

Original Article

# The Process and Development Mechanism of Age-related Fibrosis in the Pancreatic Islets of Sprague-Dawley Rats: Immunohistochemical Detection of Myofibroblasts and Suppression Effect by Estrogen Treatment

Masako Imaoka<sup>1\*</sup>, Toshimasa Jindo<sup>1</sup>, and Wataru Takasaki<sup>1</sup>

<sup>1</sup> Medicinal Safety Research Laboratories, Daiichi Sankyo Co., Ltd., 1-16-13, Kita-Kasai, Edogawa-ku, Tokyo 134-8630, Japan

**Abstract:** The mechanism of spontaneous islet fibrosis in Sprague-Dawley rats was investigated. Using sections of the pancreas in naive males aged 26 to 102 weeks old and 26-week-old males injected with  $\beta$ -estradiol 3-benzoate (EB), the incidence of lesions and histological scores of fibrosis were examined in conjunction with immunohistochemistry for  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA), platelet-derived growth factor receptor- $\alpha$  (PDGFR $\alpha$ ) and estrogen receptor- $\alpha$  (ER $\alpha$ ). The incidence of islet fibrosis increased in 78-week-old animals compared to the 26-week-old animals, and the incidence of atrophy in the fibrotic islet increased in animals over 52 weeks old.  $\alpha$ -SMA and PDGFR $\alpha$  were positively stained mainly in fibrotic/inflammatory islets, and the histological score of  $\alpha$ -SMA in the fibrotic islet decreased age-dependently. Notably,  $\alpha$ -SMA and PDGFR $\alpha$  were co-expressed in inflammatory islets with a high score at all ages. The positive index of ER $\alpha$  in the EB-treated group increased when compared with that of the naive group. However, it was independent of the existence of fibrosis. In contrast, the score of  $\alpha$ -SMA and PDGFR $\alpha$  decreased in the EB-treated group. In conclusion, it was clarified that a part of age-related fibrosis in islets became atrophy with age, and  $\alpha$ -SMA-positive myofibroblasts were considered to contribute to the development of fibrosis. Strong PDGFR $\alpha$  stainability in fibrotic/inflammatory islets may imply that myofibroblasts were stimulated by PDGF to produce an extracellular matrix. Although estradiol has been known to suppress fibrosis/inflammation in the islet, nuclear-located ER-dependent signaling was considered not to be involved in the suppression mechanism. EB possibly affected the inhibition of the appearance of myofibroblasts. (DOI: 10.1293/tox.26.1; J Toxicol Pathol 2013; 26: 1–10)

**Key words:** pancreatic islet, fibrosis, myofibroblast,  $\alpha$ -smooth muscle actin, platelet-derived growth factor receptor- $\alpha$ , Sprague-Dawley rat

## Introduction

We have previously reported about the spontaneous lesion in the pancreatic islet in Sprague-Dawley (SD) rats<sup>1</sup>. The initial change is histopathologically characterized as a micro-hemorrhage and macrophage infiltration in the islet and is firstly observed at 12 weeks of age in both sexes. The lesion develops with age and is accompanied by inflammatory cell infiltration and/or fibrosis with the predominant incidence in males until 26 weeks of age. As the process continues, the ratio of fibrous tissue to islet cells is known to increase, and some islets are reduced to scattered small islands of cells embedded in a mass of scar tissue at 26 months of age<sup>2</sup>. However, the detailed mechanism of this fibrosis has

not been investigated.

In humans, pancreatic stellate cells (PSCs) in the interstitium of the pancreas are known to play a pivotal role in fibrogenesis in chronic pancreatitis<sup>3</sup>. In the normal pancreas, PSCs are estimated to exist at 4–7% of all parenchymal cells<sup>4,5</sup>. When PSCs are stimulated by oxidative stress or various mediators, mainly transforming growth factor (TGF)- $\beta$  or platelet-derived growth factor (PDGF), they are considered to transform into myofibroblasts to produce an extracellular matrix (ECM)<sup>4,6–8</sup>. However, islet fibrosis has not been reported in humans.

On the other hand, islet fibrosis as a part of pancreatitis has been reported in rat diabetic models. For example, Otsuka Long-Evans Tokushima Fatty rats are known to show pancreatitis and pancreatic fibrosis, which develop from islet fibrosis<sup>9</sup>. TGF- $\beta$ 1 has been suggested to be a key mediator for initiation of the lesion, which leads to the accumulation of myofibroblasts and ECM production (fibrosis). From the above information, it was speculated that myofibroblasts and related cytokines could be involved in spontaneous fibrosis/inflammation in the pancreatic islet. It is effective to investigate such a mechanism in the spontaneous lesion of

---

Received: 31 August 2012, Accepted: 21 September 2012

\*Corresponding author: M Imaoka (e-mail: imaoka.masako.hf@daiichisankyo.co.jp)

©2013 The Japanese Society of Toxicologic Pathology

This is an open-access article distributed under the terms of the Creative Commons Attribution Non-Commercial No Derivatives (by-nc-nd) License <<http://creativecommons.org/licenses/by-nc-nd/3.0/>>.

naive animals.

The development of the islet lesion is known to be affected by estrogen. In our previous work, subcutaneous injection of  $\beta$ -estradiol 3-benzoate (EB) for 20 weeks suppressed the occurrence of inflammation and the incidence of fibrosis in the islet of male rats<sup>10</sup>. Genetic and immunohistochemical expressions of estrogen receptors (ERs) have been shown in the pancreatic islet in animals and humans<sup>11,12</sup>, and there is a possibility that ER-dependent signaling contributes to the suppression of these lesions. However, the mechanism of this has also not been clarified. Although estrogen-related fluctuation was examined in our previous work, there was no difference in expression between naive and EB-treated groups<sup>10</sup>.

In this study, we explored the development mechanism of fibrosis in the islets of SD rats. First, to elucidate whether islet fibrosis was correlated with the appearance of myofibroblasts, histological incidence and severity of lesions were examined in conjunction with retrospective immunohistochemistry for  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) and cytokines possibly related to the fibrogenic process in 26- to 102-week-old male rats and in 26-week-old EB-treated males from the previous study. Afterward, immunohistochemistry for ER was reexamined in the animals to clarify whether there was a relationship between ER expression and the incidence of islet lesions.

## Materials and Methods

### *Animals and experimental design*

Experiment I: The male Crl:CD(SD) rats evaluated in this experiment were used for background data collection in our laboratory. A total of 150 animals were purchased from Charles River Laboratories Japan, Inc. (Yokohama, Japan) at 5 weeks of age, housed two or three animals per wire mesh cage in an air-conditioned room (temperature,  $23 \pm 2^\circ\text{C}$ ; relative humidity,  $55 \pm 20\%$ ) with a twelve-hour light/dark cycle. They were allowed free access to a commercially standard diet (CRF-1, Charles River Laboratories Japan, Inc.) and chlorinated tap water during the experimental period, including a quarantine and acclimation period of one week, before being sequentially euthanized under deep anesthesia at 39, 52, 78, or 102 weeks old (the number of sacrificed animals was 30, 30, 40, and 50, respectively). In the present study, paraffin blocks for 5 animals in each age group were randomly selected and used. Additionally, 5 naive animals in Experiment II were used as samples at 26 weeks of age (described later).

