Peroxisome proliferator-activated receptor γ ligand prevents the development of chronic pancreatitis through modulating NF-κB-dependent proinflammatory cytokine production and pancreatic stellate cell activation

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Abstract

Purpose: Thiazolidinedione derivatives (TZDs) are known to be ligands of peroxisome proliferator-activated receptor γ (PPARγ). In this study, we investigated the effect of a TZD, troglitazone, on inflammation and fibrogenesis in the pancreas of an experimental model of chronic pancreatitis.

Material and methods: Male WBN/Kob rats with spontaneous chronic pancreatitis were fed rat chow containing 0.2% troglitazone from 1 to 4 months of age. Immunohistochemical studies of rat pancreas were carried out with monoclonal mouse antibody against human alpha-smooth muscle actin (α-SMA) or rabbit polyclonal antibody against collagen type I, collagen type III, or fibronectin. Cytokine production was measured by enzyme-linked immunosorbent assay. The inhibitory action of troglitazone on nuclear factor-κB (NF-κB) binding activity in activated macrophages was also investigated.

Results: Long-term administration of troglitazone reduced inflammatory cell infiltration and fibrosis in the pancreas of WBN/Kob rats, and expression of α-SMA, procollagen I, III, and fibronectin was significantly reduced by troglitazone. The increase in TNF-α production by activated macrophages was significantly decreased by troglitazone. Peritoneal macrophages isolated from WBN/Kob rats produced a large amount of TNF-α, whereas those from troglitazone-treated WBN/Kob rats produced only a marginal amount of TNF-α. Lipopolysaccharide-induced NF-κB binding activity in peritoneal macrophages was also significantly reduced by troglitazone.

Conclusions: Troglitazone prevented the progression of chronic pancreatitis via inhibition of ECM synthesis and proinflammatory cytokine production mediated by the inhibition of NF-κB activity.

Key words: thiazolidinedione derivatives, chronic pancreatitis, PPARγ, TNF-α, NF-κB.

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Introduction

Chronic pancreatitis is an irreversible, progressive disease characterized by pancreatic exocrine and endocrine dysfunction and by morphological damage, including inflammatory responses, subsequent fibrosis, and destruction of acinar and duct cells. Thiazolidinedione derivatives (TZDs) are known antidiabetic agents and ligands for peroxisome proliferator-activated receptor γ (PPARγ), a member of the nuclear receptor superfamily of transcription factors [1-5]. We previously reported that a TZD, troglitazone, improves exocrine pancreatic function in rats with streptozotocin-induced diabetes mellitus and inhibits the progression of pancreatic damages and pancreatic exocrine insufficiency in an animal model of chronic pancreatitis through morphological improvement of inflammation and fibrogenesis [6,7]. Pancreatic stellate cells (PSCs) have been characterized as the major source of extracellular matrix and cytokine production and as playing a pivotal role in the fibrogenesis in chronic pancreatitis [8]. We have reported finding that troglitazone prevents the progression of chronic pancreatitis by interfering with the fibrogenic action of PSCs in an *in vitro* study [9], and PPARγ ligands have been reported to inhibit inflammatory cytokine production [10,11]. Su et al. found that PPARγ ligands reduce colonic inflammation in a mouse model of inflammatory bowel disease by down-regulating proinflammatory cytokine production at the transcriptional level through inhibition of activation of a transcription factor, nuclear factor-κB (NF-κB) [12]. In the present study, we found that long-term

administration of a PPARγ ligand prevents the progression of chronic pancreatitis by modulating the pancreatic inflammatory and fibrogenic response through inhibition of NF-κB-dependent proinflammatory-cytokine production.

Material and methods

Animals and experimental protocol

One-month-old male WBN/Kob rats were fed 0.2% troglitazone (supplied by Sankyo Company Ltd, Tokyo, Japan) containing rat chow for 3 months, and male WBN/Kob rats and male Wistar rats were fed the same rat chow without troglitazone, as controls. For the morphological studies, pancreata were fixed overnight in freshly prepared 4% paraformaldehyde in PBS (pH 7.2) before embedding them in paraffin by the standard method.

