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Ramipril treatment suppresses islet fibrosis in Otsuka Long–Evans Tokushima fatty rats

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Abstract

We evaluated whether ramipril, one of long-acting ACEIs, has a direct effect on pancreas islets in animal model of type 2 diabetes. OLETF rats were treated with ramipril for 24 weeks. We assessed the body weight, glucose tolerance, and the amount of islet fibrosis. RT-PCR and Western blot analysis of transforming growth factor-β with its downstream signals were performed from the pancreas. Ramipril treatment remarkably reduced weight gain and the area under the curve of glucose. Islet fibrosis and the expression of TGF-β with its downstream signal molecules were significantly reduced in the pancreas of ramipril-treated group than in control and paired-feeding group. These beneficial effects of ramipril might be related to the downregulation of TGF-β and its downstream signals in OLETF rats. To our knowledge, this is the first report suggesting the potential effect of ramipril on the prevention of islet destruction by fibrosis in the animal model of type 2 diabetes mellitus.

Keywords: Ramipril; Otsuka Long–Evans Tokushima fatty rat; Islet fibrosis; Transforming growth factor-β; α-Smooth muscle actin

Diabetes mellitus is a common group of metabolic disorders resulting from hyperglycemia. The chronic complications of diabetes affect many organ systems and are responsible for most morbidity and mortality. Considering the recent dramatic increase in the number of type 2 diabetic patients and the enormous costs for their medical care, many clinical trials have been undertaken to prevent type 2 diabetes mellitus before onset [1]. Recently reported Heart Outcomes Prevention Evaluation (HOPE) study and Losartan Intervention for Endpoint reduction in hypertension study (LIFE) clearly showed that ramipril and losartan remarkably reduced the incidence of diabetes [2–4]. But their exact mechanism is not clarified.

Otsuka Long–Evans Tokushima fatty (OLETF) rats form a well-known animal model of type 2 diabetes mellitus, developing extensive atrophy with connective tissue proliferation of the pancreas [5,6]. Morphologically, the glomerular sclerotic changes seen in diabetic nephropathy are similar to those of the pancreatic islets of OLETF rats, and TGF-β1 is involved in the pathogenesis of pancreatic fibrosis in this model [6]. In addition to this animal model, fibrotic change of pancreas, especially amyloid deposition with islet fibrosis, is often observed in humans with long-standing diabetic mellitus [7,8]. Until now, so many evidences exist that angiotensin converting enzyme inhibitors (ACEIs) prevent the progression of diabetic nephropathy or renal fibrosis. In addition, previous reports demonstrated that ACEIs attenuated pancreatic inflammation and fibrosis in rats, and pancreatic stellate cells expressing α-smooth muscle actin (α-SMA) are also associated with the development of pancreatic fibrosis [6,9,10].

Moreover, some studies have described the presence of angiotensin II (AT II) receptors on the surface of

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pancreatic islet β-cells in rats [11–13]. In the human pancreas, the AT1 receptor has been identified in islets [14], especially on β-cells and endothelial cells, although this requires additional clarification. Thus, ACEIs might have a direct influence on pancreatic islets in animal models or in patients suffering type 2 diabetes mellitus.

We hypothesized that long-term treatment using ACEI might attenuate the progression or development of type 2 diabetes mellitus through its direct effect on pancreatic islets. This study aimed to determine whether ramipril could prevent the deterioration of glucose tolerance and the fibrotic changes in pancreatic islets by modulating the gene expression of TGF-β and its downstream signal molecules in OLETF rats.

Materials and methods

Materials. Forty-eight male OLETF rats (Otsuka Pharmaceutical, Tokushima Research Institute, Japan) aged 20 weeks, weighing between 400 and 450 g, were randomly allocated to either treatment with ramipril (Aventis Pharmaceutical, Germany) or no treatment for 24 weeks (O-RAM5, 5 mg/kg ramipril dissolved in their drinking water, $n = 16$: O-PF, paired-feeding group to O-RAM5 without ramipril treatment, $n = 8$: O-RAM1, 1 mg/kg ramipril, $n = 8$: and O-CON, control rats fed ad libitum without ramipril, $n = 16$). Long–Evans Tokushima Otsuka (LETO) rats, weighing between 350 and 400 g, were also randomized as OLETF rats (L-RAM5, $n = 16$; L-PF, paired-feeding group, $n = 8$; L-RAM1, $n = 8$; and L-CON, $n = 16$) and used as non-diabetic controls. We added the paired-feeding group to clarify the effect of ramipril, independent of body weight. For paired-feeding, the amount of consumed chow in O-RAM5 and L-RAM5 groups was calculated and this amount of food was used for O-PF and L-PF groups to match with their corresponding groups.

Body weights were measured weekly. After 24 weeks of ramipril treatment, all experimental rats were sacrificed except for a half of those of O-RAM5 ($n = 8$) and O-CON ($n = 8$) of OLETF rats and L-RAM5 ($n = 8$) and L-CON ($n = 8$) of LETO rats. Remnant OLETF and LETO rats were then fed with an additional 30% sucrose in their drinking water to exacerbate their hyperglycemic status from the 24th to the 32nd week. Oral glucose tolerance test (OGTT) was performed before and after this loading period, and all animals were euthanized.

Oral glucose tolerance test. After overnight fasting, an oral glucose tolerance test using 25% glucose solution (2 g/kg) was performed. Blood samples were obtained by tail snipping. Blood glucose levels were measured using a glucose oxidase method (Glucometer Precision, MEDISENSE Contract Manufacturing, UK). The area under the curve of glucose (AUCg) was calculated by trapezoidal estimation from the values obtained at OGTT.

Fasting insulin level and homeostasis model assessment of insulin resistance index. For measuring fasting plasma insulin concentrations, blood samples were obtained after overnight fasting, at the same time as the first OGTT sample. The plasma insulin level in the sample was measured in duplicate by radioimmunoassay (Dainabot Corporation kit, Tokyo, Japan).

For estimation of the degree of insulin resistance, we calculated the homeostasis model assessment of insulin resistance (HOMA-IR) as follows: HOMA-IR = fasting insulin concentration (μIU/ml) × fasting glucose concentration (mmol/L)/22.5 [15]. HOMA of β-cell function (HOMA-B) was calculated as 20 × fasting insulin concentration/(fasting glucose concentration −3.5) [16].