Experiment II: As previously reported<sup>10</sup>, male Crl:CD(SD) rats, purchased from Charles River Laboratories Japan, Inc. were divided into two groups (the non-treatment and EB-treatment groups) of 15 animals each. EB was obtained from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan), dissolved in corn oil (Wako Pure Chemical Industries, Ltd.) at  $100 \mu\text{g/ml}$  immediately before administration and subcutaneously administered at  $50 \mu\text{g}/0.5 \text{ ml}$  corn oil/animal once weekly for up to 20 weeks (7 to

26 weeks of age). Housing and feeding conditions were the same as in Experiment I. Animals were euthanized under deep anesthesia at 26 weeks of age. In the present study, paraffin blocks for 5 animals in each group were randomly selected and used.

### *Light microscopy*

The pancreas was carefully removed and immediately fixed in 10% neutral buffered formalin. The tissues were trimmed, embedded in paraffin wax, cut at  $4 \mu\text{m}$  in thickness, stained with hematoxylin and eosin (H&E) and examined light microscopically. In addition, Masson's trichrome stain was applied to the representative sections.

### *Incidence of lesions*

In our previous study, histological findings in the pancreatic islets were described as hemorrhage, fibrosis, and inflammation, and the incidence of these lesions (numbers of affected islets/numbers of total examined islets on the slide) were counted<sup>10</sup>. In Experiment I, the incidence of fibrosis and inflammation was calculated for the total number of the islets examined by a similar method (numbers of fibrotic or inflammatory islets/numbers of total examined islets on the slide) to focus on the purpose of the study. The individual incidence was used for calculation of the group mean incidence. The total numbers of islets examined in the sections ranged from  $48.6 \pm 8.4$  to  $96.6 \pm 19.1$ , with no significant differences among the different ages. Moreover, since atrophy of the islets coexisted with fibrosis, the incidence of atrophy (numbers of atrophic islets/numbers of fibrotic islets on the slide) was counted in Experiment I and in naive animals in Experiment II as samples at 26 weeks of age.

### *Immunohistochemistry*

In Experiment I, immunohistochemical staining of pancreas sections for  $\alpha$ -SMA, PDGF receptor- $\alpha$  (PDGFR $\alpha$ ), TGF- $\beta$ 1, TGF- $\beta$  receptor I (TGF- $\beta$ RI) and ER $\alpha$  was performed using immunoglobulin conjugated to a peroxidase-labeled dextran polymer (EnVision FLEX/HRP, DakoCytomation Co., Ltd., Kyoto, Japan) with 3,3'-diaminobenzidine H<sub>2</sub>O<sub>2</sub> as the chromogen. The sections were then counterstained with hematoxylin. In Experiment II, staining for  $\alpha$ -SMA, PDGFR $\alpha$  and ER $\alpha$  was performed similarly. Detailed immunohistochemical information including antibody dilution is listed in Table 1.

### *Histological score*

To evaluate severity, histological score for fibrosis in H&E sections were taken in animals of Experiment I and naive animals of Experiment II, according to the criteria shown in Table 2. The total of the individual score was divided by the total number of fibrotic islets on the slides in each group, and expressed as a group mean score. In addition, the incidence of each score in the fibrotic islets on the slides was calculated in each group. As for immunohistochemistry in Experiments I and II, the stainabilities of  $\alpha$ -SMA and PDGFR $\alpha$  were scored according to the criteria

**Table 1.** Antibodies Evaluated in the Present Study

Antigen	Abbreviated name	Host species	Clonality	Dilution	Antigen retrieval	Staining system	Manufacturer (city, state, country)	Positive control
$\alpha$ -Smooth muscle actin	$\alpha$ -SMA	Mouse	Monoclonal	Ready to use	Incubated at 97°C for 20 min in target retrieval solution (pH 9.0; Dako)		DAKO Japan, Inc. (Tokyo, Japan)	Rat aorta
Platelet-derived growth factor receptor- $\alpha$	PDGFR $\alpha$	Rabbit	Polyclonal	1:50				
Transforming growth factor- $\beta$ 1	TGF- $\beta$ 1	Rabbit	Polyclonal	1:200	Incubated at 97°C for 40 min in target retrieval solution (pH 9.0; Dako)	DAKO EnVision FLEX/HRP	Santa Cruz Biotechnology, Inc. (Santa Cruz, CA)	Rat uterus
Transforming growth factor- $\beta$ receptor I	TGF- $\beta$ RI	Rabbit	Polyclonal	1:200				
Estrogen receptor- $\alpha$	ER $\alpha$	Mouse	Monoclonal	1:100				
							Novocastra Laboratories (Newcastle upon Tyne, UK)	Rat uterus

**Table 2.** Histological Scores set in the Present Study

Lesion/immunohistochemistry	Scores	Histological characteristics
Fibrosis	0	No fibrosis in the islet
	1	Thin fibrotic tissues are observed focally in/around the islet.
	2	Fibrotic septa dissect the islet into small nests, and the margin of islet is irregular.
	3	Fibrous septa are densely formed, and the islet cells are partly embedded in the septa.
$\alpha$ -SMA and PDGFR $\alpha$	0	Positive reactions are sporadically observed in the islet along the capillary.
	1	The positively stained fibers branch out in the islet.
	2	The branches of positively stained fibers are thick, and the stainability is strong.

shown in Table 2. The islets on the slides were divided into three categories: intact (without fibrosis and inflammation), possessing fibrosis, and possessing inflammation. Individual scores were recorded for the islets on the slides. The total of individual score was divided by the number of islets in each category and expressed as a group mean score in each lesion.

#### Analysis of immunolocalization of ER $\alpha$

In Experiment I and II, the numbers of ER $\alpha$ -positive cells (nuclei) and total numbers of cells in the islet were counted by observation of 10 randomly selected intact and fibrotic islets/rat at 200-fold magnification, and the ER $\alpha$ -positive index (ER $\alpha$ -PI) was calculated. The mean ER $\alpha$ -PI was calculated as the average of each group.

#### Statistical analyses

The quantitative values (the incidence of islets having fibrosis, inflammation and atrophy, and ER $\alpha$ -PI) were expressed as the group mean  $\pm$  standard deviation (S.D.) and statistically analyzed by a Dunnett's multiple comparison test versus the 26-week-old groups. ER $\alpha$ -PI in Experiment II was analyzed by a Student's *t*-test. The histological scores of fibrosis and immunohistochemistry for  $\alpha$ -SMA and PDGFR $\alpha$  were analyzed by a Wilcoxon rank-sum test in Experiments I and II. A *p* value less than 5% (two-tailed) was considered to be significant.

