR* transgene were found even among as many as 139 mice. We named the rescued EpoR mutant pups resulting from the crosses with lines Tg-A and Tg-B as Rescued-A and Rescued-B lines of mice, respectively. Genomic Southern blot analysis of tail DNA from the $EpoR^{-/-}$::*Tg-A* (data not shown) and $EpoR^{-/-}$::*Tg-B* mice (Figure 1B, lanes 7, 8) clearly indicated that these rescued mice contained the knockout allele and the *EpoR* transgene. These results thus demonstrate that transgenic expression of EpoR cDNA under the influence of *GATA-1–HRD* rescued the germ-line EpoR-deficient mice from embryonic lethality.

Expression of transgenic EpoR in various mouse tissues and interbreeding experiments

Expression of the *EpoR* transgene was examined in various tissues and organs of the rescued mice, particularly in hematopoietic tissues. We used highly sensitive RT-PCR analysis using a radiolabeled probe, and the primer sets used are shown in Figure 1C. Analysis revealed specific expression of EpoR mRNA from the *HG1-EpoR* transgene in the spleen and bone marrow of Tg-A, Tg-B, and Rescued-B mice (Figure 1D, middle panel). In agreement with previous reports,[7,8,11,12,18,19] endogenous expression of EpoR mRNA was detected in most of the tissues examined in wild-type, Tg-A and Tg-B mice, but not in Rescued-B mice (Figure 1D, top panel). Importantly, as we generated transgenic mouse lines with full-length EpoR cDNA, we expected that the transgene-rescued mice would express the full-length EpoR mRNA exclusively. Indeed, we did not detect any truncated form of EpoR mRNA by Northern blot and RT-PCR analyses (data not shown). These results demonstrate that hematopoietic lineage-specific expression of EpoR rescued the *EpoR* germ-line mutant mice from embryonic lethality.

We then performed interbreeding experiments with the rescued male and female mice. We expected that 3 quarters of F1 embryos would survive because of the *HG1-EpoR* transgene. Indeed, the intercross resulted in 23 viable pups, all of which possessed the

Table 1. Genotype of transgene-rescued EpoR mutant mouse offspring

Crossing	Litters	Pups	Transgene	*EpoR* genotype +/+ (%)	*EpoR* genotype +/− (%)	*EpoR* genotype −/− (%)
$EpoR^{+/-}$::*Tg-A* and $EpoR^{+/-}$	8	90	+	15 (24)	28 (45)	19 (31)
			−	6 (21)	22 (79)	0 (0)
$EpoR^{+/-}$::*Tg-B* and $EpoR^{+/-}$	7	49	+	5 (17)	16 (53)	9 (30)
			−	4 (21)	15 (79)	0 (0)
$EpoR^{-/-}$::*Tg-B* and $EpoR^{-/-}$::*Tg-B*	4	23*	+	—	—	23 (100)
			−	—	—	0 (0)

Two lines of mice containing the *HG1-EpoR* transgene (*Tg-A* and *Tg-B*) were used for the rescue experiments. The genotypes of pups derived from each parent were determined 3 to 4 weeks after birth by PCR, Southern blot analysis, or both.

*Twelve male and 11 female pups were derived from the interbreeding of Rescued-B mice.

HG1-EpoR transgene, whereas no pups lacking the transgene were born (Table 1). The male-to-female ratio was approximately 1:1, and the pups lived for more than 1 year. Development, growth, mating, pregnancy, and childbirth of the rescued mice were within a normal range, and no apparent morphologic abnormality was observed (data not shown).

Rescue of primitive and definitive erythropoiesis in EpoR-null mutant embryos

Primitive erythropoiesis was also affected by a lack of EpoR,[20] as seen in the paleness of the yolk sac of the E10.5 $EpoR^{-/-}$ embryo shown in Figure 2C. In comparison, the yolk sacs of Rescued-B embryos (Figure 2D) were not anemic and displayed a color similar to those of wild-type and Tg-B embryos (Figure 2A-B).

Only a small number of primitive erythrocytes were observed in the E11.5 $EpoR^{-/-}$ embryonic yolk sacs, whereas the endothelium surrounding the blood islands appeared normal (Figure 2E). In contrast, significantly more primitive erythrocytes were observed in the blood islands of Rescued-B yolk sacs (Figure 2F). Thus, EpoR expressed from the *EpoR* transgene effectively recovered primitive erythropoiesis in the yolk sacs of $EpoR^{-/-}$ embryos.

On gross examination, embryonic livers of E12.5 wild-type (Figure 2G) and Tg-B (Figure 2H) mice were red, indicating active definitive erythropoiesis. In contrast, the $EpoR^{-/-}$ embryonic livers were pale (Figure 2I), and the number of erythrocytes was markedly decreased (data not shown). Erythropoiesis in EpoR-null mutant livers recovered on transgenic expression of EpoR, as indicated by the liver color, which was similar to that found in wild-type embryos (Figure 2J).

Because Tg-A mice were injected with *HG1-EpoR* and *HG1-GFP* transgenes, we examined the embryonic livers under a fluorescence microscope. As can be seen by the green fluorescence, the *HG1-GFP* transgene was specifically expressed in the livers of E12.5 transgenic (Figure 2L) and rescued (Figure 2N) embryos, but not in the livers of wild-type (Figure 2K) and $EpoR^{-/-}$ (Figure 2M) embryos. These results indicate that the *HG1-EpoR* transgene was efficiently expressed in the embryonic liver.

Rescue of hematopoietic cells from apoptotic cell death by transgenic EpoR

GFP-positive erythrocytes (dark purple) and GFP-positive megakaryocytes (arrowheads) were observed in Tg-A (Figure 3B) and Rescued-A (Figure 3D) embryonic livers, but not in the livers of wild-type or $EpoR^{-/-}$ embryos (Figure 3A,C). Histologic examination thus revealed that the *EpoR*-transgene was expressed in hematopoietic cells of the liver.

TUNEL staining allowed a clearer evaluation of the extent of apoptosis in the embryonic livers (Figure 3E-H). Rescued embryonic livers (Figure 3H) harbored a significantly lower number of

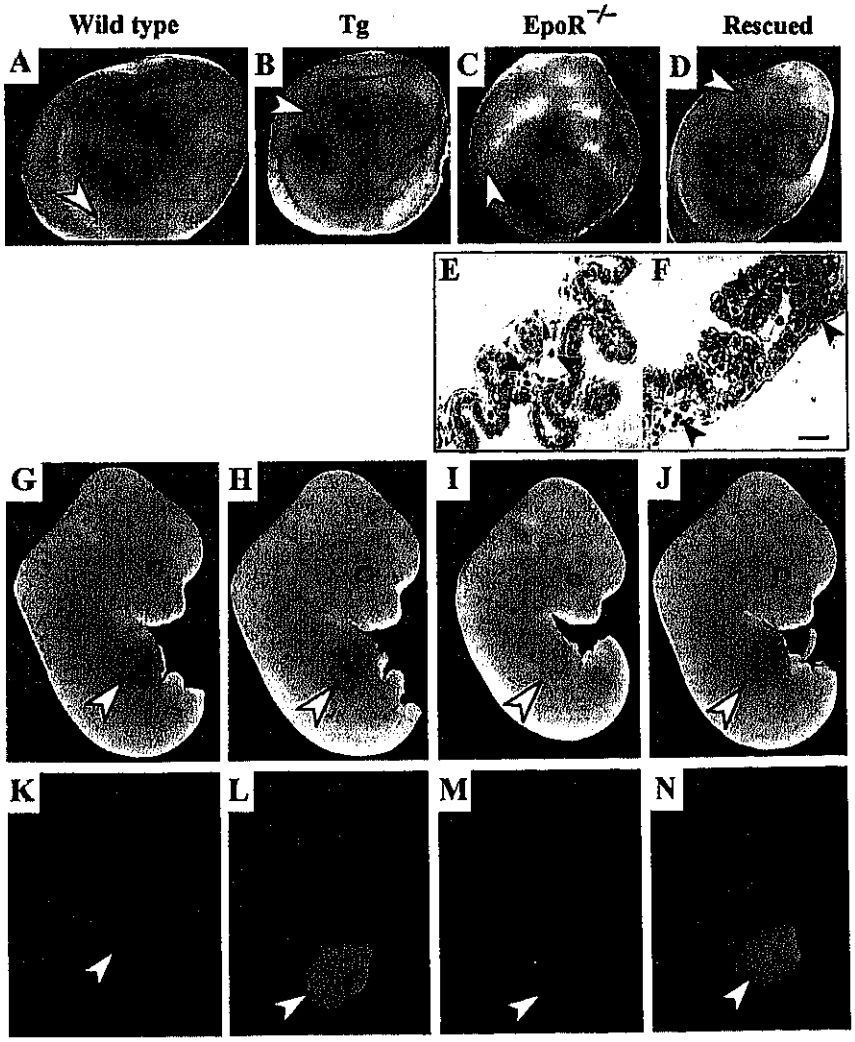


Figure 2. EpoR-null embryos were rescued from severe anemia and embryonic death by hematopoietic lineage-specific expression of the *EpoR* transgene. A single litter obtained by crossing $EpoR^{+/-}$::*Tg* with $EpoR^{+/-}$ mice was used in this study. Wild-type (A), Tg-B (B), $EpoR^{-/-}$ (C, E), and Rescued-B (D, F) embryos are shown. Panels A to D show whole E10.5 embryos, whereas panels E and F show sections of the yolk sac of E11.5 embryos. Note that the yolk sac blood vessels of the rescued embryo were filled by nucleated erythrocytes (arrowheads in panel F), whereas $EpoR^{-/-}$ embryo contained only a small number of erythrocytes (E). Scale bar, 50 μm. Whole E12.5 embryos are shown in panels G to J. In contrast to the $EpoR^{-/-}$ embryo (I), the size and redness of the liver (arrowhead) in the Rescued-B embryo (J) were similar to those of the Tg-B (H) and wild-type (G) embryos. The E12.5 embryos shown in the fluorescence images K to N were from a single litter obtained by crossing $EpoR^{+/-}$::*Tg-A* with $EpoR^{+/-}$ mice. Because *HG1-EpoR* and *HG1-GFP* transgenes were coinjected, transgenic EpoR expression could be monitored by the intensity of green fluorescence. Green fluorescence was detected only in the livers (arrowheads) of Tg-A (L) and Rescued-A (N) embryos.

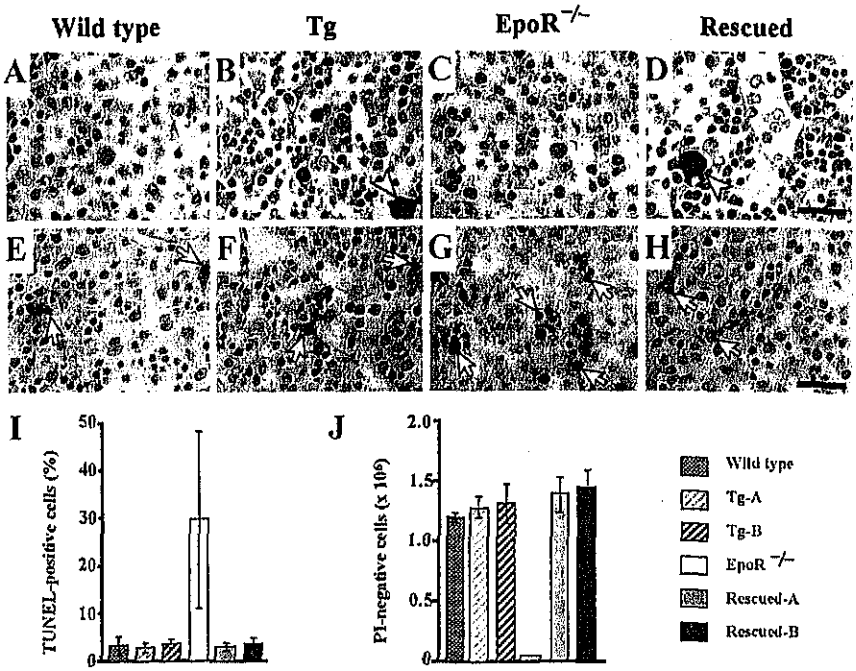


Figure 3. Rescue of hematopoietic cells from apoptotic cell death by transgenic expression of EpoR in E12.5 embryonic livers. (A-D) Sections of E12.5 embryonic livers were stained with anti-GFP antibody. GFP+ cells (stained purple) are expected to coexpress the *EpoR* transgene. Megakaryocytes (arrowheads) and erythroid cells were stained positive in Tg-A (B) and Rescued-A (D) embryos only. (E-H) TUNEL (terminal transferase-mediated dUTP nick-end labeling) assay of E12.5 embryonic liver cells. Nuclei of TUNEL-positive cells were stained purple (arrows). Although most cells in the *EpoR*$^{-/-}$ liver stained TUNEL positive (G), livers of the rescued (H), wild-type (E), and Tg-A (F) embryos contained only a small number of positive cells. Scale bar equals 50 μm (A-H). (I) TUNEL-positive cells as a percentage of the total liver cells counted in each section. (J) Number of PI-negative cells assessed by FACS analysis.

apoptotic cells than *EpoR*$^{-/-}$ embryonic livers (Figure 3G). Although the frequency of TUNEL-positive cells varied among the knockout embryos, the number of TUNEL-positive cells was always several-fold higher on average than that of wild-type, transgenic, and rescued embryos (Figure 3I). We also examined the number of PI staining-negative cells, which represent living cells, in the knockout and rescued embryonic livers. *EpoR*$^{-/-}$ embryos contained 10-fold fewer PI-negative cells than wild-type, transgenic, and rescued embryos (Figure 3J). These results demonstrate that apoptosis is prevalent in the livers of *EpoR*$^{-/-}$ embryos, where most cells are erythroid. In addition, the transgenic expression of EpoR transduces signals that effectively protect hematopoietic cells from apoptotic cell death.

