Significant correlation between endothelial nitric oxide synthase (eNOS) expression and alveolar repair in elastase-induced rat pulmonary emphysema

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Original Article

Significant Correlation between Endothelial nitric oxide synthase (eNOS) Expression and Alveolar Repair in Elastase-induced Rat Pulmonary Emphysema

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Running head: Increased eNOS and Alveolar Repair

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Key words

neovascularization, emphysema, eNOS, angiogenic factor, G-CSF

Abstract

Background and Purpose:

Angiogenic factors, such as endothelial nitric oxide synthase (eNOS), are thought to play an important role in the repair of pulmonary emphysema (PE); yet, the correlation of the factors involved has not been investigated. We conducted this study to clarify the positive correlation between eNOS expression and alveolar repair in PE recovery.

Methods:

We used elastase to induce PE in rats, which were divided into Groups A (Control), B (G-CSF), C (PE) and D (PE + G-CSF). G-CSF was injected for 12 days, 4 weeks after which the alveolar walls, arterioles, and angiogenic factors including eNOS were examined histopathologically and by western blotting.

Results:

In comparing Groups A, B, C, and D, the alveolar density was 2.4+/-0.2, 2.4+/-0.1, 1.8+/-0.1, 2.5+/-0.1 per 100 μm^2, respectively (C vs. others; $p<0.00001$) and the number of arterioles was 4.5+/-1.0, 5.6+/-0.6, 3.2+/-0.5, 5.5+/-0.7 /mm^2, respectively (C vs. others; $p<0.05$). Immunohistochemical staining (IHC) revealed different eNOS expression in Group D vs. Group C ($p<0.0001$) and western blotting revealed different eNOS, VEGF, and FLT-1 expression in Group D vs. Group C ($p<0.01$, $p<0.05$, $p<0.001$), reflecting the contribution of angiogenesis to PE repair. eNOS showed a significantly positive correlation to alveolar density and arteriole repair.

Conclusion:

Alveolar repair was correlated positively to eNOS expression by vascular regeneration in elastase-induced rat pulmonary emphysema.

Introduction

Pulmonary emphysema (PE) is one of the most common causes of respiratory failure and a frequent cause of death. Severe PE can predispose to pneumonias and the perioperative management of respiratory conditions complicating severe PE is difficult in thoracic surgery. Postoperatively, persistent PE leads to poor pulmonary function and dyspnea on effort, restricting the activities of daily life. Patients who undergo lung resection with PE suffer even further compromised quality of life (QOL)[1]. PE involves decreased elasticity of the airway and alveolar wall destruction, including chronic inflammation, with remodeling of the alveolar area [2]. The precise mechanisms behind these pathological changes and lung repair remain unclear [3]. A better understanding of the mechanism of regeneration from PE will make lung resection safer and improve QOL after surgery.

Recent studies have found that bone marrow-derived cells and angiogenic cytokines are related to lung repair in PE. Cytokines such as G-CSF, vascular endothelial growth factor (VEGF), angiopoietin-1, and erythropoietin are increased by the inflammation and ischemia in acute lung disease, triggering the mobilization of EPCs from the bone marrow to the injured lung tissue [4]. The importance of vascular regeneration in lung repair in PE has also been shown [5-7]. Endothelial nitric oxide synthase (eNOS), one of the most important angiogenic factors, is located downstream of VEGF. Reduced eNOS expression and fewer endothelial cells in the pulmonary vasculature of PE have been reported [8]. Conversely, enhanced eNOS expression in the residual lung after pneumonectomy with alveolar wall regeneration has been reported [9]. However, although the importance of eNOS expression in lung repair is recognized, the

correlation between direct alveolar repair and eNOS expression through neovascularization remains unclear. We hypothesized that alveolar repair in PE is positively correlated to eNOS expression by vascular regeneration. In this study, G-CSF was used as the angiogenic factor inducing mobilizing endothelial progenitor cells (EPCs) [10] and enhancing eNOS.

