Effects of thrombopoietin on growth of hepatocellular carcinoma: Is thrombopoietin therapy for liver disease safe or not?

doi: 10.1111/hepr.12006

Title Page

Title: Effects of thrombopoietin on growth of hepatocellular carcinoma: Is thrombopoietin therapy for liver disease safe or not?

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Abstract

Aim: Liver cirrhosis (LC) is the end stage of chronic liver disease. No definitive pharmacological treatment is currently available. We previously reported that thrombopoietin (TPO) promoted liver regeneration and improved liver cirrhosis by increasing platelet count. TPO is therefore considered to be a therapeutic agent for LC; however, it is unclear whether TPO has proliferative effects on hepatocellular carcinoma (HCC), which arises frequently in cirrhotic livers. In this study we examined the effects of TPO on growth of HCC.

Methods: Expression of the TPO receptor, myeloproliferative leukemia virus oncogene (*MPL*) was examined in various liver tumor cell lines and liver cell types. In an *in vitro* study, the effects of TPO on signal transduction, cell proliferation, migration, and invasion were examined in Huh7 cells, in which *MPL* is highly expressed. In an *in vivo* study, we subcutaneously transplanted Huh7 cells into nude mice that were divided into a TPO-treated group and a control group, and the tumor volume of each group was measured.

Results: *MPL* was expressed strongly in hepatocytes but not in other cell types. Among liver tumor cell lines, Huh7 showed the highest expression of *MPL*. In Huh7, the addition of TPO activated Akt phosphorylation but not cell proliferation, migration, or

invasion. In the mouse experiment, there was no significant difference in tumor volume between the 2 groups.

Conclusions: TPO had no proliferative effect on HCC *in vitro* or *in vivo*, and could therefore be useful in the treatment of liver cirrhosis.

Keywords: Akt; hepatocellular carcinoma; Huh7; *MPL*; thrombopoietin

Introduction

Liver cirrhosis (LC) is the end stage of chronic liver disease. It carries a poor prognosis, and liver transplantation remains the only curative option. However, there are difficulties associated with liver transplantation, including a shortage of donors, high cost, transplant rejection, and surgical complications. At present, no definitive pharmacological treatment for LC is available, thus increasing the need for development of effective therapeutic agents.

In our previous studies, we demonstrated that platelets promote liver regeneration in *in vitro*[1, 2] and *in vivo*[3, 4] models. We also revealed that platelets reduced liver fibrosis *in vivo*, and platelet-derived adenosine 5'-triphosphate suppressed activation of human hepatic stellate cells, which play a critical role in liver fibrosis.[5-7] Blood transfusion and splenectomy are the traditional methods used to increase platelet counts. However, these approaches are associated with various difficulties, such as high costs, short storage periods, and platelet transfusion refractoriness for transfusions,[8] and infection and thrombosis for splenectomies.[9] Thrombopoietin (TPO) can increase platelet counts independently of blood transfusions and splenectomies, and we therefore considered using TPO as a treatment for LC.

TPO is the major hematopoietic growth factor involved in the proliferation and

differentiation of megakaryocytes.[10] Hepatocytes are the primary site of TPO synthesis.[11, 12] The TPO receptor (TPO-R), myeloproliferative leukemia virus oncogene (*MPL*), is reported to be expressed in liver sinusoidal endothelial cells (LSECs) in mice and in liver progenitor cells in rats, and TPO promotes the proliferation of both cell types.[13, 14] Hepatocellular carcinoma (HCC) develops frequently in cirrhotic livers.[15] HCC carries a poor prognosis, and it recurs at high rates even after treatment. Therefore, it is very important that therapeutic agents for LC have no proliferative effect on HCC. It has been reported that TPO increases platelets in the short term clinical study which includes HCC patients.[16] However, there is no report of long term clinical study. Since the impact of TPO is unclear in this regard, this study examined the effects of TPO on the growth of HCC both *in vitro* and *in vivo*.

Methods

Patients and samples

HCC and non-cancerous liver specimens from 14 patients who underwent hepatectomy from August 2006 to March 2009 at Tsukuba University Hospital were collected and stored at −80 °C for subsequent analysis by real-time reverse transcription polymerase chain reaction (RT-PCR). Liver sections of HCC from these patients were also collected and fixed in 10% buffered formalin for later immunohistological analyses. The study was approved by the hospital ethics committee and informed consent was obtained from all patients. *MPL* expression was examined in 5 cancerous and 5 non-cancerous samples from patients with HCC and hepatitis C virus hepatitis, 3 cancerous and 3 non-cancerous samples from patients with HCC and hepatitis B virus hepatitis, and 6 normal liver samples from patients with metastatic tumors.

Cell culture

Human hepatoblastoma cell lines (HepG2, Hep3B), a human well-differentiated HCC cell line (Huh7), human primary hepatocytes, and an immortalized human hepatic stellate cell line (TWNT-1) were used in cell cultures. TWNT-1 was kindly donated by Dr Kobayashi, Okayama University.[17] HepG2, Hep3B, Huh7, and TWNT-1 were

cultured in Dulbecco's modified Eagle's medium (DMEM; Wako Pure Chemical Industries, Ltd., Osaka, Japan) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Road Logan, UT, USA) and 1% penicillin and streptomycin (Invitrogen, Grand Island, NY, USA). The primary hepatocytes, which were purchased from XenoTech, LLC (Lenexa, KS, USA), were cultured in Williams' E medium (Sigma, St. Louis, MO, USA) supplemented with 200 μM L-glutamine (Invitrogen), 10% FBS, 100 μM dexamethasone (Sigma), 1× ITS liquid media supplement (Sigma), and 1% penicillin and streptomycin. All cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

Total RNA extraction and real-time RT-PCR

Total RNA was isolated from frozen tissue samples using Isogen reagent (Nippon Gene, Tokyo, Japan), and that of whole cells was isolated using the FastPure RNA Kit (Takara BIO Inc., Otsu, Japan), in both cases according to the manufacturer's instructions. RNA concentrations were determined by measuring the absorbance at 260/280 nm with a NanoDrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The synthesis of complimentary DNA was performed using AMV Reverse Transcriptase (Promega, Madison, WI, USA) and random primer (Takara