Hematopoietic cell populations in EpoR-deficient and transgene-rescued fetal livers

We assessed the population of hematopoietic cells in the livers of transgenic and rescued E12.5 embryos by determining the amount of cells positive for various markers of hematopoietic differentiation. FACS analysis showed that, whereas most of the cells in the wild-type and Rescued-B livers were c-Kit$^-$ (Figure 4A, middle panel), c-Kit$^+$ hematopoietic progenitors were predominant in the *EpoR*$^{-/-}$ livers, albeit the total number of the cells was low (data not shown). In the FACS analysis, CD44^{low}/Ter119$^+$ cells represent a population of an erythroid cell lineage. Compared with wild type (88.3%) and Rescued-B (88.6%), this fraction of erythroid cells was less abundant in the *EpoR*$^{-/-}$ liver (16.0%; Figure 4A, top), suggesting that erythroid differentiation is suppressed by the absence of EpoR.

To elucidate the function of transgenic EpoR in erythroid cell development, an in vitro colony formation assay was undertaken. Embryonic liver cells were cultured in a methylcellulose medium containing various concentrations of Epo for 2 days. Benzidine-positive colonies were scored as CFU-E–derived colonies. At all Epo concentrations used, fetal liver cells from Tg-A, Tg-B, Rescued-A, and wild-type embryos showed a comparable number of erythroid colonies (Figure 4B), indicating a similar sensitivity of CFU-E progenitors to Epo among these embryos. Although liver cells from Rescued-B embryos formed a

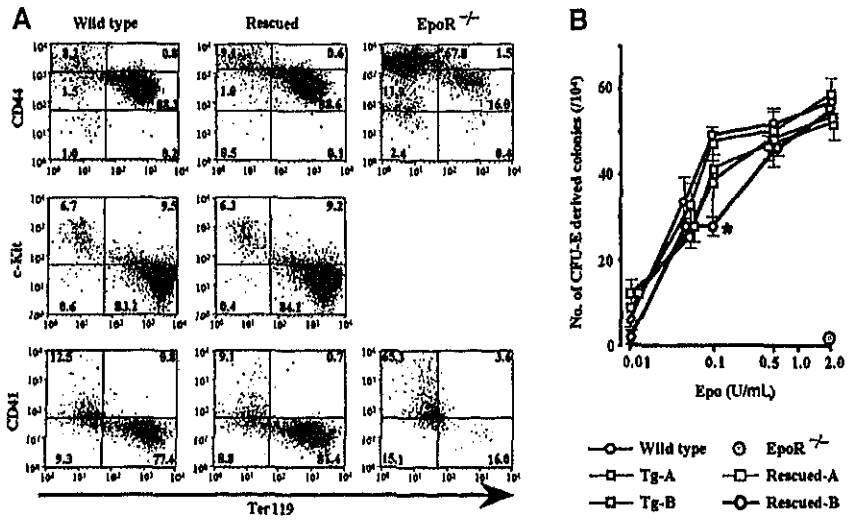


Figure 4. Fetal liver hematopoiesis in rescued embryos. (A) Analysis of E12.5 fetal liver hematopoietic cell populations. Single cell suspensions were stained with anti-c-Kit, -Ter119, -CD41, and -CD44 antibodies, and these cells (1×10^4) were analyzed by FACS. PI-negative cells were gated, and the intensity of fluorescence was measured. The ratio of cells in each quadrangle is shown. (B) Fetal liver cells were cultured for 2 days in methylcellulose medium containing various concentrations of Epo, and erythroid colonies were counted after benzidine staining. *$P < .01$ compared with wild-type mouse.

smaller number of erythroid colonies in the presence of 0.1 U/mL Epo, the erythroid colony number recovered on incubation with high concentrations of Epo. These observations suggest that livers from wild-type, Rescued-B, and other genotypes of embryos contain a similar number of CFU-E (approximately 50 CFU-E per 1×10^4 fetal liver cells). In contrast, no benzidine-positive colony was detected in the $EpoR^{-/-}$ embryonic liver cells, indicating that transgenic expression of EpoR efficiently rescued CFU-E activity in the $EpoR^{-/-}$ embryonic liver.

EpoR has been reported to play a functional role in the formation of megakaryocytes.[21] Furthermore, our study showed the *EpoR* transgene to be expressed in megakaryocytes in the livers of transgenic and rescued embryos (Figure 3B-D). We therefore examined the frequency of megakaryocytes in E12.5 embryonic livers. Despite overexpression of the *EpoR* transgene in megakaryocytes of Tg-B embryos and no expression at all in megakaryocytes of $EpoR^{-/-}$ embryos, the frequency of $CD41^+$ megakaryocytes in these livers was comparable to that of the wild type (Figure 4A, bottom panel, and data not shown). The number of circulating platelets did not change in Tg-B or transgene-rescued mice (below). These results suggest that EpoR is dispensable in megakaryocyte development.

Rescue of heart development by hematopoietic expression of EpoR

Because Epo activates angiogenesis,[22] we examined the distribution of endothelial cells in E12.5 embryos. After whole-mount staining with PECAM-1 antibody, embryos were sectioned and histologic examination was performed. The lateral sides of somites (Figure 5A-D) and livers (Figure 5E-H) are shown. In somites and liver, the endothelial cells in $EpoR^{-/-}$ and rescued embryos appeared to be similar to those in the wild-type embryos, suggesting that the Epo-EpoR signaling pathway is not necessary for endothelial cell development.

It has been reported that the Epo-EpoR signaling pathway plays an important role in cardiac morphogenesis.[7] Indeed, in E12.5 $EpoR^{-/-}$ embryonic hearts, epicardium detachment and a lack of defined capillary structures in the endocardium were observed (Figure 5K). However, the epicardium and capillary development in transgene-rescued mice were normal (Figure 5L) and were similar to those in wild-type (Figure 5I) and transgenic embryonic hearts (Figure 5J).

Because Rescued-A embryos were coinjected with the GFP transgene, embryos were examined by specific staining with anti-GFP antibody. The results clearly revealed that transgenic GFP, hence transgenic EpoR, was expressed in fetal liver (Figure 5M) but not in heart. Transgenic GFP expression was not detectable in the endocardium, epicardium, or pericardium of the embryos, where EpoR expression is usually detected, even at higher magnifications (data not shown). These results suggest that the Epo-EpoR system is not a crucial signal transduction pathway for normal development of the heart but rather that severe anemia might be pertinent to the blockage of heart development, perhaps through a reduction in the oxygen supply to the developing heart. Thus, we propose that the Epo-EpoR system does not relate directly to epicardium or capillary development in the embryonic heart.

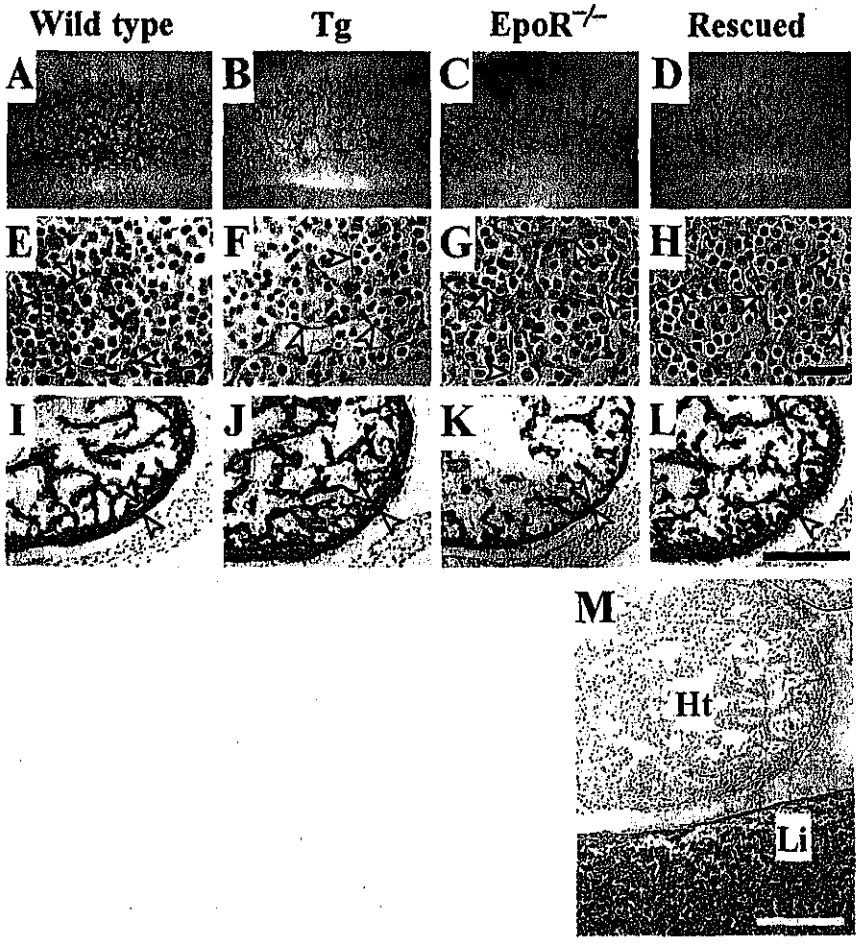


Figure 5. Analyses of nonhematopoietic embryogenesis. Whole E12.5 embryos and sections were stained with anti–PECAM-1 antibody. The morphology of the blood vessels (stained purple) in the lateral sides of somites (A-D, upside is dorsal) and fetal livers (E-H, arrowheads) of wild-type, transgenic (Tg), EpoR-null, and rescued embryos were compared. No differences were found among the embryos. In the embryonic heart sections (I-L), the smooth muscles of the left ventricles were stained with anti–alpha sarcomeric muscle actin antibody. Although the compact layer (between the arrowheads) of the rescued embryo (L) was normal, the compact layer of the $EpoR^{-/-}$ embryo (K) was considerably thinner than that of wild-type (I) and transgenic (J) embryos. (M) A section including the heart (Ht) and liver (Li) of a Rescued-A embryo was stained with anti-GFP antibody. GFP^+ cells were not detected in the heart, but most of cells in the liver were GFP^+, as seen by the purple staining. Scale bar, 50 μm (E-H) and 200 μm (I-M).

Analysis of hematopoietic cells in transgene-rescued adult *mouse bone marrow*

Although EpoR expression has been demonstrated in hematopoietic stem cells and progenitors of the bone marrow,[23] we envisage that expression of the *HG1-EpoR* transgene cannot recapitulate EpoR expression in such cells. To address the question as to whether EpoR is indispensable for the differentiation and maintenance of stem cells and progenitors, we performed FACS and RT-PCR analyses of Rescued-A mice containing *Tg-A* and *HG1-GFP* transgenes. Approximately 10% of bone marrow cells from the Rescued-A animals expressed GFP, and the population and intensity of fluorescence was similar to that of Tg-A mice (Figure 6A and data not shown).

$CD34^+$ progenitors, which were shown to express EpoR,[24] did not express GFP at all (Figure 6A, left). Thus, EpoR expression in the $CD34^+$ hematopoietic progenitors does not appear to be essential for normal hematopoiesis. Importantly, a GFP^{high} fraction was included in the c-Kit^+ fraction (Figure 6A, middle), which contains *BFU-E* and *CFU-E erythroid progenitors* (N. Suwabe, N.S., M.Y., unpublished observation, December 2001). We conclude that transgenic expression of EpoR in the $CD34^-$/c-Kit^+ fraction rescued the differentiation of EpoR-null erythroid progenitors to give rise to $Ter119^+$ mature erythroid cells.

RT-PCR analysis was performed on the GFP-positive and -negative fractions of bone marrow from Tg-A and Rescued-A mice. Although the Rescued-A mouse did not express endogenous EpoR mRNA at all, GFP^- cells of the Tg-A mouse expressed endogenous EpoR mRNA weakly (Figure 6B). As expected, the GFP^- fraction of the Rescued-A mouse did not express transgenic EpoR mRNA. GATA-1 mRNA was also enriched in the GFP^+ fraction compared with the GFP^- fraction of Tg-A and Rescued-A

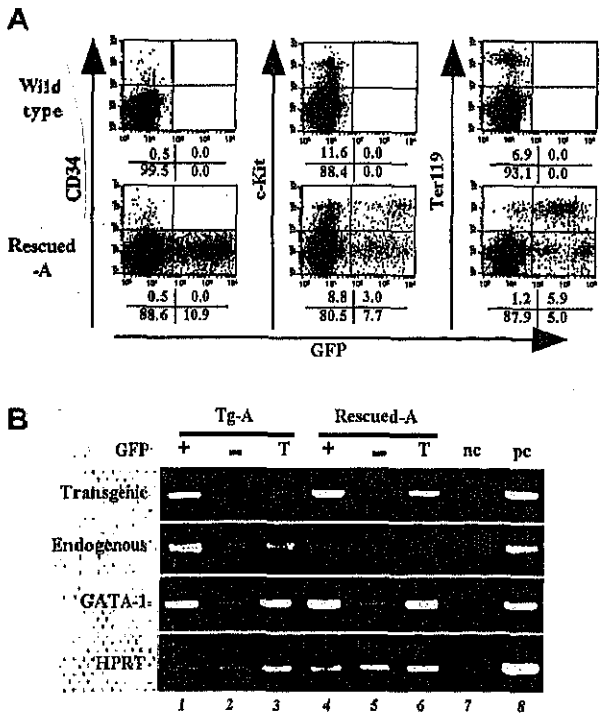


Figure 6. Analyses of bone marrow hematopoiesis in transgenic-EpoR rescued mice. (A) FACS analysis of the mononucleated cells in Rescued-A mice. PI-negative cells were gated, and fluorescence intensities were analyzed. (B) RT-PCR analysis of sorted bone marrow cells from Tg-A and Rescued-A mice. GFP-positive (+), GFP-negative (-), and total (T) fractions were sorted, and RNA was extracted. Endogenous, EpoR, transgenic EpoR, and GATA-1 transcripts were detected by RT-PCR. Negative (nc) and positive (pc) controls were loaded in lanes 7 and 8, respectively.

