Combination Therapy with an Angiotensin-Converting Enzyme Inhibitor and an Angiotensin II Receptor Blocker synergistically suppresses Chronic Pancreatitis in Rats

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Abbreviations:

Wistar Bonn/Kobori: WBN/Kob, renin-angiotensin system: RAS, angiotensin-converting enzyme: ACE, angiotensin-converting enzyme inhibitor: ACEI, angiotensin II: AT-II, angiotensin II receptor blocker: ARB, transforming growth factor-ß1: TGF-ß1, pancreatic stellate cells: PSC, myeloperoxidase: MPO, reverse transcription-polymerase chain reaction: RT-PCR, α-smooth muscle actin: α-SMA, tumor necrosis factor-α: TNF-α, platelet-derived growth factor: PDGF, platelet-derived growth factor-receptor ß: PDGF-R ß.

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Abstract

Background & Aims: We recently demonstrated that both lisinopril and candesartan, respectively an angiotensin-converting enzyme inhibitor and angiotensin II type 1 receptor blocker, attenuate pancreatic inflammation and fibrosis in male WBN/Kob rats. The purpose of the present study was to assess whether combination therapy with low doses of both, ineffective when given alone, might synergistically exert protective effects. *Methods*: Lisinopril, candesartan, or a combination of both in drinking water were administered to 10-week-old male WBN/Kob rats for 10 weeks. Parameters of inflammation and fibrosis, positive immunostaining for α-smooth muscle actin, and gene expression of cytokine and growth factors were assessed, as well as circulating renin-angiotensin system components. Dose-dependent effects of combination therapy were also investigated. *Results*: Only combination therapy attenuated gross alterations in the pancreas, as quantitatively confirmed by increases in pancreatic weights and decreases in myeloperoxidase activity, hydroxyproline content, histologic scores, relative fibrosis area, and relative area of α-smooth muscle actin positive cells. Combination therapy suppressed upregulation of tumor necrosis factor-α, platelet-derived growth factor-receptor ß, and transforming growth factor-ß1 mRNA in the pancreas. Dose-dependence of combination therapy was recognized with reference to improvement in these parameters. *Conclusion*: Combination therapy synergistically alleviated pancreatic inflammation and fibrosis in male WBN/Kob rats. This effect may be related to suppression of tumor necrosis factor-α, platelet-derived growth factor-receptor ß, transforming growth factor-ß1 mRNA. Compared with the either therapy alone, combination therapy with an angiotensin-converting enzyme inhibitor and an angiotensin II type 1 receptor blocker may be more beneficial for treating

chronic pancreatitis.

Introduction

The relative paucity of easily reproducible animal models has limited our understanding of the pathogenetic mechanisms responsible for pancreatic inflammation and fibrosis. Male Wistar Bonn/Kobori (WBN/Kob) rats consistently and spontaneously develop chronic pancreatitis, which is accompanied by parenchymal destruction and its replacement with fibrosis, resulting in both endocrine and exocrine dysfunction (Mori et al., 1988). We have demonstrated that acinar cell apoptosis is associated with infiltration of T cells and that tacrolimus, an immunosuppressant, attenuates the apoptosis and chronic inflammation in this model (Yamada et al., 2001). It is well established that T cells infiltrate into the pancreas in human chronic pancreatitis (Okazaki et al., 2000). We thus consider that the present model may be useful for exploring therapeutic strategies for chronic pancreatitis in human, including the autoimmune disease now recognized as a new entity (Okazaki et al., 2000).