Immunohistochemistry. Pancreases were removed and weighed. One set (approximately one-third of the tissues) was stored at −70 °C for subsequent reverse transcription polymerase chain reaction (RT-PCR) analysis. One-third of the pooled tissues were used for Western blot analysis. The remaining tissues were fixed and then embedded in paraffin. For the measurement of β-cell mass, an immunohistochemical staining was performed using the streptavidin–biotin–peroxidase method. After overnight incubation with insulin antibody (Linco Research, St. Charles, MO, USA), sections were developed with diaminobenzidine tetrahydrochloride (DAB) and then counterstained with hematoxylin. To investigate the degree of matrix accumulation and fibrosis in the pancreatic islets, sections were stained using Masson's trichrome method [17]. Sections were also stained using a polyclonal rabbit anti-human TGF-β antibody (Santa Cruz, CA, USA) [18] and mouse smooth muscle α-actin (α-SMA, Sigma, St. Louis, MO) using a commercial kit (DAKO, Denmark), and developed with DAB.

β-Cell mass quantification in pancreatic islets. The relative volumes of the β-cells were measured by the point counting method [19] using an Olympus BH-2 microscope connected to a video camera equipped with a color monitor with a 90-point transparent overlay under 200× magnification. The β-cells were counted in the slide after immunostaining with anti-insulin antibody. An average of 398.8 fields and 35896.3 points in non-overlapping fields were counted systematically from each section; one section was counted per tissue block. The relative β-cell volume in pancreatic tissue was described as follows: number of points corresponding to the anti-insulin antibody-stained area/number of points corresponding to the remaining pancreatic area. Cell masses were calculated by multiplying the relative percentages of β-cells by the total pancreatic weight [20].

Measurement of fibrosis and of the α-SMA-stained area in pancreatic islets. To calculate the relative percentage of blue-stained areas within islets in trichrome-stained slides, each section was processed by planimetry using an image analyzer (Optimas 6.51, Media Cybertics, Tempe, AR, USA). The amount of collagen deposition was calculated from a mean of 496 non-overlapping islets per pancreas. The amount of islet fibrosis is presented as a percentage of the total islet area and was determined as (area of fibrosis/total area of islets) × 100.

The α-SMA-stained areas were calculated as (area of α-SMA-stained area/total area of islet) × 100. Because the fibrosis of islets was observed predominantly in large islets, we analyzed islet fibrosis and the α-SMA-stained areas only in islets that were larger than the median islet size of the LETO control rats (15,957 μm^2).

RT-PCR analysis of TGF-β and its downstream regulators. Total RNA from the frozen pancreatic tissues of each group was extracted using Trizol (Gibco-BRL, Grand Island, NY, USA). Total RNA was identified using gel electrophoresis and quantified by spectrophotometry. Total RNA (4 μg) was used to synthesize first-strand cDNA according to the protocol of the SuperScript preamplification system (Gibco-BRL) [11]. After incubating total RNA with 0.5 μg oligo$(dT)_{12–18}$ primer at 70 °C for 10 min, the reaction was carried out in a mixture of 5 μl first-strand buffer in a final volume of 20 μl. The mixed contents of the tube were incubated and Superscript II transcriptase was added. After denaturation at 70 °C for 15 min, the final mixture was used directly for PCR amplification. The relative expression levels of genes isolated from pancreatic tissue were determined according to the expression level of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene. The gene-specific primers were designed on the basis of published sequences. The PCR primers were as follows: TGF-β, sense 5′-ATCGACATGGAGCTGGTGA-3′, antisense 5′-TTGGCATGGTAGCCCTTGG-3′; connective tissue growth factor (CTGF), sense 5′-CACTCGGTGAGGCTGAAG-3′, anti-sense 5′-ACTGCCTCCCAAACCAGT-3′; anti-sense 5′-AGGGAAGAAG AGGAAGCAG-3′; fibronectin, sense 5′-GCCTCCAGACCCTACT GT-3′, anti-sense 5′-TCCACACGGTATCCAGTC-3′; and GAPDH, sense 5′-ACCACAGTCCATGCCATCAC-3′, anti-sense 5′-TCCACC ACCCTGTTGCTGTA-3′. Reactions were carried out in a volume of 25 μl containing the corresponding sense and anti-sense primer sequences using a PCR reagent system (Gibco-BRL). The PCR conditions were as follows: after boiling at 94 °C for 4 min, amplification was repeated for 30 cycles at 94 °C for 1 min, 55 °C for 1 min, and 72 °C for

2 min. The mixture was then incubated at 72 °C for 10 min to complete DNA elongation and finally stored at 4 °C. The density of each band was measured using a VDS densitometer (Pharmacia Biotech, CA, USA).

Western blot analysis. Frozen pancreatic tissue pieces were homogenized using a Polytrol homogenizer in ice-cold lysis buffer. Protein concentrations were measured using the Bradford method. Proteins (20 μg) separated by SDS–PAGE were transferred onto polyvinylidene difluoride membranes (Hybond-P, Amersham, Buckinghamshire, UK). The membranes were blocked and then incubated with anti-TGF-β (Santa Cruz, CA, USA), anti-CTGF (Santa Cruz, CA, USA), anti-fibronectin (DAKO, Denmark), and anti-a-SMA (Sigma, St. Louis, MO). After washing, the membranes were incubated with a secondary peroxidase-coupled antibody for 1 h at room temperature. An antibody detection system (ECL, Amersham–Pharmacia) was applied and the membranes were exposed to X-ray films. Protein band intensities were quantified using a VSD densitometer (Amersham–Pharmacia).

Statistical analysis. Data are expressed as means ± SD. Differences between means were evaluated using the SPSS 10.0 program (Chicago, USA). One-way analysis of variance (ANOVA) with the Bonferroni correction was used to analyze the quantitative variables among groups. A P value <0.05 was considered significant.

Results

OGTT at 24 and 32 weeks

At 32 weeks, the mean fasting and 2 h blood glucose levels of O-RAM5 group were significantly different from those of O-CON (fasting glucose, O-RAM5 vs. O-CON, 6.5 ± 0.7 vs. 8.1 ± 1.1 mmol/L, $P < 0.05$; 2 h glucose, O-RAM5 vs. O-CON, 9.9 ± 2.9 vs. 15.3 ± 7.0 mmol/L, $P < 0.05$). LETO rats showed a significant difference in terms of 2 h glucose levels (L-RAM5 vs. L-CON, 6.0 ± 2.9 vs. 7.0 ± 4.2 mmol/L, $P < 0.05$).