BIO, Inc.). Briefly, a mixture of 1 mM dNTPs (Fermentas LIFE SCIENCES, Ontario, Canada), 0.025 μg/mL random primer, 0.25 U/μL reverse transcriptase, and 500 ng total RNA was incubated at 30 °C for 10 min, 37 °C for 60 min, 95 °C for 5 min, and 4 °C before storage at −80 °C. The total RNA of primary LSECs was purchased from ScienCell Research Laboratories (Carlsbad, CA, USA)

Quantitative RT-PCR of *MPL*

Primers for RT-PCR were designed by Primer Express Software for Real-Time PCR version 3.0 (Applied Biosystems, Foster City, CA, USA) using GenBank sequences. Primers were purchased from Hokkaido System Science, Ltd. (Hokkaido, Japan). We examined *MPL* and *GAPDH* (encoding the thrombopoietin receptor and glyceraldehyde-3-phosphate dehydrogenase, respectively). RT-PCR was performed using SYBR Green Realtime PCR Master Mix-Plus (TOYOBO, Ltd, Osaka, Japan) and Applied Biosystems 7300 Real-time PCR system (Applied Biosystems), following the procedure recommended by the manufacturers. *GAPDH* was used as the endogenous control. For PCR the following primers were used for amplification: *GAPDH* (NM_002046): 5'-GGAGTCCACTGGCGTCTTCA-3', 5'-TTCACACCCATGACGAACATG-3'; *MPL* (NM_005373):

5'-GGTGACCGCTCTGCATCTAG-3', 5'-GCAGGTCTGGAAGTGAGGG-3'.

Immunohistological analysis

HCC samples were stained with the anti-TpoR/c-Mpl antibody (diluted 1:100; Millipore, Billerica, MA, USA). Can Get Signal immunostain Solution (Toyobo) was used to enhance the immunohistochemical reaction. After exposure to goat anti rabbit antibody (Zymed Laboratories, South San Francisco, CA, USA), peroxidase substrate diaminobenzidine was added for staining. All sections were counterstained with hematoxylin and observed by light microscopy.

Western blot analysis of transcriptional factors

Huh7 cells or hepatocytes were precultured in 6-well plates. The medium was then changed to serum-free medium (2 mL/well), the cells were cultured for 2 h, and 25 ng/ml recombinant human TPO (Peprotech, Rocky Hill, NJ, USA) was then added to each well. Cells were harvested at 5, 10, 15, 30, and 60 min after the addition of TPO. HepG2 and Hep3B cells without TPO were also harvested. For Western blot analysis, cell lysates were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Millipore). The following

antibodies were used as primary antibodies: extracellular signal-related kinase 1 and 2 (ERK1/2) (9102), phospho-ERK1/2 (9101), protein kinase B (Akt) (9272), phospho-Akt (9271), signal transducer and activator of transcription (STAT) 3 (9132), phospho-STAT3 (9131), STAT5 (9358), phospho-STAT5 (9359), B-cell lymphoma 2 (Bcl-2) (2872), Bcl-2-associated X protein (Bax) (2774), cleaved caspase-3 (9661) (Cell Signaling, Beverly, MA, USA), and anti-TpoR/c-Mpl antibody (06-944) (Millipore). β-actin (4970) (Cell Signaling) was used as the endogenous control. The secondary antibody that we used was anti-rabbit immunoglobulin-G horseradish peroxidase-linked antibody (Cell Signaling).

Cell proliferation

A total of 3×10^3 Huh7 cells in 100 μl of DMEM medium supplemented with 10% FBS, and 1×10^4 hepatocytes in 100 μl of Williams' E medium supplemented with 10% FBS, were seeded in 96-well plates. After incubation at 37 °C for 24 h, the medium was changed to serum-free medium and cells were cultured for an additional 2 h. Different concentrations of TPO were then added to each well. After incubation at 37 °C for 24 h, cell counts were evaluated using the Cell Counting Kit-8 (Dojin, Kumamoto, Japan) and DNA synthesis was evaluated using the bromodeoxyuridine (BrdU) assay kit (Roche

Diagnostics GmbH, Penzberg, Germany) according to the manufacturer's instructions.

Cell migration and invasion assays

Huh7 cells were assessed for their migration and invasion abilities using a 96-well cell migration assay kit (Cultrex; Trevigen, Gaithersburg, MD, USA) and 96-well collagen IV cell invasion assay kit (Cultrex; Trevigen). Briefly, to starve Huh7 cells, the cells at 80% confluence were incubated in serum-free DMEM medium for 24 h. The membranes of the upper chambers were coated with 50 μl of 0.1 × Collagen IV solution and incubated for 4 h at 37 °C in 5% CO_2 (this process was used only for the invasion assay). Next, for both assays, 5×10^4 Huh7 cells in 50 μl of serum-free DMEM medium were added to the upper compartment of a simplified Boyden chamber designed with an 8-μm polyethylene terephthalate membrane separating the top and bottom chambers. Fresh DMEM medium containing different concentrations of TPO was added to the bottom chamber. After 24 h of incubation at 37 °C and 5% CO_2, the medium was removed and the cells were washed. One hundred microliters of cell dissociation solution/calcein-AM was then added and left to incubate for 1 h. After the calcein-AM was taken up by the cells, intracellular esterases cleaved the acetomethylester moiety to generate free fluorescent calcein, which was detected by a Varioskan Flash microplate

reader (Thermo Fisher Scientific, Waltham, MA, USA) at 485-nm excitation and 520-nm emission.

Anti-apoptosis assay

A total of 1×10^4 Huh7 cells in 100 μl of DMEM medium supplemented with 10% FBS were seeded in 96-well plates. After incubation at 37 °C for 24 h, the medium was changed to serum-free medium and cells were cultured for an additional 2 h. Different concentrations of TPO and 0.1 μM of staurosporine (Sigma) were then added to each well. After incubation at 37 °C for 24 h, cell counts were evaluated using the Cell Counting Kit-8.