#### Animal welfare

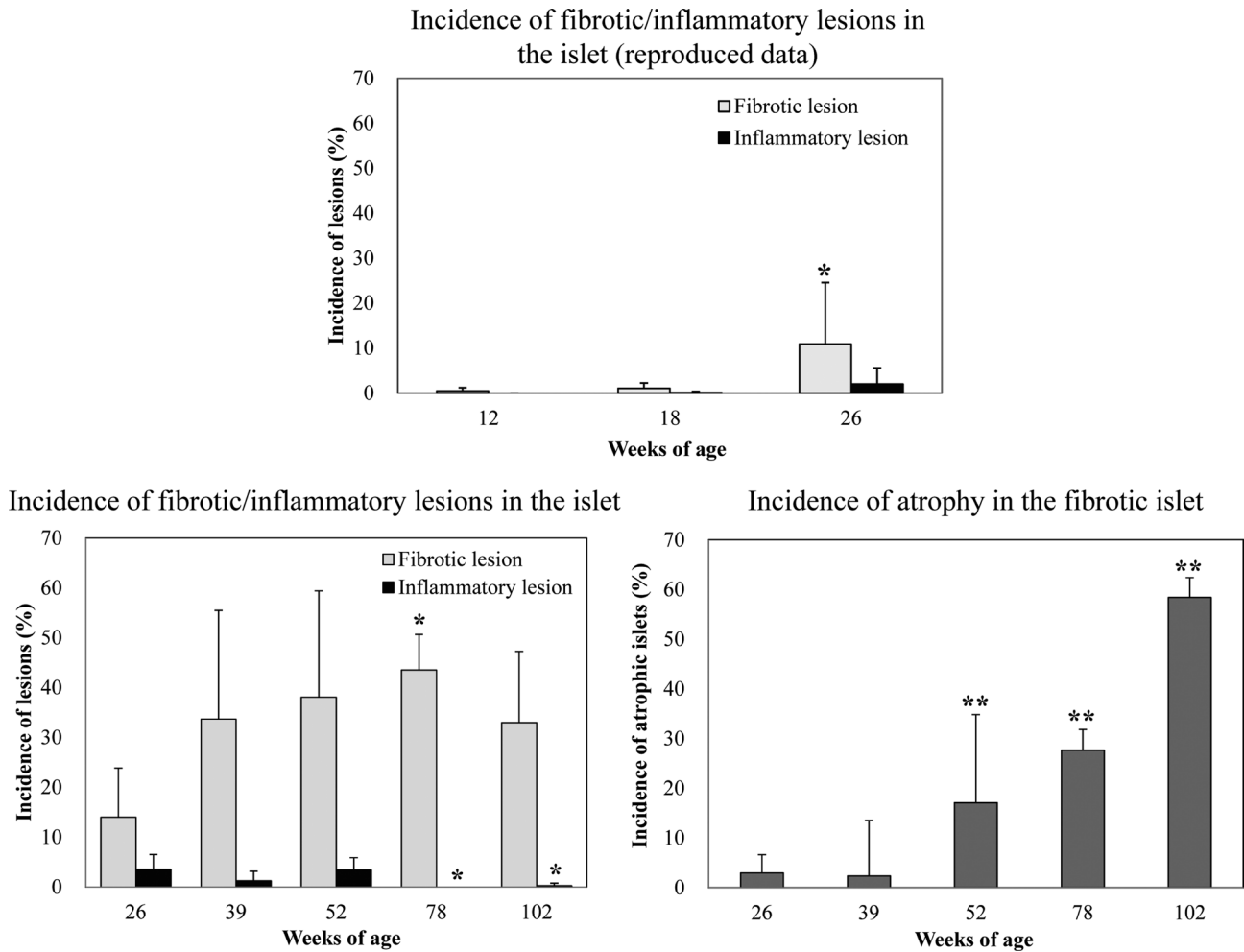
The experimental protocol was approved by the Ethics Review Committee for Animal Experiments of Daiichi Sankyo Co., Ltd. All experimental procedures were performed in accordance with the "Law Concerning the Protection and Control of Animals" and "Standards Relating to the Care and Management, etc. of Experimental Animals" in Japan.

## Results

#### Experiment I

Light microscopy and incidence of lesions: The incidence of fibrotic/inflammatory lesions in the pancreatic islet at 12 to 26 weeks of age (reproduced from our previous study<sup>10</sup>) and over 26 weeks of age in the present study is shown in Fig. 1. In the present study, a high incidence of fibrotic lesions was sustained throughout the study, and a significant increase was observed in the 78-week-old group compared to the 26-week-old group. Additionally, inflammatory lesions were rarely seen at 26 to 52 weeks of age, and 102 weeks of age with significant decreases in the 78- and 102-week-old groups. The incidence of atrophy in fibrotic islets increased at 52 weeks of age or older, as compared to the 26-week-old group.

The histological scores (grades) for fibrosis at 26 through 102 weeks of age are shown in Fig. 2. The scores were generally high (1.64, 1.78, 1.84, 1.55, and 1.45, respec-



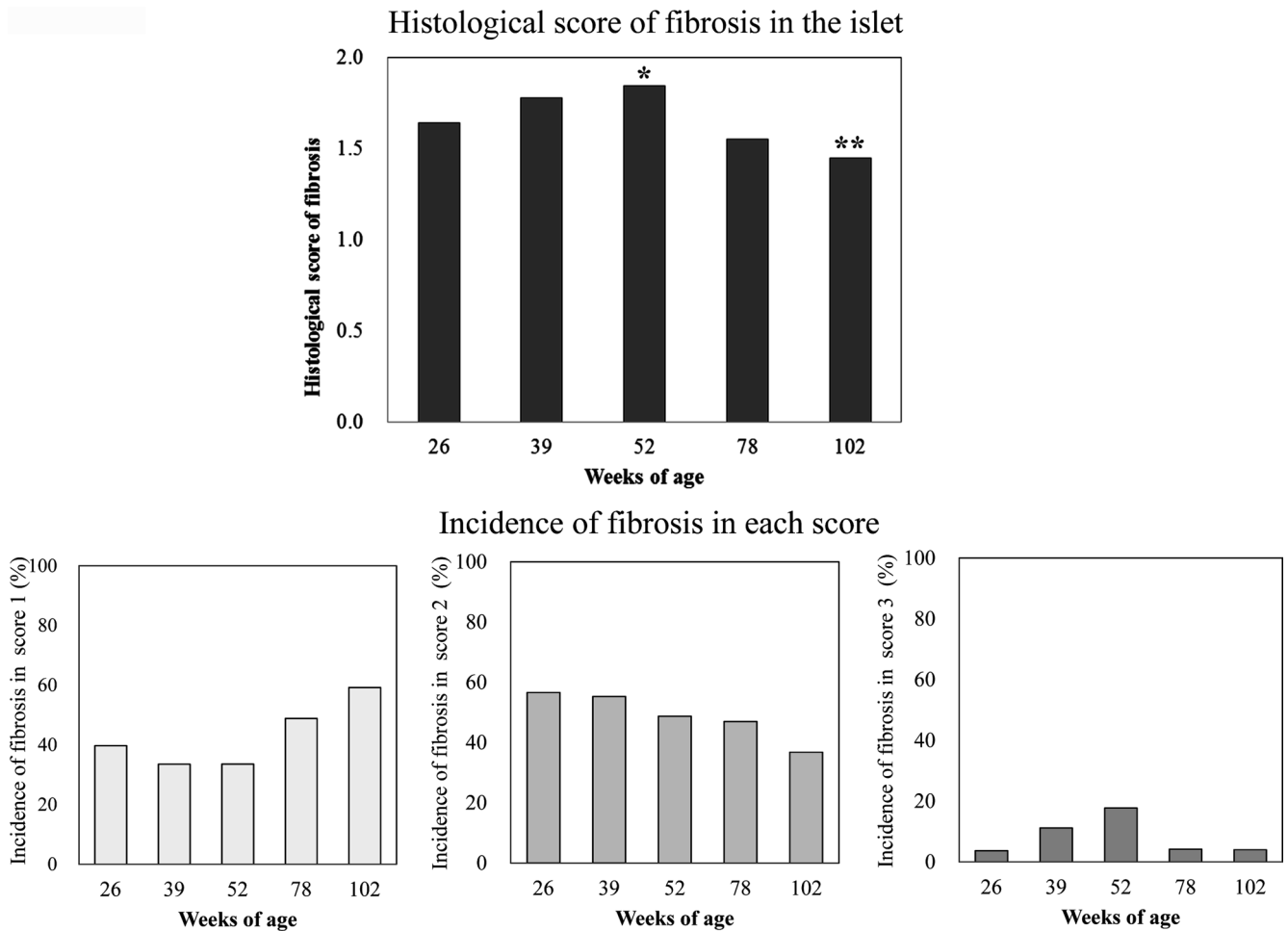
**Fig. 1.** Incidence of fibrotic and inflammatory lesions in the islet of male SD rats at 12 through 26 weeks of age (the upper panel, revised from our previous report) and at 26 through 102 weeks of age in the present study (the lower left panel). The lower right panel shows the incidence of atrophy in the islet of male SD rats aged 26 through 102 weeks. The values are expressed as the group mean  $\pm$  standard deviation (S.D.). \* $p < 0.05$ : Significantly different from the 12-week-old group for the upper panel, and significantly different from the 26-week-old group for the lower panels (Dunnett's multiple comparison test).