The mean value of the AUCg was significantly higher in O-CON group animals than in O-RAM5 group during OGTT (69.9 ± 8.8 vs. 63.4 ± 8.0 mmol/L/min, $P < 0.05$), but no difference was detected between L-RAM5 and L-CON group at 24 weeks. After exposure to sucrose for additional eight weeks, the difference in AUCg between O-RAM5 and O-CON group was more exaggerated (O-RAM5, 63.5 ± 8.5 vs. O-CON, 77.9 ± 6.6 mmol/L/min, $P < 0.05$, Fig. 1).

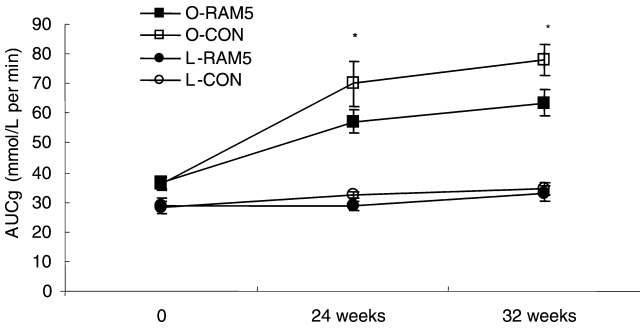

Fig. 1. Area under the curve of glucose (AUCg) at 6 months after ramipril treatment and then 8 weeks after 30% sucrose loading during OGTT. After exposure to sucrose for 8 weeks, the difference of AUCg between ramipril-treated group and control group was more exaggerated in OLETF rats. *$P < 0.05$ vs. control group of OLETF rats. ■, OLETF 5 mg/kg (O-RAM5); □, OLETF control (O-CON); ●, LETO 5 mg/kg (L-RAM5); and ○, LETO control group (L-CON).

Fasting insulin, HOMA-IR, HOMA-B, and insulinogenic index

At 32 weeks, HOMA-B and $\Delta I_{30}/\Delta G_{30}$ values (ratio of changes of insulin to glucose concentration for 30 min following oral glucose challenge) were significantly increased in O-RAM5 group in OLETF rats. In LETO rats, only $\Delta I_{30}/\Delta G_{30}$ value was significantly different between the treated and non-treated groups (Table 1). However, all the values of fasting insulin, HOMA-IR, HOMA-B, and insulinogenic index between the OLETF and LETO rat types were statistically significant (Table 1).

β-Cell mass, islet fibrosis, TGF-β, and α-SMA expression in pancreatic tissue

The mean value of the β-cell mass in the pancreases was not different among groups in OLETF and LETO rats at 24 and 32 weeks. At 24 weeks, the islets were enlarged, and connective tissue proliferation surrounding the islets was evident, especially in O-CON group. The degree of islet fibrosis declined in the order: O-CON > O-PF > O-RAM1 > O-RAM5 (Figs. 2A–D). Ramipril treatment was associated with less amount of islet fibrosis at 24 and 32 weeks in OLETF rats (O-RAM5 vs. O-CON, $P < 0.05$, Table 2). A similar overall pattern was observed in LETO rats, but structural changes of the pancreatic islets were much less prominent and islet architecture was relatively well preserved.

Immunoreactivity for TGF-β was detected in the relatively preserved or degenerated islets, in duct cells adjacent to destroyed islets, and in vessels and surrounding connective tissues. However, in O-CON and O-PF groups, TGF-β expression was noted more extensively in the whole pancreatic tissues, especially adjacent to disorganized islets and proliferating duct cells (Figs. 2F and H).

The α-SMA staining showed a characteristic pattern. In addition to normally expressed α-SMA in the vascular smooth muscle cells and exocrine tissue, multiple ring-shaped intra-islet immunostaining, suggestive of proliferating microvessels, was also observed (Figs. 2I–L). This pattern was more frequent in larger and more degenerated islets of the O-CON rats (Fig. 2L). α-SMA expression was also observed in the peri-islet area of advanced fibrotic islets. The ratio of the α-SMA-stained area to the total islet area measured by planimetry was significantly higher in O-CON group than in O-RAM5 group (Table 2).

Table 1
Fasting insulin levels and the homeostasis model assessment of insulin resistance (HOMA) index after ramipril treatment

	24 weeks				32 weeks			
	Insulin (pmol/L)	HOMA-B	HOMA-IR	$\Delta I_{30}/\Delta G_{30}$	Insulin (pmol/L)	HOMA-B	HOMA-IR	$\Delta I_{30}/\Delta G_{30}$
OLETF								
O-RAM5	8.5 ± 4.4	4.8 ± 2.9	0.39 ± 0.2	3.8 ± 1.8	7.8 ± 4.4	$7.3 \pm 3.4^{*}$	0.32 ± 0.2	$2.1 \pm 1.0^{*}$
O-CON	5.9 ± 4.8	4.6 ± 3.4	0.26 ± 0.2	2.5 ± 1.9	5.9 ± 4.9	3.5 ± 2.5	0.31 ± 0.3	1.5 ± 0.4
LETO								
L-RAM5	9.0 ± 4.9	22.7 ± 13.2	0.29 ± 0.2	10.5 ± 5.7	8.0 ± 6.1	12.0 ± 6.4	0.30 ± 0.2	$7.3 \pm 4.3^{**}$
L-CON	9.5 ± 5.3	17.8 ± 11.7	0.30 ± 0.2	8.8 ± 3.1	9.5 ± 5.3	8.9 ± 5.2	0.34 ± 0.2	5.5 ± 1.6

Data are means ± SD. HOMA-IR, homeostasis model assessment of insulin resistance; HOMA-B, homeostasis model assessment of β-cell function; and $\Delta I_{30}/\Delta G_{30}$, ratio of changes of insulin to glucose concentration for 30 min following oral glucose challenge (pmol/mol/L).

$^{*}P < 0.05$ vs. O-CON group.

$^{**}P < 0.05$ vs. L-CON group.