Animals

Female BALB/c nude mice (aged 7–8 weeks, weighing approximately 20 g; CLEA Japan, Tokyo, Japan) were used in all experiments. Mice were maintained in a temperature-controlled room on a 12 h light–dark cycle, with free access to water and standard chow. Animals were divided into 2 groups: a control group (n = 9), consisting of mice treated with saline, and a TPO-treated group (n = 8), composed of mice treated with TPO. Animal experiments were carried out in a humane manner after receiving

approval from the Institutional Animal Experiment Committee of the University of Tsukuba and in accordance with the Regulation for Animal Experiments at our university and the Fundamental Guidelines for Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Induction of thrombocytosis

To investigate the dose effect on platelet count elevation, TPO was injected intraperitoneally at doses of 5, 10, 20, and 75 μg/kg body weight into 4 mice per group, 5 days before platelet evaluation. Because platelet counts were elevated sufficiently at a TPO dose of 20 μg/kg body weight, we carried out the following experiments at this dose.

Xenograft model of human HCC cells

For the xenograft model, Huh7 cells were cultured, dissociated in trypsin, centrifuged, washed in phosphate buffered saline (PBS; Wako), and resuspended in PBS. On day 1, mice were injected subcutaneously in one rear flank with Huh7 cells, 5×10^6 cells/mouse.[18] Nude mice bearing subcutaneous xenografts of Huh7 (typically about

500 mm^3) received intraperitoneal injections of TPO (20 μg/kg body weight) or saline on days 14 and 21. Tumors were harvested on day 26. Tumor volume was calculated using the following formula: volume (mm^3) = ($d^2 \times D$)/2, where d is the smallest and D is the largest tumor diameter.[19] Tumor tissue, fixed in 10% buffered formalin, was used for histological analyses. Samples were stained in hematoxylin-eosin.

Statistical analysis

All data are expressed as the means ± standard deviations of samples. Statistical analyses were carried out with the Mann-Whitney *U*-test and one-way ANOVA, and significant data were examined using the Bonferroni-Dunn multiple comparisons post hoc test. In all cases, *p* values less than 0.05 were considered significant.

Results

Human *MPL* expression in tissues and cells

There were no significant differences in the expression of *MPL* mRNA between cancerous and non-cancerous tissues. *MPL* expression in hepatitis tissue was significantly lower than that in normal liver (Fig. 1A). *MPL* expression in hepatoma cell lines and various liver cell types were also examined using the RT-PCR technique. We used the total RNA of HepG2, Hep3B, Huh7, human primary hepatocytes, human primary LSECs, and TWNT-1. *MPL* expression was highest in primary hepatocytes. Among the hepatoma cell lines, Huh7 showed the highest expression of *MPL* (Fig. 1B). Therefore, we chose Huh7 cells in subsequent experiments. MPL protein was detected in the HCC tissue by immunohistochemistry (Fig. 1C), and in the hepatoma cell lines by Western blot analysis (Fig. 1D).

Signal transduction in Huh7 and primary hepatocytes

In Huh7, the Akt pathway was activated within 5 min after the addition of TPO, while TPO did not activate the ERK1/2, STAT3, or STAT5 pathways. In hepatocytes, the ERK1/2 and Akt pathways were activated within 15 min after the addition of TPO; the STAT3 pathway was slightly activated within 5 min after the addition of TPO, but the

STAT5 pathway was not activated by TPO addition (Fig. 2).

Effect of TPO on proliferation, migration, and invasion

Cell count did not change when Huh7 was incubated with TPO. DNA synthesis by Huh7 cells and hepatocytes was not changed by the addition of TPO (Figs. 3A, B). The ability of Huh7 cells to migrate through uncoated porous filters (Fig. 3C) or invade collagen IV-coated filters (Fig. 3D) in response to TPO was examined in a Boyden chamber. TPO did not promote the migration or invasion of Huh7.

Effect of TPO on anti-apoptosis

We evaluated downstream signals in the Akt pathway. No significant differences in levels of Bax, Bcl-2, and cleaved caspase-3 (Fig. 4A) were observed with or without TPO. Next, we evaluated the anti-apoptotic effects of TPO after adding staurosporine to Huh7 cells. No anti-apoptotic effects were observed, although apoptosis was induced in Huh7 cells by staurosporine (Fig. 4B).

Xenograft model

TPO showed a significant dose-dependent effect on platelet count elevation (Fig. 5A).

Transplanted subcutaneous tumors in both groups increased in size daily. There was no significant difference in tumor volume (2,914 mm^3 vs. 2,721 mm^3, $p = 0.793$; Fig. 5B) or the appearance of tumors (Figs. 5C, D) between the TPO-treated and control groups on day 26. Histological examination of tumor tissue from TPO-treated and control groups revealed no contrasts in morphology.

Discussion

Liver transplantation is currently the only effective treatment for LC. We previously reported that platelets are able to suppress hepatic fibrosis and promote liver regeneration. We plan to develop novel treatments for LC by using TPO to increase platelet counts in clinical settings. It is necessary to investigate the effects of TPO on HCC because HCC is frequently observed in LC patients. In this study, we revealed that in humans, *MPL* expression was lower in HCC cells than in hepatocytes. Although TPO stimulated the Akt pathway in an HCC cell line, it did not exert a proliferative effect on HCC. In addition, TPO did not promote the growth of HCC *in vivo*. We conclude, therefore, that TPO is useful in the treatment of LC even in individuals with HCC.