Morphological examination of the pancreas

After deparaffinizing the sections and rehydrating them through a xylene and ethanol series, they were stained with hematoxylin-eosin or Masson's trichrome stain and evaluated histopathologically. To measure the area of the pancreas that was fibrotic, the whole pancreas was divided into a splenic half and a duodenal half, and each half was sliced at 3 mm intervals to provide cross-sections of tissue. The area of the fibrotic tissue identified by staining with Masson's trichrome was calculated in all specimens with image analysis software (SPICA II, Olympus Optical Co., Tokyo, Japan). The proportion (%) of the pancreas that was fibrtoric was calculated thus: (fibrotic area / total area of the specimen x 100).

Immunohistochemical study

Serial sections were used for the immunohistochemical study. The protocol basically consisted using the avidin-biotin complex method and reagents provided by Vector Laboratories Inc (Burlingame, CA). Briefly, sections were incubated overnight at 4°C with monoclonal mouse antibody directed against human alpha-smooth muscle actin (α-SMA), rabbit polyclonal antibody against collagen type I, collagen type III, or fibronectin (DakoCytomation, Kyoto, Japan), and then incubated with a biotinylated secondary antibody. Peroxidase staining was performed with 3,3'-diaminobenzidine (DAB). Immunoglobulin from non-immunized animals was substituted for the primary antibody to provide a negative control.

Protein extraction and western blot analysis

The pancreas was homogenized in lysis buffer containing 0.15M NaCl, 50 mM Tris-HCl (pH 7.2), 1% deoxycholic acid, 1% Trion X-100, 0.1% SDS, and 1 mM PMSF, plus 5 μg/ml of each of the following proteinase inhibitors: pepstatin, leupeptin, chymostatin, antipain, and aprotinin. After centrifuging the homogenates at 15,000 rpm for 10 min at 4°C, the protein concentrations were determined. A 25 μg sample of protein was separated by SDS-PAGE and transferred electrophoretically to the membrane, and nonspecific binding was blocked by 1-hour incubation of the membrane in 3% low-fat milk in PBS (pH 7.6). The blots were then incubated with monoclonal mouse antibody directed against α-SMA. Following incubation with peroxidase-conjugated secondary antibodies, signals were developed by a chemiluminescence system.

Isolation of peritoneal macrophages

The abdomen was opened and lavaged with 5 ml of cooled PBS. The peritoneal lavage fluid was then collected and centrifuged at 1500 rpm for 15 min. The cell pellet was washed once in PBS and resuspended at a concentration of 2×10^6 cells/ml in RPMI 1640 medium supplemented with 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were plated in chamber slides and cultured at 37°C under a 5% CO_2 atmosphere for 2 hours. Nonadherent cells and the medium were then removed, and the adherent cells were washed three times with PBS and used as peritoneal macrophages.

Measurement of the concentration of TNF-α released by peritoneal macrophages

To measure the TNF-α released by peritoneal macrophages, cells isolated from male WBN/Kob rats were resuspended at a concentration of 1×10^5 cells/200 ml/well in 10% FBS-RPMI 1640 medium containing 100 U/ml penicillin and 100 mg/ml streptomycin and cultured at 37°C under 95% O_2 and 5% CO_2 for 3 days. The culture supernatant was used to determine the TNF-α concentration. Whether troglitazone modulates TNF-α secretion by activated macrophages was also investigated. Freshly isolated peritoneal macrophages (1×10^6 cells/ml) from male Wistar rats were grown for 18 hours in standard medium, and then for 2 hours in fresh medium containing troglitazone, after which the cells were cultured in the presence of lipopolysaccharide (LPS) (10 ng/ml) for 3 days. TNF-α in the supernatant released by the peritoneal macrophages was measured with an enzyme-linked immunosorbent assay (ELISA) kit (Genzyme, USA).