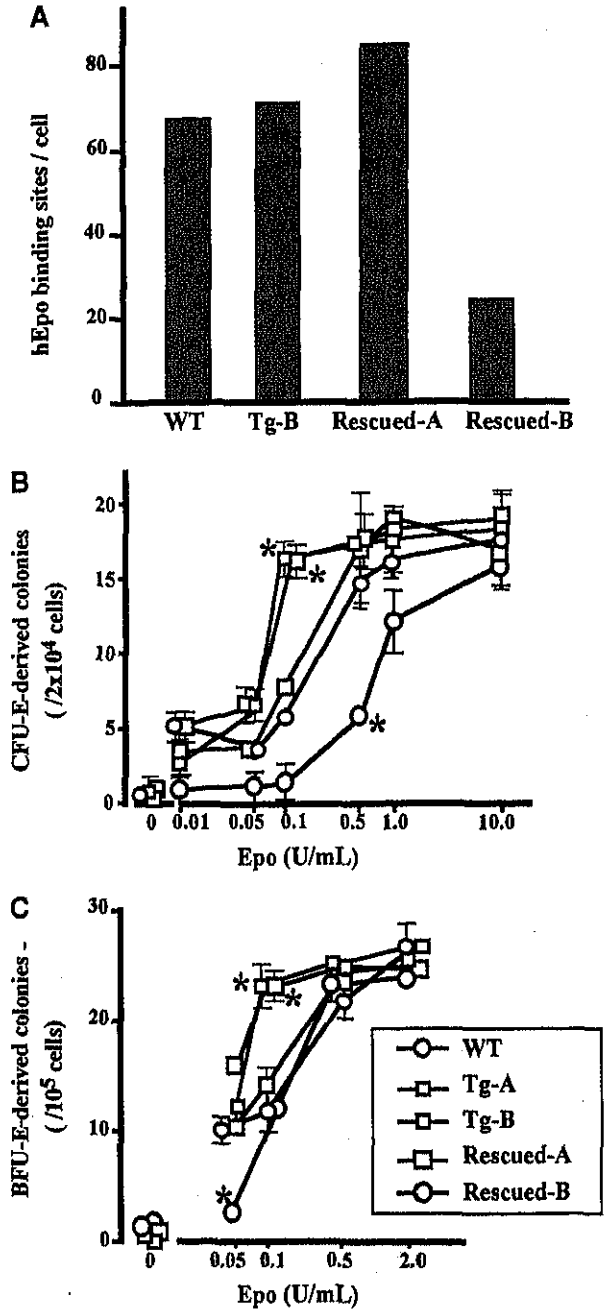


Figure 7. Number of EpoR at the bone marrow cell surface and erythroid colony formation assay in differential concentrations of Epo. (A) Number of radiolabeled recombinant human Epo binding sites at the surface of a bone marrow cell. Total mononuclear cells from the bone marrow of 4 to 8 mice at 10 to 12 weeks old were analyzed for each line. (B, C) Bone marrow cells were cultured for 3 days with different concentrations of Epo (B) or for 7 days with 50 ng/mL SCF and different concentrations of Epo (C) in methylcellulose medium. Erythroid colonies were counted by benzidine staining. $^*P < .01$ compared with wild-type mouse.

mice. Interestingly, GFP^- cells from the Rescued-A mouse express endogenous GATA-1, albeit at a low level, suggesting that *GATA-1–HRD* regulation is still missing in some population of the cells in which the endogenous *GATA-1* gene is expressed.

Expression level of EpoR determines the sensitivity of erythroid progenitors to Epo

The expression of Epo binding sites in adult mouse bone marrow cells was investigated using sodium iodide I 125-labeled recombinant human Epo (Figure 7A), which has been shown to bind to

mouse and human EpoR with comparable efficiency. The number of Epo binding sites in a bone marrow cell of the Tg-B mouse is approximately 71, almost comparable to that of the wild type (67 binding sites). In contrast, Rescued-B mice contain only 27 binding sites per cell, which represents a purely Tg-B–derived EpoR number. Rescued-A mice have 83 binding sites per cell, which is 1.2-fold that of the wild type. Transgenic and endogenous EpoR showed comparable dissociation constants against human Epo (K_d values of 201 pM and 270 pM for rescued and wild-type mice, respectively), indicating that the transgene-derived EpoR is synthesized and transferred normally to the cell surface.

The sensitivity of each erythroid progenitor to Epo was examined by erythroid colony formation assays in a methylcellulose medium containing various concentrations of Epo. For each EpoR genotype, the number of CFU-E–derived colonies in the bone marrow cells increased in a dose-dependent manner with an increasing Epo concentration (Figure 7B). Erythroid progenitors from Tg-A and Rescued-A mice (high EpoR expressors) were in plateau at 0.1 U/mL Epo, indicating that these types of erythroid progenitors were more sensitive to Epo than other types of mouse progenitors. On the other hand, erythroid progenitors from Rescued-B mice (low EpoR expressor) showed the lowest number of CFU-E–derived colonies, and those from wild-type and Tg-B mice (medium EpoR expressors) showed an intermediate number of CFU-E–derived colonies at 0.1 U/mL Epo. An important finding was that, with a higher concentration of Epo (1-10 U/mL), the colony number derived from Rescued-B mice increased and became similar to that of the wild-type and Tg-A mice. Thus, first we conclude that a comparable number of CFU-E progenitors exists among wild-type, Tg-A, Rescued-A, Tg-B, and Rescued-B mice. Second, we conclude that the ability of progenitors to differentiate toward an erythroid lineage differs among the various EpoR genotypes and depends on the expression level of EpoR.

We also examined BFU-E–derived colony formation. The number of colonies from Tg-A and Rescued-A bone marrow cells reached a plateau at a lower concentration of Epo (0.1U/mL) than did the other genotypes (Figure 7C). Thus, at a low concentration of Epo, both CFU-E– and BFU-E–derived colony numbers directly correlate with the number of Epo binding sites on the surface of erythroid progenitors, which were produced distinctly by the germ-line *EpoR*-mutation and *EpoR*-transgene. Intriguingly, though Rescued-B bone marrow cells had the lowest number of BFU-E–derived colonies at 0.05 U/mL Epo, the BFU-E number did not differ significantly from that of the other genotypes between 0.5 and 2.0 U/mL EpoR (Figure 7C). This is in clear contrast to the CFU-E case at 0.5 U/mL EpoR, in which Rescued-B bone marrow cells formed only one third the number of CFU-E colonies formed in the other genotypes (Figure 7B).

Response of transgenic EpoR-rescued mice to anemia induced by bleeding

So far, this study has demonstrated that the number of Epo binding sites in erythroid cells from Rescued-A and Rescued-B mice are approximately 120% and 40% that of the wild type, respectively. Nevertheless, both lines of transgenic mice are viable and fertile. Importantly, the erythroid progenitors of Rescued-B mice are much less sensitive to Epo than those of the wild type. In contrast, the erythroid progenitors of Tg-A and Rescued-A mice are more sensitive to Epo than those of the wild type. We wanted to know whether red blood cell production is under the influence of circulating Epo levels in these genetically engineered mice.

To this end, we first analyzed the hematopoietic indices of these mice (Figure 8A, day 1). The hematocrit (HCT), hemoglobin concentration (Hb), mean corpuscular volume (MCV), number of white blood cells (WBCs), and platelet number (PLT) remained unaffected in the 2 rescued mouse lines. However, the total number of erythrocytes (RBCs) was slightly decreased in the Rescued-B mice than in the wild type. In spite of the fact that Rescued-A mice have 20% more Epo binding sites, polycythemia was not observed in this line of mice.

Anemia was induced in these mice by daily bleeding for 4 days, and HCT values (Figure 8B) and Epo concentrations (Figure 8C) in the blood were examined over a 10-day period, starting from the first day of bleeding. Other hematopoietic indices were measured before bleeding (day 1) and after 4 days (day 4) of bleeding (Figure 8A). Using this protocol for experimentally inducing anemia, the HCT values of all 4 genotypes reached as low as 25% (Figure 8B). Furthermore, the HCT, Hb, and RBC values decreased comparably in all 4 genotypes of mice (Figure 8A-B). Other blood cell lineages, such as WBC and PLT, were also similar among the 4 different lines of mice (Figure 8A).

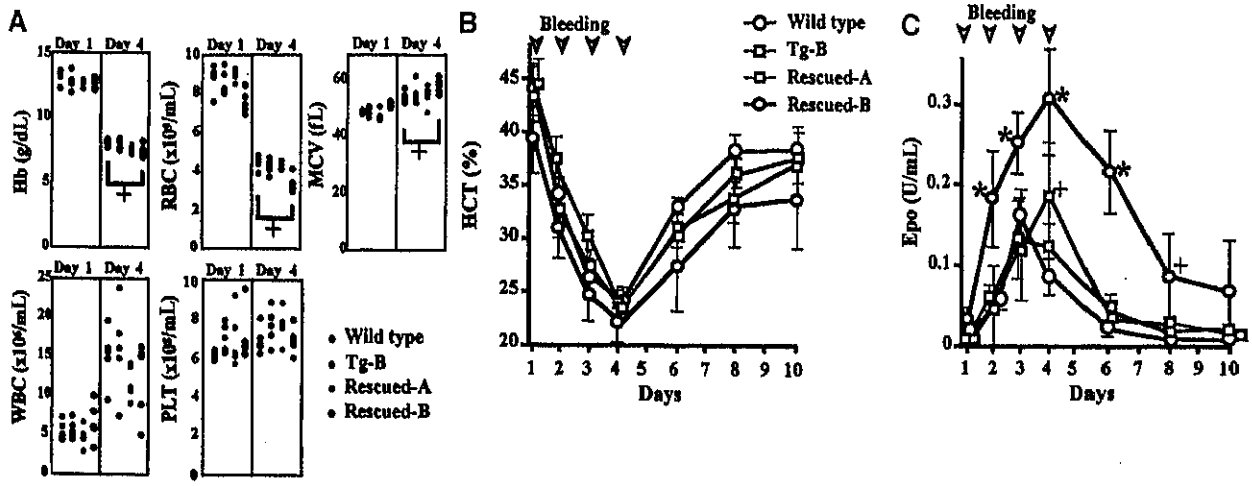


Figure 8. Effect of EpoR on recovery from anemia. Anemia was induced in 4 different EpoR genotypes of mice by withdrawing blood from the retro-orbital venous plexus every day for 4 days (day 1 to 4). Recovery from anemia was observed over a period of 1 week. (A) Peripheral blood indices of the 4 lines of mice under normal (day 1) and anemic (day 4) conditions are shown in each panel. Hb, MCV, RBC, WBC, and PLT are shown. HCT values (B) and Epo concentrations in the plasma (C) were measured daily. Results are shown with SD; n = 5 for each line. * indicates $P < .01$; +, $P < .05$ compared with wild-type mouse.

In each line, the plasma Epo concentrations increased in response to induced anemia. Notably, the daily Epo concentration of Rescued-B mice was markedly higher than that of the wild type, Tg-B, and Rescued-A mice (Figure 8C), reflecting differences in the number of Epo-binding sites among these mice. It is noteworthy that, though the plasma Epo concentration peaked at day 3 in wild-type and Tg-B mice, the peak shifted to day 4 in Rescued-A and -B mice (Figure 8C). This time delay in peak plasma Epo concentration is most likely to reflect a lack of efficient internalization and subsequent metabolic turnover of Epo in nonhematopoietic tissues through binding to EpoR, considering that the Rescued-A and -B lines of mice lack the nonerythroid expression of EpoR. An alternative possibility is that Epo production may be regulated by a negative feedback mechanism, with the circulating Epo concentration sensed by Epo-producing cells through EpoR on the cell surface. Therefore, a low level of EpoR expression may transduce the low concentration signal less efficiently, giving rise to the delay in Epo production. These results suggest that the contribution of nonhematopoietic EpoR is important in the regulation of plasma Epo concentration.