It has been reported that *MPL* is expressed in various cells and primary tissues. In the liver, *MPL* has been shown to be expressed in rodent hepatic progenitor cells[14] and LSECs[13], but there are few reports concerning these cells. In humans, several reports have demonstrated *MPL* expression in tissues derived from fetal liver and hepatoblastoma.[20-22] However, as of this writing no studies have examined human tissue derived from HCC. In the current report, *MPL* expression was recognized in HCC tissues and was lower than that observed in normal liver. Erickson-Miller et al.[23] examined the expression of *MPL* in 355 various tumor cell lines, including 5 hepatoma

cell lines (Hep3B, HepG2, SNU-182, SNU-387, and SNU-475), and found that *MPL* was expressed at very low or undetectable levels in all but 3 of these: 2 erythroleukemia cell lines and a lung tumor cell line. Similarly, in our experiment involving a hepatoma cell line, *MPL* expression was observed but was lower than that in hepatocytes. In addition, hepatocyte *MPL* expression occurred at very low levels, approximately 1/30 of those seen with a human megakaryocytic leukemia cell line (data not shown). Thus, *MPL* was expressed in HCC but we considered the expression levels to be very low.

Erythropoietin (EPO) is a hematopoietic growth factor that demonstrates amino acid homology with TPO.[24] The expression of EPO receptor (*EPOR*) has been demonstrated in many cancer cell lines and in tumor tissues.[25, 26] EPO promotes angiogenesis and proliferation and inhibits the apoptosis of tumor cells.[27] However, 3 studies have shown that EPO has no proliferative effect on cancer cell lines.[28-30] The expression of *EPOR* in tumor cells, therefore, does not necessarily reflect their capacity to accelerate tumor growth, nor does it always have a deleterious effect in cancer patients.[25, 31] Given these facts about EPO, and that expression levels of TPO in tumors are much lower than that of *EPOR*,[23] we speculated that the effects of TPO on tumors are negligible. Additionally, Wetzler et al.[32] showed that there was no correlation between *MPL* expression and functional response to TPO.

TPO is known to function as a growth factor in megakaryocytopoiesis and platelet production, and it has also been reported that TPO has proliferative effects on non-hematogenous cells, such as venous endothelial cells[33], hepatic progenitor cells[14], and LSECs[13]. The ability of TPO to stimulate the proliferation of these non-hematogenous cells plays an important role in angiogenesis, liver endothelial cell repair, and regeneration.[34] Acute myelogenous leukemia (AML) blast cells express *MPL* and its protein, and TPO exerts a proliferative effect on these cells.[35-37] Romanelli et al.[22] showed that the migratory activity of hepatoblastoma cells increased in the presence of TPO. We examined the ability of TPO to stimulate the migration and invasion of HepG2, but found that it did neither (data not shown). Furthermore, TPO affected neither DNA synthesis nor cell proliferation in an HCC cell line in our study.

It is known that TPO activates the Janus tyrosine kinase/STAT pathway, the mitogen-activated protein kinase pathway, and the phosphoinositide 3-kinase (PI3K)/Akt pathway.[38-40] PI3K/Akt constitutes an important pathway regulating the signaling of multiple biological processes such as apoptosis, metabolism, cell proliferation, and cell growth.[41] Phosphorylated Akt activates glycogen synthase kinase 3β (GSK3β) by phosphorylation at serine-9, resulting in accumulation of β-catenin and cyclin D1 in the nucleus, which induces DNA synthesis and cellular mitosis of

hepatocytes.[42, 43] On the other hand, other downstream Akt factors play critical roles in liver regeneration by regulating cell growth along with activated GSK3β.[44-46] In our study, TPO did not promote DNA synthesis of hepatocytes, but activated ERK1/2 and Akt pathways. It is now well established that the Akt signaling pathway is important for cancer growth and metastasis. Several reports have demonstrated the role of the PI3K/Akt pathway in the migration of liver cancer cells.[22, 47] In this study of Huh7, TPO activated the phosphorylation of Akt, but neither migration nor invasion activities were promoted, and the downstream anti-apoptotic signals of Akt were not activated. TPO did not have proliferative or anti-apoptotic effects on Huh7 cells despite the activation of Akt.

Before using TPO as a treatment for LC in the clinical setting, we should consider the possibility that it may promote HCC progression. With this in mind, we investigated whether or not TPO promoted tumor growth using a murine tumor transplantation model. In this experiment, the growth of subcutaneous tumor transplanted in mice was not accelerated by TPO treatment. Currently, 2 TPO-R agonists, eltrombopag and romiplostim, have been approved for the treatment of chronic immune thrombocytopenic purpura. Eltrombopag is a small-molecule, nonpeptide TPO-R agonist, while romiplostim is a peptide TPO-R agonist composed of an IgG Fc

fragment.[39] The efficacy of eltrombopag in HCV-infected patients with thrombocytopenia before initiation of pegylated-interferon and ribavirin therapy has previously been reported.[48] However, careful management is necessary, because portal vein thrombosis has been reported as a complication of romiplostim.[49] In contrast to TPO, eltrombopag does not activate the PI3K/Akt pathway.[50] Another report showed that eltrombopag has no proliferative effect in myelodysplastic syndromes and AML patients,[51] but rather inhibits the proliferation of leukemia cell lines.[52] Thus, eltrombopag would be secure to the HCC and the use of this type of TPO-R agonist can be anticipated as a novel treatment for liver disease.

Acknowledgements

This study was supported in part by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT). There exist no conflicts of interest.

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Figure Legends

Fig. 1. *MPL* expression in tissues and cells

(A) Total RNAs of cancerous and non-cancerous tissues were extracted and the expression of *MPL* was measured using real-time reverse transcription polymerase chain reaction (RT-PCR). normal liver, normal liver tissue from metastatic liver tumors (n = 6); non-ca (HCV), non-cancerous tissues from hepatitis C virus (HCV)-positive hepatocellular carcinomas (HCCs) (n = 5); ca (HCV), cancerous tissues from HCV-positive HCCs (n = 5); non-ca (HBV), non-cancerous tissues from hepatitis C virus (HBV)-positive HCCs (n = 3); ca (HBV), cancerous tissues from HBV-positive HCCs (n = 3); n.s., not significant. * $p < 0.05$ versus normal liver. Data are expressed as means ± standard deviations (SDs).