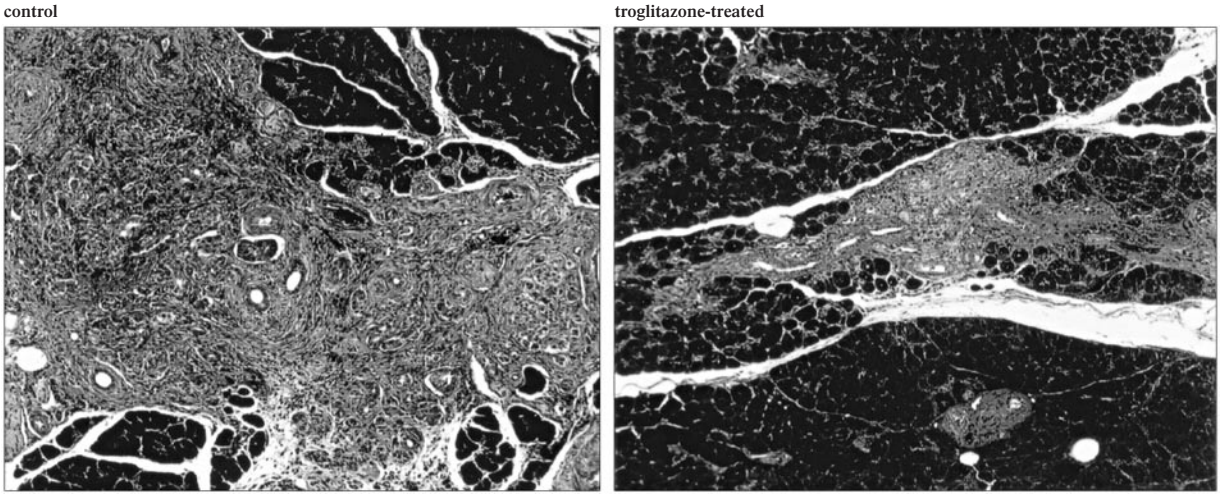
Nuclear protein extracts

Peritoneal macrophages isolated from male Wistar rats were exposed to LPS for 3 hours in the presence or absence of troglitazone, and the cells were harvested for nuclear extract preparation. Cells were washed twice in cold PBS and lysed in 1.2 ml of ice-cold hypotonic lysis buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, pH 8.0, 1 mM DTT, TritonX 0.5% containing protease inhibitor) for 30 min on ice, and centrifuged at 6500 rpm for 1 min. The pellets were resuspended in 50 μl of nuclear extraction buffer (20 mM HEPES, pH 7.9, 400 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, pH 8.0). The lysate was incubated for 20 min, and an aliquot of the culture supernatant was stored at -80°C.

Electrophoretic mobility shift assay (EMSA)

EMSA was performed by using a gel shift assay system kit (Promega, USA). Complementary oligonucleotides were ^{32}P-end-labeled with T4 polynucleotide kinase. Oligonucleotide probes were incubated with 10 μg of nuclear extracts in binding buffer with or without unlabeled competitor or noncompetitor oligonucleotide. DNA-binding protein complexes were separated with 6% DNA retardation gel (Novex, USA) at 250V and visualized by autoradiography.

Figure 1. **Histological appearance of the pancreas of WBN/Kob rats. Focal inflammatory cell infiltration, hemorrhage, and fibrosis were observed in 4-months-old WBN/Kob rats. These findings were markedly prevented by administration of troglitazone. Original magnification x100**



Statistical analysis

All data are expressed as the mean ± standard error. Statistical analysis was performed by the unpaired Student's t test or by one-way analysis of variance followed by Fisher's multiple comparison test. All differences with p values <0.05 were regarded as significant.

Results

Troglitazone prevents the progression of chronic pancreatitis in male WBN/Kob rats

Significant focal inflammatory cell infiltration, hemorrhage, and fibrosis were observed in all of the 4-month old WBN/Kob rats, and troglitazone administration for 3 months significantly limited the development of the pancreatic inflammation (*Fig. 1*). The proportion of the total area of the pancreatic specimen occupied by the fibrotic area, which stained positive with Masson's trichrome, was calculated to evaluate the effect of troglitazone on pancreatic fibrosis. The fibrotic area (10.5 ± 1.5 mm^2) accounted for only 2-3% of the total area (303.7 ± 19.3 mm^2) measured in the WBN/Kob rats fed troglitazone for 3 months, as opposed to approximately 7% in the control WBN/Kob rats (16.8 ± 0.5 mm^2/240.0 ± 11.1 mm^2). The immunohistochemical study revealed the presence of spindle-shaped α-SMA-positive cells, thought to be myofibroblasts, scattered throughout the fibrotic area in the control WBN/Kob rats. Administration of troglitazone significantly reduced the number of α-SMA positive cells, and the areas positive for collagen I, III, and fibronectin were also reduced by troglitazone (*Fig. 2*). Western blot analysis demonstrated strong expression of α-SMA in the control WBN/Kob rats, and its expression was decreased in the WBN/Kob rats fed troglitazone (*Fig. 3*). These findings suggest that PSCs may be an important target in the mechanism of the anti-fibrotic action of troglitazone.

