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From Cell Biology to Tissue Engineering

High glucose concentration-induced expression of pentraxin-3 in a rat model of continuous peritoneal dialysis

Nana Ishimatsu[1,2], Tetsu Miyamoto[1], Hiromichi Ueno[1], Emi Hasegawa[1], Akihiro Kuma[1], Yoko Fujimoto[1], Kenichiro Bando[1], Junichi Nakamata[1], Yumi Furuno[1], Ryota Serino[3], Ryoko Baba[2], Hiroyuki Morimoto[2], Yoshiaki Doi[2], Masahito Tamura[3] and Yutaka Otsuji[1]

[1]The Second Department of Internal Medicine, [2]Department of Anatomy and [3]Kidney Center, School of Medicine, University of Occupational and Environmental Health, Iseigaoka, Yahatanishi, Kitakyushu, Japan

Summary. Background: Continuous exposure to peritoneal dialysis fluids (PDFs) is associated with pathological responses such as persistent micro-inflammation, which leads to ultrafiltration failure. Pentraxin-3 (PTX3), a multifunctional soluble pattern recognition receptor, is produced at sites of inflammation by a wide range of cell types. This study investigates the *in vivo* expression of PTX3 in the peritoneal membrane of a rat continuous peritoneal dialysis (PD) model, as well as the effect of high glucose on the *in vitro* expression of PTX3.

Methods: The expression of PTX3 was analyzed using RT-PCR, real-time PCR, immunohistochemistry and western blotting in a PD rat model receiving saline or conventional PDF containing 3.86% glucose for 8 weeks. The effects of high glucose on the expression of PTX3 were examined in cultured rat peritoneal mesothelial cells (RPMCs), mouse macrophage-like cells, and mouse fibroblasts.

Results: In a rat model of PD, eight-week instillation of the conventional PDF produced increased submesothelial thickening, followed by substantially enhanced PTX3 protein levels in the submesothelial layer of peritoneal membrane. PTX3 was detected in peritoneal mesothelial cells, macrophages and fibroblasts in the thickened submesothelial area. Glucose was found to induce PTX3 protein expression in RPMCs as well as macrophage-like cells and fibroblasts.

Conclusion: Continuous exposure to conventional PDF induces PTX3 expression in the peritoneal membrane of rats. High glucose may be involved in the mechanism of PDF-induced local micro-inflammation in the peritoneum.

Key words: Pentraxin-3, Peritoneal dialysis, Peritoneal fibrosis, Micro-inflammation, Peritoneal mesothelium

Introduction

Continuous ambulatory peritoneal dialysis (PD) is a well-accepted treatment for end-stage renal disease in pediatric patients. However, long-term PD is known to result in functional and structural alterations in the peritoneal membrane. Continuous exposure to conventional peritoneal dialysis fluid (PDF) drives pathological responses, including peritoneal fibrosis and angiogenesis, resulting in a high peritoneal solute transport rate (Moinuddin et al., 2014). Both clinical and experimental evidence support the view that persistent intraperitoneal local micro-inflammation exacerbates the histological deterioration, worsening the treatment outcome of PD (Williams et al., 2002, 2003; Lambie et al., 2013; Davies, 2014). In addition to low pH and lactate buffer, the high glucose concentration of PDF is a non-physiological factor that results in the development of hyperosmolarity, toxic glucose degradation products (GDPs), and advanced glycation end products (AGEs)

Offprint requests to: Tetsu Miyamoto, The Second Department of Internal Medicine, School of Medicine, University of Occupational and Environmental Health, 1-1, Iseigaoka, Yahatanishi, Kitakyushu, 807-8555, Japan. e-mail: tetsum@med.uoeh-u.ac.jp

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(Velloso et al., 2014).

Pentraxin-3 (PTX3), first discovered by Breviario in 1992 (Breviario et al., 1992), is a multifunctional, soluble, pattern recognition receptor modulating immunoinflammatory responses and belongs to the same pentraxin superfamily as C-reactive protein (Mantovani et al., 2003). PTX3 is produced at extra-hepatic sites of inflammation by various cell types, including macrophages and fibroblasts, in response to inflammatory mediators such as lipopolysaccharide (LPS), interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) (Alles et al., 1994, Introna et al., 1996). Studies have indicated that PTX3 levels reflect disease activities in several inflammatory conditions, such as cardiovascular and infectious diseases, suggesting that the PTX3 level has clinical implications as a marker of the local inflammatory response (Moalli et al., 2011; Lech et al., 2013). Kanda et al. elucidated the expression of PTX3 in peritoneal tissue and effluent obtained from PD patients, suggesting that PTX3 may be a candidate of biomarker of peritoneal inflammation of PD patients (Kanda et al., 2013).