(B) *MPL* expression in hepatoma cell lines (HepG2, Hep3B, and Huh7) and liver cell types (human primary hepatocytes, human primary liver sinusoidal endothelial cells (LSECs), and TWNT-1) were measured using RT-PCR. * $p < 0.05$ versus hepatocyte. Data are expressed as means ± SDs.

(C) MPL protein expression in HCC tissue by immunohistochemistry. MPL-positive cells are stained brown.

(D) MPL protein expression in hepatoma cell lines by Western blot analysis.

Fig. 2. Signal transduction in Huh7 cells and hepatocytes

Huh7 cells and hepatocytes were harvested at 0, 5, 10, 15, 30, and 60 min after 25 ng/ml of TPO was added, and activation of ERK1/2, Akt, signal transducer and activator of transcription (STAT) 3, STAT5 and β-actin was examined by Western blotting.

Fig. 3. Effect of TPO on proliferation, migration, and invasion

Huh7 cells were cultured with TPO for 24 h, and the effect of TPO on Huh7 proliferation was then evaluated using bromodeoxyuridine (BrdU) (A). The effect of TPO on hepatocyte proliferation was also evaluated using BrdU (B). For each group in the proliferation assay, n = 8. Huh7 cells were cultured with TPO for 24 h, and then cell migration was analyzed with the cell migration assay kit (C) and cell invasion was analyzed with the collagen IV cell invasion kit (D). For each group evaluated with the migration and invasion assays, n = 4. Data are expressed as means ± SDs. The values indicate ratios compared to a 100% value for 0 ng/ml TPO.

Fig. 4. Effect of TPO on anti-apoptosis

Huh7 cells were harvested at 0, 5, 10, 15, 30, and 60 min after 25 ng/ml of TPO was added, and activation of Bax, Bcl-2, and cleaved caspase-3 was examined by Western blotting (A). Huh7 cells were cultured with TPO and staurosporine for 24 h, and the anti-apoptotic effect of TPO on Huh7 was evaluated using the Cell Counting Kit-8 (B).

Data are expressed as means ± SDs with n = 8 for each group. The values indicate ratios compared to a 100% value without TPO or staurosporine.

Fig. 5. Animal model

Platelet counts are indicated for groups receiving TPO at doses of 0, 5, 10, 20, or 75 μg/kg body weight, injected intraperitoneally 5 days before measurements. Data are expressed as means ± SDs, with n = 4 for each group. ** $p < 0.01$ versus normal group (A). Nude mice bearing subcutaneous xenografts of Huh7 received i.p. injections of TPO (20 μg/kg body weight) or saline on days 14 and 21. Tumor volume (mm^3) = $(d^2 \times D)/2$, where d indicates the smallest tumor diameter while D indicates the largest. Data are expressed as means ± SDs, with n = 8–9 for each group (B). Representative features of Huh7 cell grafts in control (C) and TPO-treated mice (D) on day 26.

