Angiotensin II type 1 receptor interaction is an important regulator for the development of pancreatic fibrosis in mice

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The renin-angiotensin system (RAS) is traditionally considered an endocrine system regulating the blood pressure and body fluid homeostasis (35, 36). The RAS is now recognized to be more than a circulating hormonal system that exerts hemodynamic actions but a locally active mediator in various pathophysiological processes, including the development of cardiovascular and renal diseases (20, 30, 44). Angiotensin II (ANG II) is the physiologically active mediator of the RAS. The biological roles of ANG II are mediated by high-affinity membrane-bound receptors, which have recently been classified into two subtypes: AT1 (AT1a and AT1b, respectively; see Ref. 41). The AT1 receptor mediates most of the molecular and cellular actions of ANG II (20, 41).

Recent studies have shown that the RAS is present intrinsically in the pancreas and that its level is enhanced during acute pancreatitis and chronic pancreatic hypoxia in experimental animals (11, 24–26), suggesting the role of the RAS in pancreatic diseases. However, there are several controversial reports regarding the effects of the inhibition of the RAS on acute pancreatitis. For example, Tsang et al. (42) reported that losartan, an AT1 receptor antagonist, ameliorates cerulein-induced acute pancreatitis in rats. Moreover, they (43) reported that ramiprilat, an angiotensin-converting enzyme (ACE) inhibitor, enhances acute pancreatitis in the same model. Therefore, the pathophysiological role of the RAS in pancreatic diseases has not been established.

Chronic pancreatitis is an irreversible progressive disease characterized by destruction of exocrine parenchyma and its replacement with fibrosis. It is now recognized that repeated episodes of acute pancreatitis can lead to increasing residual damage to the gland, eventually resulting in chronic pancreatitis (the necrosis-fibrosis sequence; see Refs. 1, 21, 33). Although pancreatic stellate cells (PSCs) are known to play a central role in fibrogenesis in the pancreas (2–4, 14, 28, 31, 39), the mechanisms responsible for the development of pancreatic fibrosis still remain unclear. Recently, the inhibition of the RAS with an ACE inhibitor and an AT1 receptor antagonist has been shown to attenuate pancreatic fibrosis in an animal model (22, 46), suggesting that the RAS and the ANG II-AT1 receptor pathway may play an important role in pancreatic fibrosis. One of the effective ways to investigate the precise role of the RAS is to use genetically engineered animals deficient for ANG II receptors.

The AT1 receptor, which mediates most of the biological actions of ANG II, is further subdivided into AT1a and AT1b in murine species (41). The murine AT1a is the predominantly expressed isoform in most tissues (27). Therefore, we recently generated an AT1a-deficient mouse strain that is a powerful model for analyses of the effect of ANG II blockade (16, 40). To elucidate the pathophysiological role of the ANG II-AT1a receptor system in the development of pancreatic fibrosis, we induced acute pancreatitis and pancreatic fibrosis in AT1a-deficient homozygous [AT1a(−/−)] mice and wild-type (WT) mice.

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MATERIALS AND METHODS

Animals. To obtain AT1a receptor-deficient heterozygous [AT1a(+/-)] mice that have a C57BL/6 background, a germline chimera derived from TT2 embryonic stem cells with a targeted mutation of the AT 1a gene (40) was backcrossed for five generations with C57BL/6 mice. The resulting AT1a(+/-) F5 mice were intercrossed to generate the homozygous [AT1a(-/-)] mutant mice. As WT [or AT1a(+/-)] control mice, C57BL/6 mice were obtained fromCLEA Japan (Tokyo, Japan). Male mice, 6 wk old, were used. The animals were kept in a 12:12-h light-dark cycle with free access to water and a standard mouse chow. The experiment was carried out under the guidelines of animal experiments of the University of Occupational and Environmental Health, Japan, School of Medicine.