Methods

Animals

The Animal Research Committee at the University of Tsukuba approved the experimental protocols. Rats were cared for in accordance with the Guiding Principles for the Care and Use of Animals based on the Helsinki Declaration of 1964. The 6-week-old male Wistar rats (BW: 200g, n=38, Charles River Japan, Yokohama, Japan) were divided into four groups according to the induction of PE and the use of G-CSF (GRAN®, Kirin Pharma, Japan) as follows: Group A (Control; non-PE treated with normal saline (NS), n=9), Group B (non-PE treated with G-CSF, n=11), Group C (PE treated with NS, n=9), and Group D (PE treated with G-CSF, n=9).

Surgical Procedure

Anesthesia was induced by diethyl ether (Wako Chemicals, Japan) inhalation, followed by an intraperitoneal injection of pentobarbital (Somnopentyl®: 50 mg/kg, Kyoritsu Seiyaku Corporation, Japan). A skin incision was made to expose the trachea, the front of which was punctured by a 27-gauge needle to inject porcine-derived elastase (200 U/kg, ELASTIN PRODUCTS COMPANY. INC, MO, U.S.A.). Rats were

temporarily placed upright at 70 degrees. The porcine-derived elastase was dissolved in 0.1 ml normal saline and injected over 60 s in Groups C and D. The same volume of normal saline was injected in the control non-PE groups (Groups A and B).

Experimental Protocol

Three weeks after the injection of elastase, G-CSF (100 μg/kg/day) was injected subcutaneously for 12 days. The rats were killed 9 weeks after the injection of elastase and the lungs were removed and fixed with an intratracheal injection of 10% formalin solution under constant pressure of 20 cmH_2O. We examined the specimens histopathologically (HE stain, eNOS immunohistochemistry) and also stored frozen specimens for western blotting.

Examination of the Alveolar Wall and Arterioles

PE was evaluated by the alveolar density per unit (septum frequency / 100 μm^2) and the mean linear intercept (Lm) using modified point counting methods[11], as follows: *Alveolar density per unit* (septum frequency / 100 μm^2): Alveolar density was based on the modified point counting method of Leuwerke [9, 12]. Using HE staining, the points at which the alveolar walls crossed the borders of 10×10 lattices were counted continuously. The number of crossing points was averaged by the number of lattices in efficient alveolar areas and this process was performed using light microscopy in 100 magnifications. The lattices were 100 μm in size. Lattices in non-alveolar areas containing collapsed or inflammated alveolar areas and bronchi, cartilage, and vessels were eliminated (Fig. 1a). We calculated 15 randomly selected fields.

Mean Linear Intercept (Lm): The extent of emphysematous lesions was assessed

by measuring the mean linear intercept (Lm) using the method of Thulbeck with modification [13, 14]. Briefly, Lm was defined as the linear sum of all lines in all frames counted and divided by the number of intercepts defined as an alveolar septa intersecting with a counting line. A minimum of 10 fields and 200 intercepts was measured for each rat [15].

Arterioles were defined as arteries more than 50 μm and less than 400 μm in diameter. Arterioles were evaluated by the number within 1 mm^2 in each lattice (Fig.1a).

Immunohistochemistry

Paraffin-embedded sections of the lung were deparaffinized with xylene and rehydrated with ethanol. Antigens were activated by thermal microwave radiation. Sections were blocked by 3% H_2O_2 for 15 min and incubated overnight with anti-eNOS antibody (Lab Vision Corporation, Fremont, CA, USA); then for another 30 min with the universal immuno-peroxidase polymer for rat tissue sections (Nichirei, Tokyo, Japan). The antibody-polymer-peroxidase complexes were visualized using DAB (Simple Stain DAB solution, Nichirei, Tokyo, Japan) and nuclei were counterstained with hematoxylin. Images were captured using a high-resolution digital camera mounted on a microscope (Olympus BX-40F, Tokyo, Japan). Analysis was performed on approximately eight capillaries per section of the left middle lobe of each rat lung at 1,000 times magnification. Each capillary image was converted into black and white using the graphic software Photoshop Element (Microsoft, Seattle, WA. USA). The 256-grayscale value of capillary endothelial cell bodies, excluding the nuclei, was evaluated using the graphic software ImageJ (NIH, USA). The difference in the

grayscale value of each group was regarded as the difference in eNOS expression for semi-quantitative analysis. High grayscale values indicate a high density of eNOS.