TNF-α production by peritoneal macrophages is decreased by exposure to troglitazone

We investigated the mechanism of the inhibitory action of troglitazone on proinflammatory cytokine production. Peritoneal macrophages isolated from WBN/Kob rats produced a much greater amount of TNF-α (1061 ± 28 pg/ml/10^5 cells) during three days of culture than cells from normal Wistar rats (31 ± 14 pg/ml/10^5 cells). However, the cells from troglitazone-treated rats produced only a marginal amount of TNF-α. LPS-stimulated macrophages from normal Wistar rats released a larger amount of TNF-α than unstimulated macrophages, and exposure to troglitazone significantly decreased the LPS--induced TNF-α production (*Fig. 4*).

Troglitazone dose-dependently inhibits NF-κB/Rel activation in activated macrophages

We investigated whether troglitazone affects NF-κB/Rel binding activity in LPS-stimulated macrophages. No NF-κB/Rel binding activity was detected in nuclear extracts prepared from unstimulated cells, and the specificity of the NF-κB/Rel binding activity induced by LPS was confirmed by a competition study. Increasing doses of troglitazone suppressed the NF-κB/Rel binding activity in a dose-dependent manner (*Fig. 5*).

Discussion

In previous *in vivo* studies, we found that pancreatic exocrine dysfunction in rats with streptozotocin-induced diabetes mellitus and in an animal model of chronic pancreatitis was improved by administration of troglitazone [6,7], and the severity of the morphological pancreatic damage, including inflammatory cell infiltration and fibrosis, was suppressed by the inhibitory effect of troglitazone on intrapancreatic proinflammatory cytokine expression and PSC activation. We also found

Figure 2. **Immunohistochemical staining for α-SMA, collagen I, III, and fibronectin. The area of the pancreas that stained positive for α-SMA, collagen I, III, and fibronectin in the troglitazone (TRO)-treated rats was much smaller that in the control WBN/Kob rats**

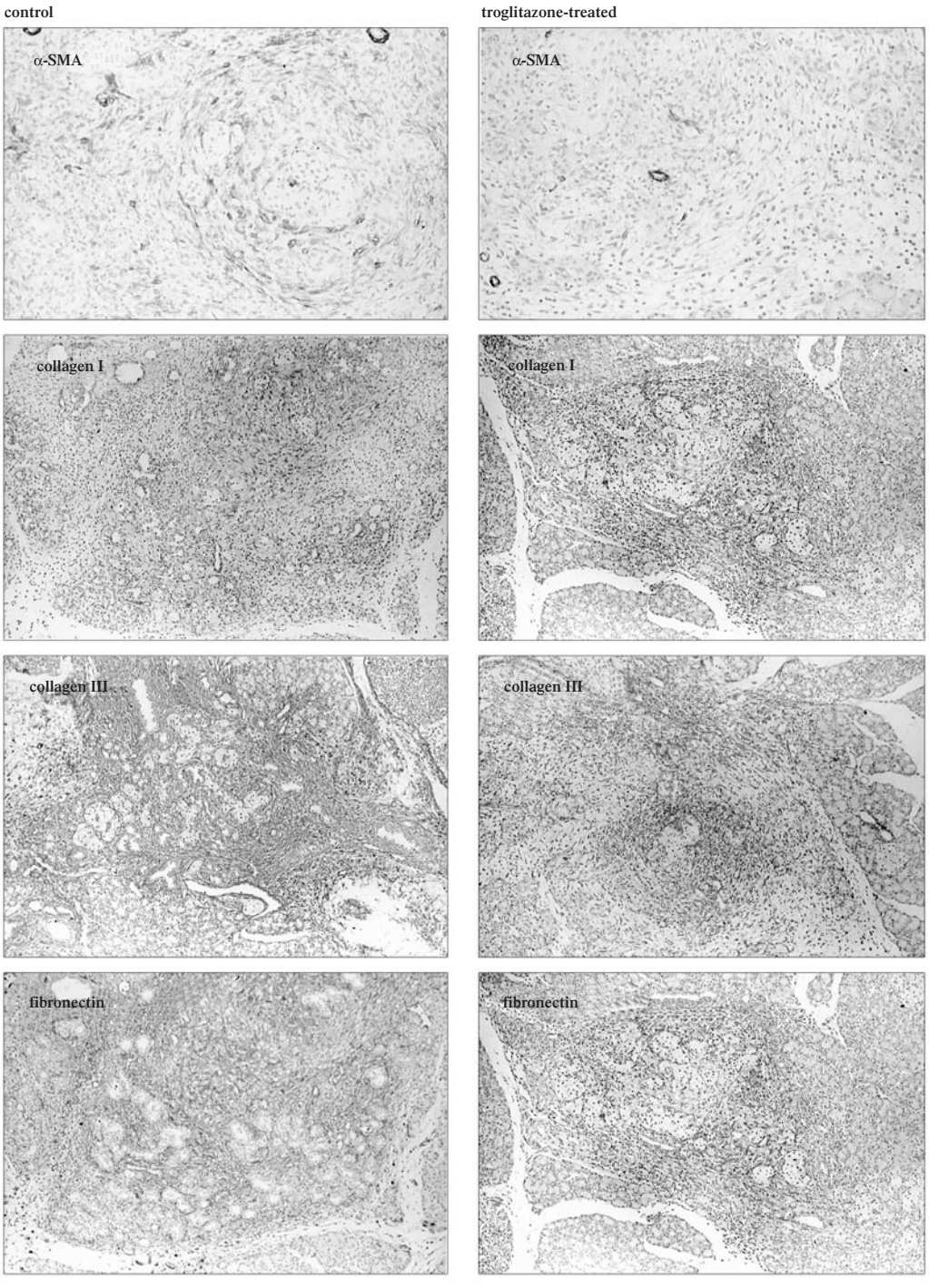
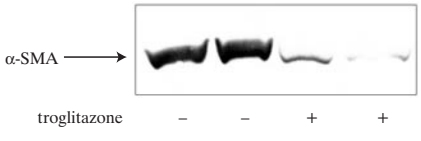