tively), and there were significant differences in the 52- and 102-week-old groups as compared to the 26-week-old group. Although the distribution of the scores was not significantly different, an increased tendency for a score of 1 was observed at 102 weeks of age, whereas the score 2 of fibrosis showed tendency of decrease. There was an increase in the tendency for a score of 3 for fibrosis at 52 weeks of age (Fig. 2). Representative photomicrographs of fibrotic and atrophic islets are shown in Figs. 3a, 3c and 3e. Microscopically, fibrosis was observed with brown pigments in the center/periphery of islets. The brownish pigments were stained positively with Prussian blue and confirmed to be hemosiderin (data not shown). In atrophic islets, the islet size was generally small, accompanying the decrease in divided nests of islet cells (Fig. 3e).

Immunohistochemistry and scores of positive reactions: Immunohistochemistry for  $\alpha$ -SMA is shown in Figs. 3b and 3d. A positive reaction was observed as branched fibers in the islets in all the tested groups, but its stainability

differed with age. At 26 weeks of age, most of the fibrotic areas were stained positively (Figs. 3a and 3b). In contrast, there was a decrease in positive reactions at older ages, while fibrosis was similarly observed (Figs. 3b and 3d). These characteristics were expressed as a decreased histological score for  $\alpha$ -SMA (Fig. 4, left panel). As compared to the 26-week-old group, an age-dependent decrease in the score was seen in the fibrotic islets. Inflammatory islets, only seen with extremely low incidence, showed a constantly high score for  $\alpha$ -SMA even at 102 weeks of age. Furthermore, the histological score for PDGFR $\alpha$  was apparently high in inflammatory islets (Fig. 4, right panel). The PDGFR $\alpha$  score sporadically increased at 52 weeks of age in fibrotic islets. A representative inflammatory islet and its immunohistochemical features are shown in Fig. 5. The positive area of  $\alpha$ -SMA corresponded to that of PDGFR $\alpha$ . With regard to TGF- $\beta$ 1 and TGF- $\beta$ RI, sufficient stainabilities were not obtained (data not shown).

ER $\alpha$ -PI and the presence of islet lesions: As shown in



**Fig. 2.** Histological score of fibrosis in the islet of male SD rats at 26 through 102 weeks of age (the upper panel). Incidence of islet fibrosis according to each score at 26 through 102 weeks of age (lower panels). \* $p < 0.05$ ; \*\* $p < 0.01$ : Significantly different from the 26-week-old group (Wilcoxon rank-sum test).

Fig. 6, immunohistochemistry for ER $\alpha$  revealed a positive reaction in the nuclei of islet cells infrequently. The ER $\alpha$ -PI was generally at the same level, and no significant differences were observed in tested ages, except for the sporadic increase of fibrotic islets in 39-week-old animals (Fig. 7). There were no differences between intact and fibrotic islets at any of the weeks of ages.

**Experiment II**

ER $\alpha$ -PI and the presence of islet lesions: For reference, the incidence of lesions in naive and EB-treated animals at 26 weeks of age is shown in Fig. 8 (left panel, reproduced from our previous study<sup>10</sup>). In the EB-treated group, fibrotic and inflammatory lesions were suppressed. The ER $\alpha$ -PI in the EB-treated groups increased significantly. However, in spite of the suppression of the lesions, there was no difference in ER $\alpha$ -PI between intact and fibrotic islets. For inflammatory islets, differences could not be examined because there were no lesions in the EB-treated group.

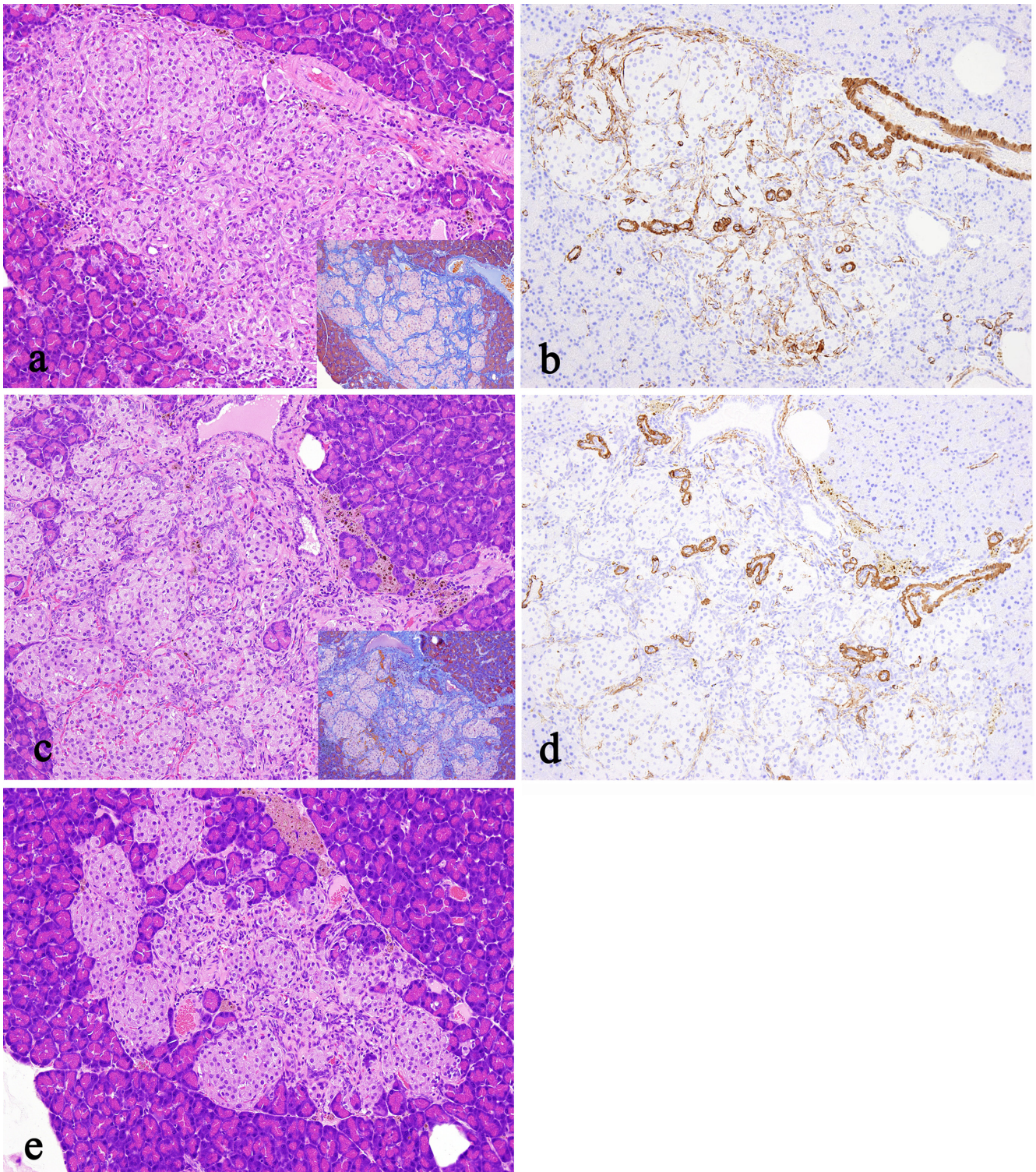
Immunohistochemistry and scores of positive reactions: The histological scores for  $\alpha$ -SMA are shown in Fig.

