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Pancreatic stellate cells reduce insulin expression and induce apoptosis in pancreatic β-cells

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ABSTRACT

Islet fibrosis, pancreatic β-cell dysfunction, and β-cell apoptosis are features of pancreatic diabetes and type 2 diabetes; however, the underlying mechanisms remain largely unknown. We hypothesized that pancreatic stellate cells (PSCs), a major profibrogenic cell type in the pancreas, might affect the phenotype of pancreatic β-cells. α-Smooth muscle actin (a marker of activated PSC)-positive cells were found within and around the fibrotic islets. Indirect co-culture with PSCs reduced insulin expression and induced apoptosis in RIN-5F pancreatic β-cells. Induction of β-cell apoptosis was associated with activation of the caspase pathway and mitochondrial depolarization. Diphenylene iodonium, an inhibitor of PSC activation, inhibited islet fibrosis and protected islets *in vivo*. Our findings suggest a novel mechanism linking PSCs, islet fibrosis, and diabetes mellitus.

1. Introduction

Secondary diabetes (type 3c diabetes) is a serious complication in patients with advanced chronic pancreatitis (CP) and pancreatic cancer [1–3]. Type 3c diabetes is believed to result from deficient insulin secretion caused by the dysfunction and destruction of β-cells due to extensive fibrosis and islet atrophy [3,4]. β-Cell dysfunction occurs much earlier in CP and pancreatic cancer, before clinical evidence of diabetes [4]. Reduced pancreatic volume and β-cell area are associated with increased apoptosis in the islet cells of patients with CP [5]. Islet fibrosis and β-cell apoptosis are also found in patients with and animal models of type 2 diabetes [6–9]. There is a 3-to-10-fold increase in β-cell apoptosis in diabetic patients compared to non-diabetic individuals [6]. Patients with type 2 diabetes also have variable intra- and peri-islet fibrosis [7]. Islet fibrosis could be important in the progression of β-cell failure because it may accelerate β-cell destruction or induce the disruption of β-cell connectivity [10]. However, the molecular mechanisms linking islet fibrosis, β-cell dysfunction, and β-cell loss remain unknown.

In 1998, star-shaped cells in the pancreas, namely pancreatic stellate cells (PSCs), were identified and characterized [11,12]. In normal pancreas, PSCs are quiescent and can be identified by the presence of vitamin A-containing lipid droplets in the cytoplasm. In response to pancreatic injury or inflammation, they are transformed (activated) from their quiescent phenotype into myofibroblast-like cells, which express α-smooth muscle actin (α-SMA), actively proliferate, and produce extracellular matrix components such as type I collagen [11–15]. PSCs play a pivotal role in the development of pancreatic fibrosis in CP and pancreatic cancer [11–15]. We hypothesized that PSCs may influence the behavior of pancreatic β-cells. By using an indirect co-culture system, we demonstrated that PSCs reduce insulin expression and induce apoptosis in β-cells. In addition, inhibition of PSC activation by diphenylene iodonium (DPI) inhibits the development of islet fibrosis *in vivo*. Our results suggest a novel mechanism linking PSCs, islet fibrosis, and diabetes.

2. Materials and methods

2.1. Materials

3-(4, 5-Dimethylthiazole-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was obtained from Dojindo (Kumamoto, Japan). Rabbit anti-α-SMA antibody was from Abcam (Cambridge, MA). Rabbit anti-insulin antibody, anti-caspase 3 antibody, anti-cleaved caspase 9 antibody, and biotinylated goat anti-rabbit IgG antibody were from Cell Signaling (Beverly, MA). Other reagents were from Sigma–Aldrich (St. Louis, MO) unless specifically described.

