Preventive Effects of Erythropoietin on Peritoneal Hypoxia and Fibrosis in Rats with Chlorhexidine Gluconate–induced Peritoneal Fibrosis

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Introduction

Peritoneal dialysis (PD) is an attractive treatment for end-stage kidney disease (ESKD). However, long-term PD is associated with the development of structural alterations in the peritoneum such as loss of mesothelial cells and marked submesothelial fibrotic thickening\(^1\)\(^\text{2}\). In particular, some patients
develop encapsulating peritoneal sclerosis (EPS) associated with high mortality, which is one of the most serious complications. Recent studies also demonstrated an increase of peritoneal vascularization with vasculopathy, that is included angiogenesis, replication of the capillary basement membrane, resulting in considerable thickening of the subendothelial tissues and culminating in occlusion of the capillary lumina, in long-term PD patients. Development of peritoneal fibrosis (PF) and vasculopathy were closely correlated with PD duration.

The authors reported that marked PF and vasculopathy with neoangiogenesis were observed in an encapsulated peritoneal sclerosis rat model. Hypoxia is one of the factors that induced angiogenesis. Therefore, it was suggested that peritoneal damage in the peritonitis model is caused by hypoxia. Recently, expressions of hypoxia-inducible factor-1α (HIF-1α) and erythropoietin receptor (EpoR) were reported to induce hypoxia in the brain and heart. HIF-1α was expressed in the primary tubular epithelial cells under hypoxic conditions and HIF-1α was required for hypoxic induction of connective tissue growth factor (CTGF) mRNA, as the down-stream mediator of TGF-β1 related peritoneal fibrosis.

Erythropoietin (Epo) is a serum glycoprotein that is synthesized mainly in the adult kidney and fetal liver. With dimerization of the EpoR by Epo binding, an intracellular signaling pathway is established to enable erythroid differentiation. Since Epo is required for erythroid development, allowing maturation of erythroid precursors by preventing apoptosis of erythroid progenitor cells, Epo promotes angiogenesis attributed to the proliferation and migration of vascular endothelial cells (VECs) and vascular smooth muscle cells (VSMCs) and inhibits their apoptosis.

Moreover, under hypoxic conditions, Epo shows neuroprotective effects by inhibition of apoptosis in neurons, including angiogenesis both in vivo and in vitro in various animal models of central nervous system diseases. Thus, upregulated Epo is a naturally self-regulated physiological protective mechanism in the mammalian brain during ischemia. Nishiya et al. reported that Epo attenuated myocardial dysfunction, interstitial fibrosis and remodeling-related gene expression in non-infarcted myocardium, and enhanced angiogenesis and reduced apoptotic cell death in the peri-infarcted myocardium. Recently, Vorobiov et al. reported that Epo prevented apoptosis of mesothelial cells induced by PD fluid.

The authors previously demonstrated that angiogenesis, thickening of interstitial fibrosis, and increase of vascular endothelial growth factor (VEGF) expression appeared in rat models. Overexpression of VEGF and imbalance of angiopoietin-1 (Ang-1) and angiopoietin-2 (Ang-2) might cause thickening of the peritoneum, and these phenomena were suppressed by neutralizing VEGF antibody. As mentioned above, it appears that there is a correlation between fibrosis and tissue hypoxia caused by vasculopathy. It is hypothesized that Epo prevented peritoneal fibrosis via sufficient oxygen supply by elevation of hematocrit, a well-established microcirculatory network and suppression of CTGF production in chlorhexidine gluconate (CG)-induced PF rats. The objectives of this study are to examine whether improvement of hypoxia using Epo prevents development of PF, and to assess the mechanism involved.