Western Blotting

The homogenized lung tissue was lysed on ice with RIPA buffer. Samples were run on SDS-PAGE, using 7.5-10% polyacrylamide gel (Wako Pure Chemical Industries, Ltd, Osaka, Japan), and electrotransferred to a polyvinylidene diflouride (PVDF, Paul Corporation, Pensacola, FL, U.S.A.) filter membrane. The PVDF membrane was blocked for 1 h at room temperature with 1% bovine serum albumin (Wako Pure Chemical Industries, Ltd, Osaka, Japan) in PBS containing 0.1% Tween 20 (TPBS). Thereafter, the PVDF membrane was incubated overnight at 4°C with primary antibodies in TPBS. After washing with TPBS, the PVDF membrane was incubated with horseradish peroxidase-conjugated anti-rabbit antibody (GE Healthcare Japan, Tokyo, Japan), and diluted to 1:6000 in TPBS at room temperature for 60 min. The blots were visualized with an enhanced chemiluminescence detection system (Amersham Biosciences, UK), exposed to X-ray film, and analyzed by the graphic software, ImageJ, using the 256-grayscale value. The following antibodies were used: Anti-eNOS antibody (AnaSpec, Inc, San Jose, CA, USA), anti-VEGF antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Flt-1 antibody (Thermo Fisher Scientific, Fremont, CA, USA), and anti-β-actin antibody (AnaSpec, Inc. San Jose, CA, USA).

Statistical Analysis

All variables are expressed as means +/- SD. All groups were analyzed statistically by the Student’s unpaired t-test with Bonferroni correction. Specific groups were compared using an unpaired Student’s test. Simple linear regression analysis was

performed for each principal factor. A difference of $p<0.05$ was considered significant. We used the statistical software SPSS (SPSS Inc., Chicago, Ill. USA).

Results

Evaluation of Alveolar Change

Alveolar Density (Septum Frequency)

The number of alveolar density crossing points on lattices in Groups A, B, C, and D was 2.4+/- 0.2, 2.4+/-0.1, 1.8+/-0.1, and 2.5+/-0.1 (unit: points/100 μm^2), respectively. There were no significant differences between Groups A and B (NS), but the number of lattices was significantly higher in Group D than in Group C ($P<0.00001$) and significantly lower in Group C than in the other three groups ($p<0.00001$; Fig. 1b).

Mean linear intercept (Lm)

The Lm in Groups A, B, C, and D were 92.4 +/- 7.0, 95.2 +/- 4.7, 125.1 +/- 8.8, and 91.7 +/- 4.4 (μm), respectively. There were no significant differences between Groups A and B (NS), but the Lm was significantly lower in Group D than in Group C ($P<0.00001$) and significantly higher in Group C than in the other three groups ($p<0.00001$; Fig. 1c).

Number of Arterioles

The average number of arterioles in Groups A, B, C, and D was 4.5+/- 1.0 /mm^2, 5.6+/-0.6 /mm^2, 3.2+/-0.5 /mm^2, and 5.5+/-0.7 /mm^2, respectively Although there were significantly fewer arterioles in Group C than in Groups A or B, there were significantly more arterioles in Group D than in Group C (Group C vs. Group A, group B, and Group

D; $p<0.05$, $p<0.0001$, and $p<0.0001$, respectively; Fig. 1d).

eNOS Expression According to Immunohistochemistry and Western Blotting of the Angiogenic Factors