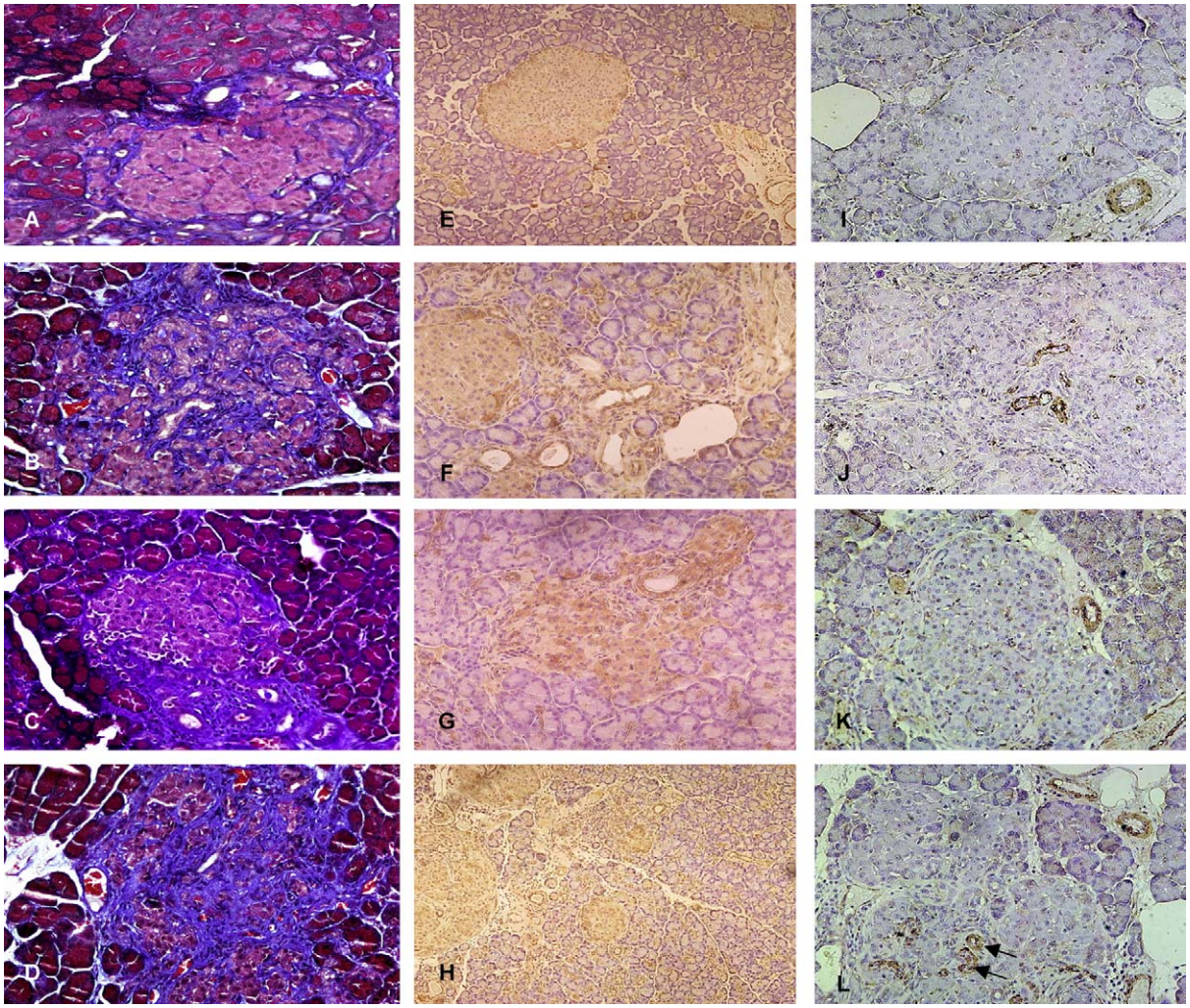

Fig. 2. Masson's trichrome method combined with immunostaining of pancreatic tissues in OLETF rats (A–D). Morphological islet fibrosis and destructive islet changes were more prominent in O-CON (D) compared with O-RAM5 (A), O-PF (B), and O-RAM1 (C) (200×). Representative findings of immunohistochemical staining of TGF-β (E–H) and α-SMA (I–L) in the pancreatic tissue of OLETF rats. In ramipril-treated groups (E,G), TGF-β expression (brown color) was relatively confined to the islets and some exocrine tissues. However, in O-CON and O-PF groups, more extensive TGF-β staining in whole pancreas was detected compared with the ramipril-treated animals (F,H). The α-SMA expression showed a similar pattern. Compared to O-RAM5 (I), more intense brown-colored α-SMA immunostaining (arrows) was observed in small ring-shaped vessels in the enlarged and disorganized pancreatic islets of O-CON (L) (200×). O-RAM5 (A,E,I); O-PF (B,F,J); O-RAM1 (C,G,K); O-CON (D,H,L).

Table 2
β-Cell mass, islet fibrosis amount, and α-SMA-stained areas after ramipril treatment

	24 weeks			32 weeks		
	β-Cell mass (mg)	Fibrosis (%)	α-SMA (%)	β-Cell mass (mg)	Fibrosis (%)	α-SMA (%)
OLETF						
O-RAM5	12.8 ± 3.4	19.9 ± 0.8*	0.4 ± 0.1	11.4 ± 1.4	20.2 ± 4.8*	1.0 ± 0.2*
O-PF	9.7 ± 3.6	25.1 ± 1.2	0.6 ± 0.1	—	—	—
O-RAM1	11.8 ± 4.3	25.8 ± 1.1	0.4 ± 0.2	—	—	—
O-CON	6.8 ± 1.0	31.5 ± 1.1	0.6 ± 0.1	8.3 ± 0.6	52.7 ± 6.9	1.4 ± 0.5
LETO						
L-RAM5	9.1 ± 2.1	5.5 ± 0.3	0.2 ± 0.1	11.0 ± 1.2	10.0 ± 2.2**	0.3 ± 0.1
L-PF	9.5 ± 3.1	10.6 ± 0.6	0.2 ± 0.1	—	—	—
L-RAM1	8.9 ± 1.6	10.1 ± 0.6	0.1 ± 0.1	—	—	—
L-CON	7.4 ± 1.1	11.5 ± 1.2	0.1 ± 0.1	10.1 ± 0.8	13.5 ± 2.8	0.2 ± 0.1

Data represent means ± SD. Sucrose feeding accelerated the islet fibrosis in control groups while ramipril protected the islet fibrosis by sucrose feeding especially in OLETF rats.

* $P < 0.05$ vs. O-CON.