Discussion

Gene ablation studies demonstrated that the Epo-EpoR signaling pathway is crucial for definitive erythropoiesis and revealed the embryonic lethal phenotype inherent in a deficiency in either Epo or EpoR.[1,3,4] This embryonic lethality made it difficult to further analyze the Epo-EpoR system in the later stages of development. As a result, many uncertainties remain regarding the function of the Epo-EpoR system in embryonic development and adult hematopoiesis. Furthermore, it is unclear whether the Epo-EpoR pathway plays a crucial role in vivo in nonhematopoietic tissues. In this study, we resolved these issues by generating transgenic lines of mice expressing EpoR cDNA exclusively in the hematopoietic lineage. *EpoR* mutant pups were successfully rescued by crossing their EpoR-null parent with one of these *EpoR* transgenic mice. Because these *EpoR* transgene–rescued mice developed normally and were fertile, we exploited them for analyzing the roles of the Epo-EpoR system in adult hematopoiesis and in nonhematopoietic tissues. For the first time, our results exposed the absolute contribution of the Epo-EpoR signaling pathway to erythropoiesis in an integrated system in vivo. We demonstrated that nonhematopoietic expression of EpoR is dispensable in the birth, fertilization, and survival of mice and that the number of EpoR on the surface of erythroid progenitors determines the sensitivity of such progenitors to Epo stimuli.

All *EpoR* gene knockout mice reported to date showed that E12.5 $EpoR^{-/-}$ embryos are pale because of a significant reduction in circulating erythrocytes.[1,3,4] Our current analysis showed very good agreement with these results. In regard to definitive hematopoiesis, colony formation assays showed that supplementation of Epo and SCF is not sufficient to produce BFU-E– and CFU-E–derived colonies from $EpoR^{-/-}$ embryonic hematopoietic cells. However, a combination of SCF and thrombopoietin (Tpo) or of interleukin-3 (IL-3), IL-11, and Tpo was found to efficiently support BFU-E colony formation from $EpoR^{-/-}$ embryonic cells.[4] In contrast, colony formation of these erythroid progenitors was completely rescued by the transgenic expression of EpoR. To identify the expression profile of GATA-1,[15,25] we generated *GATA-1–HRD-GFP* transgenic lines and identified GFP expression in a fraction containing enriched BFU-E and CFU-E (N. Suwabe, N.S., M.Y., unpublished observation, December 2001). These results thus support our contention that the target cells rescued by the *EpoR* transgene include BFU-E and CFU-E progenitors in the definitive erythroid lineage.

Closer examination of E12.5 $EpoR^{-/-}$ embryos revealed a defect in cardiac development because of ventricular hypoplasia.[7] In $EpoR^{-/-}$ embryonic hearts, the epicardial walls were detached and the capillary structures lacked definition. In contrast, transgene-rescued embryos exhibited no apparent defect or abnormality in their myocardial walls, indicating that the Epo-EpoR system is unnecessary for the initial development of the myocardial layer. Indeed, $EpoR^{-/-}$ embryonic stem cells have been shown to contribute to heart development in chimeric mice.[7] These results strongly argue that impaired heart development results from a decrease of circulating mature erythrocytes, which are responsible for supplying oxygen to cells. We therefore propose that transgenic expression of EpoR in the knockout embryos rescued primitive and definitive erythropoiesis, which in turn rescued cardiac cell development.

Several reports have described the expression of EpoR in other nonhematopoietic tissues. For instance, Epo expression is induced by hypoxia and ischemia in astrocytes, and Epo protects neurons from ischemia, trauma, and the toxicity of kainate.[12,13,26] Binding of Epo to EpoR on the surfaces of neural cells induces the expression of antiapoptotic genes after the activation of Jak2 and NF-κB.[27] EpoR is also expressed in skeletal muscle, kidney, and intestine, and Epo induces cell proliferation in these tissues.[11,18,28] These findings led to the hypothesis that the Epo-EpoR system may play important roles when cells suffer from damage or stress. Although our study demonstrated that the Epo-EpoR system is not required for normal mouse development, these broad observations suggest that further analyses under pathological conditions are necessary for a comprehensive understanding of the contribution of the Epo-EpoR signaling system in nonhematopoietic tissues.

Alternative splicing of the *EpoR* transcript produces soluble and truncated isoforms that are assumed to negatively regulate erythropoiesis in immature erythroid progenitors.[29] Therefore, we examined whether the *GATA-1–HRD* transgene bearing a truncated form of EpoR could rescue germ-line EpoR mutant mice from embryonic lethality. However, the transgenic expression of truncated EpoR could not rescue erythroid cell development in EpoR-deficient mice (N.S., N.Y., unpublished observation, June 2000). The *HG1-EpoR* transgene expresses full-length EpoR exclusively. Because the transgene rescues EpoR germ-line mutant mice from embryonic lethality and recovers the mice from experimentally induced anemia, we conclude that these splicing variants are not essential for erythroid cell formation and recovery from anemia. On the contrary, because the truncated isoform is highly expressed in cells from patients with myelodysplastic syndrome and causes ineffective erythropoiesis,[30] it is of interest to test the contribution of the *EpoR* transgene to blood cell differentiation in these patients.

Compared with wild-type mice, the amount of EpoR on the surface of bone marrow cells from Rescued-B mice is approximately 40%, and the sensitivity of erythroid progenitors to Epo in Rescued-B mice is much lower. This is consistent with the observation that CFU-E progenitors are more sensitive to Epo than BFU-E progenitors given that CFU-E progenitors contain more EpoR than BFU-E progenitors.[5,6] Nonetheless, Rescued-B mice have normal hematopoietic activity and can recover from anemia induced by bleeding. We believe that, because of the overproduction of Epo in vivo, a small number of receptors may be sufficient to sustain erythropoiesis. It should also be noted that though the erythroid progenitors in these lines of mice showed different

sensitivity to Epo, it did not result in polycythemia or anemia, indicating that the in vivo Epo level is strictly regulated. Based on these results, we conclude that the expression level of EpoR controls the sensitivity of erythroid progenitors to Epo. To our knowledge, this is the first demonstration in vivo that the number of cytokine receptors on the surface of target cells controls the sensitivity of the target cells to the cytokine ligand.

In contrast to adult mice, there was no significant difference in CFU-E colony formation in fetal livers from the different lines of mice bearing different numbers of EpoR. We speculate that erythroid progenitors in fetal liver have a higher potential for producing erythroid cells and a higher sensitivity to the Epo signaling cascade than progenitors in adult bone marrow. The notion that fetal liver erythropoiesis is under a different regulation than adult bone marrow erythropoiesis requires further examination.

In summary, we presented the usefulness of the *HG1-EpoR* transgene-rescued lines of mice for the analysis of EpoR function both in erythroid progenitors and in nonhematopoietic tissues. This study also implies that the *GATA-1–HRD-EpoR* minigene may be applicable to therapeutic use in that we anticipate an EpoR transgene under the regulation of this minigene to be expressed specifically in erythroid progenitors derived from patients with defective erythropoiesis.

Acknowledgments

We thank N. Kaneko and Y. Kikuchi for help and Drs F. Sugiyama, T. Nagasawa, and T. O'Connor for discussion and advice.

References

1. Wu H, Liu X, Jaenisch R, Lodish HF. Generation of committed erythroid BFU-E and CFU-E progenitors does not require erythropoietin or the erythropoietin receptor. Cell. 1995;83:59-67.
2. Constantinescu SN, Ghaffari S, Lodish HF. The erythropoietin receptor: structure, activation and intracellular signal transduction. Trends Endocrinol Metab. 1999;10:18-23
3. Lin CS, Lim SK, D'Agati V, Costantini F. Differential effects of an erythropoietin receptor gene disruption on primitive and definitive erythropoiesis. Genes Dev. 1996;10:154-164.
4. Kieran MW, Perkins AC, Orkin SH, Zon LI. Thrombopoietin rescues in vitro erythroid colony formation from mouse embryos lacking the erythropoietin receptor. Proc Natl Acad Sci U S A. 1996;93:9126-9131.
5. Landschulz KT, Noyes AN, Rogers O, Boyer SH. Erythropoietin receptors on murine erythroid colony-forming units: natural history. Blood. 1989; 73:1476-1486.
6. Broudy VC, Lin N, Brice M, Nakamoto B, Papayannopoulou T. Erythropoietin receptor characteristics on primary human erythroid cells. Blood. 1991;77:2583-2590.
7. Wu H, Lee SH, Gao J, Liu X, Iruela-Arispe ML. Inactivation of erythropoietin leads to defects in cardiac morphogenesis. Development. 1999;126: 3597-3605.
8. Yasuda Y, Masuda S, Chikuma M, Inoue K, Nagao M, Sasaki R. Estrogen-dependent production of erythropoietin in uterus and its implication in uterine angiogenesis. J Biol Chem. 1998;273: 25381-25387.
9. Anagnostou A, Liu Z, Steiner M, et al. Erythropoietin receptor mRNA expression in human endothelial cells. Proc Natl Acad Sci U S A. 1994;91: 3974-3978.
10. Ohneda O, Yanai N, Obinata M. Erythropoietin as a mitogen for fetal liver stromal cells which support erythropoiesis. Exp Cell Res. 1993;208:327-331.
11. Ogilvie M, Yu X, Nicolas-Metral V, et al. Erythropoietin stimulates proliferation and interferes with differentiation of myoblasts. J Biol Chem. 2000; 275:39754-39761.
12. Brines ML, Ghezzi P, Keenan S, et al. Erythropoietin crosses the blood-brain barrier to protect against experimental brain injury. Proc Natl Acad Sci U S A. 2000;97:10526-10531.
13. Sakanaka M, Wen TC, Matsuda S, et al. In vivo evidence that erythropoietin protects neurons from ischemic damage. Proc Natl Acad Sci U S A. 1998;95:4635-4640.
14. Zon LI, Youssoufian H, Mather C, Lodish HF, Orkin SH. Activation of the erythropoietin receptor promoter by transcription factor GATA-1. Proc Natl Acad Sci U S A. 1991;88:10638-10641.
15. Onodera K, Takahashi S, Nishimura S, et al. GATA-1 transcription is controlled by distinct regulatory mechanisms during primitive and definitive erythropoiesis. Proc Natl Acad Sci U S A. 1997;94:4487-4492.
16. Takahashi S, Shimizu R, Suwabe N, et al. GATA factor transgenes under GATA-1 locus control rescue germline GATA-1 mutant deficiencies. Blood. 2000;96:910-916.
17. Higuchi M, Oh-eda M, Kuboniwa H, Tomonoh K, Shimonaka Y, Ochi N. Role of sugar chains in the expression of the biological activity of human erythropoietin. J Biol Chem. 1992;267:7703-7709.
18. Westenfelder C, Biddle DL, Baranowski RL. Human, rat and mouse kidney cells express functional erythropoietin receptors. Kidney Int. 1999; 55:808-820.
19. Foresta C, Mioni R, Bordon P, Gottardello F, Nogara A, Rossato M. Erythropoietin and testicular steroidogenesis: the role of second messengers. Eur J Endocrinol. 1995;132:103-108.
20. Lee R, Kertesz N, Joseph SB, Jegalian A, Wu H. Erythropoietin (Epo) and EpoR expression and 2 waves of erythropoiesis. Blood. 2001;98:1408-1415.
21. Stohlawetz PJ, Dzirlo L, Hergovich N, et al. Effects of erythropoietin on platelet reactivity and thrombopoiesis in humans. Blood. 2000;95:2983-2989.
22. Ribatti D, Presta M, Vacca A, et al. Human erythropoietin induces a pro-angiogenic phenotype in cultured endothelial cells and stimulates neovascularization in vivo. Blood. 1999;93:2627-2636.
23. Heberlein C, Fischer KD, Stoffel M, et al. The gene for erythropoietin receptor is expressed in multipotential hematopoietic and embryonal stem cells: evidence for differentiation stage-specific regulation. Mol Cell Biol. 1992;12:1815-1826.
24. Akashi K, Traver D, Miyamoto T, Weissman IL. A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. Nature. 2000;404: 193-197.
25. Yamamoto M, Takahashi S, Onodera K, Muraosa Y, Engel JD. Upstream and downstream of erythroid transcription factor GATA-1. Genes Cells. 1997;2:107-115.
26. Masuda S, Okano M, Yamagishi K, Nagao M, Ueda M, Sasaki R. A novel site of erythropoietin production: oxygen-dependent production in cultured rat astrocytes. J Biol Chem. 1994;269: 19488-19493.
27. Digicaylioglu M, Lipton SA. Erythropoietin-mediated neuroprotection involves cross-talk between Jak2 and NF-kB signaling cascades. Nature. 2001;412:641-647.
28. Juul SE, Ledbetter DJ, Joyce AE, et al. Erythropoietin acts as a trophic factor in neonatal rat intestine. Gut. 2001;49:182-189.
29. Nakamura Y, Takano H, Osawa M, et al. Impaired erythropoiesis in transgenic mice overexpressing a truncated erythropoietin receptor. Exp Hematol. 1999;26:1105-1110.
30. Shimizu R, Komatsu N, Miura Y. Dominant negative effect of a truncated erythropoietin receptor (EPOR-T) on erythropoietin-induced erythroid differentiation: possible involvement of EPOR-T in ineffective erythropoiesis of myelodysplastic syndrome. Exp Hematol. 1999;27:229-233.