Figure 3. **Western blot analysis showed a significantly lower α-SMA protein level in the pancreas of troglitazone-treated WBN/Kob rats than in the control WBN/Kob rats**



that PSC proliferation was inhibited by troglitazone's blocking effect on cell-cycle progression beyond the G1 phase via PPARγ-dependent and independent mechanisms [9]. The present study demonstrated the area of the pancreas that was positive for collagen I, III, and fibronectin, and expression of α-SMA was markedly diminished by troglitazone in an animal model of chronic pancreatitis, suggesting that troglitazone contributes to the prevention of the progression of chronic pancreatitits by inhibiting ECM production.

Figure 4. **TNF-α production by peritoneal macrophages. Peritoneal macrophages were isolated from male Wistar rats (n=4) and exposed to LPS 10 ng/ml for 3 days. TNF-α released into the medium by the peritoneal macrophages was determined by ELISA. LPS exposure increased TNF-α secretion, and LPS-stimulated TNF-α secretion was significantly inhibited by troglitazone. Each value represents a mean ± SEM**

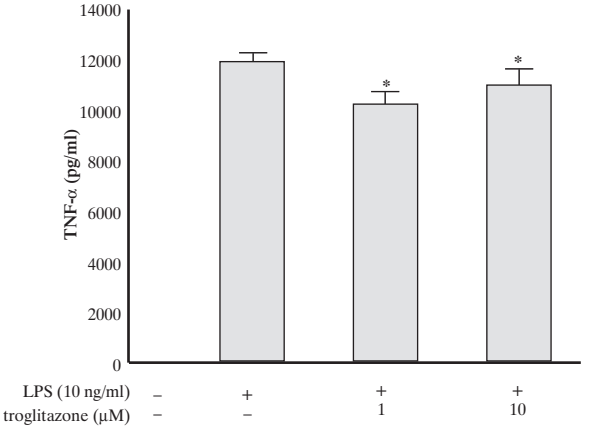
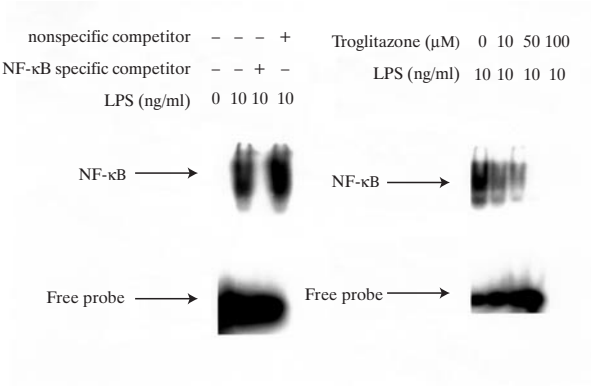