In this context, we investigated PTX3 expression in peritoneal tissue using a rat PD model, and examined PTX3 expression in cultured mesothelial cells, macrophage-like cells, and fibroblasts with a specific focus on the effect of high glucose concentrations.

Materials and methods

Peritoneal dialysis model animals

Eight-week-old Wistar rats (Charles River Japan, Yokohama, Japan) weighing 190-220 g were housed under conventional laboratory conditions of constant temperature at 22°C and a 12-h light/dark cycle. The research protocol was approved by the ethics committee of the University of Occupational and Environmental Health, and conforms to the provision of the Declaration of Helsinki, 1995 (as revised in Edinburgh, 2000). Rats were divided into 3 groups: the control group, in which rats were not instilled (n=6); the saline group, in which rats were instilled intraperitoneally with 20 mL of saline solution twice a day for 8 weeks (n=5); and the PDF group, in which rats were instilled intraperitoneally with 20 mL of lactate-buffered PDF containing 3.86% glucose (pH 5.5) (Dianeal® PD-4 4.25; Baxter Healthcare, Tokyo, Japan) twice a day for 8 weeks (n=6). The animals were anesthetized with pentobarbital, and the parietal peritoneal tissues including the muscle layer were subsequently dissected intact.

Cell culture

Rat peritoneal mesothelial cells (RPMCs) were obtained using a standard trypsin/ethylenediaminetetraacetic acid (EDTA) digestion method from nine-week-old adult Wistar rats (Hjelle et al., 1989; Tamura et al., 2003). Cells were identified as RPMCs by the cobblestone appearance and positive staining for HBME-1 and cytokeratin. Mouse macrophage-like cells (RAW264.7) and mouse fibroblasts (NIH-3T3) were purchased from Riken RBC (Ibaraki, Japan). RPMCs, RAW264.7 and NIH-3T3 were maintained in low glucose (5.6 mmol/L) Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin at 5% CO_2 and 37°C. To investigate PTX3 expression, glucose was added into the culture medium of RPMCs, RAW264.7 and NIH-3T3 at various concentrations (0, 0.1, 1.0, 2.0, 4.0 and 5.0%) for 4 hours.

Histological analysis

For light microscopic studies, parietal peritoneal tissues were fixed with 4.0% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) overnight at 4°C, dehydrated in a graded ethanol series followed by xylene, and then embedded in paraffin wax (Merck, Darmstadt, Germany). Embedded samples were cut into 5-μm-thick slices using a microtome and then stained with hematoxylin and eosin (HE) or Masson's trichrome. The fibrous areas of each peritoneal tissue were measured using ImageJ software (National Institutes of Health, Bethesda, MD) (Schneider et al., 2012).

RNA extraction and reverse transcription (RT)-PCR analysis

The mRNA expression levels of PTX3 in PD-treated rat peritoneal tissues and glucose-treated cultured cells were evaluated using RT-PCR. Total RNA was isolated from 30 mg of rat peritoneal tissue using an RNeasy Fibrous Mini Kit (Qiagen, Hilden, Germany). One microgram of total RNA was used for first strand cDNA synthesis using Super Script III reverse transcription enzyme (Thermo Fisher Scientific Inc., Waltham, MA USA) and an Oligo (dT) primer (Thermo Fisher Scientific Inc.) according to the manufacturer's protocol. One microliter of cDNA was used as the template in each PCR. PCR reactions were performed with the Go Taq Green PCR mixture (Promega, Madison, WI, USA) using the following primers:

PTX3 forward: 5'ACTGGTGGCTACCACTCTGG 3', PTX3 reverse: 5'TCTCTCAAGCCTTCCCTTCA 3', GAPDH forward: 5'GAAGGGCTCATGACCACAGT 3', GAPDH reverse: 5' GGATGCAGGGATGATGTTCT 3'.

Amplification products were separated on 1.5% agarose gels, stained with ethidium bromide, and visualized with an ultraviolet transilluminator.

Real Time PCR

Quantitative real time PCR analysis was performed using SYBR Premix Ex Taq Perfect Real Time (Takara Bio, Kyoto, Japan). The sequences of the primers used

were as follows: PTX3 forward, 5'CTGGTCTGCAG TGTTGGCTGA 3', PTX3 reverse, 5'ATGATGAA CAGCTTGTCCCACTC 3', GAPDH forward, 5'TGTG TCCGTCGTGGATCT 3', GAPDH reverse, 5'TGCTGT TGAAGTCGCAGGAG 3'.