2.2. Cell line and cell culture

RIN-5F rat pancreatic β-cells [16] were obtained from American Type Culture Collection (Manassas, VA). Cells were maintained in

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RPMI1640 supplemented with 10% FBS, penicillin sodium, and streptomycin sulfate. Rat PSCs were prepared as previously described [17] from the pancreas tissues of male Wistar rats (Japan SLC Inc., Hamamatsu, Japan) using Nycodenz solution (Nycomed Pharma, Oslo, Norway) after perfusion with 0.03% collagenase P. All animal procedures were performed in accordance with the National Institutes of Health Animal Care and Use Guidelines. Cells were maintained in Ham's F-12/DMEM (1:1) supplemented with 10% FBS, penicillin sodium, and streptomycin sulfate. All experiments were performed using rat PSCs at passage 5 or 6.

2.3. Indirect co-culture of pancreatic β-cells and PSCs

RIN-5F cells were seeded in 6- or 24-well culture plates (BD Biosciences, Bedford, MA) in RPMI1640 supplemented with 10% FBS, penicillin sodium, and streptomycin sulfate. Rat PSCs were seeded into the culture inserts of 1.0 μm pore size (BD Bioscience) in Ham's F-12/DMEM (1:1) supplemented with 10% FBS, penicillin sodium, and streptomycin sulfate. The next day, culture insets containing rat PSCs were placed into 6- or 24-well culture plates containing RIN-5F cells, and incubation was continued up to 3 days in RPMI1640 supplemented with 10% FBS, penicillin sodium and streptomycin sulfate.

2.4. Immunohistochemical analysis

Immunohistochemical staining of α-SMA and insulin was performed as previously described [18] using a streptavidin–biotin–peroxidase complex detection kit (Histofine Kit; Nichirei, Tokyo, Japan). Briefly, pancreatic tissues were removed from patients undergoing surgery for CP or pancreatic cancer and fixed by immersion in 4% paraformaldehyde overnight at 4 °C. The specimens were embedded in regular paraffin wax and cut into 4-μm sections. Tissue sections were deparaffinized and rehydrated in PBS. Following antigen retrieval with target retrieval solution (Dako, Glostrup, Denmark), endogenous peroxidase activity was blocked by incubation with 0.3% hydrogen peroxide. After immersion in 10% normal goat serum, the sections were incubated with rabbit anti-α-SMA or anti-insulin antibody overnight at 4 °C. The slides were incubated with biotinylated goat anti-rabbit IgG antibody, followed by peroxidase-conjugated streptavidin. Finally, the color was developed by incubating the slides for several minutes with diaminobenzidine (Dojindo, Kumamoto, Japan).

2.5. Sirius red staining

Following immunostaining for insulin, collagen accumulation was assessed by Sirius red staining, which preferentially labels collagen fibrils in red [19].

2.6. Insulin secretion assay

The insulin secretion activity of RIN-5F cells was assessed as previously described with minor modifications [20]. Briefly, RIN-5F cells were mono-cultured or indirectly co-cultured with rat PSCs in 24-well plates. After 72 h, culture inserts containing rat PSCs were removed, and RIN-5F cells were washed with PBS to remove released insulin. Then, 400 μL of fresh medium were added and incubation was continued for 3 h. The concentration of insulin in the supernatants was determined using the ELISA system (AKRIN-010T, Shibayagi, Gunma, Japan) according to the manufacturer's instructions. The insulin level was normalized to the cellular protein level determined by the BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL).

2.7. RNA extraction and real-time RT-PCR

RIN-5F cells were mono-cultured or co-cultured with rat PSCs for 24 h, and total RNA was extracted using the RNeasy total RNA preparation kit (Qiagen, Valencia, CA). Complementary DNA was synthesized using the GoScript reverse transcription system (Promega). Quantitative real-time PCR was performed using Taqman® Fast Universal PCR Master Mix (Applied Biosystems) and detected using the Applied Biosystems StepOnePlus 7300 Real-Time PCR System. Primers and probes were predesigned by the manufacturer (Applied Biosystems). The assay ID numbers were as follows: Rn02121433_g1 for insulin1 and Rn01775763_g1 for GAPDH. PCR was performed at 95 °C for 20 s, followed by 40 cycles of 95 °C for 1 s and 60 °C for 20 s. The absolute number of gene copies was standardized by a sample standard curve. The values of insulin mRNA were normalized to GAPDH mRNA and expressed as percentage of the mono-cultured cells.