5

Function of the Transcription Factor GATA-2

Shigehiko Imagawa

1. INTRODUCTION

Erythropoietin (EPO) gene expression is under the control of HIF-1 (hypoxia inducible factor-1) through an HIF-1 binding site in the Epo enhancer. HIF-1 is composed of a redox-sensitive HIF-1α subunit and a constitutively expressed HIF-1β subunit. Under normoxic conditions, HIF-1α is rapidly degraded, whereas under hypoxic conditions, HIF-1α is stabilized and its transcription is stimulated *(10)*. The EPO gene is negatively regulated by GATA, which binds to the GATA site of the EPO promoter in Hep3B cells *(12)*. Under normoxic conditions, EPO gene expression is not stimulated, because HIF-1α is degraded by prolyl hydroxylation *(13,14)* and EPO gene expression is suppressed by GATA. N^G-monomethyl L-arginine (L-NMMA), H_2O_2 and cadmium inhibit EPO gene expression even under hypoxic conditions through different mechanisms *(3,8,9,28)*. In this chapter, the negative regulation of EPO gene by GATA is demonstrated and a mechanism by which L-NMMA suppresses the EPO gene is proposed.

2. NEGATIVE REGULATION OF EPO GENE BY GATA

2.1. Hep3B and HepG2 Cells Express hGATA-2 mRNA and Protein

Human hepatoma cell lines Hep3B and HepG2 can be induced by hypoxia, as well as by cobalt, to produce large amounts of biologically active and immunologically identifiable EPO *(4)*. Hypoxia and cobalt also induce markedly increased levels of EPO mRNA *(4)*. The expression of hGATA tran-

From: Erythropoietin: Molecular Biology and Clinical Use
Edited by Wolfgang Jelkmann
FP Graham Publishing Co., Johnson City, TN
http://www.fpgrahamco.com

scription factors was examined in Hep3B and HepG2 cells by Northern blotting analysis *(12)*. Hep3B cells express hGATA-2 and -3 transcription factors, whereas HepG2 cells express hGATA-2. Immunohistochemical staining showed that hGATA-2 was abundantly expressed within the nucleus of Hep3B and HepG2 cells. However, hGATA-1 and -3 were not expressed *(12)*.

2.2. hGATA-1, -2, and –3 Transcription Factors Specifically Bind to the GATA Element in the Human EPO Gene Promoter

Constructs of hGATA-1, -2, and –3 transcription factors were transfected into QT6 cells by $CaPO_4$ precipitation. Binding of proteins from nuclear extracts of QT6 cells transfected with hGATA transcription factors was assessed by a gel mobility shift assay. DNA-protein interactions in nuclear extracts of cells transfected with hGATA-1, -2 and –3 and in Hep3B cells were identified as retarded complexes, using the GATA element as a probe. The addition of non-radiolabeled GATA element oligonucleotide showed that these DNA-protein interactions were specific. Furthermore, a 150 fold molar excess of unlabeled irrelevant oligonucleotide and of one of the mutant GATA oligonucleotides were not able to compete with these complexes *(12)*. These experiments showed that hGATA-1, -2, and -3 transcription factors specifically bind to the GATA element of the human EPO gene promoter.

2.3. hGATA-1, -2, and –3 Transcription Factors Decrease Expression of EPO mRNA in Hep3B Cells

To examine the effect of hGATA transcription factors on EPO mRNA in Hep3B cells, hGATA-1, -2, or -3 expression plasmids were transfected into Hep3B cells by electroporation. The level of EPO mRNA was then measured in the transfected Hep3B cells by competitive PCR. These results showed that 20 μg of hGATA-1, -2, and -3 transcription factors decreased the expression level of EPO mRNA, to 38.3%, 36.1% and 33.8%, respectively as compared with EPO mRNA levels after incubation in 1% O_2 with transfection of carrier DNA only *(12)*.

2.4. Mutation of the GATA Sequence of the EPO Promoter Interferes With Inhibition of the EPO Promoter Activity by hGATA-1, -2, and –3 Transcription Factors

To understand the regulation mechanism of the EPO promoter by hGATA transcription factors, reporter constructs (luciferase) that contain a mutated GATA sequence were prepared. Pwt is a wild-type plasmid in which the 126-bp 3' EPO enhancer and the 117-bp Epo promoter were placed upstream of the luciferase gene PXP2. There is one GATA and two CACCC motifs in the promoter region, and one HIF-1 and one hepatic nuclear factor 4 (HNF-4) binding site in the enhancer. To investigate the effect of GATA transcription factors, GATA sequence of the EPO promoter in Pwt was mutated (AGATAACAG -> ATATAAAAG) and named Pm7. Therefore, GATA transcription factors cannot bind to this mutant; however, TF II D can bind to this TATA. We transfected Pwt and Pm7 into Hep3B cells and incubated the cells under 21% (normoxia) or 1% (hypoxia) oxygen for 24 h. The hypoxic induction of Luc gene expression is represented as a hypoxia/normoxia ratio. Transient transfection of Pwt showed 46.5-fold induction by hypoxia. hGATA-1 transcription factor significantly inhibited the hypoxic induction to 13.1-fold. hGATA-2 and hGATA-3 transcription factors also inhibited hypoxic induction to 15.6-fold (33.5% compared with Pwt), 9.0-fold (19.4% compared with Pwt), respectively. However, transient transfection of the mutated GATA (Pm7) showed 77.7-fold induction by hypoxia. The hypoxic inductions of Pm7 transfected cells were higher than the hypoxic induction of the wild-type, Pwt. Furthermore, hGATA-1, -2, and also –3 transcription factors significantly interfered with the inhibition of EPO promoter activity, 49.2-fold, 98.7-fold, and 235.8-fold induction by hypoxia *(12)*. These results clearly showed that hGATA transcription factors bind to the GATA sequence of the EPO promoter, inhibit the activity of the EPO promoter, and finally, negatively regulate EPO gene expression.

This study clearly demonstrates that expression of hGATA-1, -2, and –3 transcription factors significantly inhibit EPO mRNA expression in Hep3B cells. This decrease in expression may be due to inhibition of EPO promoter activity by hGATA-1, -2, and –3 transcription factors. In Hep3B and HepG2 cells, which regulate EPO protein and mRNA expression in response to hypoxia and $CoCl_2$, the hGATA-2 transcription factor may bind the GATA element in the human EPO gene promoter, negatively regulating EPO gene expression. Because Hep3B cells already express hGATA-2, this additional

decrease in EPO expression after transient transfection of the hGATA-2 plasmid may be caused by an increase in hGATA-2 expression. The result of transient transfection of the EPO-luciferase significantly demonstrated that the EPO promoter activity was inhibited by the overexpression of hGATA transcription factors. The transient transfection of the mutated GATA of the EPO promoter with luciferase construct clearly showed that the hGATA transcription factors interfered with the inhibition of the EPO promoter activity. Furthermore, hGATA transcription factors stimulate the EPO promoter activity in the transient transfection of the mutated GATA. Because TF II D can bind to the mutated GATA (TATA) sequence more tightly than to the GATA sequence, it is tempting to speculate that hGATA transcription factors may bind TF II D or another associated molecule *(1)*.

3. L-NMMA SUPPRESSES EPO GENE EXPRESSION

The major sites of EPO production are the liver in the fetus *(31)*, and the kidney in the adult *(15)*. Peritubular capillary interstitial cells are thought to be the major site of production of EPO in the kidney *(19)*. The cause of the anemia of renal disease is believed to be damage to this site of EPO production by renal failure *(20)*. In this regard, however, it is interesting to note that there are some patients with the anemia of renal disease who still have the ability to produce EPO in response to acute blood loss and hypoxia *(2)*. On the other hand, there are also patients with renal failure who do not have anemia *(30)*. These observations suggest that chronic perturbation of oxygen sensing and/or signal transduction underlie the pathogenesis of the anemia of renal disease rather than damage at the site of EPO production. Recently, L-NMMA was reported to be undetectable in non-uremic subjects, while the concentration of L-NMMA was markedly elevated in uremic patients *(24)*. Based on this observation, we hypothesized that this substance may be a candidate uremic toxin responsible for renal anemia. However, the precise function of L-NMMA in mediating expression of the EPO gene remains to be elucidated. Since L-NMMA functions as an inhibitor of nitric oxide synthase (NOS) *(7)*, it is expected to suppress production of nitric oxide (NO) and cyclic guanosine 3', 5'-monophosphate (cGMP). We have found that GATA transcription factors bind to a GATA site in the EPO gene promoter and negatively regulate EPO gene expression in Hep3B cells *(12)*. In this study, we demonstrate that L-NMMA suppresses EPO gene expression by up-regulation of the GATA transcription factor.

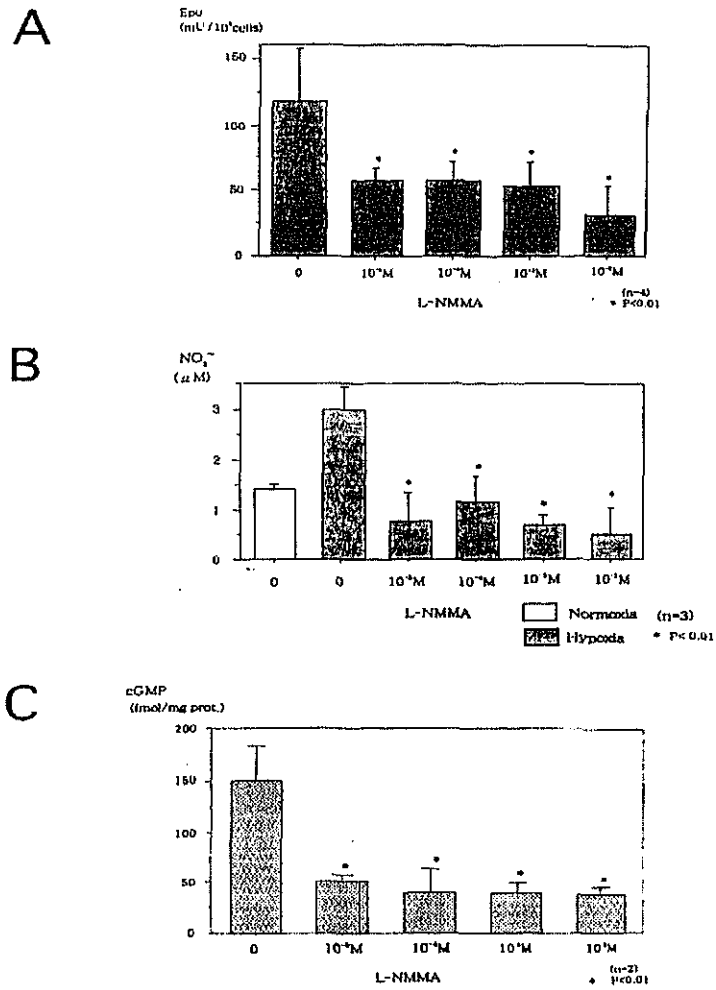


Fig. 1A. Effect of L-NMMA on EPO protein from Hep3B cells stimulated by hypoxia. Hep3B cells were incubated with different concentrations of L-NMMA under hypoxic conditions (1% O_2) for 24 h. EPO protein was measured by RIA. Four separate experiments were performed (n=4). Error bars represent one standard deviation (SD).

Fig. 1B. Effect of L-NMMA on NO from Hep3B cells. Hep3B cells were incubated under normoxic (21% O_2) or hypoxic (1% O2) conditions for 4h in the presence or absence of L-NMMA. NO was measured by the 2,3-diaminonaphthalene method. Three separate experiments were performed (n=3). Error bars represent one SD.

Fig. 1C. Effect of L-NMMA on cGMP from Hep3B cells stimulated by hypoxia. Hep3B cells were incubated with different concentrations of L-NMMA under hypoxia (1% O_2) for 2 h. cGMP was measured by EIA. Two separate experiments were performed (n=2). Error bars represent one SD.

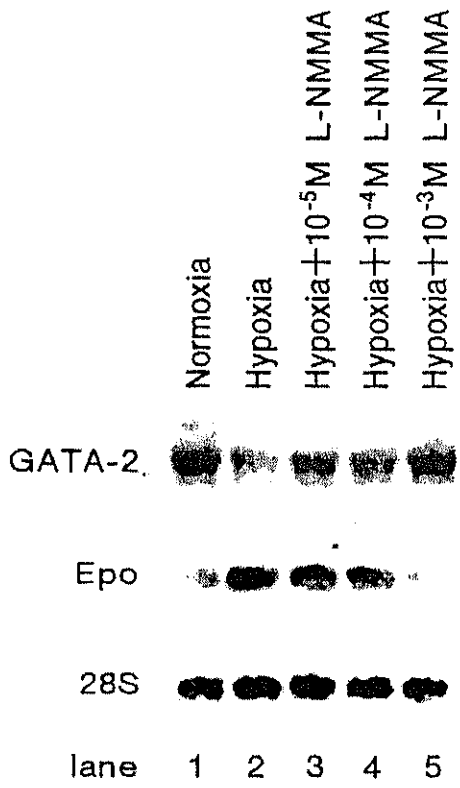


Fig. 2. Effect of L-NMMA on the expression of GATA-2 and EPO mRNA. Northern blot analysis was performed using 20 μg of RNA from Hep3B cells incubated under conditions of normoxia (21 % O_2) (lane 1), conditions of hypoxia (1 % O_2) (lane 2), hypoxia with 10^{-5} M L-NMMA (lane 3), hypoxia with 10^{-4} M L-NMMA (lane 4) and hypoxia with 10^{-3} M L-NMMA (lane 5) for 8 h. Upper, middle and lower panels show GATA-2, EPO and 28S mRNA, respectively.