DNA amplification and detection were performed using the ABI Step One Plus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). PCR amplification (40 cycles) was performed as follows: 95°C for 3 seconds and 60°C for 30 seconds. Standard curves were generated using 10-fold serial dilutions of rat c-DNA. PTX3 expression levels were normalized to GAPDH expression.

Western blotting

Protein expression of PTX3 in PD rats and cultured cell lines was analyzed by western blotting using mouse monoclonal anti-PTX3 antibody (SAB1404282, Sigma Aldrich, St. Louis, MO, USA). Anti-β-actin antibody (A5316, Sigma Aldrich) was used as a loading-control. Peritoneal tissue and cultured cells were lysed in Radio-Immunoprecipitation Assay buffer and homogenized to extract the protein. Protein lysates were clarified by centrifugation at 12,000×g for 30 minutes at 4°C and then added to 5× Laemmli sample buffer. Samples were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and then analyzed by western blotting with anti-PTX3 antibody (1:1000) using the enhanced chemiluminescence system (Amersham ECL; GE Healthcare, Buckinghamshire, UK).

Immunohistochemistry

For detection of PTX3, deparaffinized sections were immersed in 20 mM citric acid (pH 6.0) and microwaved for 20 min at 800 W (MicroMed T/T; Milestone, Sorisole, Italy). Antigen-retrieved sections were blocked with 0.1% hydrogen peroxide in methanol for 20 min to remove endogenous peroxidase and rinsed with PBS. They were then blocked with 1.0% bovine serum albumin (BSA)-PBS for 1 h at room temperature. The sections were incubated overnight at 4°C with mouse monoclonal anti-PTX3 antibody (SAB1404282, Sigma Aldrich, St. Louis, MO, USA) diluted 1:200 in 1.0% BSA-PBS. After three washes with PBS, the sections were reacted using horseradish peroxidase labeled anti-mouse IgG (Histofine Simple Stain Rat MAX-Multi-PO; Nichirei, Tokyo, Japan). The peroxidase complexes were visualized by treatment with a freshly prepared solution of 0.1 mg/ml diaminobenzidine tetrahydrochloride (DAB) in 50 mM Tris-HCl (pH 7.6) containing 0.05% hydrogen peroxide for 7 min. All sections were washed three times with PBS, stained with hematoxylin, and observed with a light microscope.

For identification of the PTX3 expressing cell, we attempted double staining immunofluorescence. Antigen-retrieved sections were blocked with 1.0% BSA-PBS for 1 h at room temperature. Beside anti-PTX3, rabbit polyclonal anti-rat C-ERC/Mesothelin (28001, IBL, Gunma, Japan) as a marker of mesothelial cell, rabbit monoclonal anti-F4/80 (ab111101, abcam, Cambridge, UK) as a marker of macrophage or rabbit polyclonal anti-S100A4 (A5114, DakoCytomation, Glostrup, Denmark) as a marker of fibroblast was used for cell type-specific antibody. The sections were incubated with the primary antibodies diluted 1:200 each in 1.0% BSA-PBS for overnight at 4°C. After being washed three times with PBS, all the sections were incubated with secondary antibodies diluted 1:500 each in 1.0% BSA-PBS for 1 hour at room temperature. As secondary antibody, Alexa Fluor 488-labeled goat anti-mouse IgG and Alexa Fluor 546-labeled goat anti-rabbit IgG (Molecular Probes, Eugene, OR, USA) were used. All sections were washed three times with PBS, mounted with a Vectashield Hard Set Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA, USA) and observed with a fluorescence microscope (Axioskop 2 plus, Carl Zeiss Inc., Göttingen, Germany). Specificity of immunoreactivity was confirmed by replacing the primary antibodies with 1.0% BSA in PBS.

Statistical analysis

Data were expressed as the mean ± SD. Differences among groups were assessed using a one-way analysis of variance, followed by a Wilcoxon-Mann-Whitney U test[1] for between group assessments (JMP11; SAS Institute, NC, USA). Differences were considered statistically significant at a p value of less than 0.05.