2.8. Cell viability assay

Cell viability was assessed by the MTT assay. RIN-5F cells were mono-cultured or co-cultured with rat PSCs in 24-well plates. After 48 h, the culture supernatants were aspirated, MTT solution was added to the cells at a final concentration of 500 μg/mL, and incubation continued at 37 °C for 4 h. The medium was then aspirated and the formazan product was solubilized with dimethylsulfoxide. Cell viability was determined by OD_{570}–OD_{690}.

2.9. Apoptosis assay

Apoptosis of RIN-5F cells was examined by cytoplasmic histone-associated DNA fragment ELISA (Cell Death Detection ELISA; Roche Diagnostics, Tokyo, Japan) and the terminal deoxyribonucleotidyl transferase-mediated dUTP nick end-labeling assay (Apoptag® *in situ* apoptosis detection kit; Millipore, Billerica, MA). Briefly, to quantify cytoplasmic histone-associated DNA fragments, cell lysates of RIN-5F cells were pipetted into a streptavidin-coated microplate. A mixture of anti-histone–biotin and anti-DNA–peroxidase antibodies was added, and the samples were incubated for 2 h. After removal of unbound antibodies, nucleosomes were photometrically detected by measuring peroxidase activity with 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) as the substrate at OD_{405}–OD_{490}.

To detect apoptosis *in situ*, the 3′-OH DNA termini were enzymatically labeled with digoxigenin-labeled nucleotide by terminal deoxynucleotidyl transferase. The incorporated nucleotides were detected by anti-digoxigenin–rhodamine under a fluorescence microscope (ZB-8000, Keyence, Osaka, Japan). Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI).

2.10. Western blotting

Cell lysates were prepared and the expression of total and cleaved caspases-3 was examined by western blotting as described [18]. Briefly, cells were lysed in SDS buffer, and cellular proteins (~100 μg) were fractionated on a 10% SDS–polyacrylamide gel (Bio-Rad, Hercules, CA). They were transferred to a nitrocellulose membrane (Bio-Rad) and the membrane was incubated overnight at 4 °C with rabbit-anti-caspase 3 antibody. After incubation with peroxidase-conjugated anti-rabbit IgG antibody, proteins were visualized using an ECL kit (Amersham Biosciences, Buckinghamshire, United Kingdom). The level of α-tubulin was examined in a similar manner.

2.11. Immunofluorescent staining

Immunofluorescent staining of cleaved caspase-9 was performed as previously reported [21]. RIN-5F cells were fixed in methanol for 10 min at −20 °C. After blocking with 10% normal goat serum, cells were incubated with rabbit anti-caspase 9 antibody overnight at 4 °C. After washing, the cells were incubated with Alexa Fluor^{488}-labeled goat anti-rabbit IgG antibody (Invitrogen, Carlsbad, CA) for 1 h. After washing, the cells were analyzed for fluorescence under a fluorescence microscope (BZ-8000). Nuclei were stained with DAPI.

2.12. Estimation of mitochondrial membrane potential ($\Delta_{\psi m}$)

The mitochondrial membrane potential was assessed using a MitoPT-JC1 assay kit (Immunochemistry, Bloomington, MN). Briefly, RIN-5F cells were incubated in assay buffer with JC-1 for 15 min at 37 °C. After washing, cells were mounted on slides with a drop of assay buffer and examined under a fluorescence microscope (BZ-8000). In healthy cells with high $\Delta_{\psi m}$, JC-1 spontaneously forms complexes known as J-aggregates with intense red fluorescence. In contrast, in unhealthy cells with low $\Delta_{\psi m}$, JC-1 remains in the monomeric form, which appears as green fluorescence [22].