The levels of eNOS expression in the capillary endothelial cells in Groups A, B, C, and D were 59.1+/- 8.9, 62.9+/-13.6, 35.1+/-6.4, and 61.7+/-7.3, respectively (Group C vs. Groups A and D; $p<0.0001$, $p<0.0005$, respectively; Fig. 2). Enhanced eNOS expression in the alveolar capillaries was accompanied by a higher number of arterioles in Group D than in Group C (Figs 1e, 2, 3). The eNOS expression in Groups C and D according to western blotting was 0.13+/-0.06 and 0.6+/-0.2, respectively ($p<0.01$; Fig. 3a). The VEGF expression in Groups C and D according to western blotting was 0.13+/-0.06 and 0.70+/-0.20, respectively ($p<0.05$; Fig. 3b). FLT-1 expression was similar, being 0.25+/-0.1 in Group C vs.1.56+/-0.2 in Group D ($p<0.001$; Fig. 3c).

Evaluation by Simple Linear Regression

Simple linear regression analyses showed significant correlations between eNOS expression and alveolar wall counts, being alveolar density ($r=0.648$; $p<0.005$; Fig. 4a); arteriole counts and alveolar wall counts, being alveolar density ($r=0.546$; $p<0.005$; Fig. 4b); and eNOS expression and arteriole counts ($r=0.670$; $p<0.005$; Fig. 4c). Moreover, e-NOS expression, the number of arterioles, and the number of alveolar walls were all positively correlated with each other.

Discussion

Elastase-induced emphysema is a simple experimental model, designed to allow us

to study emphysema[16]. It can be established within approximately 2 weeks and is particularly useful for evaluating regeneration to assess the effectiveness of drugs administered after the development of emphysema[3].

In this experiment, injecting elastase into the trachea induced PE by disrupting the alveolar wall and reducing the number of arterioles with lowered eNOS expression and VEGF axis. We administered G-CSF as an angiogenic promoter, resulting in the repair of arteriole structure and alveolar wall density. Alveolar wall density and vascular development (arteriole number) was found to be significantly correlated with the eNOS expression of the alveolar capillary endothelium. It was recently recognized that apoptosis of endothelium, leading to the loss of capillaries, may be a central mechanism in emphysema[5]. Other reports have described impaired endothelial function in the small pulmonary arteries from patients with COPD [17, 18]. Accordingly, fewer arterioles were detected in our group C than in the other groups.

G-CSF as a potent angiogenic promoter acts by mobilizing endothelial progenitor cells (EPCs) [19], which are essential for vasculogenesis [20]. This activation of angiogenesis, might have contributed to the recovery from PE. Nitric oxide (NO) is an important cellular messenger molecule with pleiotropic effects including vasodilatation, anti-oxidation, anti-inflammation, mobilization of EPC, and neovascularization[21]. The activation of eNOS and the production of NO, which is triggered by a serine/threonine protein kinase (Akt) signaling axis, is one of the most potent endothelial functions [22]. In endothelial cells, the Akt signaling axis is activated by a variety of stimuli and growth factors such as VEGF or angiopoietin-1 that regulate angiogenesis, endothelial cell survival, and vessel integrity by controlling NO synthesis [22].

Arteriogenesis is reported to be enhanced by increased eNOS expression [23],

whereas G-CSF is reported to induce eNOS in endothelial cells [10]. In this experiment, eNOS contributed to vascular regeneration in addition to the VEGF axis. In relation to PE, it has been speculated that eNOS is capable of regenerating the alveolar wall [9]. Consequently, we focused on the role of eNOS in lung repair from PE from the following perspectives: the pleiotropic effects of NO; eNOS and NO as essential factors for angiogenesis; and the regenerative effects of eNOS on cells, including cells in the alveolar wall.

Although eNOS expression in PE repair has been emphasized [3, 8], its correlation has not been proven (Fig. 4). Thus, eNOS expression in the capillaries was positively correlated with the number of alveolar walls and arterioles. Fig. 4 shows that the angiogenic effect is one of the most important aspects of eNOS in lung regeneration from PE. Other studies have found that statins have a protective effect on alveolar and capillary apoptosis in PE [8]. This effect is mediated by Akt and eNOS activation [3, 7]. The importance of the angiogenic function of eNOS on PE repair was also apparent in our study.