There is a growing body of evidence that the renin-angiotensin system (RAS) plays a crucial role in the pathophysiology of fibrosis in the heart, kidney, and liver (Kagami et al., 1994; Bataller et al., 2000; Ostrom et al., 2003). Both angiotensin-converting enzyme (ACE) inhibitors (ACEIs) and angiotensin II (AT-II) type 1 receptor blockers (ARBs) attenuate hepatic fibrosis by suppressing activation of hepatic stellate cells and reducing their production of transforming growth factor (TGF)-ß1 in experimental animals, suggesting that AT-II and its type 1 receptor interaction may be intimately involved in hepatic fibrosis (Jonsson et al., 2001; Yoshiji et al., 2001). Furthermore, it has been demonstrated that anti-aldosteronic drugs suppress cardiac and renal fibrosis in experimental animals, so that aldosterone, the last product of the RAS, may also be involved in fibrosis in these organs (Epstein et al.,

2001; Rocha et al., 2002).

Pancreatic stellate cells (PSC) which have some similar properties to their counterparts in liver are involved in the pathogenesis of pancreatic fibrosis in both experimental animals and humans (Apte et al., 1999; Haber et al., 1999). Further, the RAS is present intrinsically in the pancreas and its gene expression is elevated during acute pancreatitis and chronic pancreatic hypoxia in experimental animals, suggesting an important role of RAS in the pathogenesis of pancreatic injury (Leung et al., 2000; Chan et al., 2000). We recently demonstrated that both lisinopril, an ACEI, and candesartan, an ARB, in drinking water attenuate pancreatic inflammation and fibrosis by suppressing induction of TGF-ß1 mRNA and blocking activation of PSC in male WBN/Kob rats (Kuno et al., 2003; Yamada et al., 2003). However, the effective doses of ACEI and ARB observed in the previous studies are too high for the clinical use.

It is well documented that addition of ACEI to ARB and combination therapy with both drugs can either additively or synergistically exert cardio- and reno-protection in the experimental animals and humans (McKelvie et al., 1999; Mogensen et al., 2000; Rossing et al., 2003). These findings strongly suggested the possibility that combination therapy with ACEI and ARB may exert an additive or synergistic effect on pancreatic fibrosis, and to test this hypothesis the present study was performed with our WBN/Kob rats. The purposes were to: 1) determine whether ineffective doses of lisinopril and candesartan might in combination reduce pancreatic inflammation and fibrosis; 2) assess the effects of combination therapy on circulating components of the RAS and cytokine and growth factors mRNA in the pancreas in order to clarify mechanisms; and 3) investigate dose-dependence of combination therapy in the model.

Methods

Materials and Animals

Male WBN/Kob and Wistar rats were purchased from SLC (Hamamatsu, JAPAN) and maintained in a temperature-controlled room under constant light, with free access to water and standard laboratory feed. The study protocol was approved by the Animal Care Committee of Nagoya City University. Lisinopril and candesartan cilexetil (candesartan) were generous gifts from Shionogi Co., Ltd. (Osaka, Japan) and Takeda Co., Ltd. (Osaka, Japan), respectively. All other chemicals were of the highest quality available.

Groups of Animals and Treatment

Twenty-three WBN/Kob (10-week-old) rats were randomly divided into the four groups, receiving no treatment (n=6; untreated group), lisinopril (20 mg/L in drinking water) (n=5, lisinopril group), candesartan (10.5 mg/L) (n=5; candesartan group), and the combination of lisinopril (20 mg/L) and candesartan (10.5 mg/L) (n=7; combination group). In a second experiment, twenty-one animals (10-week-old) were divided into untreated (n=6; untreated group), and three combination therapy groups, the latter receiving lisinopril (20 mg/L) plus candesartan (10.5 mg/L) (n=5; combination group), lisinopril (6 mg/L) plus candesartan (8.4 mg/L) (n=5; medium combination group), lisinopril (3 mg/L) plus candesartan (4.2 mg/L) (n=5; low combination group).