Subjects and Methods

Animal models

Twenty-four male Sprague-Dawley rats at 8 weeks of age (284.0 ± 13.4 g body weight) (Charles River Breeding Laboratories, Kanagawa, Japan) were used in this study. They were housed in standard rodent cages at constant temperature and humidity with 12 hours of lighting each day. Drinking water and pelleted rodent food were provided ad libitum. All animal studies were performed according to the National Research Council Guidelines. These rats were divided into four groups: 1) chlorhexidine gluconate (CG) + epoietin alpha (Epo: Espo, a kind gift from Kyowa Hakko Kirin Co. Ltd., Tokyo, Japan) group, 2) CG group, 3) Epo group and 4) control group. The rats in the CG group+Epo group were administered intraperitoneally (i.p.) 1.5 ml/100g body weight of 0.1% CG and 15% ethanol dissolved in saline 3 times weekly and 20 IU/100g body weight of Epo dissolved in saline on the day after CG administration. The rats in the CG group + Epo group were administered intraperitoneally (i.p.) 1.5 ml/100g body weight of 0.1% CG and 15% ethanol dissolved in saline 3 times weekly and 20 IU/100g body weight of Epo dissolved in saline on the day after CG administration. The rats in the CG group were administered i.p. in the same amount of CG and vehicle dissolved in saline instead
of Epo. The rats in the Epo group were given i.p.
1.5 ml/100 g body weight of 15% ethanol dissolved
in saline and 20 IU/100 g body weight of Epo dis-
solved in saline on the day after ethanol adminis-
tration. The control rats were given ethanol and
vehicle in the same way. The effect of repeated
subcutaneous and intravenous injections of Epo
alone on a sufficient increase of hematocrit was ex-
amined by the previous report

The CG and CG+Epo groups consisted of seven
rats each and the Epo and control groups consisted
of five rats each. Animals showing any bacterial
infection in the ascites were excluded from this
study.

Histological assessment
The anterior abdominal walls were fixed in 10%
formalin and embedded in paraffin. The 4 μm
sections obtained were stained with Masson’s trichrome.
Parietal peritoneal surfaces were evaluated by mor-
phometry and immunohistochemistry. Thickening
of the submesothelial compact zone (SMC), an area
from the surface of the abdominal muscle to the
peritoneal cavity, was defined as interstitial fibrosis.
Quantification of SMC was performed by imaging
analysis. The image was analyzed using KS400 im-
ing system Release 3.0 (Carl Zeiss, Oberkocken,
Germany). The objective (×200) was positioned at
random on the sections, the microscopic image was
recorded at each of these 25 points and the average
was calculated. Fibrosis was defined as SMC of
more than 15 μm, the highest value recorded for
the control subjects.

Immunohistochemistry
Peritoneal tissues were immunohistochemically
stained for HIF-1α and EpoR. Using the same 4 μm
sections, immunohistochemical analyses were per-
formed. All sections were deparaffinized in xylene,
followed by 100% ethanol, and then placed in freshly
prepared methanol/0.3% H₂O₂ solution for 15 min-
utes to block endogenous peroxidase activity. Micro-
wave antigen retrieval was performed with hot
0.01 mol/l citrate buffer for 12 minutes. The sections
were returned to room temperature before subse-
quent procedures were performed, and then blocked
with blocking solution containing 2% bovine serum
albumin (BSA), 2% fetal calf serum (FCS) and 0.2%
fish gelatin in 0.01 mol/l PBS (pH 7.4) for 30 minutes,
followed by overnight incubation with rabbit anti-
human HIF-1α antibody diluted 1 : 200 (Santa Cruz
Biotechnology, Santa Cruz, CA, USA) and rabbit
anti-human EpoR antibody diluted 1 : 50 (Santa
Cruz Biotechnology) at 4°C. The sections for HIF-
1α and EpoR were incubated with goat anti-rabbit,
which were conjugated to peroxidase-labeled polymer
(Histofine Simple Stain MAX-PO, Nichirei Bioso-
ciences, Tokyo, JAPAN) at room temperature for 30
minutes. The bound antibodies were visualized with
3, 3’-diaminobenzine containing 0.003% H₂O₂. The
negative control was confirmed by incubation with-
out primary or secondary antibody to show no po-
sitive cells. The sections were washed with PBS
(pH 7.4) three times after each incubation, except
before addition of the primary antibodies. All sections
were counterstained with Mayer’s hematoxylin at
room temperature for 3 minutes before mounting
with glycergel mounting medium (MOUNT-
QUICK, Daido Sangyo, Saitama, JAPAN).