Results

Conventional PDF induces submesothelial thickening in rat peritoneal tissue

HE staining revealed significantly increased submesothelial thickness (arrow) without acute peritonitis in the PDF-treated group compared to the control and saline groups (Fig. 1A a-c). The submesothelial connective tissue showed progressive fibrosis. Masson's trichrome stain revealed that the thickening submesothelial tissue (arrow) was associated with connective tissue deposit (Fig. 1A d-f). We measured the areas per 350 μm of fibrosis in each group and compared the thickening of submesothelial compact zones (Fig. 1B). The submesothelial fibrous area in PDF-treated rats (6348.9±668.6 μm^2) was significantly greater than that in control (2614.3±246.6 μm^2) and saline-treated rats (3409.2±398.9 μm^2; $P<0.05$). The saline-treated rats showed mild fibrosis of the submesothelial connective tissue. However, the submesothelial fibrous area in saline-treated rats was not significantly different from that in the control rats ($P>0.10$).

The expression of PTX3 mRNA in rat peritoneal tissue

To examine the expression of PTX3 mRNA in rat

[1]In the original version the statistical method (student's t test) was wrong. Correction done in December 29, 2016

peritoneal tissue, total RNA was extracted from the peritoneum of each group of rats. In the RT-PCR analysis, PTX3 mRNA expression was detected in 5 out of 6 rats in the PDF group; 1 out of 5 in the saline group; and no rats in the control group (Fig. 2A). RT (-) samples (data not shown) and GAPDH (Fig. 2A) primers were used as negative- and positive-controls for the reactions. Real-time PCR analysis showed that PTX3 expression was significantly higher in PDF-treated rats (108±51 fold) compared to saline-treated and control rats (P<0.001) (Fig. 2B).

PTX3 protein expression in rat peritoneal tissue

Western blotting analysis showed that PTX3 protein was expressed in all samples of the PDF group (Fig. 3A). Although PTX3 was detected in the saline group, the expression levels (PTX3/β-actin ratio; 0.256±0.072) were lower compared with the PDF group (0.702±0.147; P<0.05) (Fig. 3B). No expression was observed in any of the control group samples.

Immunohistochemical analysis of PTX3 expression in rat peritoneal tissue

PTX3 protein was detected in the mesothelial cells and the cells of the submesothelial connective tissue in the PDF-treated rats (arrows) (Fig. 4A c). The signal intensity was higher than in the control and saline-treated groups (Fig. 5A a, b).