Figures

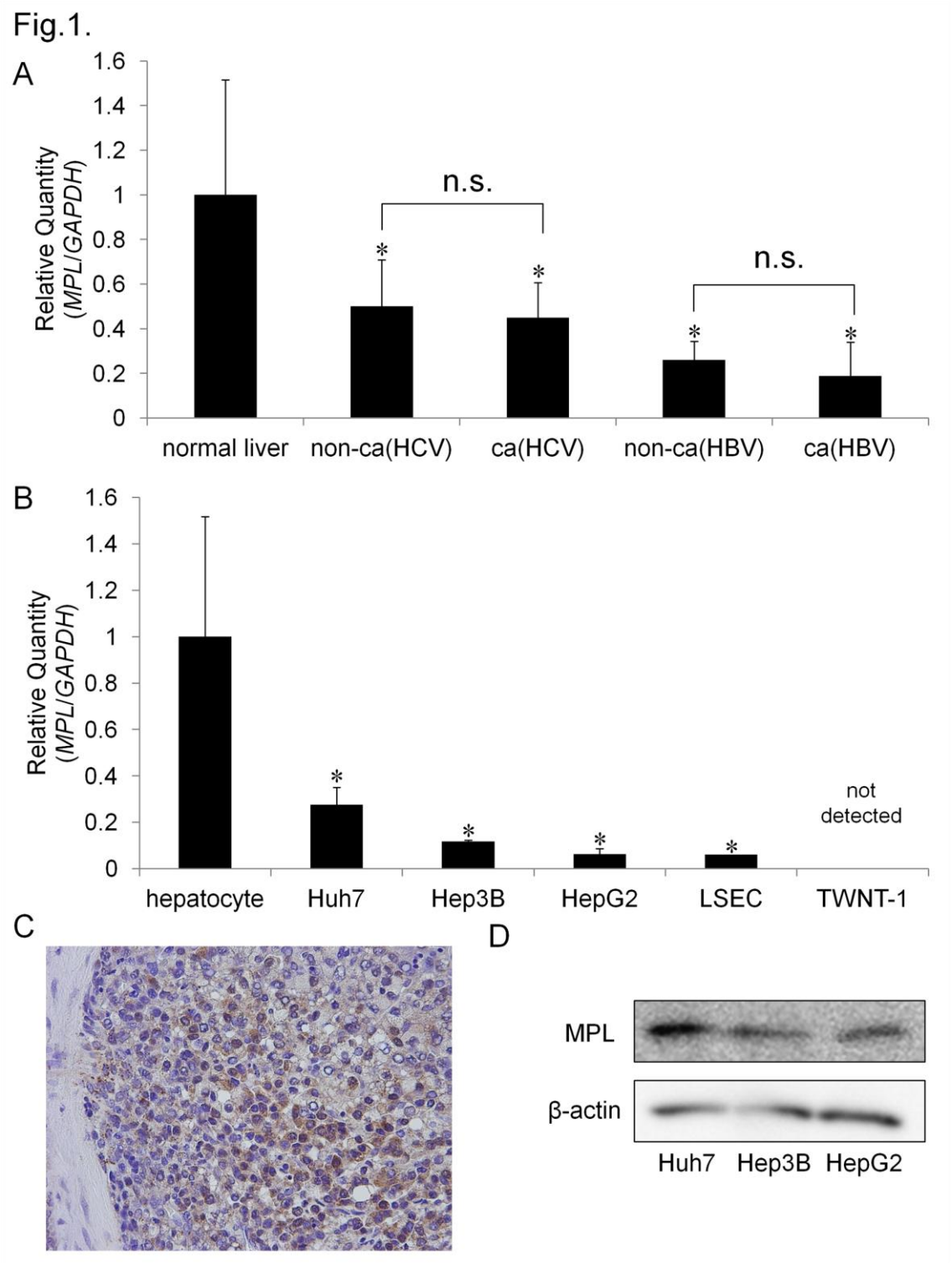


Fig.1.
A
Relative Quantity (MPL/GAPDH)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
n.s.
n.s.
*
normal liver
non-ca(HCV)
ca(HCV)
non-ca(HBV)
ca(HBV)
B
Relative Quantity (MPL/GAPDH)
not detected
hepatocyte
Huh7
Hep3B
HepG2
LSEC
TWNT-1
C
D
MPL
β-actin
Huh7
Hep3B
HepG2

Fig.2.

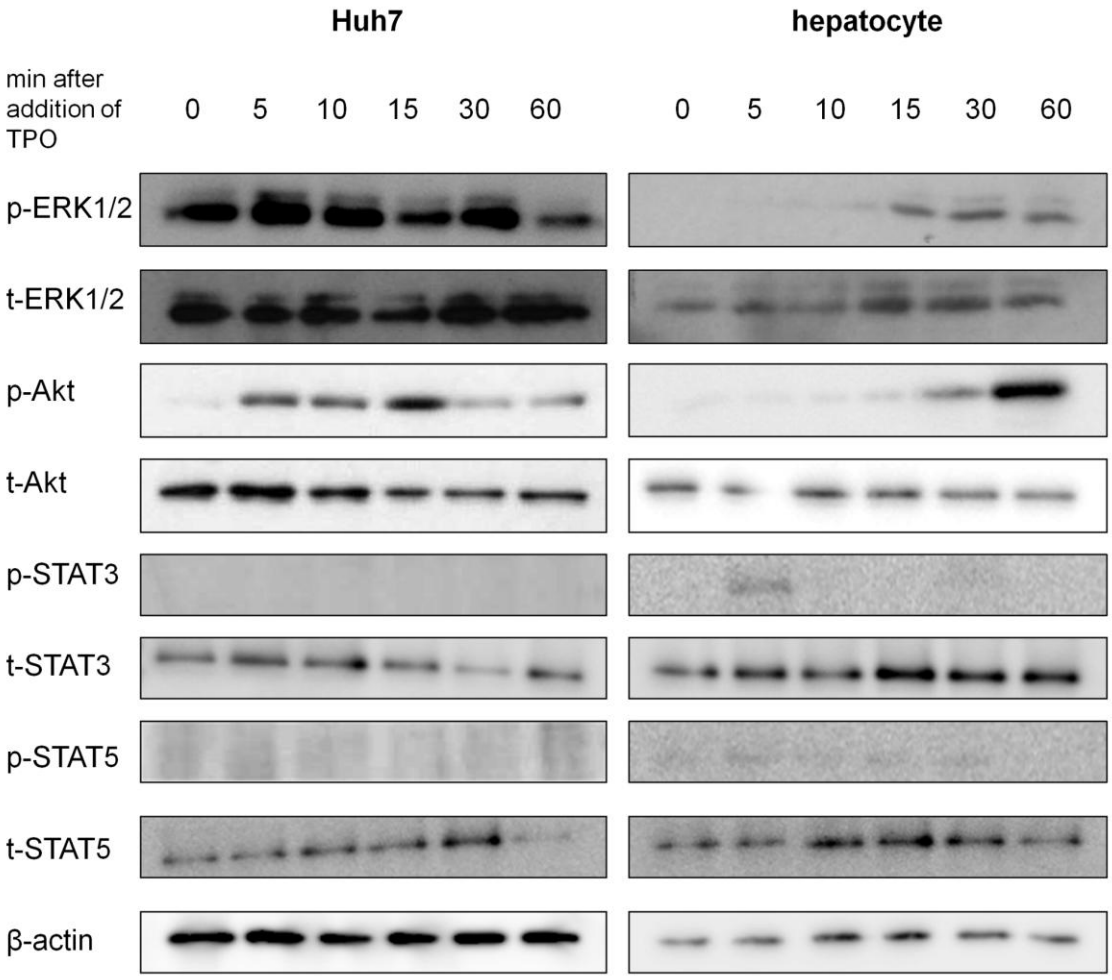

Huh7
hepatocyte
min after addition of TPO
0 5 10 15 30 60
0 5 10 15 30 60
p-ERK1/2
t-ERK1/2
p-Akt
t-Akt
p-STAT3
t-STAT3
p-STAT5
t-STAT5
β-actin

Fig.3.