Double immunohistochemical staining
Double immunohistochemistry was performed
using CD34 and α-smooth muscle actin (α-SMA)
in rat peritoneal membrane sections. Paraffin-em-
bbeded sections were previously deparaffinized as
described. Sections were incubated using goat anti-
human CD34 antibody (Santa Cruz Biotechnology)
diluted 1 : 200 as a marker of vascular endothelial
cells and vascular endothelial progenitor cells, and
mouse anti-human α-SMA antibody (Dako North
America) diluted 1 : 400 as a marker of vascular
smooth muscle cells. They were incubated at 4°C
overnight with blocking solution and then reacted
with FITC-conjugated secondary antibody (Cappel,
Costa Mesa, CA, USA), which stained positive cells
green and with rhodamine-conjugated secondary
antibody (Cappel), which stained positive cells red.
Nuclei were stained with 4.6 diamidino-2-phenylin-
odole (DAPI).

Evaluation of microvessel density (MVD) and mi-
crovessel pericyte coverage index (MPI)
For each sample, the numbers of CD34 positive
microvessels were counted at 200 magnification
using a KS400 imaging system, and MVD per mm$^2$ was obtained. MVD were calculated on five fields at 200 magnification for each sample. Pericytes were defined as a single layer of $\alpha$-SMA positive cells colocalized with CD34 expressing microvessels. Immature microvessels were not covered by pericytes and might be irregular, tortuous and leaky$^{23}$. To estimate quality of microvessels in this study, MPI was evaluated on five fields at 200 magnification for each sample, and the average percentage was obtained. To compare vascular maturity among the groups, MPI$^{23}$ was evaluated as follows:

$$\text{MPI} = \frac{(\text{number of vessels wrapped with pericytes})}{(\text{total number of vessels})} \times 100$$

Detection of hypoxia

Pimonidazole (Hypoxyprobe$^{TM}$-1 kit, Chemicon Europe, Hampshire, UK) was used as hypoxia markers, which were reductively activated and bound to macromolecules at absolute oxygen concentrations below 10mmHg$^{24}$, and intravenously injected via the tail vein (6mg/100g) in each group on day 28. Rats were sacrificed at 1 hour after the injection, and the immunostained sections were visualized according to the manufacturer’s instructions.

Morphologic semiquantitative analysis

In each sample, the numbers of HIF-1$\alpha$ and EpoR, expressing cells were counted at 200 magnification. In a semiquantitative analysis of HIF-1$\alpha$ and EpoR expressing cells, the average number of cells in each of five fields randomly selected in the SMC was estimated.

Blood sampling

Blood samples were obtained from the tail vein on days 1 and 28. Hematocrit was analyzed as an indicator of anemia using a capillary tube and centrifugation at 10,000 r.p.m. for 5 minutes.

Quantitative real-time transcription-polymerase chain reaction (real-time PCR)

Gene expressions of HIF-1$\alpha$, VEGF, Ang-1, Ang-2 and CTGF mRNA were analyzed using real-time PCR. Peritoneal samples were snap-frozen immediately after resection and stored at $-80^\circ$C for RNA extraction. Total RNA was extracted by the acid guanidium thiocyanate-phenol-chloroform method$^{25}$. One microgram of total RNA was used to prepare cDNA by reverse transcription using M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and random decamers (Ambion, Austin, TX, USA). Quantitative PCR analysis was performed using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) on the ABI Prism 7500 REAL TIME PCR System (Applied Biosystems). PCR conditions were 95$^\circ$C for 10 minutes, 40 cycles at 95$^\circ$C for 15 seconds and 60$^\circ$C for 1 minute. The specific primers are listed in Table-1. The samples were analyzed by quantification software (7500 System Software, Applied Biosystems). All data are values after normalization to the 18s expression observed in the same sample.