The findings of this study provide solid evidence that eNOS plays an important role in vascular regeneration in lung repair. Drugs enhancing eNOS may become a strategy for the treatment of PE, focusing on regeneration of the pulmonary vasculature and may help surgical patients with PE.

Conclusion

Alveolar repair is correlated positively with eNOS expression through vascular regeneration in elastase-induced rat pulmonary emphysema.

Acknowledgment

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Conflict of Interest Statement

We have no conflicts of interest.

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Figure legends

Fig.1 Alveolar walls and arterioles**. a** (upper section). Typical alveolar images of Groups A to D (HE stain, X100). The number of alveolar walls (alveolar wall density, septum frequency) were counted by point counting methods. **b, c.** G-CSF induced a recovery of the alveolar walls (Group C vs. Group D). **d.** The number of arterioles recovered significantly well following the administration of G-CSF to the rats with induced pulmonary emphysema (PE) (Group C vs. Group D, $p<0.05$).

Fig.2 Immunohistochemical findings of eNOS expression. eNOS was dyed brown inside the vascular endothelial cells (arrow). The average 256 grayscale value of eNOS expression was significantly higher in the G-CSF treated rats than in the non-treated rats with pulmonary emphysema (PE; Group C vs. Group D, $p<0.00005$).

Fig.3 Quantification of angiogenic factors. Pulmonary emphysema (PE) with G-CSF treatment significantly increased the angiogenic factors, VEGF and eNOS, vs. PE without G-CSF (Group C vs. D; Fig3a and 3b). Flt-1 expression showed the same tendency (Fig3c).

Fig.4 Evaluation by simple linear regression. Significant correlations were found between the alveolar walls and eNOS expression (a), the alveolar walls and arterioles (b), and eNOS expression and the arterioles (c). From this series of correlations, we concluded that higher eNOS expression enhances alveolar repair through recovery of the arterioles.

Figure 1

Fig.1a Counting of Alveolar walls and arterioles HE X100

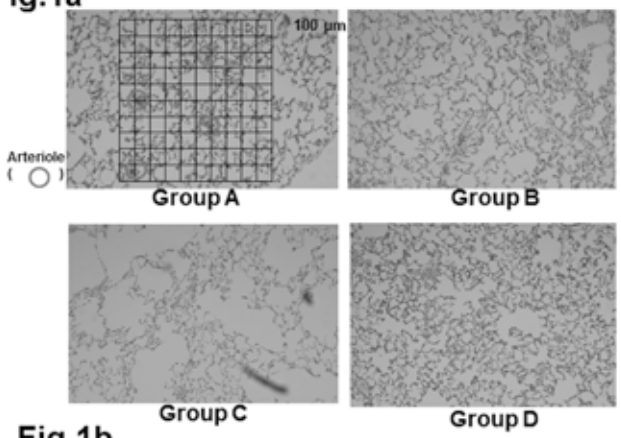

Fig.1b alveolar wall density (septum frequency)

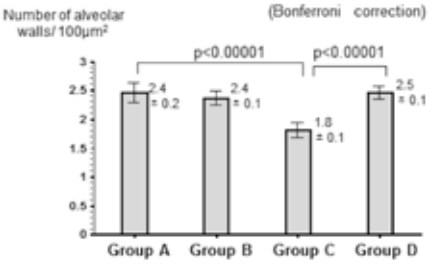

Fig.1c mean linear intercept (Lm)