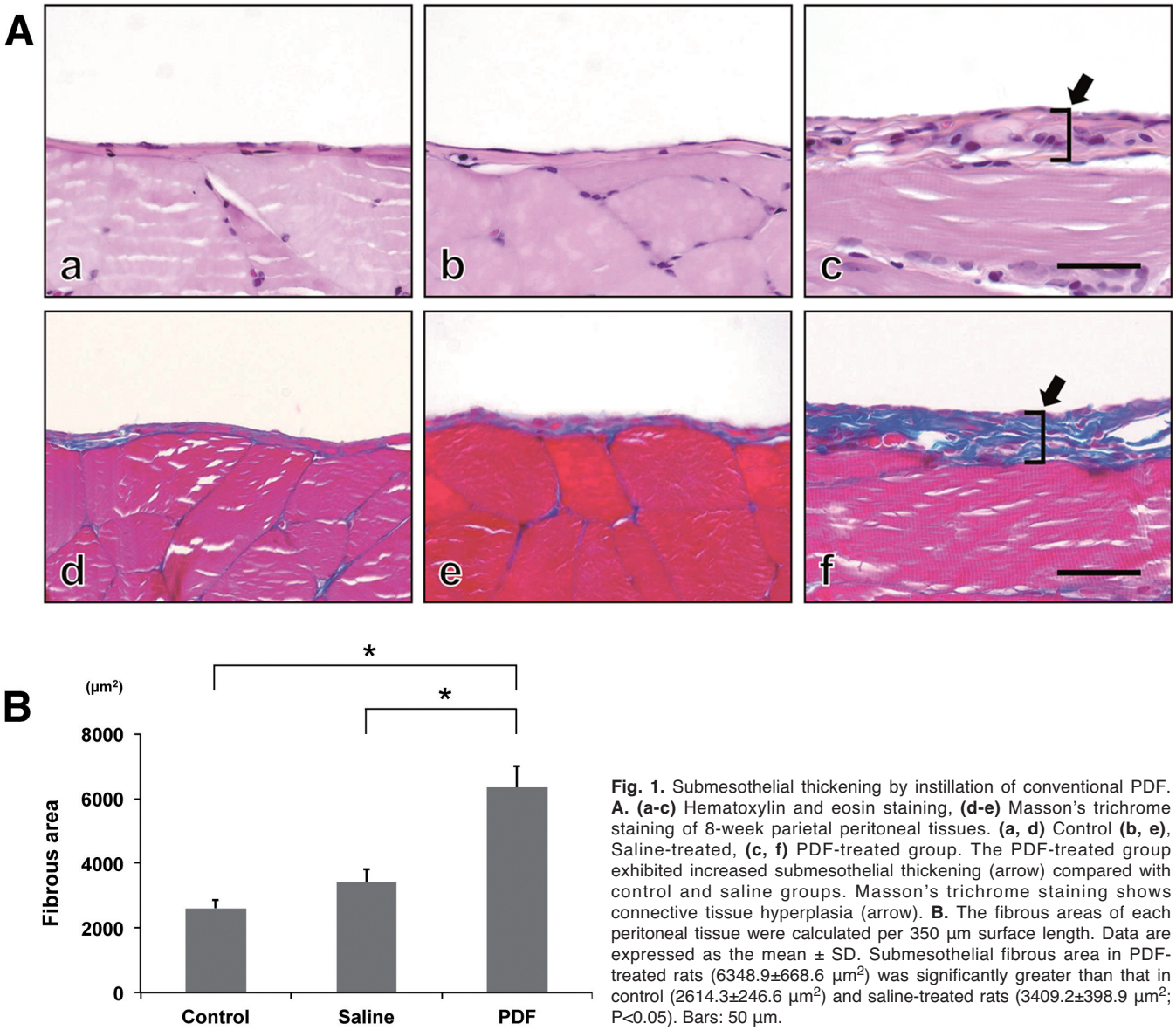

Fig. 1. Submesothelial thickening by instillation of conventional PDF. **A. (a-c)** Hematoxylin and eosin staining, **(d-e)** Masson's trichrome staining of 8-week parietal peritoneal tissues. **(a, d)** Control **(b, e)**, Saline-treated, **(c, f)** PDF-treated group. The PDF-treated group exhibited increased submesothelial thickening (arrow) compared with control and saline groups. Masson's trichrome staining shows connective tissue hyperplasia (arrow). **B.** The fibrous areas of each peritoneal tissue were calculated per 350 μm surface length. Data are expressed as the mean ± SD. Submesothelial fibrous area in PDF-treated rats (6348.9±668.6 μm^2) was significantly greater than that in control (2614.3±246.6 μm^2) and saline-treated rats (3409.2±398.9 μm^2; P<0.05). Bars: 50 μm.

In the double staining fluorescence analysis, Alexa 488 labeled PTX3 expressed in Alexa 546 labeled mesothelial cells (Fig. 4B a), macrophages (Fig. 4B b) or fibroblasts (Fig. 4B c) in the peritoneum of the PDF-treated rats.

Glucose-induced expression of PTX3 protein in cultured cells

To further investigate the regulation of PTX3 expression at the cellular level, we treated RPMCs, RAW264.7 and NIH 3T3 with various concentrations of glucose for 4 hours. Glucose enhanced PTX3 protein expression in RPMCs in a concentration-dependent manner. Glucose also induced PTX3 expression in both RAW264.7 and NIH-3T3 (Fig. 5), suggesting that mesothelial cells, macrophages, and fibroblasts could produce PTX3 with glucose treatment of peritoneal tissue.

Discussion

The present study revealed the presence of PTX3 in the peritoneal membrane of PD model rats. PTX3 was detected in peritoneal mesothelial cells, macrophages and fibroblasts in the thickened submesothelial area. Additionally, *in vitro* studies demonstrated that PTX3 expression was induced by high glucose in RPMCs, fibroblasts, and macrophage-like cells.

During PD, the peritoneal surface is continuously exposed to the non-physiological conditions of PDF, which cause the deterioration of peritoneal tissue, including mesothelial cell layer loss, angiogenesis, and submesothelial fibrosis. The fibrotic process in the peritoneum during PD is exacerbated following PD-related acute and chronic release of inflammatory mediators from peritoneal cells (Lai et al., 2007). Detection of the local inflammatory response in the peritoneal membrane during the early stages of disease progression is critical in preserving peritoneal function, since a determinant of the peritoneal solute transport rate is intraperitoneal inflammation, which is largely