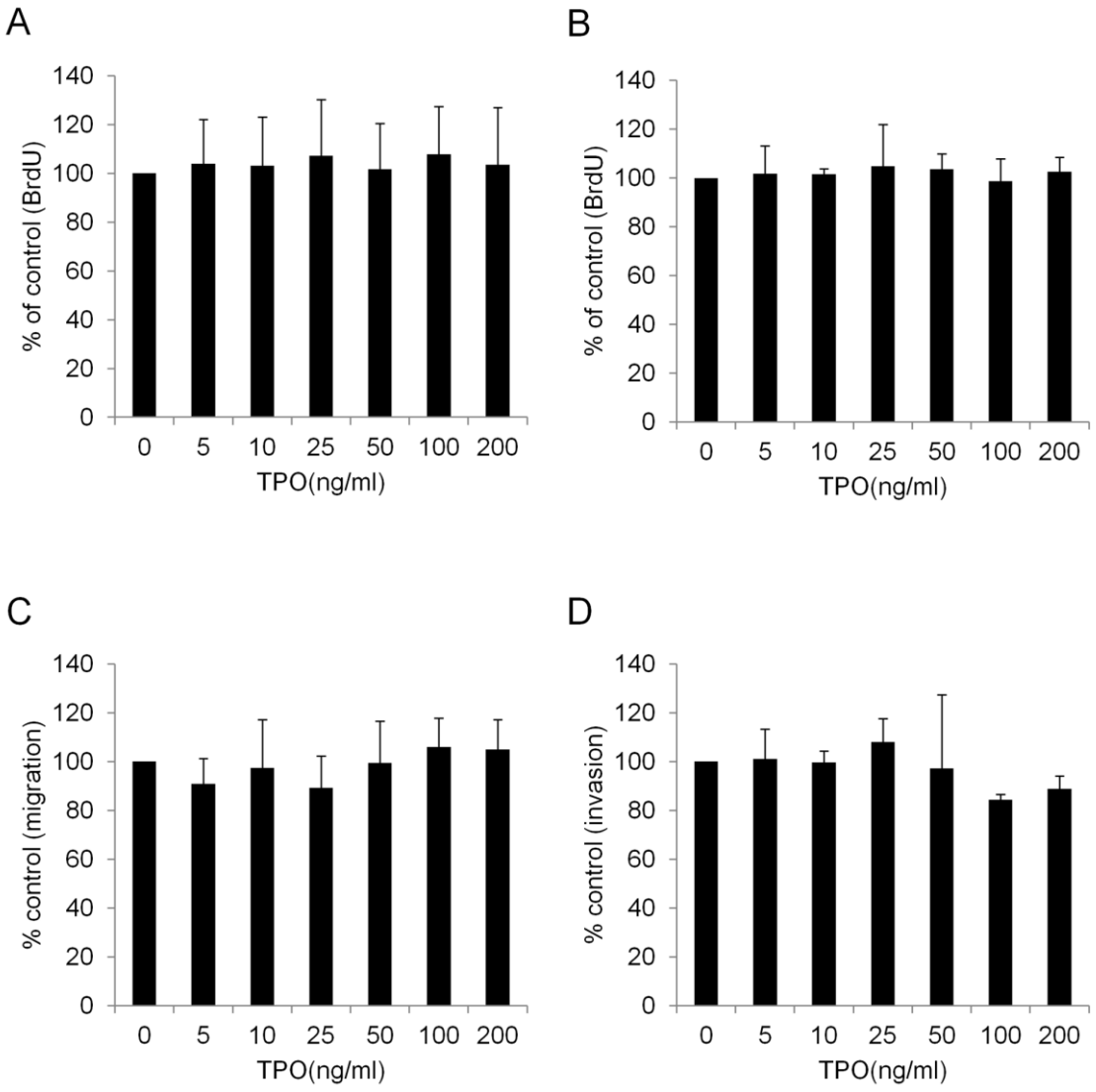

A
% of control (BrdU)
140
120
100
80
60
40
20
0
0 5 10 25 50 100 200
TPO(ng/ml)
B
% of control (BrdU)
140
120
100
80
60
40
20
0
0 5 10 25 50 100 200
TPO(ng/ml)
C
% control (migration)
140
120
100
80
60
40
20
0
0 5 10 25 50 100 200
TPO(ng/ml)
D
% control (invasion)
140
120
100
80
60
40
20
0
0 5 10 25 50 100 200
TPO(ng/ml)

Fig.4.