Statistical analysis

The data are expressed as mean±SD. Analyses were performed using the statistical program Stat View version 5.0 (SAS Institute, Cary, NC, USA). Mann-Whitney U test was used to test statistical significance. A $p$ value of less than 0.05 denoted a statistically significant difference.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
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<tr>
<td>18s</td>
<td>5′-CTTTTGGTTCGCTGCTCCTC-3′</td>
<td>5′-CTGACCGGGTGGTTTTGAT-3′</td>
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<tr>
<td>HIF-1$\alpha$</td>
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<td>5′-TATCGAGGCTGTGTCGACTGAG-3′</td>
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<td>5′-AACGAAAGGCCAAAGAAATCC-3′</td>
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<tr>
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<td>5′-TGATTTCCAGAGGGAATGT-3′</td>
</tr>
<tr>
<td>Ang-2</td>
<td>5′-CGGCCACAGTCAACAACCTCA-3′</td>
<td>5′-GCTCTTATAGTCCGGCGATGA-3′</td>
</tr>
<tr>
<td>CTGF</td>
<td>5′-CGGGTTTACCATGACAATACC-3′</td>
<td>5′-GTCGACGCCAGAAAGCTCAA-3′</td>
</tr>
</tbody>
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Table-1. Primers used for PCR
Results

Morphologic examination

Morphologic changes were assessed by Masson’s trichrome staining (Figure 1). In the control group, peritoneal tissues consisted of a mesothelial monolayer and exiguous of connective tissues under the mesothelial layer (Figure 1A). To examine the reaction of Epo, Epo alone was injected into the peritoneal cavity for 28 days, but morphologic changes were not observed and the results were quite similar to those in the control group (Figure 1B). The peritoneum in the CG group showed marked thickening of the SMC and the presence of numerous infiltrating cells was observed in the CG group (Figure 1C). The thickness of the SMC and the number of infiltrating cells in the CG+Epo group were significantly less than those in the CG group (Figure 1D). The thickness of the SMC and the number of infiltrating cells in the CG+Epo group were significantly less than those in the CG group (Figure 1E). The thickness of the SMC in the CG group was greater than that in the control group and the Epo group (280.4±56.4 μm, 12.0±1.8 μm, 12.6±2.8 μm, respectively, p<0.01 vs the CG group, Figure 1E). The thickness of the SMC in the CG+Epo group (178.5±53.3 μm) was prevented compared with that in the CG group, and there was a remarkable difference in the thickness of the peritoneum between the CG group and the CG+Epo group (p<0.01, Figure 1E).

Expression of HIF-1α and EpoR

The results of immunohistochemical analysis of HIF-1α and EpoR are shown in Figures 2 and 3. HIF-1α expressed mesothelial cells were observed in the control and Epo groups (Figure 2A and B). In the CG group, many HIF-1α positive cells were observed in the thickened peritoneal tissues (Figure 2C), while HIF-1α positive cells were reduced in the CG+Epo group (Figure 2D and 3A, p<0.01).

EpoR expression was found in the vascular endothelial cells and infiltrating mononuclear cells (Figure 2G and H). The number of EpoR expressing cells was increased in the thickened peritoneum in the CG group, but significantly decreased in the CG+Epo group (Figure 3B, p<0.01). Some mesothelial cells expressed EpoR in the CG and CG+Epo groups (Figure 2E and F).

Immunohistochemistry for pimonidazole at day 28

Strong signals of pimonidazole were detected in the thickened SMC by administration of CG (Figure 4A D) on day 28. In the CG group, many pimonidazole expressing cells in the SMC were observed in the diffusely thickened peritoneum (Figure 4A and C). Compared with the CG group, extensive staining of pimonidazole was decreased in the CG+Epo group (Figure 4B and D).

Figure 1 Histological features of the anterior abdominal wall on day 28

In the control group, peritoneal tissue was composed of a mesothelial monolayer and thin connective tissue (A). Morphologic findings were quite similar to those in the Epo group (B). Peritoneal tissue in the CG group indicated marked thickening with vasculopathy and collagen deposition and cellularity (C). Peritoneal thickening and marked cell infiltration in the submesothelial compact zone (SMC) were prevented in the CG+Epo group (D).