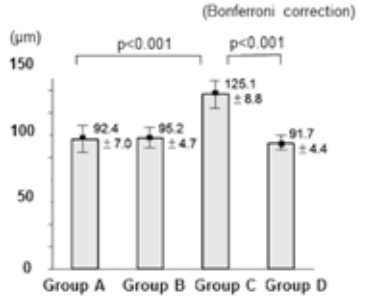

Fig.1d arterioles

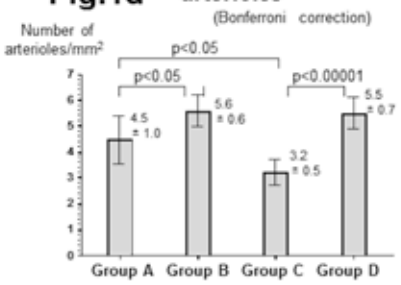

Figure 2

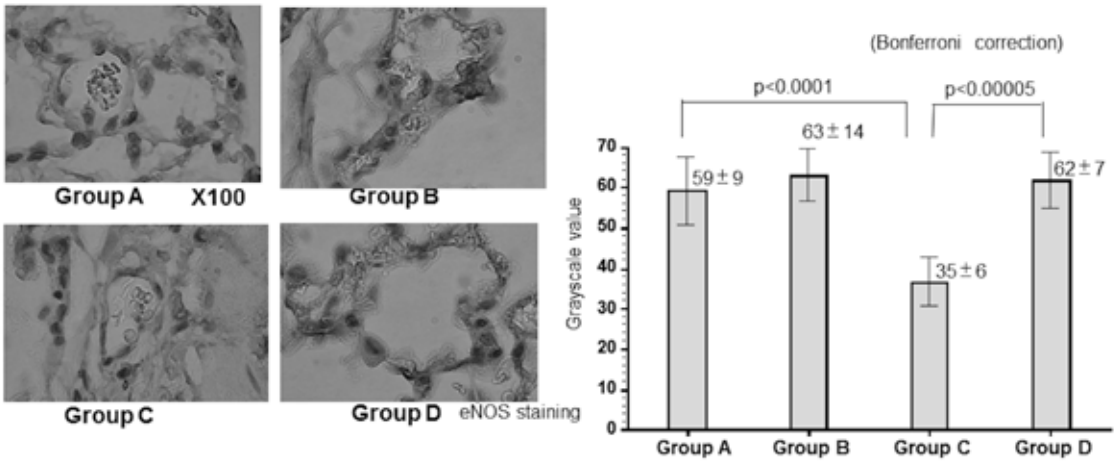

Figure 3

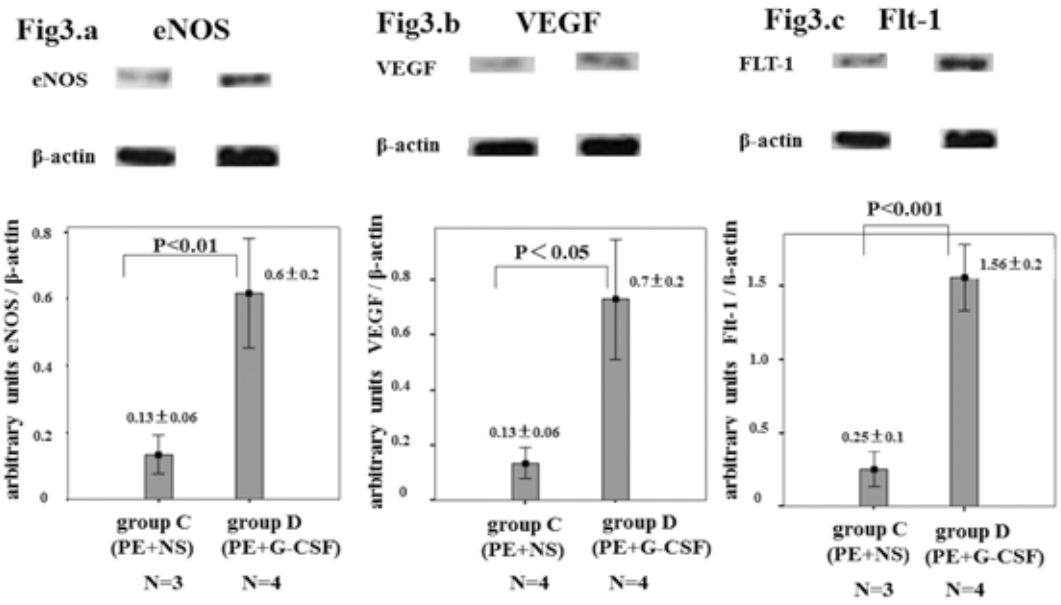

Figure 4