2.13. Effect of DPI on the development of islet fibrosis in vivo

After an adaptation period of 3 week, DPI was continuously administered in drinking water (1 mg/kg body weight/day) to 10-week-old male WBN/Kob rats until they were euthanized after 20 week of age. This dose is well tolerated and effective in long-term studies of rats [23]. Control rats received drinking water containing vehicle (0.02% dimethylsulfoxide). Each group consisted of 6 animals. There were no significant differences in body weight development between the two groups. Pancreas specimens were routinely fixed in 4% paraformaldehyde in PBS and embedded in paraffin. Tissue sections were stained with H&E or Sirius red.

2.14. Statistical analysis

Results were expressed as mean ± SD. Experiments were performed at least 3 times and similar results were obtained. The differences between groups were analyzed by the two-tailed unpaired Student's *t* test or ANOVA, followed by the Dunnett test for post hoc analysis. A *P* value less than 0.05 was considered statistically significant.

3. Results and discussion

3.1. Presence of α-SMA-positive activated PSCs in fibrotic islets

First, we examined the presence of islet fibrosis in patients with CP or pancreatic cancer using a combination of immunostaining for insulin and Sirius red staining, which preferentially labels collagen fibrils [19]. As shown in Fig. 1, variable intra- and peri-islet fibrosis was found on resected pancreatic specimens from patients with CP or pancreatic cancer. The development of pancreatic fibrosis is a major component in several diseases of the pancreas including CP, pancreatic cancer, and type 2 diabetes mellitus, but its actual role in the progression of these disorders remains unknown. Although fibrosis in CP and pancreatic cancer predominately involves exocrine pancreatic tissue, evidence from both animal models and human patients suggests that islet fibrosis also occurs and is a component in the progression of type 2 diabetes mellitus [6–9].

Because activated PSCs play a critical role in the development of pancreatic fibrosis [11–15], we examined whether PSCs are involved in islet fibrosis. We performed immunostaining of α-SMA, a marker of activated PSCs [11,12]. α-SMA expression was found around and within the islets, suggesting a role of PSCs in islet fibrosis.

3.2. Co-culture with PSCs decreased insulin expression in RIN-5F cells

Because PSCs and islet cells are closely located, we hypothesized that PSCs might have direct effects on the behavior of β-cells. We examined the effect of co-culture with PSCs on the insulin

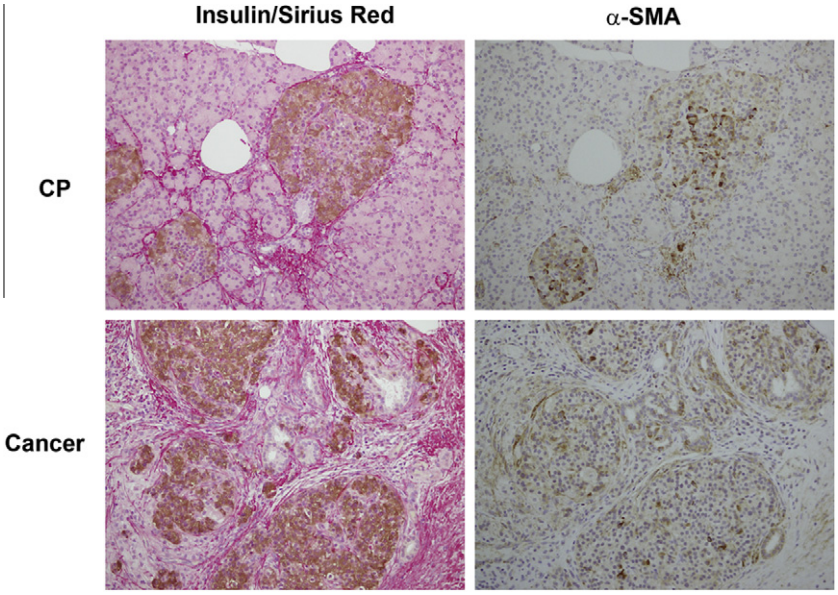

Fig. 1. Activated PSCs are present within and around the fibrotic islets. (A) Sirius red staining (red) was performed on resected pancreatic specimens from patients undergoing surgery for CP or pancreatic cancer, following immunostaining for insulin (brown). (B) In serial sections, immunostaining for α-SMA was performed. Nuclear staining was performed using hematoxylin. Original magnification: ×200. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