9. Decreased  $\alpha$ -SMA score in the fibrotic islet was observed in the EB-treated group. The scores for inflammatory islets in the naive group were high. The PDGFR $\alpha$  score was 0 in all animals (data not shown).

**Discussion**

The process and mechanism of spontaneous islet fibrosis were investigated. The incidence of fibrosis was generally high, and the tendency was sustained during the study in the animals at 26 weeks old and older. The total score for fibrosis increased once, at 52 weeks of age, and then decreased at 102 weeks of age. This could be explained by the increased tendency of fibrosis with scores of 3 and 1 at each age examined. At 52 weeks of age or older, the incidence of atrophy in these fibrotic islets increased. These results implied that fibrosis was exacerbated once and that a part of the fibrotic tissue was absorbed until 102 weeks of age. The progression of the lesions was consistent with a general consequence of tissue injury and repair.

In the present study, we detected the involvement of



**Fig. 3.** Micrographs of the pancreatic islets of male SD rats. a: A 26-week-old animal with fibrosis in the islet. The islet was dissected into small segments by abundant collagen fibers. H&E,  $\times 200$ . Inset: blue staining revealed collagen fibers. Masson's trichrome stain,  $\times 200$ . b: Immunohistochemistry for  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) in the same islet as Fig. 3a. A positive reaction was clearly observed in the fibrotic region of the islet.  $\times 200$ . c: A 102-week-old animal with fibrosis in the islet. Fibrous septa was similarly observed with those of the 26-week-old animal shown in Fig. 3a. H&E,  $\times 200$ . Inset: blue staining revealed collagen fibers. Masson's trichrome stain,  $\times 200$ . d: Immunohistochemistry for  $\alpha$ -SMA in the same islet as Fig. 3c. A positive reaction was rarely seen in the islet.  $\times 200$ . e: A 102-week-old animal with fibrosis and atrophy in the islet. The islet size was generally small, accompanying the decrease in divided nests of islet cells. H&E,  $\times 200$ .

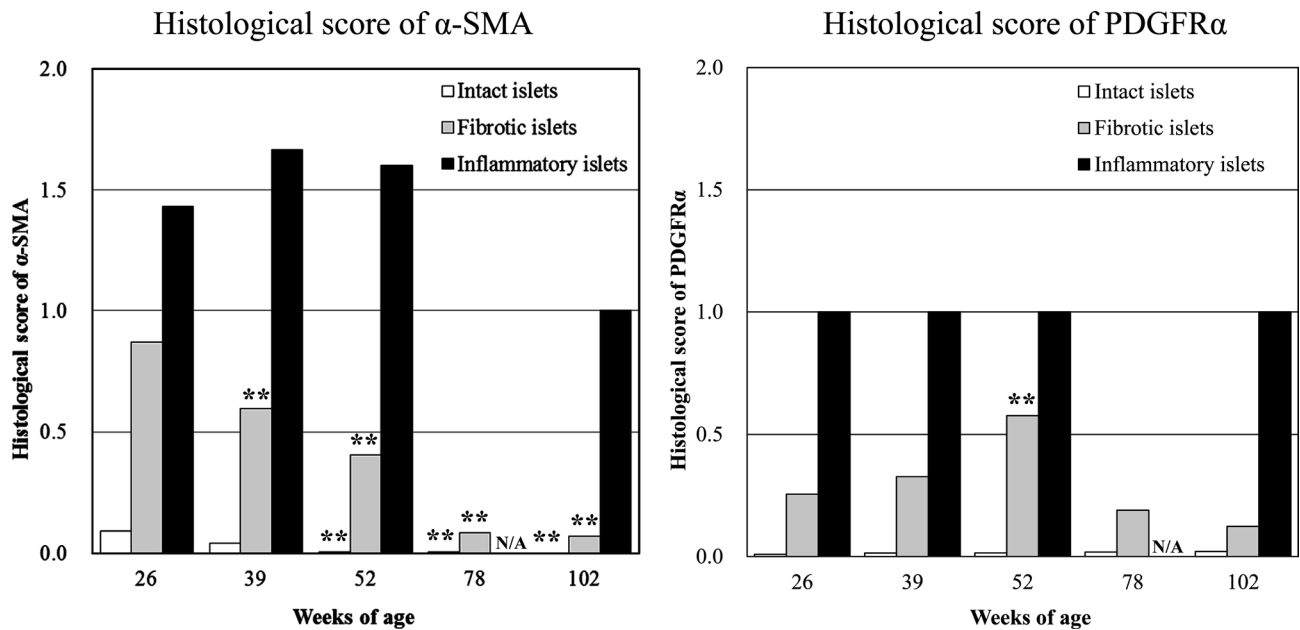


Fig. 4. Histological scores of  $\alpha$ -SMA and platelet-derived growth factor- $\alpha$  (PDGFR $\alpha$ ) in the islet. N/A: not applicable because of the absence of the lesion. \*\* $p$ <0.01: Significantly different from the 26-week-old group (Wilcoxon rank-sum test).

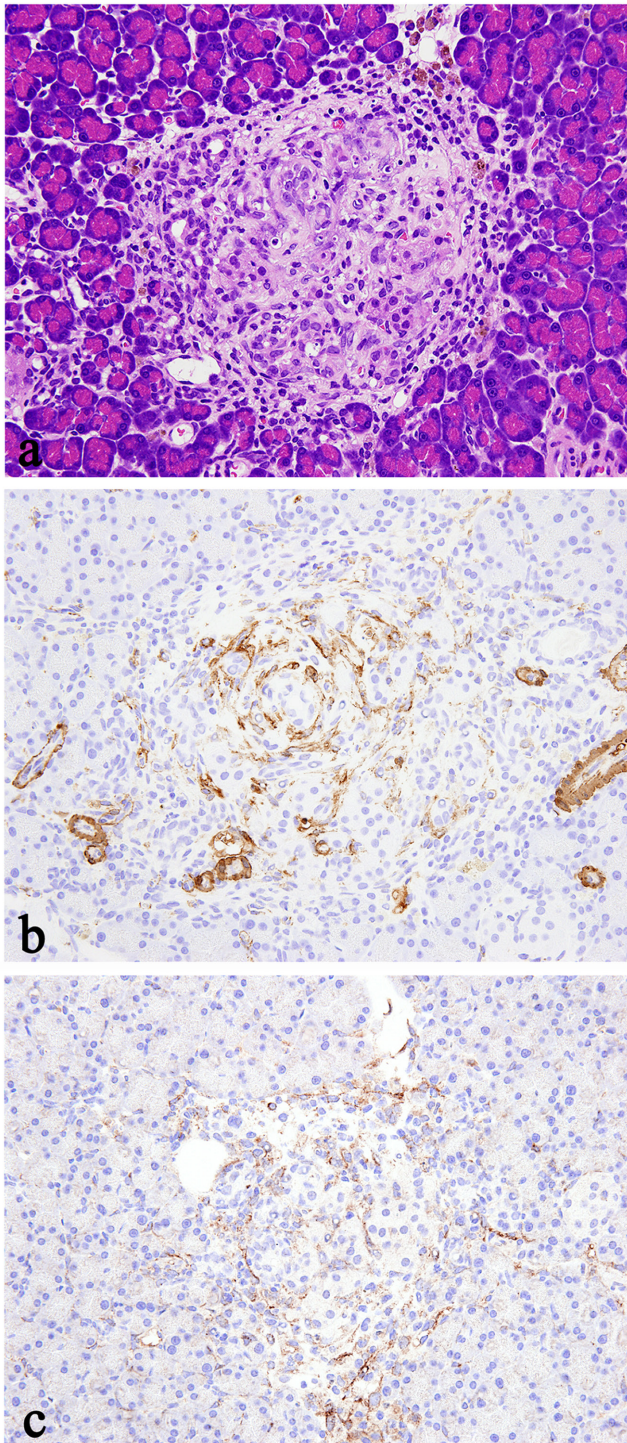
$\alpha$ -SMA-positive cells (myofibroblasts) in islet fibrosis. Although fibrosis was seen with a steadily high incidence at 26 weeks of age or older, an age-dependent decrease in the  $\alpha$ -SMA score was observed. It was considered that myofibroblasts contributed to the fibrogenic process to fix the lesion with “mature” fibrous tissue. Similar to the present results, previous studies have demonstrated that the number of myofibroblasts is inversely correlated with the pathological stage of fibrotic disease in animals and humans<sup>13–16</sup>.