The concentrations of lisinopril and candesartan in drinking water, 20 mg/L and 10.5 mg/L and more than twice of those were earlier found to be ineffective for attenuating pancreatic inflammation and fibrosis in the present model (Kuno et al.,

2003; Yamada et al., 2003). The estimated doses in the single therapy groups were 2 and 1 mg/kg/day, respectively (Kuno et al., 2003; Yamada et al., 2003). In the present experiments, lisinopril was dissolved in drinking water and candesartan was dissolved in 1:1 mix of ethanol and polyethylene glycol, warmed to 60 centigrade, and then 1 N sodium bicarbonate solution was added before being dissolved in drinking water (Yamada et al., 2003). Untreated group was given water containing equal volume of ethanol, polyethylene glycol, and sodium bicarbonate solution without lisinopril and candesartan. Fresh solutions were given three times a week, for a total of 10 weeks, and the amount consumed was calculated. Body weights were recorded weekly. Five male Wistar rats (20-week-old) were used as non-pancreatitis controls.

Tissue Sampling

All WBN/Kob rats treated for 10 weeks were killed with an overdose of pentobarbital sodium (Abbott Laboratories, North Chicago, IL, USA) after taking blood from the abdominal aorta. Each pancreas was immediately removed and samples were stored at –80 centigrade for determination of myeloperoxidase (MPO) activity and hydroxyproline content, or in liquid nitrogen for eventual determination of cytokine and growth factors mRNA levels by reverse transcription-polymerase chain reaction (RT-PCR). Tissue was also fixed in 10% buffered formalin for histological assessment and immunohistochemistry. Serum and plasma containing ethylenediamine tetraacetic acid sodium were kept at –80 centigrade for eventual determination of ACE (serum), AT-II (plasma), and aldoseterone (serum). In addition, pancreases from 20-week-old Wistar rats were immediately placed in liquid nitrogen for RT-PCR analyses.

Histologic Analyses

Pancreas tissues, obtained from both duodenal and splenic lobes, were fixed with 10% buffered formalin and routinely processed for embedding in paraffin, sectioned and stained with hematoxylin-eosin and Azan. Histologic observation was performed using a microscope having the object lens of x10, intermittent lens of x4, and eyepiece lens of x10 (Olympus BH-2, Olympus Co., Tokyo, Japan). The histologic status of inflammation in each animal was evaluated by a pathologist who was unaware of the groups, with reference to the grades of inflammatory cell infiltration, interstitial edema, acinar cell necrosis, hemorrhage, and fibrosis, as reported previously (Yamada et al., 2001; Kuno et al., 2003; Yamada et al., 2003). Each factor was graded as none, mild, moderate, and severe, scored as 0, 1, 2 and 3, respectively. Quantity of fibrosis was analyzed under the microscope (Olympus BH-2, Olympus Co., Tokyo, Japan) connected with the 3CCD Color Video Camera (DXC-950, Sony Co., Tokyo, Japan) with the aid of an image processor (Image Processor for Analytical Pathology; Sumica Technoservise, Osaka, Japan). The percentage of aniline-blue positive fibrous tissue per total area in whole Azan-stained pancreas section was measured except lymph nodes and major vessels, if included (Kuno et al., 2003; Yamada et al., 2003). The results were then used to calculate values relative to the control cases, set at 100%.

Immunohistochemistry for α-Smooth Muscle Actin

Tissues were stained with mouse anti-human α-smooth muscle actin (SMA) mAb (Dako, Carpiteria, CA) which can detect rat α-SMA as described previously (Kuno et al., 2003; Yamada et al., 2003). The relative area of α-SMA-positive cells per total area was also evaluated as described for "Histologic Analyses".

Measurement of Pancreatic Myeloperoxidase Activity

Pancreatic MPO activity, an indirect quantitative index of granulocyte infiltration, was determined as described previously (Yamada et al., 2001; Kuno et al., 2003; Yamada et al., 2003). MPO activity was determined by measuring H_2O_2-dependent oxidation of 3,3',5,5' tetramethylbenzidine and expressed as units per gram wet weight of pancreas.