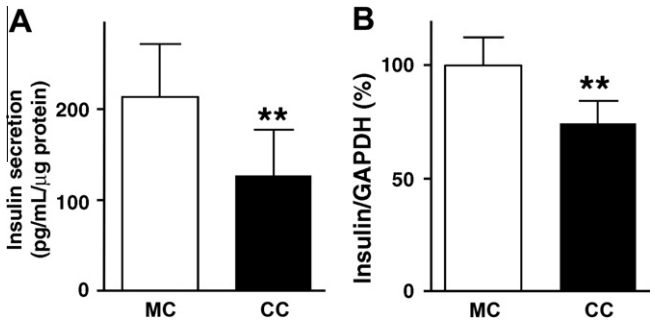

Fig. 2. PSCs decreased insulin expression in RIN-5F cells RIN-5F cells were mono-cultured (MC) or indirectly co-cultured (CC) with rat PSCs. (A) After 72 h, culture inserts containing rat PSCs were removed and the medium was replaced with fresh. After 3-h incubation, the insulin concentration in the culture supernatant was determined by ELISA. $^{**}P < 0.01$ vs. mono-culture ($n = 8$). (B) After 24 h, total RNA was prepared from RIN-5F cells, reverse-transcribed, and mRNA levels of insulin and GAPDH were determined by real-time PCR. The values of insulin mRNA were normalized to GAPDH mRNA and expressed as a percentage of mono-cultured cells. $^{**}P < 0.01$ vs. monoculture ($n = 6$).

expression in RIN-5F pancreatic β-cells. RIN-5F cells were mono-cultured or indirectly co-cultured with rat PSCs, and insulin secretion was determined by ELISA. Compared with the mono-culture, co-culture with PSCs decreased insulin secretion from RIN-5F cells (Fig. 2A). Inhibition of insulin expression by co-culture with PSCs was also shown at the mRNA level by quantitative real-time PCR (Fig. 2B). Thus, PSCs caused a reduction in the functional capacity of β-cells.

3.3. PSCs induced apoptosis in RIN-5F cells

Because the apoptosis of β-cells is associated with pancreatic diabetes and type 2 diabetes [3–9], we examined whether PSCs induced apoptosis in RIN-5F cells. Co-culture of RIN-5F cells with PSCs resulted in decreased cell viability as assessed by the MTT assay (Fig. 3A), and increased apoptosis (Fig. 3B). The induction of apoptosis in RIN-5F cells by co-culture with PSCs was confirmed by the terminal deoxyribonucleotidyl transferase-mediated dUTP nick end-labeling assay (Fig. 3C).

The interactions between PSCs and other cell types in the pancreas have attracted increasing attention from researchers. In general, PSCs promote the proliferation of neighboring cells or protect them from apoptosis. For example, PSCs increased the proliferation and migration of human umbilical vein endothelial cells, suggesting a role of PSCs in angiogenesis [24]. PSCs increased the proliferation of pancreatic cancer cells, suggesting that PSCs promote the progression of pancreatic cancer [25]. PSCs protect pancreatic cancer cells from apoptosis induced by gemcitabine, H_2O_2 [25], and radiation [26]. Therefore, the induction of apoptosis in RIN-5F cells by PSCs is in striking contrast to previous reports on the interactions between PSCs and neighboring cells, and rather specific to that between PSCs and pancreatic β-cells.