3.1. Inhibition of EPO Protein by L-NMMA

Incubation of Hep3B cells for 24 h with 10^{-2} M L-NMMA under hypoxic conditions caused an 80% inhibition of EPO (measured by radioimmunoassay), whereas 10^{-5} M, 10^{-4} M and 10^{-3} M L-NMMA each showed a 60% inhibition of EPO (Fig. 1A). These results suggest that L-NMMA specifically

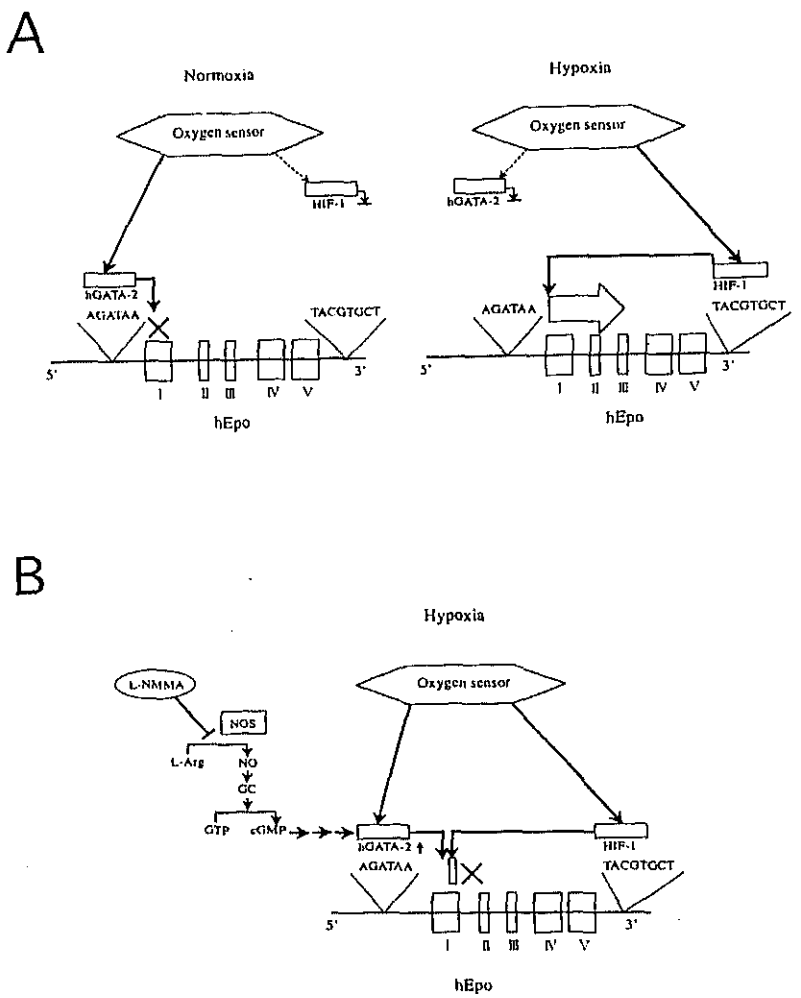


Fig. 3A. Scheme of regulation of EPO gene expression by HIF-1 and GATA.
Fig. 3B. Scheme of negative regulation of EPO gene expression by L-NMMA.

inhibited the production of EPO protein in Hep3B cells (Fig. 1A).

3.2. Inhibition of NO and cGMP by L-NMMA

L-NMMA is known to be an NOS inhibitor. In agreement with this finding, L-NMMA inhibited the hypoxia-induced secretion of NO by Hep3B cells (Fig. 1B). Furthermore, NO is known to stimulate guanylate cyclase (GC) to

produce cGMP *(21)*. In agreement with this finding, L-NMMA inhibited the secretion of cGMP from the cells (Fig.1C).

3.3. Inhibition of Serum EPO by L-NAME

L-NMMA is reported to be catabolyzed by N^G-dimethylarginine dimethylaminohydrolase (DDHA) in the intact kidney *(16,17)*. However, L-NAME is not catabolyzed by this enzyme *(22)*. To identify the effect of L-NAME on EPO production in vivo, BDF1 mice were injected i.p. with L-NAME (0.2 ml, 10mg/ml) PBS, or 0.2 ml of PBS as a control. Blood samples (0.3 ml) were obtained from the orbital vein immediately after (0 h) and at 12 and 24 h after the injection of L-NAME. Serum EPO levels in the L-NAME-injected mice (28.5 mU/ml at 12 h and 98.8 mU/ml at 24 h after the injection) were significantly lower than those of the control (66.4 and 276.8 mU/ml, respectively) *(28)*. These *in vivo* results are comparable with those obtained from the *in vitro* incubation of Hep3B cells.

3.4. Inhibition of EPO Promoter Activity by L-NMMA

Hypoxic induction from Pwt was 55.8 fold higher than that from normoxic Pwt. Interestingly, the addition of L-NMMA inhibited hypoxic induction of the Luc reporter gene expression from Pwt with hypoxia/normoxia ratio of only 33.0 fold, 59.1% of that from Pwt incubated without L-NMMA *(28)*. These results indicate that the hypoxic induction of the EPO gene expression is suppressed by L-NMMA through the EPO gene regulatory regions.

We examined the contribution of the GATA site to the L-NMMA suppression of hypoxic EPO gene induction. To this end, Hep3B cells were co-transfected with Pwt and an hGATA-2 expression vector. The expression of hGATA-2 resulted in inhibition of the hypoxic induction of the EPO gene. The Luc reporter gene was induced in the presence of hGATA-2 by 21.5 fold, 43.2 % of that from Pwt incubated without hGATA-2. Furthermore, the exposure of Hep3B cells co-transfected with Pwt and hGATA-2 expression vector to L-NMMA resulted in further suppression, with hypoxic induction of the reporter gene of only 14.2 fold, 25.4 % of that from the cells incubated without L-NMMA and hGATA-2 *(28)*. These results suggest that hGATA-2 acts as a repressor of the hypoxic induction of the EPO gene and that the GATA sequence in the promoter mediates L-NMMA suppression.

3.5. The GATA Element in the Promoter Only Contributes to L-NMMA Suppression

The contribution of the GATA element was further tested by using a reporter plasmid, Pm7, which contains GATA site mutations. We previously found that this GATA mutation alone affects the basal level expression of Luc reporter activity *(12)*. As was the case for Pwt, Luc expression was also strongly induced following the exposure of transfected cells to hypoxia, 96.9 fold. However, L-NMMA failed to affect the GATA mutant Pm7 Luc activity with hypoxic induction of 103.7 fold, 107% of that from Pm7 only. Transfection of Pm7 into Hep3B cells which express hGATA-2 resulted in hypoxic induction of 147.3 fold, 152 % of that from Pm7 only. Furthermore, exposure of the cells co-transfected with Pm7 and hGATA-2 expression vector to L-NMMA showed 123.5 fold, and 127.5% of that from Pm7 only *(28)*. These results suggest that L-NMMA inhibits EPO gene expression through the GATA site in the EPO promoter rather than through the enhancer activity. To clearly identify whether this inhibitory effect of L-NMMA on EPO gene expression was due to a GATA-2 and/or HIF-1 binding site, we used a construct that contained the EPO promoter only. We then transfected Δ18pXP2 (wild type) or Δ18m7pXP2 (GATA site mutant) plasmids into Hep3B cells and incubated the cells both with or without additional L-NMMA under 21% or 1% oxygen for 24 h. Hypoxic induction from Δ18pXP2 was 7.6 fold higher than that from normoxic Δ18pXP2. The addition of L-NMMA significantly inhibited the hypoxic induction of the Luc reporter gene expression from Δ18pXP2 with hypoxia/normoxia ratio of only 4.0 fold, 52.6% of that from Δ18pXP2 incubated without L-NMMA. Hypoxic induction from Δ18m7pXP2 was 7.3 fold higher that from normoxic Δ18m7pXP2. L-NMMA failed to affect the Δ18m7pXP2 Luc activity with hypoxic induction of 8.2 fold, 112.3% of that from Δ18m7pXP2 only *(28)*. These results clearly indicate that the inhibitory effect of L-NMMA on EPO gene expression was due to GATA-2, not HIF-1.

3.6. Enhancement of GATA-2 Binding Activity by L-NMMA

To examine whether L-NMMA treatment affects the binding activity of hGATA-2, nuclear extracts were prepared from the cells stimulated by L-

NMMA for 1 h under normoxic or hypoxic conditions, and electrophoretic mobility shift assays (EMSA) were performed with an oligonucleotide containing the wild-type GATA element (AGATAA). L-NMMA induced the binding activity of GATA-2 under normoxic and hypoxic conditions. This binding activity was abolished by self-competitor. To confirm that the band was GATA-2, nuclear extracts were prepared from Hep3B cells under hypoxia with 10^{-4} M L-NMMA for 1 h, and incubated with 0.5 or 1.0 μl monoclonal antibodies of hGATA-1, 2 and 3, and then EMSA was performed. The control revealed a band of increased intensity, and the addition of FCS further increased the intensity of this band, though the mechanism of this increase is unknown. The addition of monoclonal antibodies of hGATA-1, and -3 resulted in bands of similar intensities; however, the band disappeared with the addition of monoclonal hGATA-2 antibody *(28)*. These results strongly suggest that the band was a specific GATA-2 band.

The effects of L-NMMA on the binding activity of HIF-1, HNF-4, chicken ovalbumin upstream promoter-transcription factor 1 (COUP-TF1), NF-κB were measured by EMSA. L-NMMA did not alter the binding activity of any of these transcription factors.

3.7. GATA-2 mRNA Expression was Induced by L-NMMA

To examine whether GATA-2 and EPO mRNA expression levels were affected by the addition of L-NMMA, Northern blot analysis was performed. Northern blot analysis showed hypoxia-induced EPO mRNA expression. However, the addition of L-NMMA inhibited this induction of EPO mRNA (Fig. 2A, middle panel). In contrast, hypoxia reduced GATA-2 mRNA expression, while L-NMMA induced the expression of GATA-2 mRNA (Fig. 2 A, upper panel and B). The 28S used as a control revealed a constant level of mRNA expression from the cells incubated under normoxia or hypoxia and with or without L-NMMA (Fig. 2A, lower panel).

We hypothesized that NO produced by NOS, which behaves as an intracellular or extracellular messenger, activates GC and, through stimulation of cGMP, consequently upregulate Epo gene expression in peritubular cells. However, in chronic renal failure, we suspect that an NOS inhibitor such as L-NMMA suppresses EPO gene expression through inhibition of NO production. In the present study, we found that L-NMMA decreased the expression of NO and cGMP, and increased expression of GATA-2 mRNA and the

level of GATA-2 binding activity, thereby inhibiting EPO promoter activity and causing a decrease in the expression of EPO protein (Fig. 3A). L-NMMA did not alter binding activity of HIF-1, HNF-4, COUP-TF1, or NF-κB at all. Neither EMSA nor EPO reporter gene transfection experiments showed any effect of L-NMMA on EPO enhancer activity. L-NMMA did not affect Luc activity when the GATA site in the EPO promoter was mutated (Pm7). Recently, Kimura et al. reported that L-NAME did not interfere with hypoxia-induced VEGF promoter (HIF-1 binding site) activation *(18)*. This result is compatible with our data. These results strongly suggest that L-NMMA affects GATA-2 binding.

The effect of NO on VEGF expression via HIF-1 is controversial. Some recent reports show an inhibitory effect of NO on VEGF expression. Huang et al. *(10)* and Sogawa et al. *(26)* have demonstrated that sodium nitroprusside (SNP, NO donor) suppresses hypoxia-induced VEGF gene activation and HIF-1 binding activity. SNP inhibits the hypoxic induction of the VEGF gene in a dose-dependent manner, in contrast to the effects of S-nitroso-N-acetyl-D, L-penicillamine (SNAP) and 3-(hydroxy-1-(1-methylethyl)-2-nitrosohydrazino)-1-propanamine (NOC5) (another NO donor) as shown in Kimura et al. *(18)*. To explain these discrepancies, Fandrey et al. tested several NO donors with diverse chemical structures and NO-releasing half-lives *(25)*. They found that NO donors have the diverse effects of NO that appears as a result of time- and –concentration dependent delivery, and concurred with Kimura et al. in showing NO donor-induced HIF-1α protein accumulation *(25)*. As to the effect of NO on Epo, Ohigashi et al. reported that serum levels of EPO in hypoxic polycythemic mice were significantly increased after injections of 200 μg/kg SNP *(23)*. Furthermore, cGMP levels in hypoxic Hep3B cells were also elevated. SNP (10 and 100 μM) and NO (2 μM) increased cGMP levels in Hep3B cells *(23)*. These results are compatible with our data, and strongly suggest that L-NMMA inhibits EPO production via the GATA transcription factor. Recently, Umetani et al. found a novel cell adhesion inhibitor (K-7174) as a specific GATA inhibitor. This substance should be examined to determine whether it has a role in the anemia with renal disease *(29)*.