Measurement of Pancreatic Hydroxyproline Content

Hydroxyproline content, an indicator of collagen deposition, was determined as reported previously (Kuno et al., 2003; Yamada et al., 2003). Hydroxyproline levels were calculated using a standard curve made with 4-hydroxy-1-proline and expressed as micrograms per gram tissue.

Measurement of Serum Angiotensin-Converting Enzyme Activity, Plasma Angiotensin II Levels, and Serum Aldosterone Levels

Serum ACE activity was determined by using a commercially available kit, ACE color (Fujirebio Co., Tokyo, Japan), and expressed as IU/L.

AT-II in the plasma containing ethylenediamine tetraacetic acid sodium was determined by radioimmunoassay as reported previously (Iwahana et al., 1996). Briefly, plasma was incubated with a rabbit anti-AT-II antibody and ^{125}I-labelled AT-II and then a goat antibody for rabbit immunoglobulin. After adding polyethylene glycol, the radioactivity of the pellet was measured using a counter. The amount of AT-II was calculated using a standard curve generated using angiotensin II human.

Serum aldosterone was determined with a commercially available aldosterone

RIA kit II (Dinabott Co., Tokyo, Japan) and expressed as pg/mL.

Reverse Transcription-Polymerase Chain Reaction

Total RNA was extracted from frozen pancreatic tissue with Trizol reagent (Invitrogen Co., Carlsbad, CA. USA) and 2 mg was reverse transcribed into cDNA using an Oligo (dT) 12-18 Primer (Invitrogen Co.), Superscript II RNase H-Reverse Transcriptase (Invitrogen Co.), and an RNase inhibitor (TOYOBO Co., LTD., Osaka, Japan). The PCR was performed with reaction mixtures containing 2.5 mM dNTP, 10 mM sense and anti-sense primers, and 5 units/mL Taq DNA polymerase (TAKARA SHUZO Co. Otsu, Japan) in a thermal cycler for 1 min at 94 centigrade, 1 min at 55 [tumor necrosis factor (TNF)-α, platelet-derived growth factor (PDGF)-B, PDGF-receptor ß (PDGF-Rß), ß-actin], or 62 [transforming growth factor (TGF)-ß1] degrees centigrade, and 2 min at 72 centigrade, for 34 (TNF-α), 28 (PDGF-B), 30 (PDGF-Rß), 32 (TGF-ß1), and 22 (ß-actin) cycles, and then an extension reaction was carried out at 72 centigrade for 5 min.

Sequences of primers for TNF-α, PDGF-B, PDGF-Rß, TGF-ß1, and ß-actin were listed in Table 1. PCR products were electrophoresed on 1% agarose gels, visualized by ethidium bromide staining and photographed under ultraviolet light.

The relative expression intensity of each mRNA band was determined with an image analyzer (NIH image) and semiquantitative analyses relative to ß-actin were preformed.

Statistics

Data are expressed as arithmetic means ± standard deviations. Statistical

differences among groups were identified using the one-way analysis of variance, followed by multiple comparisons using the least significance difference method. The Mann-Whitney's U-test was employed for statistical analyses of the histologic scores, relative fibrosis area, and relative area of α-SMA positive cells.

Results

Doses of Lisinopril and Candesartan, Body Weights, and Pancreas Weights

The average doses of lisinopril and candesartan in the single therapy groups calculated from water consumption and body weights were 2.34 ± 0.18 and 0.94 ± 0.05 mg/kg/day, respectively, during the experimental period. Those of lisinopril and candesartan in the combination group were 2.40 ± 0.07 and 1.26 ± 0.04 mg/kg/day, respectively. Body weights were significantly lower in the lisinopril and combination groups compared with the untreated group (Table 2). The pancreas weights were significantly higher in the combination group than in the other groups.

Macroscopic Findings

The pancreases in the untreated, lisinopril, and candesartan groups had widespread brown and red foci (Fig. 1A, 1B, and 1C, respectively). In sharp contrast, the pancreases in the combination group had almost intact appearances (Fig. 1D) as observed in the pancreas in Wistar rat (figure not shown).