3.4. PSC-induced β-cell apoptosis was associated with activation of the caspase pathway and mitochondrial depolarization

To clarify the mechanisms responsible for PSC-induced apoptosis in β-cells, we examined activation of the caspase pathway. Co-culture with PSCs increased the amount of cleaved caspase-3 and caspase-9 in RIN-5F cells, as shown by western blotting and immunofluorescence staining (Fig. 3D and E). In addition, co-culture with PSCs resulted in the loss of mitochondrial membrane potential (Fig. 3F), suggesting mitochondria-dependent apoptosis. Previous studies have shown an association between caspase-3 activation and pancreatic β-cell apoptosis induced by streptozotocin [27] and c-Myc activation [28]. Caspase-9 is activated in RIN5F cells upon apoptotic induction with streptozotocin [29]. Thus, PSCs

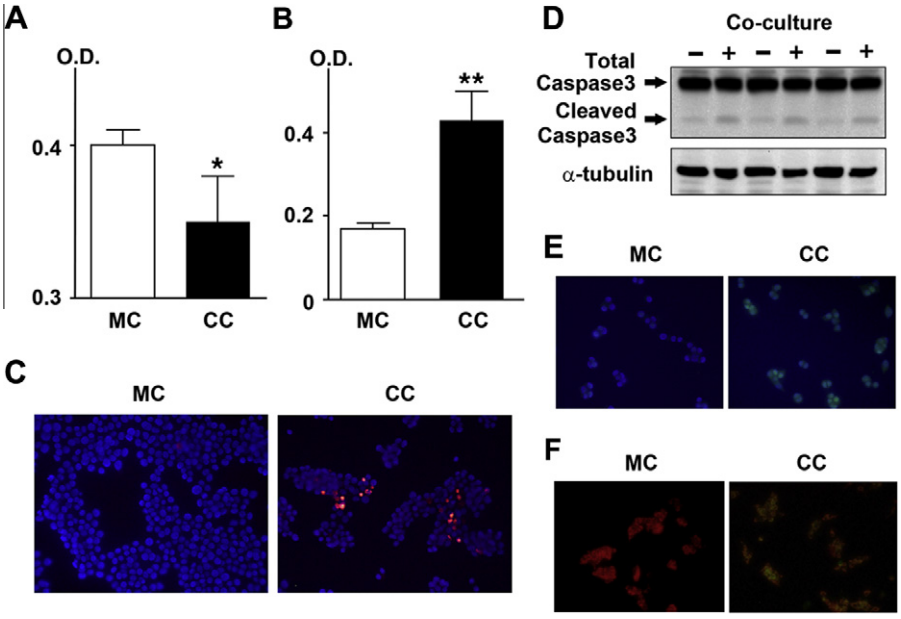

Fig. 3. PSCs induced apoptosis in RIN-5F cells RIN-5F cells were mono-cultured (MC) or indirectly co-cultured (CC) with rat PSCs. (A) After 48 h, cell viability was assessed by the MTT assay. $^{*}P < 0.05$ vs. monoculture ($n = 6$). (B and C) After 72 h, apoptosis of RIN-5F cells was examined by cytoplasmic histone-associated DNA fragment ELISA (B) and Apoptag® *in situ* apoptosis detection kit (C). $^{**}P < 0.01$ vs. monoculture ($n = 6$). Nuclei were counterstained with DAPI. Original magnification: ×200. (D) After 24 h, total cell lysates were prepared and expression of caspase-3 was examined by Western blotting. α-tubulin serves as a loading control. (E) Expression of cleaved caspase-9 was examined by fluorescent immunocytochemistry. Nuclei were counterstained with DAPI. Original magnification: ×100. (F) Mitochondrial membrane potential was assessed using the MitoPT-JC1 assay kit. In healthy cells with high $\Delta_{\psi m}$, JC-1 spontaneously forms complexes known as J-aggregates with intense red fluorescence. In contrast, in unhealthy cells with low $\Delta_{\psi m}$, JC-1 remains in the monomeric form, appearing as green fluorescence. Original magnification: ×200. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