** $P < 0.05$ vs. L-CON.

RT-PCR for TGF-β and its downstream signals at 24 and 32 weeks

RT-PCR analysis demonstrated significant up-regulation of TGF-β mRNA in O-CON rats compared with the other 3 groups of OLETF and LETO rats at 24 weeks (Fig. 3A). The decreased gene expression was remarkable in the O-RAM5 group, but we did not observe any difference between O-PF and O-RAM1 group. The increased expressions of TGF-β and CTGF mRNAs paralleled the appearance of pancreatic fibrosis. In addition, the mRNA of fibronectin was also higher in the O-CON rats than in the O-RAM5 rats (Fig. 3A). At 32 weeks, the differences of their gene expressions were more prominent and significant (Fig. 3B). In LETO rats, differences in gene expression were similar to those of OLETF rats (data not shown).

Western blotting at 24 and 32 weeks

Based on band densities, pancreatic TGF-β, CTGF, fibronectin, and α-SMA protein expressions were significantly elevated in O-CON rats compared with the O-RAM5 and O-RAM1 animals at 24 weeks ($P < 0.05$, Fig. 4A). The TGF-β, fibronectin, and α-SMA expressions were reduced more so in O-RAM5 than O-RAM1, and quantification of density of the band from control rats was approximately about 2 times greater than in samples from O-RAM5 animals ($P < 0.05$). These findings corresponded with the results of mRNA expressions. Their protein expressions were also significantly elevated in O-CON group at 32 weeks compared to O-RAM5 group (Fig. 4B).

Body weight change during paired-feeding

Long-term high-dose (5 mg/kg) ramipril treatment significantly suppressed weight gain in OLETF (9.8 ± 2.3%) and LETO (14.3 ± 8.4%) rats compared with the control groups. Body weights were well matched in paired-feeding group (O-PF) and high-dose ramipril-treated group. Thus, it suggested that the effect of high-dose ramipril (5 mg/kg) on body weight was related to a reduced appetite. One milligram per kilogram of ramipril administration tended to suppress the weight gain only in OLETF rats but there was no statistical significance.

Discussion

This study showed that long-term ACE inhibition caused by high-dose (5 mg/kg) ramipril treatment protected against the pancreatic islet destruction and fibrosis caused by chronic hyperglycemia in OLETF rats. Moreover, these effects correlated with decreased gene expressions of TGF-β and its downstream signals, such as CTGF, fibronectin, and α-SMA.

The clinical background of this experimental study design was based on HOPE study [2]. Remarkably, the study observed a marked reduction in the incidence of complications related to diabetes and in the incidence of new cases of diabetes [2,4], although these results require confirmation. These findings suggest that ACEIs have an influence on insulin sensitivity, insulin resistance, and pancreatic function directly or indirectly.

In general, obesity, one component of metabolic syndrome, induces more insulin resistant condition. It may aggravate some burden to pancreatic islets to secrete more insulin for compensation of insulin resistance. As a result, it accelerates islet dysfunction. So we added the paired-feeding group not only to clarify the effect of ramipril on reduced weight gain in OLETF rats but also to test the direct effect of ramipril on the islets, independent of body weight.

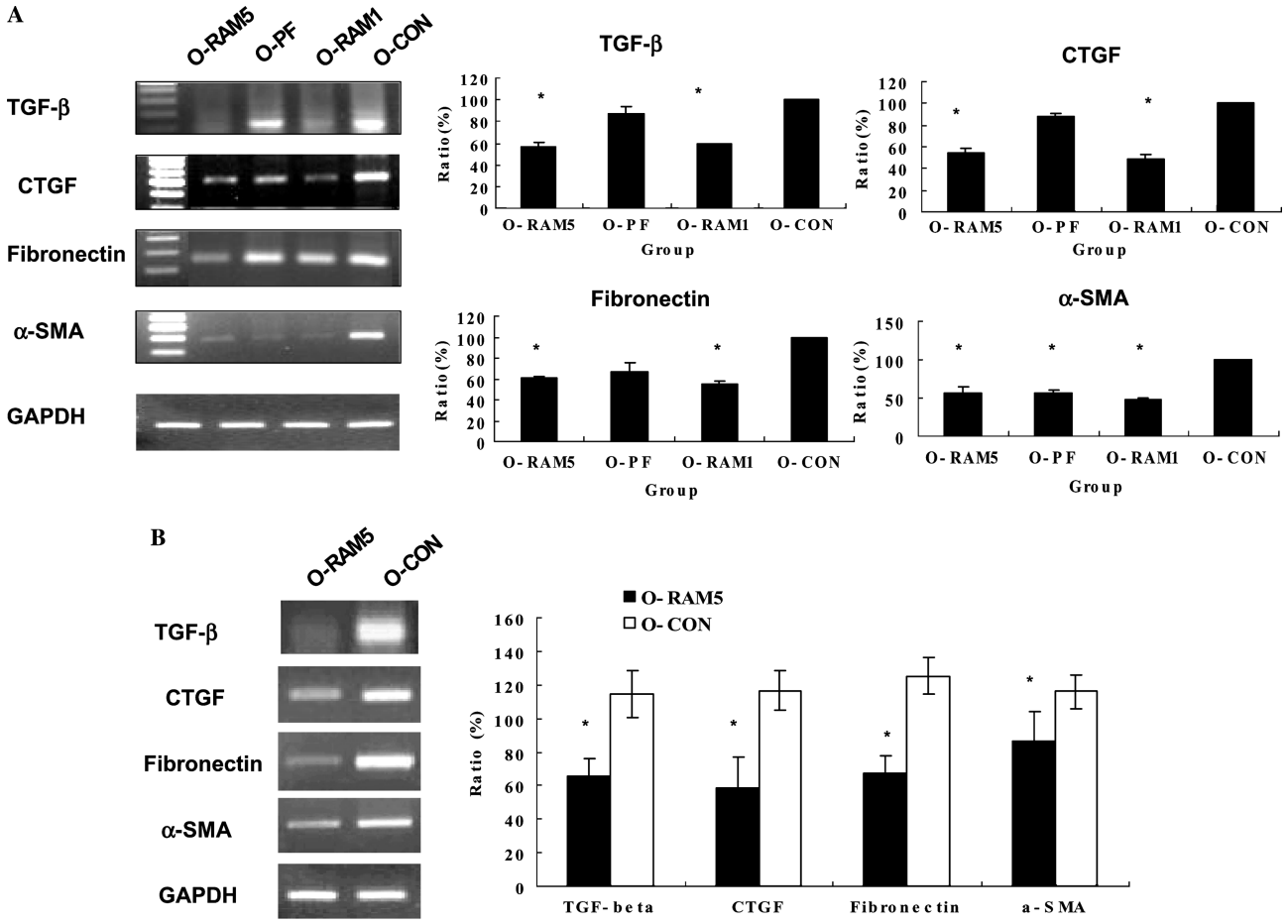

Fig. 3. (A) Reverse transcription polymerase chain reaction analysis after ramipril treatment in OLETF rats for the expression of TGF-β, CTGF, and fibronectin mRNAs at 24 weeks. O-RAM5, lane 1; O-PF, lane 2; O-RAM1, lane 3; and O-CON, lane 4. (B) At 32 weeks, ramipril treatment showed a clear reduction of expression of mRNAs compared with the control group in the pancreas of OLETF rats. O-RAM5, lane 1; O-CON, lane 4. *$P < 0.05$ vs. O-CON group.