Figure 5. **Analysis of NFκB binding activity by the EMSA. Peritoneal macrophages were activated by LPS, and NFκB binding activity was analyzed by EMSA with a ^{32}P-end labeled NFκB oligomer. Specific binding activity was determined by an excess amount of unlabeled NF-κB oligonucleotide or a nonspecific oligomer containing the SP-1 DNA recognition motif. Peritoneal macrophages were incubated in medium at a concentration of 1, 50, or 100 μM of troglitazone for 2 hours and then exposed to LPS 10 ng/ml for 3 hours**



When PSCs are stimulated by various soluble mediators released by activated macrophages and aggregating platelets, the phenotype of quiescent fat-storing cells converts to a myofibroblast-like cell phenotype that produces collagen type I, III, and IV, and fibronectin [13,14], and transforming growth factor (TGF)-β and TNF-α accelerate the change in cell phenotype. Platelet-derived growth factor is a potent stimulator of cell proliferation, and TGF-β, PDGF, and basic fibroblast growth factor (bFGF) stimulate ECM synthesis [14,15]. Macrophage activation is closely associated with the fibrogenic action of PSCs. The present study revealed that the TNF-α release by peritoneal macrophages isolated from WBN/Kob rats was significantly suppressed by troglitazone, and similar results were obtained in LPS-stimulated peritoneal macrophages isolated from normal Wistar rats. PPARγ ligands have been reported to inhibit inflammatory cytokine gene expression and promotor activity by antagonizing the activity of the transcription factors of AP-1, STAT, and nuclear factor-κB (NF-κB) [10,11], and PPARγ ligands inhibit the nuclear translocation and subsequent DNA binding of NF-κB by inhibiting the degradation of IκB-α [11, 16-18]. NF-κB is a transcription factor that binds to enhancer elements involved in a variety of inflammatory cytokine genes, such as the genes encoding TNF-α, IL-6, IL-8, and inducible nitric oxide synthase. NF-κB/Rel is known to be activated in animal models of acute pancreatits, including cerulein-induced pancreatitis and taurocholate pancreatitis [19,20], and we found that LPS-induced NF-κB/Rel activation in peritoneal macrophages isolated from rats with chronic pancreatitis was inhibited by troglitazone, suggesting that one of the mechanisms by which troglitazone decreases inflammatory cytokine production is mediated by the inhibition of NF-κB/Rel activation.

The matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) are crucial modulators of fibrogenesis and fibrolysis. PSCs express not only the ECM but membrane type-1 MMP, MMP-2, and MMP-9, which are known to degrade basement membrane collagen, and MMP-13, which degrades fibrillar collagen, and their inhibitors, TIMP-1 and TIMP-2 [14,21]. Activated PSCs also express activated TGF-β1, which up-regulates collagen I and down-regulates MMP-3 and MM-9 [14]. Recent reports suggest that pioglitazone and rosiglitazone inhibit pancreatic cancer cells invasiveness by suppressing their gelatinolytic and fibrinolytic activity though inhibition of MMP-2 activity [22], and a study on hepatic stellate cells reported that pioglitazone prevents the activation of hepatic stellate cells, thereby resulting in a reduction in expression of type I procollagen, MMP-2, TIMP-1, and TIMP-2 mRNA and acceleration of fibrolysis in the liver [23]. While the regulation of fibrogenic and fibrolytic action on ECM by the combination of MMPs and TIMPs is complicated, the balance between ECM synthesis and degradation by MMPs and TIMPs may be a candidates for the mechanism that regulates the anti-fibrogenic effect of TZDs in chronic pancreatitis.

Prevention of inflammation and fibrosis through inhibition of proinflammatory cytokine production and activation of PSCs is a major target of the treatment of chronic pancreatitis. Our recent study suggests that PSCs act as resident phagocytic cells and that scavenger receptor CD36 promotes troglitazone-induced phagocytic activity via activation of endogenous PPARγ [24]. TZD may be a useful drug for the treatment of chronic pancreatitis.

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