Histologic Analyses

Focal severe inflammation in the pancreas was evident in the untreated group (Fig. 2A). This was characterized by neutrophil and lymphocyte infiltration, interstitial edema, hemorrhage, and occasional acinar cell necrosis. Fibrosis was noted as replacing acini, being also found between remaining acini. Neither lisinopril nor candesartan had any significant effects on these inflammatory changes (Fig. 2B and 2C). However, the combination therapy dramatically reduced the inflammatory changes,

resulting in limited foci of lymphocyte infiltration with slight fibrosis (Fig. 2D). No histologic alteration in the pancreas was present in Wistar rat (Fig. 2E). Significant reduction of inflammatory scores was observed for almost all parameters in the combination group compared with the untreated, lisinopril, and candesartan groups (Table 3). Relative fibrosis area calculated by Azan stained area in the pancreas tended to be decreased to 56.8 ± 35.4 % in the combination group compared with 100% set for the untreated group.

Immunohistochemistry for α-Smooth Muscle Actin

α-SMA positive cells with the morphology of activated PSCs were localized in the peri-acinar fibrotic areas and vascular walls in the untreated, lisinopril, and candesartan groups (figure not shown). In contrast, α-SMA positive cells were observed only in the vascular walls in the combination group. Decrease in the relative area of α-SMA positive cells was noted, although it did not reach statistical significance (Table 3).

Pancreatic Myeloperoxidase Activity

Pancreatic MPO activity, an indirect index of granulocyte infiltration, was significantly suppressed in the combination group compared with the untreated, lisinopril, and candesartan groups (Table 2).

Pancreatic Hydroxyproline Content

The combination therapy significantly suppressed the increase in pancreatic hydroxyproline content, an indeirect index of collagen deposition, compared with the

untreated, lisinopril, and candesartan groups (Table 2).

Serum Angiotensin-Converting Enzyme Activity, Plasma Angiotensin II Levels, and Serum Aldosterone Levels

Serum ACE activity and plasma AT-II levels were significantly decreased in the lisinopril and combination groups compared with the untreated and candesartan groups, respectively. Serum aldosterone levels were not significantly decreased in the combination group compared with the other groups (Table 4).

Expression of Tumor Necrosis Factor-α, Platelet-Derived Growth Factor B, Platelet-Derived Growth Factor-Receptor ß, and Transforming Growth Factor-ß1 mRNA

RT-PCR revealed TNF-α, PDGF-R ß, and TGF-ß1 mRNA to be overexpressed in the pancreas in the untreated group, while they were detected at only low levels in male Wistar rats (Fig. 3). However, PDGF B mRNA was not upregulated in the untreated groups compared with male Wistar rats. While lisinopril and candesartan alone did not alter TNF-α, PDGF-R ß, and TGF-ß1 mRNA levels, the combination therapy suppressed upregulation of all three mRNAs. Semiquantitative analysis of each mRNA relative to ß-actin revealed that TNF-α, PDGF-R ß, and TGF-ß1 mRNA were significantly upregulated in the untreated group compared with Wistar rats and they were suppressed in the combination group (Table 5).

Doses of Lisinopril and Candesartan, Body Weights, and Pancreas Weights in the Dose-dependence Study

Next, the dose-dependent effects of the combination therapy were assessed. The average doses of lisinopril in the low and medium combination and combination groups were 0.29 ± 0.01, 0.62 ± 0.06, and 2.27 ± 0.12 mg/kg/day, respectively. Those of candesartan were 0.41 ± 0.01, 0.87 ± 0.08, and 1.19 ± 0.06 mg/kg/day, respectively. The body weights were significantly lower in all three combination groups than in the untreated group, and the pancreas weights were significantly higher (Table 6).