Experimental protocol. Pancreatic fibrosis was induced by repeated acute pancreatitis episodes, as described previously (33). Acute pancreatitis was elicited by the hourly (6 times) intraperitoneal injections of 50 μg/kg body wt cerulein (Sigma-Aldrich, Tokyo, Japan). Mice were subjected to three episodes of acute pancreatitis per week for four consecutive weeks. At 4 wk, venous blood was collected, and the mice were killed by administration of a lethal dose of pentobarbital. Pancreatic tissues were harvested, fixed in 4% paraformaldehyde buffered with 0.01 M sodium phosphate, pH 7.4 (PBS) overnight at 4°C, and embedded in paraffin or frozen immediately in liquid nitrogen for extractions of hydroxyproline, protein, and mRNA.

To assess the effect of AT1a receptor deletion on acute pancreatic injury, blood samples were collected for measurement of amylase activity at 12 h after a single episode of cerulein-induced acute pancreatitis, and the mice were killed. For morphological studies, the pancreas was removed rapidly, fixed in 4% paraformaldehyde buffered with PBS overnight at 4°C, and embedded in paraffin.

Histological examination. Paraffin sections (5 μm) were stained with hematoxylin and eosin and Azan-Mallory or subjected to immuno histochemical staining using antibodies against collagen type I (LSL, Tokyo, Japan), fibronectin (DAKO Cytomation, Kyoto, Japan), α-smooth muscle actin (α-SMA; DAKO Cytomation), and AT1 receptor (Santa Cruz). Immunohistochemical staining of α-SMA was performed as described previously (48). A DAKO EnVision system (DAKO Cytomation) was used for collagen type I, fibronectin, and AT1 receptor immunostaining.

To further clarify the sites of action of ANG II in the process of pancreatic fibrosis, we performed dual-immunofluorescence staining for α-SMA and AT1 receptor. Sections were incubated with anti-α-SMA antibody and with anti-AT1 receptor antibody for 60 min at room temperature. The sections were then incubated for 60 min at room temperature with a rhodamine-conjugated (DAKO Cytomation) and an FITC-conjugated (DAKO Cytomation) secondary antibody to detect activated PSCs and AT1 receptors, respectively. Dual-immunostained sections were analyzed using a confocal fluorescence microscopy (LSM 410; Carl Zeiss).

Severity of acute pancreatitis was blindly graded by a semiquantitative assessment of inflammatory cell infiltration, vacuolization, and acinar necrosis according to the score described previously (34): 0 = absent or <5%, 1 = 5–15%, 2 = 15–35%, 3 = 35–50%, 4 = >50% of cells involved.

Western blot analysis. Frozen pancreatic tissue was homogenized with a Polytron homogenizer in ice-cold lysis buffer (pH 7.4) containing 25 mM Tris-HCl, 25 mM NaCl, 0.5 mM EGTA, 10 mM NaF, 1 mM Na2VO4, 10 mM sodium pyrophosphate, 1 mM phenylmethylsulfonl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 0.1 mg/ml soybean trypsin inhibitor. Samples were then centrifuged at 15,000 rpm for 10 min at 4°C. Protein concentration was determined by the Bradford (8) method using BSA as a standard. Supernatant was prepared for one-dimensional SDS-PAGE. Proteins (20 μg/each lane) were then separated by 8% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Hybond-P; Amersham Pharmacia Biotech). Membranes were blocked with 10% fat-free dry milk for 1 h in PBS (pH 7.4) and then incubated with anti-α-SMA antibody at a 1:4,000 dilution in PBS containing 0.05% Triton X-100 (pH 7.4) for 1 h at room temperature. After being washed, membranes were incubated with appropriate IgG antibody conjugated with horseradish peroxidase in PBS for 1 h at room temperature. Antibody binding was detected by the enhanced chemiluminescence detection system (ECL Plus; Amersham Pharmacia Biotech) and exposed to X-ray films (Scientific Bio-Imaging Film; Kodak). For quantitation of α-SMA expression, we calculated the density of the band using the analysis software National Institutes of Health Image 1.62, as described previously (48).