The histological score for  $\alpha$ -SMA in the inflammatory islet was high throughout the study period. PDGFR $\alpha$  was also seen mainly in the inflammatory islet with high scores, and the stainability in both antibodies was histopathologically corresponded. This co-expression of  $\alpha$ -SMA and PDGFR $\alpha$  led us to the speculation that the transformation/activation of myofibroblasts might be caused by an inflammatory reaction like cell infiltration and release of PDGF, while TGF- $\beta$ 1 and its receptor's expressions were not detected successfully in the present study. During tissue repair and inflammatory processes, it has been revealed that PDGF is secreted by various cells including platelets and activated macrophages<sup>17</sup>. Since erythrocyte-containing macrophages were observed in the fibrotic islet ultrastructurally and macrophages are known to be one of the major sources of PDGF, it is possible that there was a slight inflammatory reaction followed by a fibrotic response in the islets<sup>1,17</sup>. PDGF- $\alpha$  and  $\beta$  are known as the most potent modulators of myofibroblasts, extracellular matrix protein synthesis and degradation, resulting in the formation of fibrosis and tissue repair<sup>6–8,14,15,18</sup>. In the pancreatic tissue from patients with alcoholic chronic pancreatitis, the inflammation at an early pathological stage is characterized by extensive myofibroblast appearance

around necrotic foci, and the myofibroblasts showed a positive reaction in PDGFR immunohistochemistry<sup>14</sup>.

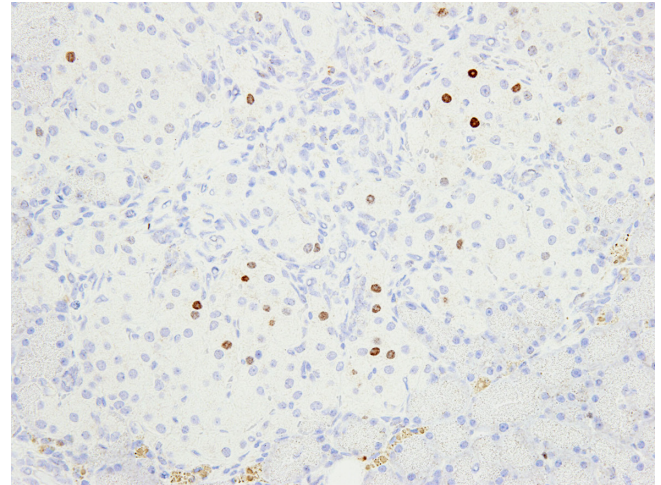
Similar mechanisms of fibrosis has been reported in various organs/tissues: myofibroblast-like transformation from activated hepatic stellate cells (Ito cells) in acute and chronic liver injuries<sup>19</sup>, mesangial and endothelial cells in diabetic renal interstitial fibrosis<sup>20</sup> and pericytes in renal fibrosis following ureteral obstruction or ischemia-reperfusion injury<sup>21</sup> or cutaneous fibrosis<sup>22</sup>. The common mechanisms, transformation into myofibroblasts and ECM production, seem to resemble each other. However, the origin of these ‘myofibroblasts’ have been reported differently by organs. With regard to the pancreas, it is currently unclear whether activated PSCs are different from resident myofibroblasts, smooth muscle cells and pericytes in blood vessels<sup>23,24</sup>. Especially, there are abundant capillaries and pericytes in the islet, and they partially expressed  $\alpha$ -SMA and PDGFR in the present study. The present results are not sufficient to determine the origin of the cells, and further studies will be required.

There are reports demonstrating the suppressive effect of estrogen on fibrosis in several organs. For instance, estradiol treatment resulted in reduced dimethylnitrosamine-induced hepatic fibrosis or angiotensin II-induced cardiac fibrosis in rats by inhibiting the hepatic stellate cell or cardiac fibroblast transformation into myofibroblasts<sup>25,26</sup>. The mechanisms of these phenomena have been partly explained by attenuation of TGF- $\beta$ 1 and/or PDGF signaling. In streptozotocin-induced diabetic rats, 17 $\beta$ -estradiol ( $E_2$ ) diminished glomerular fibrosis in combination with decreases in TGF- $\beta$  and TGF- $\beta$ R expression in the glomerulus<sup>27</sup>.  $E_2$  also inhibits PDGF-induced proliferation and migration of vas-

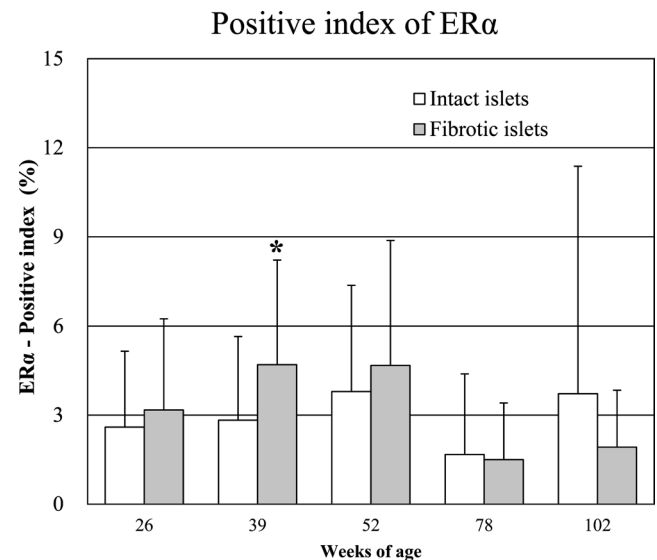


**Fig. 5.** Micrographs of the pancreatic islets of male SD rats. a: A 39-week-old animal. Inflammatory cell infiltration and cell debris were observed in the islet. H&E,  $\times 400$ . b: Immunohistochemistry for  $\alpha$ -SMA in the same islet as Fig. 5a. Similar to Fig. 3b, a positive reaction was seen in the islet.  $\times 400$ . c: Immunohistochemistry for PDGFR $\alpha$  in the same islet as Figs. 5a and 5b. A positive reaction was observed in the fibrotic area corresponding to the  $\alpha$ -SMA positive region.  $\times 400$ .

cular smooth muscle cells *in vitro*<sup>28</sup>. In our present work, a decreased histological score was seen for  $\alpha$ -SMA in the EB-



**Fig. 6.** Immunohistochemistry for estrogen receptor- $\alpha$  (ER $\alpha$ ) in the pancreatic islet cells of a 26-week-old male SD rat. The positive reaction was localized in the islet nucleus.  $\times 400$ .