Dose-dependence of the Combination Therapy Effects on Histologic Findings

In line with earlier studies, untreated animals showed severe inflammation and fibrosis in the pancreas (Fig. 4A). Limited foci of severe and moderate focal inflammation remained in the low and medium combination groups, respectively (Fig. 4B and 4C), so that protective effects assessed by histologic scores were observed with regard to acinar cell necrosis in the low combination group and acinar cell necrosis and fibrosis in the medium combination group, respectively (Table 7). In contrast, significant reduction was noted for all histologic scores in the combination group (Fig. 4D and Table 7).

Dose-dependence of the Combination Therapy Effects on Pancreatic Myeloperoxidase Activity and Pancreatic Hydroxyproline Content

Pancreatic MPO activity and pancreatic hydroxyproline content were significantly decreased in the three combination groups as compared with the untreated group (Table 6).

Discussion

The present study for the first time demonstrated synergistic preventive effects of combination therapy with ACEI and ARB on chronic inflammation and fibrosis in a visceral organ, the pancreas. The combination therapy with lisinopril and candesartan, ineffective when given alone, attenuated pancreatic inflammation and fibrosis in male WBN/Kob rats. This was quantitatively reflected in increased pancreatic weights and decreased MPO activity (an index of granulocyte infiltration), hydroxyproline content (an index of collagen deposition), histologic scores, relative area of α-SMA positive cells (an index of stellate cell activation), and relative fibrosis area. Furthermore, the combination therapy suppressed upregulation of TNF-α, PDGF-R ß, and TGF-ß1 mRNAs in the pancreas. We speculate that suppression of TNF-α mRNA may lead to decreased production of cytokine and reduced inflammatory response. Furthermore, suppression of PDGF-R ß and TGF-ß1 mRNA might account for prevention of pancreatic stellate cell activation and pancreatic fibrosis. We additionally demonstrated dose-dependence of the protective effects of combination therapy.

We recently reported that lisinopril, an ACEI, and candesartan, an ARB, in drinking water suppress pancreatic inflammation and fibrosis in the present model (Kuno et al., 2003; Yamada et al., 2003). However, the doses were much higher than the maximal clinical doses (lisinopril; 21.3 mg/kg/day, 65-fold and candesartan; 13.9 mg/kg/day, 70-fold). Since ACEI and ARB suppress the RAS by pharmacologically different mechanisms, the combination therapy with ACEI and ARB might be expected to be safer and more effective in inhibiting AT-II function than single therapy with twice doses of either agent. It was well documented that the combination therapy with ACEI and ARB has advantages over either therapy alone at low doses for treating heart and

kidney diseases in the experimental models and humans (McKelvie et al., 1999; Mogensen et al., 2000; Rossing et al., 2003; Nakamura et al., 2003). These findings prompted us to explore the effect of the combination therapy on chronic pancreatitis. Since the single therapies with more than twice of the present single doses were reported to be ineffective for treating chronic pancreatitis (lisinopril; 5.1 ± 0.5 mg/kg/day, Kuno et al., 2003; candesartan; 4.3 ± 0.3 mg/kg/day, Yamada et al., 2003), we defined the protective effect of the combination therapy as synergistic.

The serum levels of lisinopril and candesartan in the combination therapy are estimated to be around 18 and 55 ng/mL, respectively, from the data of the previous studies (Kuno et al., 2003; Yamada et al., 2003). Since pancreatic weights were increased and pancreatic MPO activity, pancreatic hydroxyproline content, and some histologic scores were decreased in the low and medium combination groups compared with the untreated group in the study of dose dependence, the combination therapy with lower doses of both drugs could still exert protective effects. Considering that the calculated doses of lisinopril and candesartan were 0.29 ± 0.01 and 0.41 ± 0.01 mg/kg/day in the low combination group, it is possible that combination therapy with the maximal clinical doses of both drugs (lisinopril; 0.33 mg/kg/day and candesartan; 0.20 mg/kg/day) may alleviate pancreatic inflammation and fibrosis in cases of human chronic pancreatitis.