4. H_2O_2 SUPPRESSES EPO GENE EXPRESSION

Goldberg et al. *(5)* have proposed that Hep3B cells have an oxygen-sensing

mechanism in which hypoxia or $CoCl_2$ treatment results in a ligand-dependent conformational change in heme protein, subsequently inducing EPO gene expression. It was further found that this heme protein may change its conformation depending on the ambient O_2 tension, suggesting that this conformational change induces EPO gene expression through some unknown intracellular signal *(5)*. Studies on EPO-producing HepG2 cell lines suggested the involvement of a b-type cytochrome in the oxygen-sensing process *(6)*. The presence of an NADPH oxidase-like heme protein in the oxygen-sensing system has been suggested by the observation that H_2O_2 is formed in these cells following exposure to oxygen. Using HepG2 cells, Fandrey et al. *(3)* found that H_2O_2 decreased EPO mRNA and EPO protein. Since H_2O_2 is able to freely diffuse within cells, it becomes one of the most likely candidates for an intracellular messenger molecule *(3)*. In this respect, H_2O_2-mediated inhibition of EPO gene expression has been reported to, at least partly, prevent the binding of HIF-1 to the HIF-1 binding site in the 3'-enhancer of the gene *(9)*. Moreover, addition of H_2O_2 has been found to inhibit hypoxia-induced EPO mRNA and EPO protein production in Hep3B cells *(11)*. We reported the effect of H_2O_2 on EPO gene expression in Hep3B cells through the GATA site, using transient transfection analysis *(27)*. EPO promoter-luciferase and promoter/enhancer-luciferase constructs (both wild type and a GATA site mutant promoter) were transfected into Hep3B cells and incubated with or without H_2O_2. Hypoxic induction was found to be suppressed by co-transfection with a human GATA-2 cDNA expression plasmid. Transfection of Hep3B cells with a reporter gene bearing a mutation at the promoter GATA binding site was found to be only mildly affected by the addition of H_2O_2. Electrophoretic gel mobility shift assays (EMSAs), using the EPO promoter GATA site as a probe and the GATA-2 protein extracted from Hep3B cells, showed that addition of H_2O_2 enhanced the binding of GATA-2, while addition of catalase inhibited this binding. From these results, we conclude that H_2O_2 increases the binding activity of GATA-2 in a specific manner, thereby suppressing the activity of the EPO promoter and thus inhibiting EPO gene expression *(27)*. Therefore, H_2O_2 administration not only inhibits HIF-1 binding activity, but also increases the binding of GATA-2 to a down-regulated site at the EPO promoter, thus providing another explanation for the mechanism by which H_2O_2 inhibits EPO gene transcription.

5. CADMIUM SUPPRESSES EPO GENE EXPRESSION

Horiguchi et al. reported that the induction of binding activity of HIF-1 transcription factor and EPO mRNA expression and protein production were suppressed by cadmium *(8)*. The binding activity of GATA was not affected by cadmium (our unpublished data). Wild type and a GATA site mutant EPO promoter/enhancer luciferase constructs were transfected into Hep3B cells. No significant difference in EPO promoter activity in these two types of cells was observed in the presence of cadmium (our unpublished data).

6. SUMMARY

1. Hypoxia-induced EPO gene expression is markedly up-regulated by HIF-1 through a binding site in the EPO enhancer. However, under normoxic conditions, GATA-2 negatively regulates EPO gene expression (Fig. 3A).
2. L-NMMA inhibits production of NO, cGMP and stimulates GATA-2 binding activity and GATA-2 mRNA expression, then inhibits EPO promoter activity. However, L-NMMA does not affect HIF-1 (Fig. 3B).
3. H_2O_2 treatment was found to inhibit EPO promoter activity and increased the binding activity of GATA-2. As GATA-2 binds to the GATA site at the EPO promoter and down-regulates EPO gene expression in Hep3B cells, this suppression of EPO gene expression by H_2O_2 is partly due to the enhanced GATA-2 binding activity. This effect may be attributed to post-transcriptional regulation of GATA-2 by H_2O_2, since the expression of GATA-2 mRNA and protein was unchanged upon the addition of H_2O_2.
4. Cadmium inhibits HIF-1 binding activity and EPO promoter activity, then suppresses EPO mRNA and protein production. However, cadmium did not affect GATA-2 binding activity.

REFERENCES

1. Aird WC, JD Parvin, PA Sharp and RD Rosenberg (**1994**) The interaction of GATA-binding proteins and basal transcription factors with GATA box-containing core promoters. *J. Biol. Cem.* 269, 882-889.
2. Eschbach JW and JW Adamson (**1985**) Anemia of end-stage renal disease. *Kidney Int.* 28, 1-5.
3. Fandrey J, S Frede and W Jelkmann (**1994**) Role of hydrogen perox-

ide in hypoxia-induced erythropoietin production. *Biochem. J.* 303, 507-510.

4. Goldberg MA, GA Glass, JM Cunningham and HF Bunn (**1987**) The regulated expression of erythropoietin by two human hepatoma cell lines. *Proc. Natl. Acad. Sci. USA* 84, 7972-7976.
5. Goldberg MA, SP Dunning and HF Bunn (**1988**) Regulation of the erythropoietin gene: evidence that the oxygen sensor is a heme protein. *Science* 242, 1412-1415.
6. Görlach A, G Holtermann, W Jelkmann, JT Hancock, SA Jones, OTG Jones and H Acker (**1993**) Photometric characteristics of heme proteins in erythropoietin-producing hepatoma cells (HepG2). *Biochem. J.* 290, 771-776.
7. Gray GA, C Schott, G Julou-Schaeffer, I Fleming, JR Paratt and J Stoclet (**1991**) The effect of inhibitors of the L-arginine/nitric oxide pathway on endotoxin-induced loss of vascular responsiveness in anesthetized rats. *Br. J. Pharmacol.* 103, 1218-1224.
8. Horiguchi H, F Kayama, E Oguma, WG Willmore, P Hradecky and HF Bunn (**2000**) Cadmium and platinum suppression of erythropoietin production in cell culture: clinical implications. *Blood* 96, 3743-3747.
9. Huang LE, Z Arany, DM Livingston and HF Bunn (**1996**) Activation of hypoxia-inducible transcription factor depends primarily upon redox-sensitive stabilization of it's α-subunit. *J. Biol. Chem.* 271, 32253-32259.
10. Huang LE, J Gu, M Schau and HF Bunn (**1998**) Regulation of hypoxia-inducible factor 1α is mediated by an O_2-dependent degradation domain via the ubiquitin-proteasome pathway. *Proc. Natl. Acad. Sci. USA* 95, 7987-7992.
11. Imagawa S, M Yamamoto, M Ueda and Y Miura (**1996**) Erythropoietin gene expression by hydrogen peroxide. *Int. J. Hematol.* 64, 189-195.
12. Imagawa S, M Yamamoto and Y Miura (**1997**) Negative regulation of the erythropoietin gene expression by the GATA transcription factors. *Blood* 89, 1430-1439.
13. Ivan M, K Kondo, H Yang, W Kim, J Valiando, M Ohh, A Slic, JM Asara, WS Lane and WG Kaelin Jr (**2001**) HIF1α targeted for VHL-mediated destruction by proline hydroxylation: implications for O_2

sensing. *Science* 292, 464-468.

14. Jaakkola P, DR Mole, Y-M Tian, MI Wilson, J Gielbert, SJ Gaskell, AV Kriegsheim, HF Hebestreit, M Mukherji, CJ Schofield, PH Maaxwell, CW Pugh and PJ Ratcliffe (**2001**) Targeting of HIF-α to the von Hippel-Lindau ubiquitylation complex by O_2-regulated prolyl hydroxylation. *Science* 292, 468-472.
15. Jacobson LO, E Goldwasser, W Fried and L Plzak (**1957**) Role of the kidney in erythropoiesis. *Nature* 179, 633-634.
16. Kimoto M, H Tsuji, T Ogawa and K Sasaoka (**1993**) Detection of N^G, N^G-dimethylarginine dimethylaminohydrolase in the nitric oxide-generating systems of rats using monoclonal antibody. *Arch. Biochem. Biophys.* 300, 657-662.
17. Kimoto M, GS Whitley, H Tsuji and T Ogawa (**1995**) Detection of N^G, N^G-dimethylarginine dimethylaminohydrolase in human tissues using a monoclonal antibody. *J. Biochem.* 117, 237-238.
18. Kimura H, A Weisz, Y Kurashima, K Hashimoto, T Ogura, F D'Acquisto, R Addeo, M Makuuchi and H Esumi (**2000**) Hypoxia response element of the human vascular endothelial growth factor gene mediates transcriptional regulation by nitric oxide: control of hypoxia-inducible factor-1 activity by nitric oxide. *Blood* 95, 189-197.
19. Lacombe C, J-L Da Silva , P Bruneval, J-G Fournier, F Wendling, N Casadevall, J-P Camilleri, J Bariety, B Varet and P Tambourin (**1988**) Peritubular cells are the site of erythropoietin synthesis in the murine hypoxic kidney. *J. Clin. Invest.* 81, 620-623.
20. McGonigle RJS, JD Wallin, RK Shadduck and JW Fisher (**1984**) Erythropoietin deficiency and erythropoiesis in renal insufficiency. *Kidney Int.* 25, 437-444.
21. Moncada S, RMJ Palmer and EA Higgs (**1991**) Nitric oxide: physiology, pathology, and pharmacology. *Pharmacol. Rev.* 43, 109-142.
22. Ogawa T, M Kimoto and K Sasaoka (**1989**) Purification and properties of a new enzyme, N^G , N^G-dimethylarginine dimethylaminohydrolase, from rat kidney. *J. Biol. Chem.* 264, 10205-10209.
23. Ohigashi T, J Brookins and JW Fisher (**1993**) Interaction of nitric oxide and cyclic guanosine 3',5'-monophosphate in erythropoietin production. *J. Clin. Invest.* 92, 1587-1591.
24. Ribeiro ACM, NB Roberts, C Lane, M Yaqoob and JC Ellory (**1996**) Accumulation of the endogenous L-arginine analogue N^G-

monomethyl-L arginine in human end-stage renal failure patients on regular haemodialysis. *Exp. Physiol.* 81, 475-481.

25. Sandau KB, J Fandrey and B Brüne (**2001**) Accumulation of HIF-1α under the influence of nitric oxide. *Blood* 97, 1009-1015.
26. Sogawa K, K Numayama-Tsuruta, M Ema, M Abe, H Abe, Y Fujii-Kuriyama (**1998**) Inhibition of hypoxia-inducible factor 1 activity by nitric oxide donors in hypoxia. *Proc. Natl. Acad. Sci. USA* 95, 7368-7373.
27. Tabata M, T Tarumoto, K Ohmine, Y Furukawa, K Hatake, K Ozawa, H Mukai, M Yamamoto and S Imagawa (**2001**) Stimulation of GATA-2 as a mechanism of hydrogen peroxide suppression in hypoxia-induced erythropoietin gene expression. *J. Cell. Physiol.* 186, 260-267.
28. Tarumoto T, S Imagawa, K Ohmine, T Nagai, M Higuchi, N Imai, N Suzuki, M Yamamoto and K Ozawa (**2000**) N^G-monomethyl-L-arginine inhibits erythropoietin gene expression by stimulating GATA-2. *Blood* 96, 1716-1722.
29. Umetani M, H Nakao, T Doi, A Iwasaki, M Ohtaka, T Nagoya, C Mataki, T Hamakubo and T Kodama (**2000**) A novel cell adhesion inhibitor, K-7174, reduces the endothelial VCAM-1 induction by inflammatory cytokines, acting through the regulation of GATA. *Biophys. Biochem. Res. Commun.* 272, 370-374.
30. Walle AJ, GY Wong, GK Clemons, JF Garcia and W Niedermayer (**1987**) Erythropoietin-hematocrit feedback circuit in the anemia of end-stage renal disease. *Kidney Int.* 31, 1205-1209.
31. Zanjani ED, J Poster, H Burlington, LI Mann and LR Wasserman (**1977**) Liver as the primary site of erythropoietin formation in the fetus. *J. Lab. Clin. Med.* 89, 640-644.

研究組織

研究者代表 ： 今川　重彦　（筑波大学）

研究分担者 ： 山本　雅之　（筑波大学）

交付決定額（配分額）

	直接経費	合計
平成１３年度	2,500 千円	2,500 千円
平成１４年度	800 千円	800 千円
平成１５年度	800 千円	800 千円
総計	4,100 千円	4,100 千円

研究発表

(1) 学会誌

Tabata M, Tarumoto T, Ohmine K, Furukawa Y, Hatake K, Yamamoto M, **Imagawa S.**
Stimulation of GATA-2 as a mechanism of hydrogen peroxide suppression in hypoxia-induced erythropoietin gene expression.
J Cell Physiol 186: 260-267, 2001.

Imagawa S, Yamaguchi Y, Higuchi M, Neichi T, Hasegawa Y, Mukai HY, Suzuki N, Yamamoto M, Nagasawa T.
Levels of vascular endothelial growth factor are elevated in patients with obstructive sleep apnea-hypopnea syndrome.
Blood 98: 1255-1257, 2001.

Ohmine K, Ota J, Ueda M, Ueno S, Yoshida K, Yamashita Y, Kirito K, **Imagawa S**, Nakamura Y, Saito K, Akutsu M, Mitani K, Kano Y, Komatsu N, Ozawa K, Mano H.
Characterization of stage progression in chronic myeloid leukemia by DNA microarray analysis with purified hematopoietic stem cells.
Oncogene 20: 8249-8257, 2001.