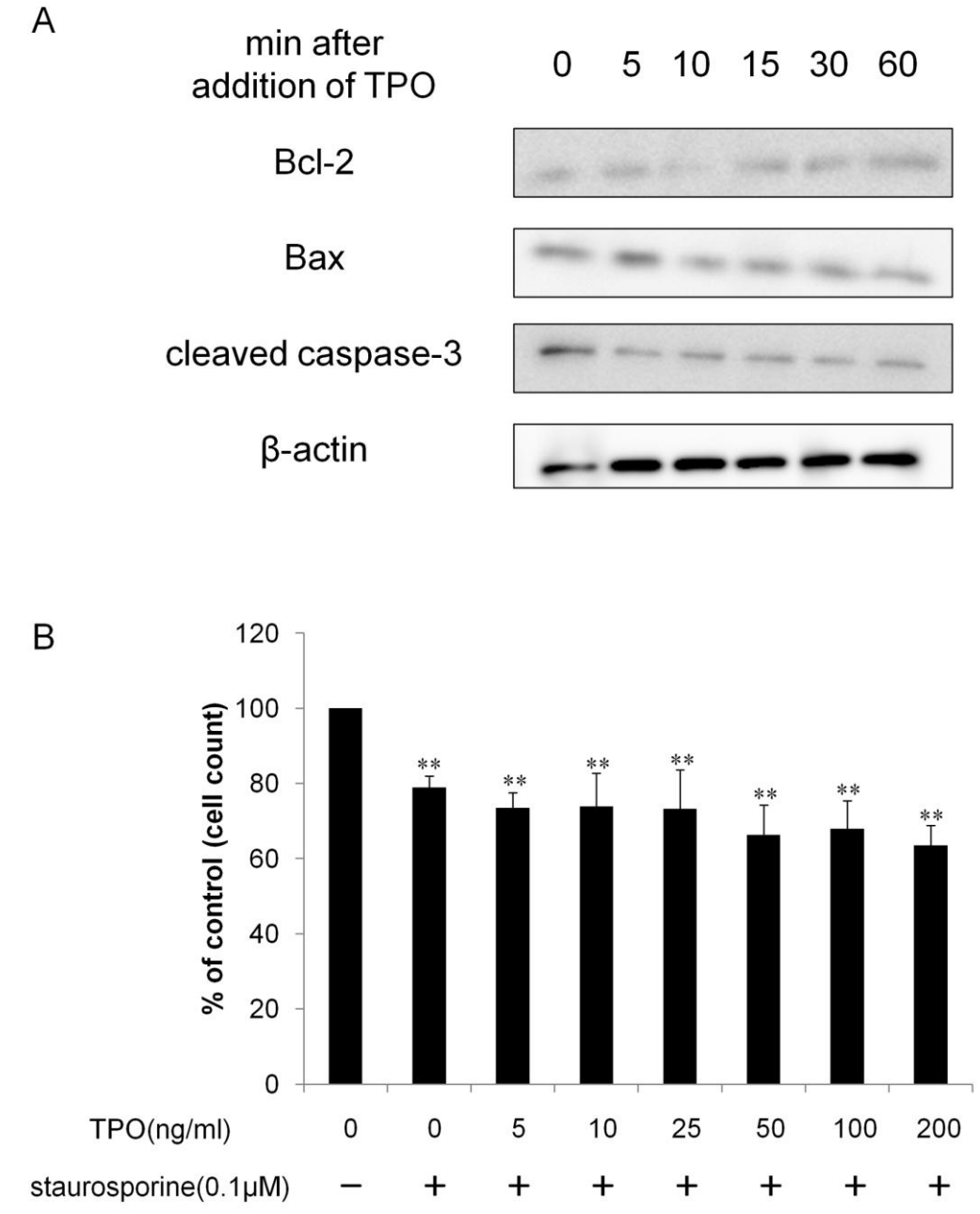
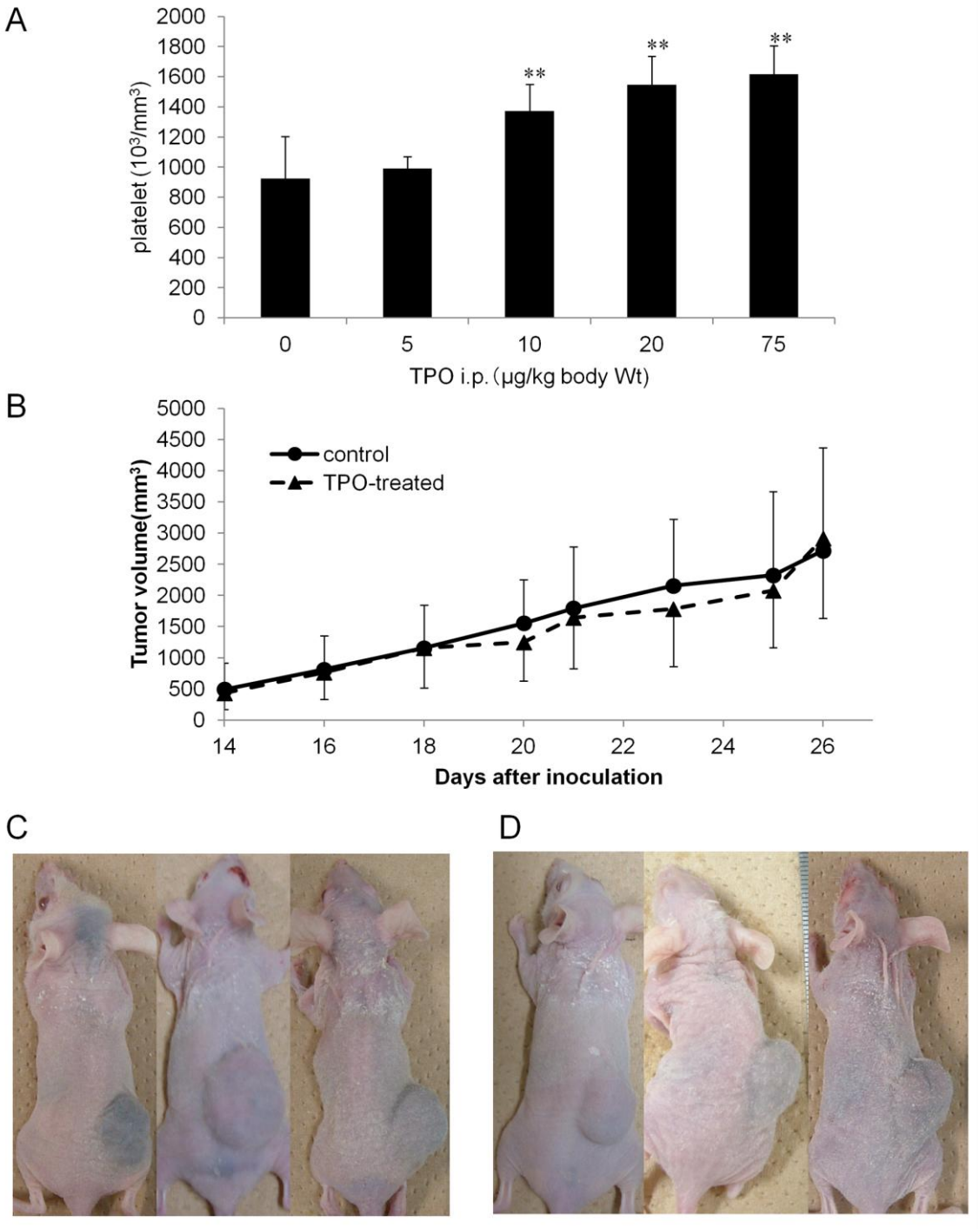

A
min after addition of TPO
0 5 10 15 30 60
Bcl-2
Bax
cleaved caspase-3
β-actin
B
% of control (cell count)
120
100
80
60
40
20
0
**
TPO(ng/ml) 0 0 5 10 25 50 100 200
staurosporine(0.1μM) − + + + + + + +

Fig.5.


A
platelet (10³/mm³)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
**
**
**
0
5
10
20
75
TPO i.p. (μg/kg body Wt)
B
Tumor volume(mm³)
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
control
TPO-treated
14
16
18
20
22
24
26
Days after inoculation
C
D