Quantitative analysis of pancreatic fibrosis. A semi quantitative evaluation of fibrosis in the pancreatic specimen was performed using an Axioskop microscope (Carl Zeiss) connected to an IBAS image analysis system (Carl Zeiss), as described previously (48). Five nonoverlapping fields per Azan-Mallory-stained pancreatic specimen (n = 4) were randomly selected at ×20 magnification by an investigator who was unaware of the sample identity. The rate of pancreatic fibrosis was indicated as a percentage of total pancreatic specimen. We also determined collagen contents in pancreatic tissue quantitatively by measuring pancreatic hydroxyproline content according to the method reported by Woessner (45).

Measurement of serum amylase. Serum amylase activity was determined by a chromogenic method with the Phadebas amylase test (10) and expressed as Somogyi units.

RT-PCR. Total RNA was extracted from each tissue using a guanidium thiocyanate-phenol solution (ISOGEN; Nippon gene, Toyama, Japan), quantified by measuring absorption at 260 nm, and subject to RT-PCR. Single-stranded cDNA was synthesized from 1 μg total RNA using random primers and RNase H− RT (Toyobo, Osaka, Japan), and subjected to RT-PCR. Primer sequences used in RT-PCR are shown in Table I. PCR products were resolved by agarose-gel electrophoresis and visualized by ethidium-bromide staining. The relative expression levels of the amplified bands were calculated using the Photodensitometry method (ImageMaster 2D Platinum; Amersham Pharmacia Biotech). The relative expression was determined by normalizing the expression of each PCR product to that of GAPDH. The relative expression of AT1a mRNA was significantly increased in the pancreas of WT mice but not in that of AT1a(-/-) mice. AT1a mRNA was expressed in both WT and AT1a(-/-) mice. Total RNA from kidney and adrenal gland was used for positive controls of AT1a and AT1b, respectively.
Osaka, Japan) and was subjected to PCR. The synthesized cDNA was amplified using specific sets of primers for AT1a receptor (sense 5'-TCACCTGCATCATCTGG-3' and antisense 5'-AGCTGG-TAAGAATGATTAGG-3'), AT1b receptor (sense 5'-AGCTGGTGAGAAATAAACG-3' and antisense 5'-GAGAATAATAACG-3'), AT1b mRNA was not expressed in the pancreas of WT and AT1a receptor in AT1a mice, whereas it was expressed in that of WT mice (Fig. 1). AT1b mRNA expression was observed in both AT1a mice and WT mice (Fig. 1). Effects of AT1α receptor deletion on acute pancreatic injury induced by cerulein. To address the possibility that the chronic fibrosing response to injury was related to the degree of acute pancreatic injury, we evaluated serum amylase activity and histological changes after a single course of acute pancreatitis. Six hourly intraperitoneal injections of cerulein increased serum amylase levels and produced histological evidence of pancreatic injury, including inflammatory cell infiltration, acinar cell vacuolization, and acinar cell necrosis in AT1a mice and WT mice. There were no significant differences in these parameters between WT and AT1a mice (Figs. 2 and 3). Histological severity of acute pancreatitis assessed by the score was not significantly different between WT and AT1a mice [inflammatory cell infiltration 4.0 ± 0.0 vs. 3.8 ± 0.2, vacuolization 2.0 ± 0.3 vs. 1.8 ± 0.4, and acinar necrosis 1.0 ± 0.0 vs. 1.0 ± 0.0 in WT and AT1a mice, respectively].

RESULTS

Expressions of AT1α and AT1β mRNA. To confirm the absence of AT1a receptor in AT1a(−/−) mice and the presence of AT1a receptor in WT mice, we first performed RT-PCR analysis. AT1α mRNA was not expressed in the pancreas of AT1a(−/−) mice, whereas it was expressed in that of WT mice (Fig. 1). AT1b mRNA expression was observed in both AT1a(−/−) and WT mice (Fig. 1).

Statistical analysis. Data are expressed as means ± SE. Differences between the groups were evaluated by Student’s t-test. The histological scores were analyzed using the Mann-Whitney U-test. A P value <0.05 was considered to be statistically significant.