Our preliminary data showed that high-dose ramipril treatment tended to reduce weight gain in this animal model of type 2 diabetes. We tried to clarify the drug effect by adding a paired-feeding group to ramipril (5 mg/kg) group. Reduced weight gain completely disappeared in the paired-feeding. Long-term treatment with high-dose ramipril, therefore, significantly reduced weight gain, suggestive of an anorexic effect.

At 24 weeks, the GTT showed no significant difference between groups. However, after feeding with an additional 30% sucrose for a further 8 weeks, responses to exaggerated hyperglycemia revealed some meaningful results. Fasting and 2 h glucose levels as shown by the AUCg were clearly lower in the long-term ramipril-treated OLETF group than in the control rats. Morphologically, in non-treated group, prominent islet fibrosis was observed without any differences in fasting insulin concentration, β-cell mass or the degree of insulin resistance between groups. But HOMA-B and $\Delta I_{30}/\Delta G_{30}$ index were significantly improved in ramipril-treated group. This seems to mean that insulin secretory function was improved with long-term use of ramipril, despite no changes of β-cell mass, accompanied by protection of islet disorganization. This suggests that islet fibrosis with accompanying secretory impairment of insulin had an important role in the deterioration of glucose tolerance status in OLETF control rats.

Several reports have presented experimental data on animal models of diabetes regarding the effects of ACE inhibition on insulin sensitivity. Some demonstrated that ACEIs have beneficial effects on insulin sensitivity [21,22], glucose disposal [23], and increased islet blood flow of rat pancreas [24]. In rats, the mRNA expressions of angiotensinogen, renin, and the AT II receptor subtypes have been determined in the pancreas and localized to the epithelium of pancreatic ducts and the endothelium of blood vessels [12,13,25]. In the human pancreas, the AT 1 receptor has been identified in islets [14], especially in β- and endothelial cells, though this requires additional clarification. Thus, ACEIs might have a direct influence on the pancreatic islets in animal models or in human patients with type 2 diabetes mellitus.

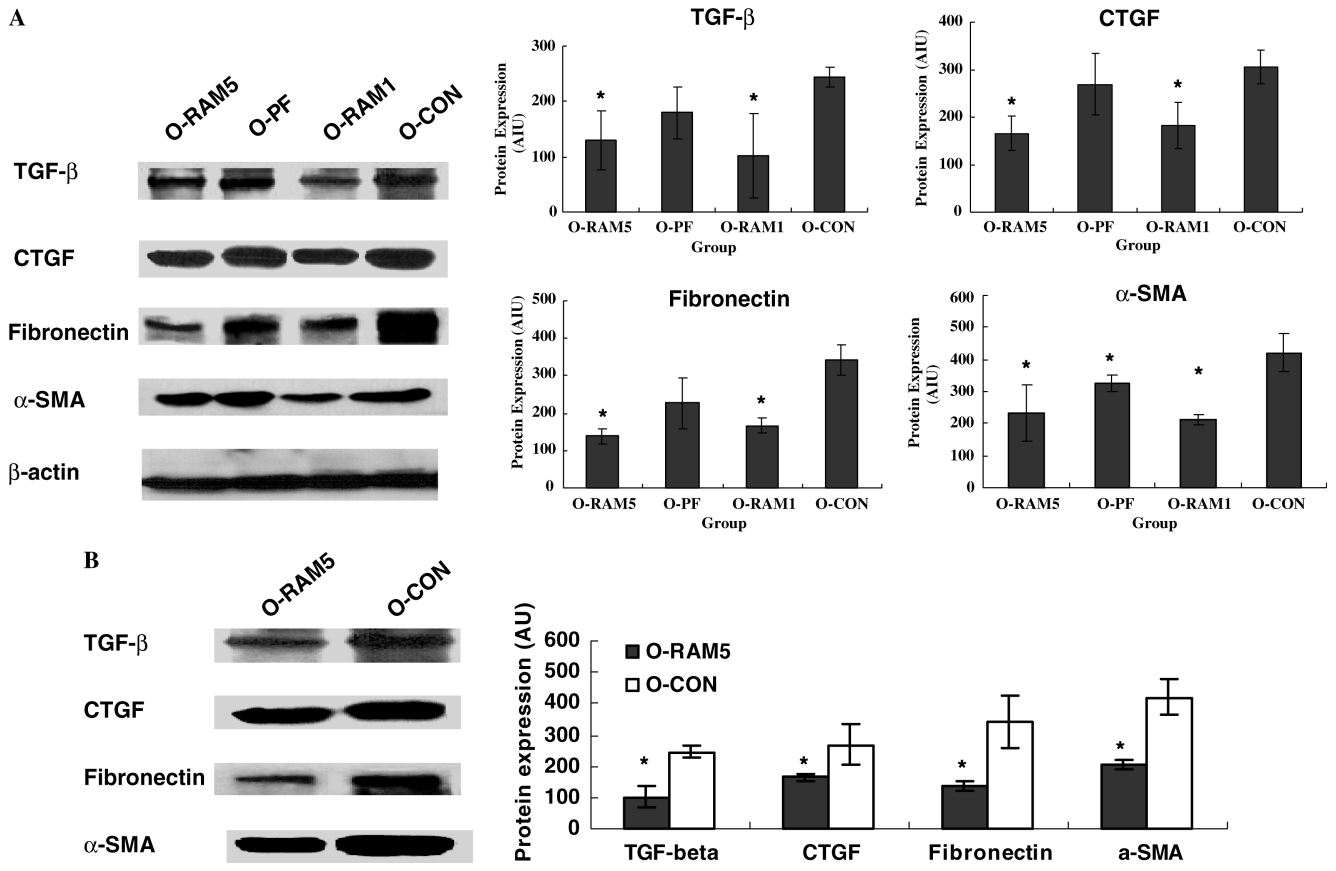

Fig. 4. Western blot analysis of TGF-β, CTGF, fibronectin, and α-SMA proteins in the OLETF rat pancreas. After autoradiography, signals of appropriate molecular weight were detected. Ramipril treatment showed a clear reduction in the protein expressions compared with their control group at 24 weeks (A) and 32 weeks (B). *$P < 0.05$ vs. control group.