Thickening of the SMC in the abdominal wall was statistically significant (E). CG stimulation was involved in significant peritoneal thickening compared with the control ($: p<0.01$) and Epo group ($$:p<0.01). Thickening induced by CG stimulation was markedly prevented in the CG+Epo group ($$:p<0.01). All sections, Masson’s Trichrome stain. Magnification, ×200.
Blood samples
On day 1, no difference was observed in the hematocrit among the control, Epo, CG and CG+Epo groups. Hematocrit in the Epo and CG+Epo groups was significantly increased compared with that in the control and CG groups on day 28 (Table 2, p<0.05).

Microvessel density (MVD) and microvessel pericyte coverage index (MPI)
MVD in the CG+Epo group was significantly higher than that in the CG group (176.9±43.7/mm² and 137.9±31.1/mm², respectively, p<0.01, Figure 5D). MPI in the peritoneum was determined by quantifying the percentage of microvessels that colocalized vascular endothelial cells (Figure 5A and B, in green) and pericytes by immunostaining (Figure 5B, in red). MPI in the CG+Epo group was significantly higher than that in the CG group (45.0±14.0%, 26.3±11.7%, respectively, p<0.01, Figure 5C).

Assessment of mRNA expression
Gene expressions of HIF-1α, VEGF, Ang-1, Ang-2 and CTGF in the peritoneal tissues were analyzed by real-time PCR (Figure 6A-E). The levels of HIF-1α, VEGF, Ang-2 and CTGF mRNA expression in the CG and CG+Epo groups were significantly increased compared with those in the control group (p<0.05). Increased expressions were suppressed in the CG+Epo group (p<0.05, Figure 6A, B, D, E). The expression of Ang-1 mRNA
in the CG+Epo group was significantly higher than that in the CG group (p<0.05, Figure-6 C).

### Discussion

In the present study, we demonstrated that intraperitoneal administration of Epo markedly prevented development of PF and improved hypoxia on CG induced PF rat model. Real-time PCR study revealed that Epo suppressed expressions of CTGF and HIF–1α mRNA. These findings indicated that Epo has a role in preventing progression of PF directly and indirectly.

Pathological changes in the peritoneal membrane in patients on long-term PD are characterized by a decrease or loss of mesothelial cells, thickening of the SMC due to interstitial fibrosis associated with collagen accumulation and vasculopathy\(^1\)\(^-\)\(^4\). Many factors in the peritoneal dialysate such as the content of endotoxin\(^2\)\(^7\) and lactate\(^2\)\(^7\) and high concentrations of glucose and glucose degradation products (GDPs)\(^2\)\(^8\), which are produced during the process of heat sterilization, are considered to be involved in the development of PF. Advanced glycation end-products (AGEs)\(^2\)\(^9\) are also formed in the peritoneal cavity during PD. Since ESKD patients have thickened peritoneum compared with the peritoneum in healthy controls, uremia is considered to cause PF\(^1\). Inflammatory cytokines, which are induced in the peritoneal cavity during peritonitis, may further promote chronic inflammation and fibrosis\(^3\)\(^0\). These stimuli enhance the production of fibrogenic and angiogenic mediators in the mesothelial cells, infiltrating cells and vascular cells. Although CG is not used clinically in peritoneal dialysis, we employed CG as a nonspecific inducer of experimental PF to examine pathogenesis. It can be assumed that certain common pathways exist during the development of PF in our model and CAPD patients. In fact, chronic CG exposure elicits morphologic changes, similar to those in the peritoneum of CAPD patients\(^3\)\(^1\)\(^3\)\(^2\). The PF rat model with repeated intraperitoneal injections of CG should be appropriate for our purpose of clarifying the relationship between development of interstitial fibrosis and tissue hypoxia caused by microcirculatory failure. In the present study, we demonstrated that HIF–1α and EpoR positive cells and hypoxic cells, expressing pimonidazole, increased in the SMC in the CG group. These findings indicated that repeated CG exposure resulted in not only fibrosis and non-specific inflammation but also hypoxia in the peritoneum.