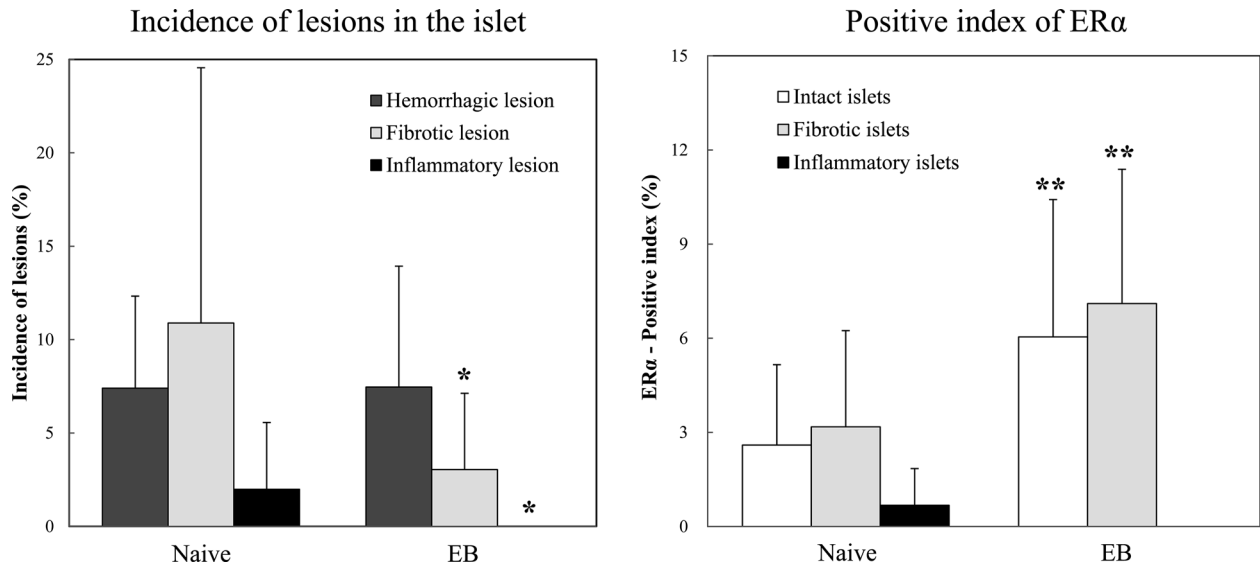


**Fig. 7.** Positive index of ER $\alpha$  in the pancreatic islet of male SD rats. The values are expressed as the group mean  $\pm$  S.D. \* $p < 0.05$ : Significantly different from the 26-week-old group (Dunnnett's multiple comparison test).

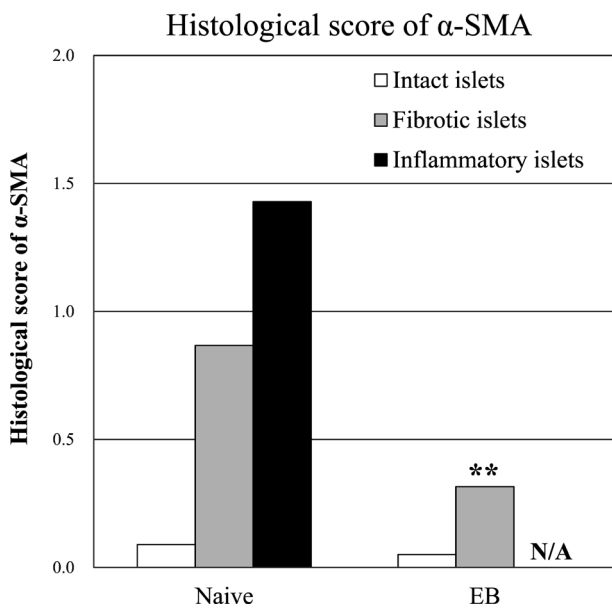
treated group. Moreover, there was no score for PDGFR $\alpha$  in this group. There is a possibility that EB affects the source of the cells directly, to inhibit the transformation into myofibroblasts. On the contrary, the ER $\alpha$ -PI in the EB-treated group was high regardless of the existence of fibrosis. It was suggested that the nuclear-located ER and possibly its downstream signaling were not involved with the islet fibrosis.

In conclusion, it was clarified that a part of age-related fibrosis in islets became atrophy with age, and  $\alpha$ -SMA-positive myofibroblasts were considered to contribute to the development of fibrosis. Strong PDGFR $\alpha$  stainability in fibrotic/inflammatory islets may imply that myofibroblasts





**Fig. 8.** Incidence of lesions in the islet of non-treated (naive) or  $\beta$ -estradiol 3-benzoate (EB)-treated male SD rats aged 12 through 26 weeks of age (left panel, revised from our previous report) and positive index of ER $\alpha$  in the pancreatic islet of naive or EB-treated male SD rats at 26 weeks of age (right panel). The values are expressed as the group mean  $\pm$  S.D. \* $p$ <0.05; \*\* $p$ <0.01: Significantly different from the naive group (Student's  $t$ -test).



**Fig. 9.** Histological scores of  $\alpha$ -SMA in the islet of non-treated (naive) or EB-treated male SD rats at 26 weeks of age. N/A: not applicable because of the absence of the lesion. \*\* $p$ <0.01: Significantly different from the naive group (Wilcoxon rank-sum test).

were stimulated by PDGF to produce an extracellular matrix. Although estradiol has been known to suppress the fibrosis/inflammation in the islet, nuclear-located ER-dependent signaling was considered not to be related to the suppression mechanism. EB possibly affected the inhibition of the appearance of myofibroblasts.

**Acknowledgment:** The authors thank Dr. T. Shibutani for her valuable advice and expert assistance with immunohistochemistry.

**References**

1. Imaoka M, Satoh H, and Furuhashi K. Age- and sex-related differences in spontaneous hemorrhage and fibrosis of the pancreatic islets in Sprague-Dawley rats. *Toxicol Pathol.* **35**: 388–394. 2007. [Medline] [CrossRef]
2. Dillberger JE. Age-related pancreatic islet changes in Sprague-Dawley rats. *Toxicol Pathol.* **22**: 48–55. 1994. [Medline] [CrossRef]
3. Shimizu K. Pancreatic stellate cells: Molecular mechanism of pancreatic fibrosis. *J Gastroenterol Hepatol.* **23**: S119–S121. 2008. [Medline] [CrossRef]
4. Apte MV, Haber PS, Applegate TL, Norton ID, McCaughan GW, Korsten MA, Pirola RC, and Wilson JS. Periacinar stellate shaped cells in rat pancreas: identification, isolation, and culture. *Gut.* **43**: 128–133. 1998. [Medline] [CrossRef]
5. Bachem MG, Schneider E, Gross H, Weidenbach H, Schmid RM, Menke A, Siech M, Beger H, Grünert A, and Adler G. Identification, culture, and characterization of pancreatic stellate cells in rats and humans. *Gastroenterology.* **115**: 421–432. 1998. [Medline] [CrossRef]
6. Apte MV, Haber PS, Darby SJ, Rodgers SC, McCaughan GW, Korsten MA, Pirola RC, and Wilson JS. Pancreatic stellate cells are activated by proinflammatory cytokines: implications for pancreatic fibrogenesis. *Gut.* **44**: 534–541. 1999. [Medline] [CrossRef]
7. Shek FW, Benyon RC, Walker FM, McCrudden PR, Pender SL, Williams EJ, Johnson PA, Johnson CD, Bateman AC, Fine DR, and Iredale JP. Expression of transforming growth factor-beta 1 by pancreatic stellate cells and its implications for matrix secretion and turnover in chronic pancreatitis.