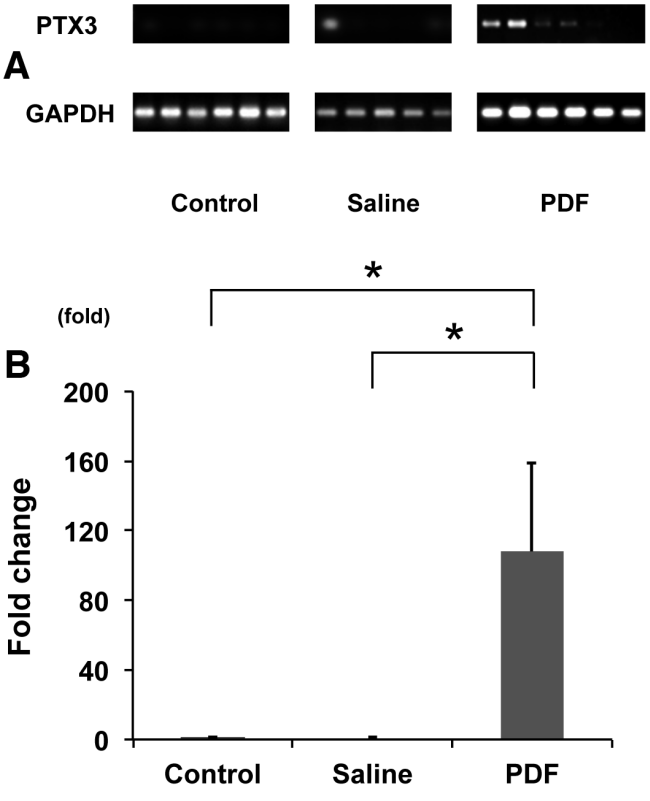

Fig. 2. PTX3 mRNA induction in peritoneal tissues by 8-week PDF treatment. **A.** RT-PCR was used to analyze PTX3 expression in mRNA samples extracted from peritoneal tissues of control (n=6), saline-treated (n=5), and PDF-treated (n=6) groups. PTX3 expression was detected in 5 out of 6 rats in the PDF group and 1 out of 5 in the saline group. PTX3 mRNA expression was not observed in the control groups. **B.** Quantitative analysis of PTX3 mRNA expression in rat peritoneal tissue by real-time PCR. Data are expressed as fold increase relative to the mean of the control group. The results shown are representative of two independent experiments. The expression of PTX3 was higher in PDF-treated rats (108±501 fold) than in the control (1.0±0.8 fold) and saline-treated (0.9±0.3 fold) rats (P<0.001).

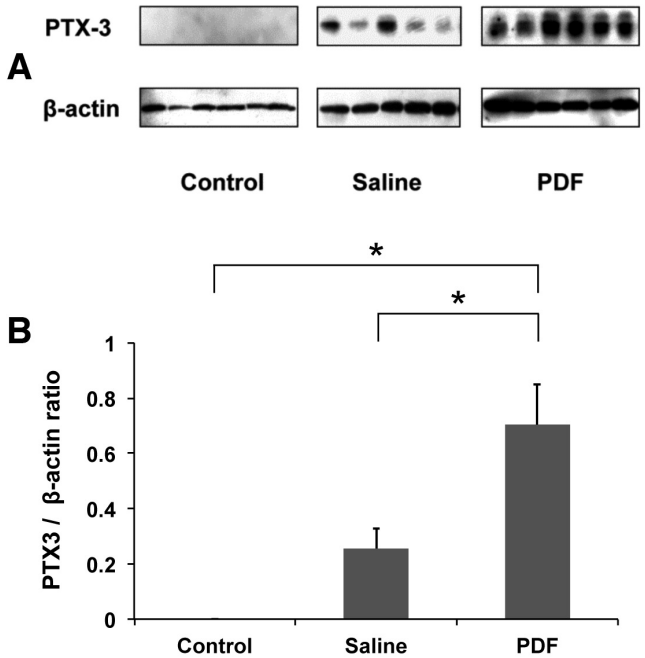

Fig. 3. PTX3 protein up-regulation in peritoneal tissue by 8-week PDF treatment. **A.** PTX3 protein expression was analyzed in samples extracted from the peritoneal tissues of the control (n=6), saline-treated (n=5), and PDF-treated (n=6) groups by western blotting. PTX3 protein was expressed in all samples from the PDF and saline groups. PTX3 protein was not detected in the control group. **B.** The PTX3/β actin ratio of the saline group (0.256±0.072) was significantly lower compared with the PDF group (0.702±0.147; P<0.05).

independent of systemic inflammation (Davies, 2014).