Fig. 2. Serum amylase levels in WT (open bars) and AT1a(−/−) mice (closed bars) after a single course of acute pancreatitis. Mice were given 6 hourly injections of cerulein and killed at 12 h after the induction of acute pancreatitis. Serum amylase was determined as described in MATERIALS AND METHODS. No significant difference of serum amylase level was found between the 2 groups.

Fig. 3. Histological changes of the pancreas in WT and AT1a(−/−) mice after a single course of acute pancreatitis. Mice were given 6 hourly injections of cerulein and killed at 12 h after the induction of acute pancreatitis. Pancreas sections were stained with hematoxylin and eosin. Representative micrographs are shown from pancreas in control WT (A) and AT1a(−/−) (B) mice. C and D: pancreas from WT (C) and AT1a(−/−) (D) mice injected with cerulein. Acinar cell vacuolization, edema, and inflammatory cell infiltration were similarly seen in the 2 groups. Original magnification, ×200.
Effects of AT₁a receptor deletion on histological changes after repeated courses of acute pancreatitis. Three episodes of cerulein-induced acute pancreatitis per week for four consecutive weeks induced tissue remodeling in the pancreas in WT mice. Azan staining and immunostainings for collagen type I and fibronectin showed extracellular matrix (ECM) protein deposition mainly in the periacinar and the interlobular areas with pancreatic atrophy (Fig. 4, A, E, and G). In addition, intra-acinar lumina became dilated and some acinar units appeared to redifferentiate into tubular complexes. PSCs are known to mediate fibrogenesis in the pancreas (2–4, 14, 28, 31, 39). When activated by profibrogenic mediators, PSCs transform into myofibroblast-like cells expressing α-SMA. The fibrotic area was also stained positively by an antibody against α-SMA-positive area was reduced in AT₁a(−/−) mice compared with that in WT mice. Original magnification, ×200.

Fig. 4. Histological changes of the pancreas in WT and AT₁a(−/−) mice after repetitive episodes of acute pancreatitis. Mice were submitted to three episodes of acute pancreatitis per week for 4 wk. Pancreas sections were histologically examined by Azan staining (A and B) or by immunostaining with antibodies against α-smooth muscle actin (α-SMA; C and D), collagen type I (E and F), or fibronectin (G and H). In AT₁a(−/−) mice, pancreatic fibrosis was clearly reduced compared with that in WT mice. Moreover, α-SMA-positive area was reduced in AT₁a(−/−) mice compared with that in WT mice. Original magnification, ×200.
significant decreases in AT_1a (–/–) mice compared with in WT mice. Data are means ± SE of 4 mice/group. *P < 0.05 compared with WT mice.

α-SMA (Fig. 4C). In contrast, in AT_1a (–/–) mice, pancreatic fibrosis was significantly attenuated, as indicated by both Azan staining and immunostainings (Fig. 4, B, F, and H). Moreover, the α-SMA-positive area was also significantly decreased in AT_1a (–/–) mice compared with that in WT mice (Fig. 4, D vs. C). Western blot analysis also demonstrated that the expression of α-SMA was significantly decreased in AT_1a (–/–) mice compared with that in WT mice (Fig. 5, A and B).

**Effects of AT_1a receptor deletion on fibrosis area and hydroxyproline content in the pancreas.** Semiquantitative analysis of pancreatic fibrosis by image analysis showed that the percentage of fibrosis area in AT_1a (–/–) mice was significantly lower than that in WT mice (Fig. 6A). For quantitative comparison, we measured the hydroxyproline content in the pancreas. The amount of hydroxyproline in WT mice treated with cerulein was 6.6 times higher than that found in the control untreated WT mice (Fig. 6B). The amount of hydroxyproline in AT_1a (–/–) mice treated with cerulein was significantly less than that in WT mice (Fig. 6B).