- Am J Pathol. **160**: 1787–1798. 2002. [[Medline](#)] [[CrossRef](#)]
8. Luttenberger T, Schmid-Kotsas A, Menke A, Siech M, Berger H, Adler G, Grünert A, and Bachem MG. Platelet-derived growth factors stimulate proliferation and extracellular matrix synthesis of pancreatic stellate cells: implications in pathogenesis of pancreas fibrosis. *Lab Invest*. **80**: 47–55. 2000. [[Medline](#)] [[CrossRef](#)]
  9. Yoshikawa H, Kihara Y, Taguchi M, Yamaguchi T, Nakamura H, and Otsuki M. Role of TGF-beta1 in the development of pancreatic fibrosis in Otsuka Long-Evans Tokushima Fatty rats. *Am J Physiol Gastrointest Liver Physiol*. **282**: G549–G558. 2002. [[Medline](#)]
  10. Imaoka M, Kato M, Tago S, Gotoh M, Satoh H, and Manabe S. Effects of estradiol treatment and/or ovariectomy on spontaneous hemorrhagic lesions in the pancreatic islets of Sprague-Dawley rats. *Toxicol Pathol*. **37**: 218–226. 2009. [[Medline](#)] [[CrossRef](#)]
  11. Winborn WB, Sheridan PJ, and McGill HC. Estrogen receptors in the islets of Langerhans of baboons. *Cell Tissue Res*. **230**: 219–223. 1983. [[Medline](#)] [[CrossRef](#)]
  12. Adachi T, Yasuda K, Mori C, Yoshinaga M, Aoki N, Tsujimoto G, and Tsuda K. Promoting insulin secretion in pancreatic islets by means of bisphenol A and nonylphenol via intracellular estrogen receptors. *Food Chem Toxicol*. **43**: 713–719. 2005. [[Medline](#)] [[CrossRef](#)]
  13. Kuroda N, Toi M, Nakayama H, Miyazaki E, Yamamoto M, Hayashi Y, Hiroi M, and Enzan H. The distribution and role of myofibroblasts and CD34-positive stromal cells in normal pancreas and various pancreatic lesions. *Histol Histopathol*. **19**: 59–67. 2004. [[Medline](#)]
  14. Detlefsen S, Sipos B, Feyerabend B, and Klöppel G. Fibrogenesis in alcoholic chronic pancreatitis: the role of tissue necrosis, macrophages, myofibroblasts and cytokines. *Mod Pathol*. **19**: 1019–1026. 2006. [[Medline](#)]
  15. Detlefsen S, Sipos B, Zhao J, Drewes AM, and Klöppel G. Autoimmune pancreatitis: expression and cellular source of profibrotic cytokines and their receptors. *Am J Surg Pathol*. **32**: 986–995. 2008. [[Medline](#)] [[CrossRef](#)]
  16. McGowan SE, Grossmann RE, Kimani PW, and Holmes AJ. Platelet-derived growth factor receptor-alpha-expressing cells localize to the alveolar entry ring and have characteristics of myofibroblasts during pulmonary alveolar septal formation. *Anat Rec (Hoboken)*. **291**: 1649–1661. 2008. [[Medline](#)] [[CrossRef](#)]
  17. Ross R. Platelet-derived growth factor. *Lancet*. **1**: 1179–1182. 1989. [[Medline](#)] [[CrossRef](#)]
  18. Masamune A, Kikuta K, Satoh M, Kume K, and Shimosegawa T. Differential roles of signaling pathways for proliferation and migration of rat pancreatic stellate cells. *Tohoku J Exp Med*. **199**: 69–84. 2003. [[Medline](#)] [[CrossRef](#)]
  19. Yoshida K, and Matsuzaki K. Differential regulation of TGF-beta/Smad signaling in hepatic stellate cells between acute and chronic liver injuries. *Front Physiol*. **3**: 53. 2012. [[Medline](#)]
  20. Li J, Qu X, and Bertram JF. Endothelial-myofibroblast transition contributes to the early development of diabetic renal interstitial fibrosis in streptozotocin-induced diabetic mice. *Am J Pathol*. **175**: 1380–1388. 2009. [[Medline](#)] [[CrossRef](#)]
  21. Chen YT, Chang FC, Wu CF, Chou YH, Hsu HL, Chiang WC, Shen J, Chen YM, Wu KD, Tsai TJ, Duffield JS, and Lin SL. Platelet-derived growth factor receptor signaling activates pericyte-myofibroblast transition in obstructive and post-ischemic kidney fibrosis. *Kidney Int*. **80**: 1170–1181. 2011. [[Medline](#)] [[CrossRef](#)]
  22. Juniantito V, Izawa T, Yuasa T, Ichikawa C, Yamamoto E, Kuwamura M, and Yamate J. Immunophenotypical analyses of myofibroblasts in rat excisional wound healing: possible transdifferentiation of blood vessel pericytes and perifollicular dermal sheath cells into myofibroblasts. *Histol Histopathol*. **27**: 515–527. 2012. [[Medline](#)]
  23. Erkan M, Adler G, Apte MV, Bachem MG, Buchholz M, Detlefsen S, Esposito I, Friess H, Gress TM, Habisch HJ, Hwang RF, Jaster R, Kleeff J, Klöppel G, Kordes C, Logsdon CD, Masamune A, Michalski CW, Oh J, Phillips PA, Pinzani M, Reiser-Erkan C, Tsukamoto H, and Wilson J. StellaTUM: current consensus and discussion on pancreatic stellate cell research. *Gut*. **61**: 172–178. 2012. [[Medline](#)] [[CrossRef](#)]
  24. Hayden MR, Karuparthi PR, Habibi J, Wasekar C, Lastra G, Manrique C, Stas S, and Sowers JR. Ultrastructural islet study of early fibrosis in the Ren2 rat model of hypertension. Emerging role of the islet pancreatic pericyte-stellate cell. *JOP*. **8**: 725–738. 2007. [[Medline](#)]
  25. Shimizu I, Mizobuchi Y, Yasuda M, Shiba M, Ma YR, Horie T, Liu F, and Ito S. Inhibitory effect of oestradiol on activation of rat hepatic stellate cells in vivo and in vitro. *Gut*. **44**: 127–136. 1999. [[Medline](#)] [[CrossRef](#)]
  26. Wu M, Han M, Li J, Xu X, Li T, Que L, Ha T, Li C, Chen Q, and Li Y. 17beta-estradiol inhibits angiotensin II-induced cardiac myofibroblast differentiation. *Eur J Pharmacol*. **616**: 155–159. 2009. [[Medline](#)] [[CrossRef](#)]
  27. Dixon A, and Maric C. 17beta-Estradiol attenuates diabetic kidney disease by regulating extracellular matrix and transforming growth factor-beta protein expression and signaling. *Am J Physiol Renal Physiol*. **293**: F1678–F1690. 2007. [[Medline](#)] [[CrossRef](#)]
  28. Kappert K, Caglayan E, Huntgeburth M, Bäumer AT, Sparwel J, Uebel M, and Rosenkranz S. 17Beta-estradiol attenuates PDGF signaling in vascular smooth muscle cells at the postreceptor level. *Am J Physiol Heart Circ Physiol*. **290**: H538–H546. 2006. [[Medline](#)] [[CrossRef](#)]