Unlike short pentraxins (C-reactive protein and amyloid P) which are mainly produced by the liver, PTX3 is a long pentraxin that is produced at inflammatory sites by a wide range of cell types, such as macrophages, vascular endothelial cells, and fibroblasts, in response to toll-like receptor agonists, LPS, and proinflammatory signals like IL-1β and TNF-α (Mantovani et al., 2003; Doni et al., 2006; Imamura et al., 2007; Deban et al., 2011). PTX3 has been reported to be a potent biomarker of cardiovascular disease (Norata et al., 2010), infectious diseases (Liu et al., 2014), chronic kidney disease and others (Tong et al., 2007; Suliman et al., 2008; Miyamoto et al., 2011; Moalli et al., 2011; Lech et al., 2013). PTX3 is also reported to be related to matrix deposition, angiogenesis and tissue repair (Lech et al, 2013), events that also occur in the disease process of peritoneal fibrosis. The results of the present study suggest the involvement of PTX3 in the pathological tissue changes induced by bioincompatible PDFs and support the results of the clinical study (Kanda et al., 2013) which examined the potential of PTX3 as a new biomarker of peritoneal inflammation and fibrosis.

In addition to repeated episodes of infection, the

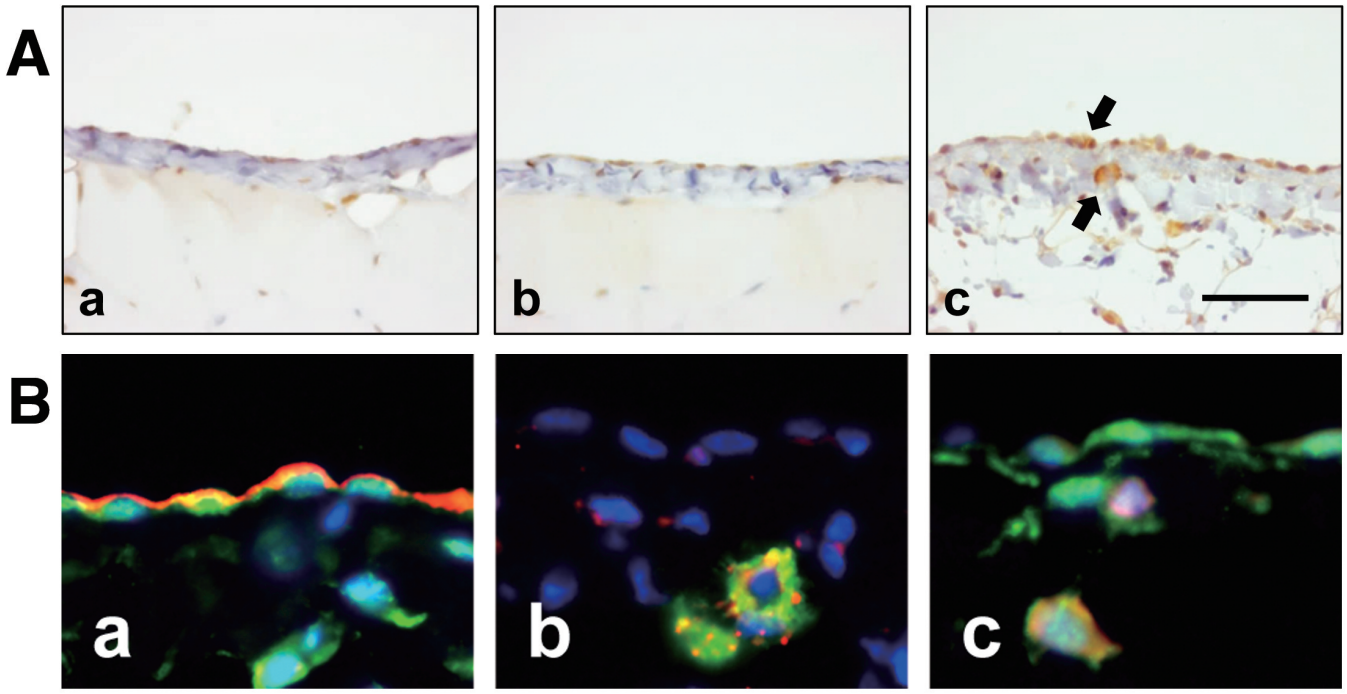

Fig. 4. Immunohistochemical analysis of PTX3 protein expression in the mesothelium. **A. (a)** Control **(b)**, Saline-treated, **(c)** PDF-treated group. PTX3, visualized as dark brown coloration, was detected in the mesothelial cells and the cells of the submesothelial tissue of PDF-treated rats (arrows). **B.** PTX3 was visualized with Alexa 488 (green). The peritoneal mesothelial cells, macrophages or fibroblasts were visualized with Alexa 546 (red). Nuclei were stained with DAPI (blue). Bars: 50 μm.