**Activated PSCs express AT1 receptors.** To investigate the sites of action of ANG II in the process of pancreatic fibrosis, we evaluated the localization of AT_1 receptors in the pancreas of the control and cerulein-treated WT mice by immunostaining. The blood vessels could be identified by positive staining for α-SMA, whereas the epithelial cells of the pancreatic ducts were negative for α-SMA (Fig. 7A). In the control pancreas, AT_1 receptor staining was seen in the blood vessels and the epithelial cells of the pancreatic ducts (Fig. 7B). Cerulein treatment for 4 wk induced the proliferation of α-SMA-positive activated PSCs that are responsible for pancreatic fibrosis (Fig. 7C, red fluorescence). Interestingly, dual-immunofluorescence staining for α-SMA and AT_1 receptor revealed that the major site of the expression of AT_1 receptors was the activated PSCs (Fig. 7E, yellow fluorescence). AT_1 receptor (green fluorescence) was observed in the blood vessels and the infiltrated cells as well as in the activated PSCs (Fig. 7D).

**Effects of AT_1a receptor deletion on TGF-β1 mRNA expression in the pancreas.** Because TGF-β1 is known to be an important cytokine in the development of pancreatic fibrosis, we examined the effect of AT_1a receptor deletion on the TGF-β1 mRNA expression in the pancreas. TGF-β1 mRNA was detected faintly in the control pancreas, whereas it was overexpressed in cerulein-treated WT mice (Fig. 8). AT_1a (–/–) mice treated with cerulein showed lower expression of TGF-β1 compared with WT mice treated with cerulein (Fig. 8).

**DISCUSSION**

Although the RAS is an important system in regulating vascular homeostasis and fibrogenesis in the heart and kidney (20, 32, 35, 36), the precise roles of the RAS and the ANG II-AT_1 receptor pathway in the development of pancreatic fibrosis still remain unclear. Recently, some investigators have...
demonstrated that an ACE inhibitor and an AT1 receptor antagonist attenuate pancreatic fibrosis in an animal model (22, 46). However, the direct evidence for the sites of action of ANG II in the process of pancreatic fibrosis has not been demonstrated. To elucidate these issues, we took advantage of using genetically modified AT1a (H11002/H11002/H11002) mice that we had generated recently (16, 40). In the present study, we demonstrated that the ANG II-AT1 receptor pathway plays an important role in the development of pancreatic fibrosis. Moreover, attenuation of pancreatic fibrosis in AT1a (H11002/H11002/H11002) mice was mediated by suppressing activation and proliferation of PSCs.

The ANG II-AT1 receptor pathway is shown to be implicated in fibrosis of critical organs, including the heart, kidney, and liver. Pharmacological ANG II blockade attenuated fibrosis in these organs (17, 38, 47). Moreover, the studies using AT1a (−/−) mice have demonstrated that the deletion of AT1a receptor attenuates cardiac fibrosis after myocardial infarction (15), renal fibrosis induced by unilateral ureteral obstruction (37), and liver fibrosis induced by CCl4 (19). In the present study, we found that the progression of pancreatic fibrosis was significantly attenuated in AT1a (−/−) mice compared with that in WT mice, as assessed not only by histology but also by quantitation of the hydroxyproline content in the pancreas. These results suggest that the ANG II-AT1a receptor pathway is also responsible for regulating the development of pancreatic fibrosis.

In the pancreas, PSCs play a pivotal role in fibrogenesis, and PSC activation has been recognized as a key event in fibro-

Fig. 7. Colocalization of α-SMA and AT1 receptors. Pancreas sections of control WT mice (A and B, original magnification, ×400) or WT mice treated with cerulein for 4 wk (C-E, scale bar = 25 μm) were histologically examined by immunostaining with antibodies against α-SMA and AT1 receptor. A and B: in control mouse pancreas, α-SMA staining was observed in blood vessels (bv), and AT1 receptor staining was in the blood vessels and the epithelial cells of the pancreatic ducts (d). C: in mice treated with cerulein for 4 wk, an increased number of α-SMA-positive activated PSCs (red) were observed in fibrotic area. D: immunofluorescence staining for AT1 receptor (green) was observed in the activated PSCs, the blood vessels, and the infiltrated cells. E: merge image revealed that the major site of the expression of AT1 receptors was the activated PSCs (yellow).