The local role of the renin–angiotensin system in tissues during the pathogenesis of diabetic complications is relatively well described in the field of diabetic nephropathy. ACE inhibitors and AT II receptor blockers attenuate progressive glomerulosclerosis in disease models and slow disease progression in humans [26], regardless of their hemodynamic effects. Some vasoactive factors such as AT II, endothelin I, thromboxane, and hyperglycemia clearly play a part in growth stimulation and profibrotic activity during diabetic renal disease through the secondary induction of TGF-β, and the reduced expression of TGF-β receptors following ACE inhibition [27].

The multifunctional cytokine TGF-β is recognized as a mediator of matrix accumulation and fibrosis, and as a regulator of cell growth and differentiation. In addition to mediating renal fibrosis, TGF-β plays an important role in the development of pancreatic fibrosis, such as chronic pancreatitis, acute necrotizing pancreatitis, and pancreatic regeneration [28–30]. CTGF, originally identified in human endothelial cells, is an important downstream mediator of TGF-β activity [31]. CTGF seems to be upregulated by TGF-β, which subsequently modulates fibroblast proliferation, growth, and extracellular matrix overproduction. Although many pathophysiological mechanisms associated with the diabetic state interact in concert to induce the changes, it seems that AT II and TGF-β are also critical molecules in the pathogenesis of destructive changes of pancreatic islets in type 2 diabetes mellitus. In our results, TGF-β, CTGF, and fibronectin gene expressions were significantly increased in non-treated group. Even in 1 mg/kg ramipril group, these gene expressions were decreased, suggestive of beneficial effect of anti-hypertensive dose of ramipril on islet fibrosis.

Pancreatic stellate cells have now been identified and have been shown to differentiate into myofibroblast-like cells expressing α-SMA and producing collagen types I and III, laminin, and fibronectin in response to various inflammatory cytokines [32,33]. These cells are involved in the pathogenesis of pancreatic fibrosis and have receptors for cytokines, growth factors, and inflammatory mediators, including AT II [34]. Morphologically, disorganized or fibrotic changes of pancreatic islets in this model were correlated with the expression of α-SMA. It was suggested that α-SMA contributes to the pathogenesis of islet fibrosis.

In summary, we observed that long-term treatment with an ACEI attenuated the destructive changes of pancreatic islets in an animal model of type 2 diabetes mellitus. We showed that TGF-β and AT II might act as key mediators of the pathogenesis of islet fibrosis in this disease. In addition to the control of hyperglycemia, therefore, inhibition of TGF-β by ACEIs might be a promising therapeutic route towards the prevention of pancreatic islet fibrosis in patients with long-standing diabetes mellitus. Clarification of the signaling pathways associated with TGF-β and AT II could assist in developing therapies for the prevention or treatment of type 2 diabetes mellitus, although the clinical implications of these molecules remain to be determined.

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References

[1] J. Sidorov, R. Shull, J. Tomcavage, S. Girolami, N. Lawton, R. Harris, Does diabetes disease management save money and improve outcomes?: a report of simultaneous short-term savings and quality improvement associated with a health maintenance organization-sponsored disease management program among patients fulfilling health employer data and information set criteria, Diabetes Care 25 (2002) 684–689.

[2] S. Yusuf, P. Sleight, J. Pogue, J. Bosch, R. Davies, G. Dagenais, Effects of an angiotensin-converting enzyme inhibitor, ramipril, on cardiovascular events in high-risk patients. The heart outcomes prevention evaluation study investigators, N. Eng. J. Med. 34 (2000) 145–153.

[3] L.H. Lindholm, H. Ibsen, B. Dahlof, R.B. Devereux, G. Beevers, U. de Faire, F. Fyhrquist, S. Julius, S.E. Kjeldsen, K. Kristiansson, O. Lederballe-Pedersen, M.S. Nieminen, P. Omvik, S. Oparil, H. Wedel, P. Aurup, J. Edelman, S. Snapinn, LIFE study group, cardiovascular morbidity and mortality in patients with diabetes in the losartan intervention for endpoint reduction in hypertension study (LIFE): a randomised trial against atenolol, Lancet 23 359 (9311) (2002) 1004–1010.

[4] S. Yusuf, H. Gerstein, B. Hoogwerf, J. Pogue, J. Bosch, B.H.R. Wolffenbuttel, B. Zinman, HOPE study investigators, ramipril and the development of diabetes, JAMA 286 (2001) 1882–1885.

[5] K. Kawano, T. Hirashima, S. Mori, T. Natori, OLETF (Otsuka Long–Evans Tokushima fatty) rat: a new NIDDM rat strain, Diabetes Res. Clin. Pract. 24 (1994) S317–S320.

[6] H. Yoshikawa, Y. Kihara, M. Taguchi, T. Yamaguchi, H. Nakamura, M. Otsuki, Role of TGF-β1 in the development of pancreatic fibrosis in Otsuka Long–Evans Tokushima fatty rats, Am. J. Physiol. Gastrointest. Liver Physiol. 282 (2002) G549–G558.

[7] A. Clark, C.A. Wells, I.D. Buley, J.K. Cruickshank, R.I. Vanhegan, D.R. Matthews, G.J. Cooper, R.R. Holman, R.C. Turner, Islet amyloid, increased A-cells, reduced B-cells and exocrine fibrosis: quantitative changes in the pancreas of type 2 diabetes, Diabetes Res. 9 (1988) 151–159.

[8] K.H. Yoon, S.H. Ko, J.H. Cho, J.M. Lee, Y.B. Ahn, K.H. Song, S.J. Yoo, M.I. Kang, B.Y. Cha, K.W. Lee, H.Y. Son, S.K. Kang, H.S. Kim, I.K. Lee, S. Bonner-Weir, Selective beta-cell loss and alpha-cell expansion in patients with type 2 diabetes mellitus in Korea, J. Clin. Endocrinol. Metab. 88 (2003) 2008–2300.