HIF–1α is an oxygen-labile protein that is very rapidly stabilized under hypoxia. Upon stabilization, the HIF–1 heterodimer binds to specific DNA sequences located in hypoxia response elements associated with oxygen-regulated genes such as Epo and VEGF, which induce angiogenesis\(^6\). Expression of HIF–1α was significantly correlated with VEGF expression, indicating that HIF–1α may be an upstream regulator of VEGF expression\(^3\)\(^3\). In the present study, suppressive effect of Epo in the expressions of HIF–1α and VEGF mRNA was observed in the CG+Epo group. We considered that VEGF mRNA was decreased by suppression
of HIF–1α mRNA through improvement of hypoxia.

We have reported that angiogenesis, a thickening of interstitial fibrosis and increase of VEGF expression were observed in the PF rat model. Ang–1 mRNA levels were decreased, although the levels of VEGF and Ang–2 mRNA expression increased progressively in the late phase. According to these results, it was postulated that the sprouted vessels were destabilized and immature in the PF rat model. We suspected that the imbalance between Ang–1 and Ang–2 during progression of neoangiogenesis induced by overexpressed VEGF might involve obstruction of the immature vascular structure that was contributed to circulation insufficiency.

Transgenic overexpression of Ang–1 in mouse skin resulted in a dramatic increase in the number, size and branching pattern of blood vessels. Overexpression of VEGF induced angiogenesis and increased vascular permeability in skin of transgenic mice. Furthermore, Ang–1 may have a beneficial effect in maintaining normal endothelial cell integrity during intracoronary irradiation and mannitol therapy through the antiapoptotic effect of Ang–1 in endothelial cells. It was reported that Epo has a mitogenic effect on VSMCs, which induced Ang–1, inhibits VSMCs apoptosis and promotes the proliferation of VSMSc. Ang–2, which was up-regulated by hypoxia, induced severe vascular defects including disruption of vessel integrity.

In our study, we demonstrated that MVD in the CG+Epo group was higher than that in the CG group. MPI in the CG+Epo group was also higher than that in the CG group, and then the expression of Ang–1 mRNA was increased in the CG+Epo group compared with the CG group. The expressions of Ang–1 mRNA in the CG group were lower than those in the CG+Epo group.

Figure 6 Gene expression of hypoxia-inducible factor–1α (HIF–1α), vascular endothelial growth factor (VEGF), angiopoietin–1 (Ang–1), angiopoietin–2 (Ang–2) and connective tissue growth factor (CTGF) by real-time PCR. The levels of HIF–1α, VEGF, Ang–1, Ang–2 and CTGF mRNA in both the CG and CG+Epo groups were increased compared with those in the control group. The levels of HIF–1α, VEGF, Ang–2 and CTGF mRNA in the CG group were higher than those in the CG+Epo group. The expressions of Ang–1 mRNA in the CG group were lower than those in the CG+Epo group.

Data represents the mean±SD. §: p<0.05 vs. the control group, #: p<0.05 vs. the CG group.
mature vascular structure and elevation of hematocrit might inhibit HIF–1 mRNA expression by improvement of hypoxia. Since Epo is an effective medication for renal anemia or anemia due to chronic inflammation, Epo has been used in most PD patients to improve moderate hematocrit. In this study, an extremely high dosage of Epo compared with the clinical dosage was injected into the peritoneum and as a result, the high dosage of Epo induced a highly elevated hematocrit. Recently, asialo-Epo, a short-lived Epo metabolite that retains most of the biological functions of Epo, except for erythropoiesis, was developed. We expect that peritoneal damage caused by ischemia is improved by administration of asialo-Epo to prevent a very highly elevated level of hematocrit.