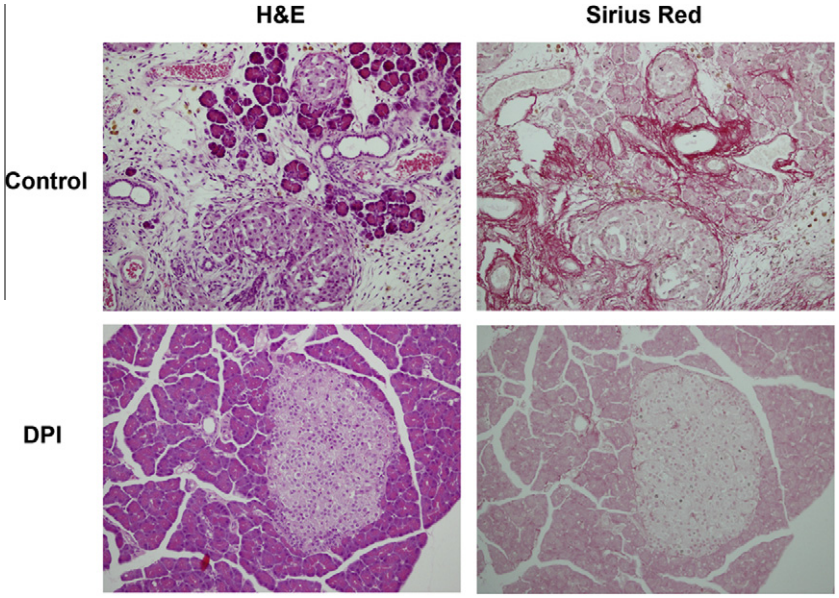

Fig. 4. DPI inhibited the development of islet fibrosis in WBN/Kob rats DPI was administered in drinking water (1 mg/kg body wt/day) to 10-week-old male WBN/Kob rats for 10 week. Representative histological appearance of the pancreas in the control and DPI-treated groups (H&E staining and Sirius red staining). Original magnification: ×200.

induced apoptosis in RIN-5F cells at least in part through the activation of caspases and mitochondrial depolarization, common machineries for β-cell apoptosis.

The interaction between β-cells and elements of their local microenvironment including extracellular matrix components is essential in the activation of intracellular signaling pathways that regulate cell proliferation, survival, and function. The loss of cell-to-cell communications associated with increased intra-islet fibrosis may reduce the secretory efficiency of the islets and promote islet cell apoptosis [10]. Our results suggest that mediator(s) produced by PSCs directly decreased insulin expression and induced apoptosis in β-cells. The mediator(s) responsible for PSC activity remains unknown. Previous studies have shown that oxidative and inflammatory cytokines such as IL-1β, TNF-α, and IFN-γ induced apoptosis in pancreatic β-cells [30]. However, antibodies against these cytokines did not inhibit PSC-induced apoptosis or the decrease in insulin expression in RIN-5F cells in this study (data not shown). Nitric oxide produced by inducible nitric oxide synthase might play a role [31], but co-culture with PSCs did not increase the expression of inducible nitric oxide synthase in RIN-5F cells (data not shown).

3.5. DPI, an inhibitor of PSC activation, inhibited the development of islet fibrosis

Finally, we examined whether inhibition of PSC activation inhibited the development of islet fibrosis. We previously demonstrated that DPI, an inhibitor of NADPH oxidase, inhibited the activation and function of PSCs [32]. We examined the effects of DPI on the development of islet fibrosis in male WBN/Kob rats, an experimental model of spontaneous CP [32,33]. Control rats had infiltration of inflammatory cells, degeneration and disappearance of acinar cells, and intra- and peri-islet fibrosis (Fig. 4). DPI inhibited collagen accumulation and protected islets, as assessed by H&E and Sirius red staining (Fig. 4).

Anti-fibrosis therapy targeting PSCs might be a novel therapeutic option for the treatment of pancreatic cancer and CP. Our results suggest anti-fibrosis therapy might be useful for the treatment of diabetes through the inhibition of islet fibrosis and protection of β-cells. It was recently shown that reagents that inhibit the activation and function of PSCs such as thiazolinediones [34], angiotensin converting enzyme inhibitor [35], and antioxidants [36], inhibited islet fibrosis and improved β-cell function. Experiments along this line are underway in our laboratory.

4. Conclusions

By using an indirect co-culture system, we demonstrated that PSCs reduced insulin expression and induced apoptosis in pancreatic β-cells. Our findings suggest a novel mechanism linking PSCs, islet fibrosis, and diabetes.

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