[9] P.S. Haber, G.W. Keogh, M.V. Apte, C.S. Moran, N.L. Stewart, D.H. Crawford, R.C. Pirola, G.W. McCaughan, G.A. Ramm, J.S. Wilson, Activation of pancreatic stellate cells in human and experimental pancreatic fibrosis, Am. J. Pathol. 155 (1999) 1087–1095.

[10] T. Yokuyasu, W. Denham, K. Murayama, C. Pelham, R. Joehl, R.H. Bell, Pancreatic stellate cell activation and MMP production in experimental pancreatic fibrosis, J. Surg. Res. 104 (2002) 106–111.

[11] P.S. Leung, W.P. Chan, T.P. Wong, C. Sernia, Expression and localization of the renin-angiotensin system in the rat pancreas, J. Endocrinol. 160 (1999) 13–19.

[12] P.O. Carlsson, The renin–angiotensin system in the endocrine pancreas, J. Pancreas 2 (2001) 26–32.

[13] P.S. Leung, P.O. Carlsson, Tissue renin–angiotensin system: its expression, localization, regulation and potential role in the pancreas, J. Mol. Endocrinol. 26 (2001) 155–164.

[14] M. Tahmasebi, J.R. Puddefoot, E.R. Inwang, G.P. Vinson, The tissue renin–angiotensin system in human pancreas, J. Endocrinol. 161 (1999) 317–322.

[15] S.M. Haffner, H. Miettinen, M.P. Stern, The homeostasis model in the San Antonio heart study, Diabetes Care 20 (1997) 1087–1092.

[16] M. Kanauchi, M. Nakajima, Y. Saito, K. Kanauchi, Pancreatic beta-cell function and insulin sensitivity in Japanese subjects with impaired glucose tolerance and newly diagnosed type 2 diabetes mellitus, Metabolism 52 (2003) 476–481.

[17] P. Masson, Trichrome stainings and their preliminary technique, J. Tech. Methods 2 (1929) 75–90.

[18] J.J. Pimentel, C.L. Sundell, S. Wang, J.B. Kopp, A. Montero, M.M. Martinez, Role of angiotensin II in the expression and regulation of transforming growth factor-beta in obstructive nephropathy, Kidney Int. 48 (1995) 1233–1246.

[19] E.R. Weibel, Stereologic methods, in: Practical Methods for Biologic Morphometry, vol. 1, Academic Press, London, 1978, pp. 101–161.

[20] S. Bonner-Weir, β-cell turnover: its assessment and implications, Diabetes 50 (suppl. 1) (2001) S20–S24.

[21] M. Uehara, H. Kishikawa, S. Isami, K. Kisanuki, Y. Ohkubo, N. Miyamura, T. Miyata, T. Yano, M. Shichiri, Effect on insulin sensitivity of angiotensin converting enzyme inhibitors with or without a sulphydryl group: bradykinin may improve insulin resistance in dogs and humans, Diabetologia 37 (1994) 300–307.

[22] E. Torlone, A.M. Rambotti, G. Periello, G. Botta, F. Santeusanio, P. Brunetti, G.B. Bolli, ACE-inhibition increases hepatic and extrahepatic sensitivity to insulin in patients with type 2 (non-insulin-dependent) diabetes mellitus and arterial hypertension, Diabetologia 34 (1991) 119–125.

[23] A.A. Mehta, S. Patel, D.D. Santani, R.K. Goyal, Effect of nifedipine and enalapril on insulin-induced glucose disposal in spontaneous hypertensive and diabetic rats, Clin. Exp. Hypertension 21 (1999) 51–59.

[24] P.O. Carlsson, C. Berne, L. Jansson, Angiotensin II and the endocrine pancreas: effects on islet blood flow and insulin secretion in rats, Diabetologia 41 (1998) 127–133.

[25] P.S. Leung, H.C. Chan, L.X.M. Fu, P.Y.D. Wong, Localization of angiotensin II receptor subtype AT1 and AT2 in the pancreas of rodents, J. Endocrinol. 153 (1997) 269–274.

[26] E.J. Lewis, L.G. Hunsicker, R.P. Bain, R.D. Rohde, Collaborative study group, the effect of angiotensin-converting enzyme inhibition on diabetic nephropathy, N. Engl. J. Med. 329 (1993) 1456–1462.
[27] G. Wolf, E. Mueller, R.A.K. Stahl, F.N. Ziyadeh, Angiotensin II-induced hypertrophy of cultured murine proximal tubular cells is mediated by endogenous transforming growth factor-β, J. Clin. Invest. 92 (1993) 1372–1377.
[28] J.L. van Laethem, J. Deviere, A. Resibois, F. Rickaert, P. Vertongen, H. Ohtani, M. Cremer, K. Miyazono, P. Robberecht, Localization of transforming growth factor beta 1 and its latent binding protein in human chronic pancreatitis, Gastroenterology 108 (1995) 1873–1881.
[29] A. Menke, H. Yamaguchi, T.M. Gress, G. Adler, Extracellular matrix is reduced by inhibition of transforming growth factor beta1 in pancreatitis in the rat, Gastroenterology 113 (1997) 295–303.
[30] Y. Kihara, M. Tashiro, H. Nakamura, T. Yamaguchi, H. Yoshikawa, M. Otsuki, Role of TGF-beta1, extracellular matrix, and matrix metalloproteinase in the healing process of the pancreas after induction of acute necrotizing pancreatitis using arginine in rats, Pancreas 23 (2001) 288–295.
[31] A. Igarashi, H. Okochi, D.M. Bradham, G.R. Grotendorst, Regulation of connective tissue growth factor gene expression in human skin fibroblasts and during wound repair, Mol. Biol. Cell 4 (1993) 637–645.
[32] M.V. Apte, P.S. Haber, S.J. Darby, S.C. Rodgers, G.W. McCaughan, M.A. Korsten, R.C. Pirola, J.S. Wilson, Pancreatic stellate cells are activated by proinflammatory cytokines: implications for pancreatic fibrogenesis, Gut 44 (1999) 534–541.
[33] F.W. Shek, R.C. Benyon, F.M. Walker, P.R. McCrudden, S.L. Pender, E.J. Williams, P.A. Johnson, C.D. Johnson, A.C. Bateman, D.R. Fine, J.P. Iredale, 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 (2002) 1787–1798.
[34] D.W. Powell, R.C. Mifflin, J.D. Valentich, S.E. Crowe, J.I. Saada, A.B. West, Myofibroblasts. I. Paracrine cells important in health and disease, Am. J. Physiol. 277 (1 Pt 1) (1999) C1–C9.