Infiltrating inflammatory cells and endothelial cells promote various cytokines and growth factors. These cells play a role in tissue injury via hypoxia. It appears that hypoxia is caused by peritonitis, biocompatible PD fluid and accumulation of AGEs, which are common conditions in long-term PD patients. In the present study, expressions of HIF–1α and CTGF mRNA increased by CG were suppressed in the CG+Epo group. Higgins et al. reported that HIF–1α was expressed in primary tubular epithelial cells under hypoxic conditions and HIF–1α was required for hypoxic induction of CTGF mRNA. These findings suggested that Epo might suppress peritoneal fibrosis by inhibition of HIF–1α mRNA. CTGF is a downstream effector molecule of profibrotic activities of TGF–β1 in the maintenance and repair of connective tissues and in the development of fibrotic disease. Zarrinkalam et al. demonstrated that CTGF was produced by peritoneal mesothelial cells in vitro and was present in the peritoneal cavity of PD patients. CTGF has also been implicated in the pathogenesis of peritoneal fibrosis in PD patients. The marked increase of CTGF levels by factors implicated in the development of PF suggests its involvement in underlying pathophysiologic mechanisms.

It appears that Epo may play a critical role in prevention of PF. This study suggested that Epo might directly or indirectly prevent acceleration of peritoneal fibrosis in a PF animal model.

Conclusions

Epo prevented peritoneal fibrosis in CG-induced PS rats via a sufficient oxygen supply by an increase in hematocrit, a well-established vascular network and suppression of CTGF production.

Acknowledgement

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References

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抄録

腹膜硬化モデルラットにおけるエリスロポエチンの抗線維化作用についての検討

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Preventive effects of erythropoietin on peritoneal hypoxia and fibrosis in rats with chlorhexidine gluconate-induced peritoneal fibrosis

腹膜硬化モデルラットにおけるエリスロポエチンの抗線維化作用についての検討

脇内: 長期腹膜透析患者では、腹膜表面を覆う中皮細胞の脱落と線維・静脈病変、間質の線維性肥厚を中心とする組織学的変性を認め、腹膜透析の除水不全の一因となっている。腎性貧血患者に投与するerythropoietin（Epo）は、造血作用以外にも血管新生作用を有することが報告されている。今回、クロールヘキシジングルコネート（CG）を用いた腹膜硬化モデルラットにEpoを併用することで、組織侵襲期におけるEpoの血管新生の組織への影響について検討を行った。

方法: 24匹の8週齢のSprague-Dawleyラットを4群に分け、①腹腔内にCGとEpoの投与を行ったCG+Epo群、②CG投与のみを行ったCG群、③Epo投与のみを行ったEpo群と④対照群を作成した。投与開始28日後に屠殺し、採取した壁側腹膜を用い、hypoxia-inducible factor-1α（HIF-1α）、erythropoietin receptor（EpoR）、pimonidazoleの発現を免疫組織学的に検索した。HIF-1α、vascular endothelial growth factor（VEGF）、angiopoietin-1（Ang-1）、angiopoietin-2（Ang-2）、connective tissue growth factor（CTGF）のmRNA発現をReal-time PCRで評価した。またCD34とα-smooth muscle actin（α-SMA）の蛍光二重染色を行い、血管密度・成熟度を計測した。

結果: CG群において、腹膜の線維性肥厚が認められ、Epoの併用で抑制された。CG+Epo群のヘマトクリットはCG群に比べ著名に上昇した。CG群の肥厚した腹膜内にHIF-1α、EpoR、pimonidazole陽性細胞が多数存在し、Epoの併用により減少した。CG群では、CG+Epo群に比べHIF-1α・VEGF・Ang-2・CTGFmRNA発現の亢進を認め、CG+Epo群と比べると、これらのmRNA発現の有意な抑制を認めた。CG群のAng-1mRNAの発現は、CG+Epo群に比べると有意に抑制されていた。CG+Epo群の血管密度と血管成熟度は、CGのそれらに比べ有意な上昇を示した。

結語: CG群の投与を行った腹膜硬化モデルラットにEpoを併用することで腹膜の線維性肥厚は、抑制された。その理由として、Epoの造血作用と成熟血管網の構築を高めることで、虚血組織への酸素供給の上昇が期待できる。また、Epo投与は低酸素状態を改善し、HIF-1α発現抑制を介したCTGF発現抑制が期待される。