Fig. 8. Expression of transforming growth factor-β1 (TGF-β1) mRNA in the pancreas of WT and AT1a (−/−) mice treated with cerulein for 4 wk. Ethidium bromide-stained 2% agarose gels of the RT-PCR products are shown.

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genesis (2–4, 14, 28, 31, 39). In response to pancreatic injury or inflammation, PSCs transform into a myofibroblast-like phenotype expressing α-SMA (activated state), proliferate, synthesize, and secrete increased amounts of ECM proteins, particularly collagens and fibronectin. In the current study, the number of activated PSCs was also decreased in AT1a(−/−) mice compared with that in WT mice, suggesting that attenuation of pancreatic fibrosis in AT1a(−/−) mice is mediated by suppressing activation and proliferation of PSCs. In the liver, hepatic stellate cells (HSCs), which exhibit similar features of PSCs, play a central role in fibrogenesis (12). Recent in vivo and in vitro studies showed that ANG II acts as a fibrogenic mediator via HSCs in the liver (5, 6, 19, 23, 47). In fact, activated human and rat HSCs express AT1 receptors, and ANG II induces proliferation and collagen synthesis in HSCs through the AT1 receptors (5, 6, 23). Therefore, we hypothesized that activated PSCs also express AT1 receptors and that the ANG II-AT1 receptor pathway plays an important role in pancreatic fibrogenesis. Expectedly, dual-immunofluorescence staining in WT mice revealed that α-SMA-positive activated PSCs actually express AT1 receptors. The data of this study and those from previous studies suggest that the ANG II-AT1a receptor pathway plays an important role in pancreatic fibrogenesis through the AT1 receptors (5, 6, 23).

Repeated episodes of acute pancreatitis can lead to increasing residual damage to the gland, eventually resulting in chronic pancreatitis that is characterized by destruction of exocrine parenchyma and its replacement with fibrosis (the necrosis-fibrosis sequence; see Refs. 1, 21, 33). Therefore, we chose this model to investigate the role of the ANG II-AT1a receptor pathway in the development of pancreatic fibrosis. Because there is a possibility that attenuation of acute pancreatic injury might lead to less severe progression of pancreatic fibrosis in AT1a(−/−) mice than in WT mice, we examined the effects of AT1a deletion on acute pancreatic injury. There were no significant differences in the severity of acute pancreatic injury evaluated by serum levels of amylase activity and histological alternations in the pancreas between AT1a(−/−) and WT mice. These results suggest that the ANG II-AT1a receptor pathway has little involvement in acute pancreatic injury induced by ceruline.

TGF-β1 is a major cytokine in the regulation of the production, degradation, and accumulation of ECM (29). A number of factors have been shown to modulate TGF-β1 expression. In various in vivo models of fibrotic diseases, ANG II-mediated TGF-β1 induction has been shown to contribute to progression of fibrosis (7, 23, 47). Moreover, in vitro, ANG II has been shown to stimulate TGF-β1 mRNA expression in a broad range of cell types, including mesangial cells (18), vascular smooth muscle cells (13), cardiac fibroblasts (9), and HSCs (47). In this study, TGF-β1 mRNA expression was less in AT1a(−/−) mice than in WT mice, suggesting that AT1a receptor is involved in the production of TGF-β1 in the pancreas.

In conclusion, our results demonstrate that the ANG II AT1a receptor pathway is not essential for the local pancreatic injury in acute pancreatitis but plays an important role in the development of pancreatic fibrosis through the ANG II-mediated PSC activation and proliferation. Our study provides not only novel insights into the pathogenesis of pancreatitis but also an important clinical implication. This study would open up a new therapeutic avenue targeting the ANG II action in patients with chronic pancreatitis. Because PSC activation and proliferation are key events in the development of pancreatic fibrosis, the use of an AT1 receptor antagonist at the early stage of chronic pancreatitis may suppress PSC activation and proliferation, resulting in prevention or retardation of the progression of chronic pancreatitis.

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