Imagawa S, Tarumoto T, Suzuki N, Mukai HY, Hasegawa Y, Higuchi M, Neichi T, Ozawa K, Yamamoto M, Nagasawa T.
L-arginine rescues decreased erythropoietin gene expression by stimulating GATA-2 with L-NMMA.
Kidney Int 61: 396-404, 2002.

Imagawa S, Yamaguchi Y, Higuchi M, Neichi T, Hasegawa Y, Mukai HY, Suzuki N, Yamamoto M, Nagasawa T.
Elevation of vascular endothelial growth factor in patients with obstructive sleep apnea-hypopnea syndrome is not due to increased platelet counts.
Blood 99: 393-394, 2002.

Imagawa S, Suzuki N, Ohmine K, Obara N, Mukai HY, Ozawa K, Yamamoto M, Nagasawa T.
GATA-2 suppresses erythropoietin gene expression through GATA site in mouse erythropoietin gene promoter.
Int J Hematol 74: 376-381, 2002.

Suzuki N, Ohneda O, Takahashi S, Higuchi M, Mukai H, **Imagawa S**, Yamamoto M.
Erythroid-specific expression of erythropoietin receptor rescued its null mutant mice from lethality.
Blood 100: 2279-2288, 2002.

Okoshi Y, **Imagawa** S, Higuchi M, Yoshida C, Shimizu S, Mukai HY, Komeno T, Hasegawa Y, Ninomiya H, Kojima H, Nagasawa T.

A patient with acquired pure red cell aplasia showing a positive antiglobulin test and the presence of inhibitor against erythroid precursors.

Internal Med 41: 589-592, 2002.

Imagawa S.

Function of transcription factor GATA-2.

Erythropoietin: Moleculay Biology and Clinical Use

F.P. GRAHAM PUBLISHING CO., Johnson City, TN (W.Jelkmann Eds) pp129-144, 2002.

Katsura Y, Suzukawa K, Kojima H, Yoshida C, Shimizu S, Mukai H, Hasegawa Y, **Imagawa S**, Mori N, Nagasawa T.

Cytotoxic T-cell lymphoma arising in Behcet disease.

Int J Hematol 77: 282-285, 2003.

Obara N, **Imagawa S**, Nakano Y, Suzuki N, Yamamoto M, Nagasawa T.

Suppression of erythropoietin gene expression by cadmium depends on inhibition of HIF-1, not stimulation of GATA-2.

Arch Toxicol 77: 267-2273, 2003.

Imagawa S. Nakano Y, Obara N, Suzuki N, Doi T, Kodama T, Nagasawa T, Yamamoto M.

A GATA-specific inhibitor (K-7174) rescues anemia induced by IL-1β, TNF-α or L-NMMA.

FASEB J 17: 1742-1744, 2003.

Suzuki N, Suwabe N, Ohneda O, Obara N, **Imagawa S**, Pan X, Motohashi H, Yamamoto M.

Identification and characterization of 2 types of erythroid progenitors that express GATA-1 at distinct levels.

Blood 15: 3575-3583, 2003.

Obara N, **Imagawa S**, Nakano Y, Yamamoto M, Noguchi T, Nagasawa T.

Hematological aspects of a novel 9-aminoanthracycline, amrubicin.

Cancer Science 94:1104-1106, 2003.

Imagawa S, Yamaguchi Y, Ogawa K, Obara N, Suzuki N, Yamamoto Y, Nagasawa T.

Interleukin-6 and tumor necrosis factor α in patients with obstructive sleep apnea-hypopnea syndrome.

Respiration 71: 24-29, 2004.

今川　重彦、樽本　高寿、向井　陽美、長谷川　雄一、鈴木　教郎、樋口 正人、今井　信雄、山本　雅之、長澤　俊郎.

N^G-monomethyl L-arginine (L-NMMA)の GATA-2 促進による Epo 産生低下は L-arginine により改善する.

医学のあゆみ 196: 229-230, 2001.

今川　重彦、鈴木　教郎、大嶺　謙、小原　直、向井　陽美、長谷川　雄一、山本　雅之、長澤　俊郎.

マウス GATA-2 によるマウスエリスロポエチン遺伝子の発現抑制.

医学のあゆみ 198: 731-732, 2001.

今川　重彦、中野　陽子、小原　直、鈴木　教郎、土肥　武、児玉　龍彦、長澤　俊郎　山本　雅之.

新規エリスロポエチン発現促進剤 (K-7174)：腎性貧血への試み.

医学のあゆみ 204: 291-292, 2003.

今川　重彦.

血液学からみたドーピング.

臨床スポーツ医学 20(2):195-200, 2003.

今川　重彦.

慢性骨髄増殖性疾患 (RV, ET, MF).

Modern Physician 23(2): 181-185, 2003.

今川　重彦、中野　陽子、小原　直、鈴木　教郎、土肥　武、児玉　龍彦、長澤　俊郎、山本　雅之.

二次性貧血に対する新規治療薬の開発.

医学のあゆみ 204: 903-904, 2003.

今川　重彦.

診療の秘訣：慢性骨髄性白血病慢性期の治療.

Modern Physician 23 (8): 1288, 2003.

今川　重彦.

GATA 転写因子によるエリスロポエチン発現制御機構.

分子細胞治療 2 (5): 516-521, 2003.

今川　重彦.

私の処方：鉄欠乏性貧血.

Modern Physician 23 (10):1538-1539, 2003.

中野　陽子、**今川　重彦**、小原　直、鈴木　教郎、長澤　俊郎、山本　雅之.
新規 GATA 阻害薬の経口投与による慢性貧血治療の試み.
医学のあゆみ 208: 177-178, 2004.

(2) 口頭発表

Imagawa S, Yamamoto M, Nagasawa T.
N^G-monomethyl L-arginine inhibits erythropoietin gene expression by stimulating GATA-2.
Oxygen club california 2001, March. 7-10, 2001, Santa Barbara, USA.

Imagawa S, Suzuki N, Ohmine K, Obara N, Neichi T, Yamamoto M, Nagasawa T:
Negative regulator of mouse erythropoietin.
30th Annual meeting of the international society for experimental hematology.
August 25-28, 2001,Tokyo, Japan.

今川　重彦、山口　祐司、長谷川　雄一、向井　陽美、小島　寛、樋口　正人、山本　雅之、長澤　俊郎.
重症睡眠時無呼吸症候群における一過性低酸素刺激による erythropoietin と vascular endothelial growth factor の反応動態.
第 63 回日本血液学会総会、平成 13 年 4 月 21 日、名古屋.

今川　重彦.
エリスロポエチンと低酸素応答.
第 5 回日本適応医学会学術集会　シンポジウム　平成 13 年 6 月 2 日. 大阪.

今川　重彦、樽本　高寿、小原　直、樋口　正人、根市　知宏、鈴木　教郎、山本　雅之、長澤　俊郎.
腎性貧血の新規治療法の開発: 腎性貧血研究会.
平成 12 年度報告会、平成 13 年 6 月 9 日、東京.

今川　重彦.
難治性貧血とエリスロポエチン.
茨城県医師会講演、平成 13 年 6 月 23 日、水戸.

森田　理一郎、金子　公一、中村　聡美、赤石　亨、山崎　庸弘、**今川　重彦**、奥田　諭吉、許　俊鋭.
肺癌患者における IL-6、VEGF の血清濃度.
日本外科学会総会, 4 月, 2002、京都.

今川　重彦.
エリスロポエチンの基礎と臨床-up-to-date.
中外製薬育成研究所　講演、平成14年2月1日、東京.

今川　重彦.
エリスロポエチンの基礎と臨床-up-to-date.
和歌山県立医科大学　講演、平成14年3月28日、和歌山.

今川　重彦.
エリスロポエチンの基礎と臨床-up-to-date.
第10回腎疾患ネットワーク　講演、平成14年4月16日、筑波.

今川　重彦、土肥　武、小原　直、中野　陽子、樋口　正人、鈴木　教郎、山本　雅之、
長澤　俊郎.
新規エリスロポエチン発現促進薬(K-7174): 腎性貧血への試み.
第64回日本血液学会総会、平成14年9月13日、横浜.

小原　直、**今川　重彦**、中野　陽子、樋口　正人、鈴木　教郎、山本　雅之、
長澤　俊郎.
カドミウムによるエリスロポエチン遺伝子発現抑制機序.
第64回日本血液学会総会、平成14年9月13日、横浜.

中野　陽子、**今川　重彦**、小原　直、樋口　正人、鈴木　教郎、山本　雅之、
長澤　俊郎.
2 oxoglutarate による VEGF・Epo 産生抑制.
第64回日本血液学会総会、平成14年9月13日、横浜.

Imagawa S, Nakano Y, Obara N, Suzuki N, Doi T, Kodama T, Yamamoto M and Nagasawa T.
A GATA-specific inhibitor (K-7174) rescues anemia induced by IL-1β, TNF-α or L-NMMA.
American Society of Hematology. 44th annual meeting(Philadelphia) 12月, 2002 (Blood 100(11): 657a, 2002).

Imagawa S, Nakano Y, Obara N, Suzuki N, Doi T, Nagasawa T, Yamamoto M.
K-7174 (A GATA-Specific Inhibitor) rescues anemia of chronic disease.
6th International Lübeck Conference on the Pathophysiology and Pharmacology of Erythropoietin and other Hemopoietic Growth Factors. Lübeck , Germany, June 26-29, 2003 (Annals of Hematology 82(6): S116, 2003).

今川　重彦.
貧血治療薬の現状と今後の展望.
興和（株）東京創薬第二研究所　講演、平成15年7月18日、東京.

中野　陽子、**今川　重彦**、小原　直、鈴木　教郎、長澤　俊郎、山本　雅之.
新規GATA特異的阻害薬の経口投与による慢性貧血改善の試み.
第65回日本血液学会総会、平成15年8月30日、大阪.

Imagawa S.
A Novel GATA-Specific Inhibitor (GSI) rescues anemia of chronic disease by oral administration. Oxygen and the Cell. Harnack-Haus, Berlin, Germany, September 6-9, 2003.

今川　重彦.
腎性貧血対策の現況：ラジオ短波　平成15年9月21日22時30分-22時50分

今川　重彦.
腎性貧血対策の現況：BSCテレビ　平成15年10月7日21時20分-21時40分

Nakano Y, **Imagawa S**, Obara N, Suzuki N, Nagasawa T, Yamamoto M.
A GATA-Specific Inhibitor (GSI) rescues anemia of chronic disease by oral administration.
The 76th Annual Meeting of the Japanese Biochemical Society, Yokohama, Japan, October 16, 2003.

今川　重彦、小原　直、中野　陽子、長澤　俊郎、鈴木　教郎、大根田　修、山本　雅之.
BAC (Bacterial Artificial Chromosome) トランスジェニックマウスを用いたエリスロポエチン (Epo) 産生細胞の同定.
第12回腎とエリスロポエチン研究会、平成15年11月 8日、東京.

Imagawa S. Nakano Y, Obara N, Suzuki N, Nagasawa T, Yamamoto M.
Novel GATA-Specific Inhibitor (GSI) rescues anemia of chronic disease by oral administration. 45th American Society of Hematology, San Diego, December 5-9, 2003.

今川　重彦.
エリスロポエチン開発の現状と問題点（腎性貧血と慢性貧血の治療の現状と今後の展開）
アルギニンによる保存期腎性貧血の治療に関するセミナー、平成16年1月12日、東京.

(3) 出版物

今川　重彦.

赤芽球分化の転写制御. 造血にかかわる転写因子と腫瘍.

Molecular Medicine. 38 (7): 770-777, 2001. 中山書店（平井　久丸編集）

今川　重彦、樽本　高寿、向井　陽美、長谷川　雄一、鈴木　教郎、樋口　正人、根市　知宏、山本　雅之、長澤　俊郎.

L-arginine による Erythropoetin 発現促進効果　第 9 回腎とエリスロポエチン研究会　Proceedings. 161-167, 2001.

今川　重彦.

溶血性貧血.

難病の指針. 318-324, 2002. 茨城県医師会.

今川　重彦、山口　祐司、小原　直、長谷川　雄一、向井　陽美、樋口　正人、山本　雅之、長澤　俊郎.

重症睡眠時無呼吸症候群における一過性低酸素刺激による Erythropoietin と Vascular Endothelial Growth Factor の反応動態　第 10 回腎とエリスロポエチン研究会　Proceedings. 155-160, 2002.

Wolfgang Jelkmann. (監修)　**今川　重彦.**

Molecular and Clinical Use of Erythropoietin: An update

第 10 回腎とエリスロポエチン研究会　Proceedings. 181-188, 2002.

今川　重彦.

Evidence Based Hematology. EBM 血液疾患の治療 2003-2004.　押味　和夫、別所　正美、岡本　真一郎、加藤　淳.　編集. P48-55, 中外医学社.

今川　重彦.

造血システムにおける自己複製と分化機構の解析

平成 10 年度-平成 13 年度科学研究費補助金（特定領域研究(A)（1））研究成果報告書

平成 15 年 1 月　研究代表者　須田　年生.（慶応義塾大学医学部教授）p202-204.

今川　重彦.

腎性貧血対策の現況.

MEDICAL CORNER, 114: 26-29, 2004.

今川　重彦.

エリスロポエチン. ホルモンの事典. 清野　裕、千原　和夫、名和田　新、平田　結喜緒. 編集. P397-404, 朝倉書店.