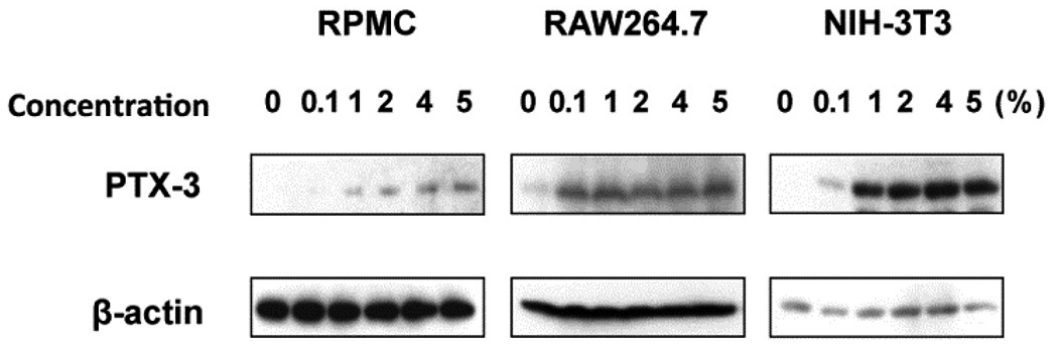

Fig. 5. PTX3 protein induction by glucose treatment in cultured cell lines. Various concentrations of glucose were added to the culture medium of RPMCs, RAW264.7 and NIH-3T3. After 4-hour incubation, PTX3 protein expression in RPMCs, RAW264.7, and NIH-3T3 was analyzed by western blotting. Glucose induced PTX3 protein expression in RPMCs, RAW264.7 and NIH-3T3.

non-physiological content of PDF could be a stimulus for the progression of tissue fibrosis. We previously showed that the high glucose concentration in PDF suppresses integrin-mediated adhesion of mesothelial cells, followed by impaired wound healing of the mesothelial cell layer (Tamura et al., 2003; Miyamoto et al., 2010; Matsumoto et al., 2012). High glucose levels also induce the production of various proinflammatory cytokines such as TNF-α and IL-6 in peritoneal mesothelial cells (Wang et al., 1995; Ogata et al., 2001; Lambie et al., 2013). A previous study showed that a classical nuclear factor-κB (NF-κB) complex can functionally interact with the "long pentraxin" hPTX3 promoter in human fibroblasts after exposure to TNF-α (Basile et al., 1997). AGEs, metabolites of GDPs in PDF, interact with the receptor for advanced glycation endproducts (RAGE), thereby up-regulating NF-κB (Goldin et al., 2006, Kihm et al., 2008). The up-regulation of NF-κB induced by proinflammatory cytokines and GDPs in PDF is proposed as a mechanism of glucose-induced expression of PTX3. Our data demonstrated that high glucose induces PTX3 expression in mesothelial cells, macrophages, and fibroblasts. PTX3 gene expression might be promoted by proinflammatory mediators such as TNF-α and NF-κB. PTX3 mRNA and protein were weakly expressed in peritoneal tissue of saline-treated rats in this study. Nonphysiological factors of the saline administration, including toxicity of normal saline for peritoneal tissue (Breborowicz and Oreopoulos, 2005) and fluid flow stress (Aoki et al., 2011) may explain the expression of PTX3 in the saline-treated rats.

Several limitations of this study should be recognized. First, our PD model does not produce conditions of uremia. Uremia is reported to be related to a systemic inflammatory state, which could influence the peritoneal expression of PTX3. Second, the effects of factors other than glucose, such as lactic acid and low pH, were not investigated. Third, the pathway regulating PTX3 induction by glucose was not examined.

In conclusion, we confirmed conventional PDF-induced expression of PTX3 in a rat PD model. A high glucose concentration is one of the non-physiological factors promoting the production of PTX3 in peritoneal tissue. This study provides a possible strategy for the detection of micro-inflammation in the peritoneum of PD patients by focusing on long pentraxin.

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Conflicts of Interest. The authors of this article have no conflicts of interest to declare. The results presented in this paper have not been published previously in whole or part, except in abstract format.

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