Since both lisinopril and candesartan are widely used as antihypertensive drugs in human, one might speculate that they may decrease blood pressure in the present model. However, the combination therapy with much higher doses of ACEI and ARB did not cause significant decrease in blood pressure in rats with myocardial infarction (Nakamura et al., 2003). It has been well documented that the combination therapy

with ACEI and ARB did not cause excessive decrease in blood pressure in human (McKelvie et al., 1999; Mogensen et al., 2000; Rossing et al., 2003). Taken together, it was speculated that the combination therapy may not cause excessive decrease in blood pressure in the present study.

TNF-α, a proinflammatory cytokine, is expressed in acinar cells and infiltrating macrophages in acute pancreatitis (Gukovskaya et al., 1997). It is reported that TNF-α released from acinar cells is involved in the development of pancreatitis by mediating early inflammatory reactions (Gukovskaya et al., 1997; Xie et al., 2001a). Its inhibition reduces acute pancreatic damage and improves survival with acute pancreatitis in rats (Hughes et al., 1996). Moreover, tissue expression of TNF-α is involved in the onset of chronic pancreatitis in the present model (Xie et al., 2001b). Thus, we assessed tissue TNF-α mRNA levels and confirmed upregulation in the untreated group. Only combination therapy suppressed this.

PDGF, a dimerised protein secreted by infiltrated inflammatory cells, including macrophages and platelets, induces proliferation of PSC (Apte et al., 1999; Luttenberger et al., 2000). It consists of two polypeptide chains linked via disulphide bonds and has three isoforms, PDGF-A, PDGF-B, and PDGF-AB, PDGF-B having the most potent effects in vitro (Apte et al., 1999). The PDGF receptor is composed of two types α and ß which recognize all three types of PDGF and PDGF-B, respectively. PDGF-B and PDGF-R ß, but not PDGF-R α, are closely associated with fibrosis in human and experimental pancreatitis (Ebert et al., 1998; Haber et al., 1999). Furthermore, PDGF-B mRNA is upregulated in dibutyltin dichloride-induced chronic pancreatitis (Inoue et al., 2002). We here found PDGF-R ß mRNA to be upregulated in the present model, while PDGF-B mRNA was unchanged. Again, only combination therapy

suppressed this upregulation of PDGF-R ß mRNA.

It is well established that TGF-ß1, a multifunctional cytokine, mediates fibrosis in various organs (Waltenberger et al., 1993; Yoshioka et al., 1993; Bedossa et al., 1995) and TGF-ß1 precursors and its latent binding protein are observed in inflamed lesions with fibrosis in human chronic pancreatitis (Van Laethem et al., 1995). TGF-ß1 promotes pancreatic fibrosis and inhibition of its expression results in reduction of extracellular matrix protein in experimental animals (Van Laethem et al., 1996; Menke et al., 1997). As reported previously, TGF-ß1 mRNA was here found to be upregulated and the combination therapy suppressed this.

AT-II directly induces proliferation of pancreatic stellate cells as well as digestive enzyme secretion by acinar cells in vitro, both of which were abolished in the presence of ARBs (Reiner et al., 2004; Hama et al., 2004; Tsang et al., 2004). We speculate that inhibition of AT-II by the combination therapy may have similar effects in the present model. Moreover, AT-II is a potent vasoconstrictor and ACEIs increase vasodilatory factors, including bradykinin, nitric oxide, and prostaglandin (Campbell et al., 1994). The combination therapy could thus increase pancreatic blood flow by both blocking AT-II function and increasing vasodilatory factors, thereby improving pancreatic ischemia, which was reported to be one element in the pathogenesis of chronic pancreatitis (Mori et al., 1988). Moreover, interaction between AT-II and its type 1 receptor may result in secretion of aldosterone, which may be involved in cardiac and renal fibrosis in the experimental animals (Epstein et al., 2001; Rocha et al., 2002). In fact, combination therapy with an ACEI and an ARB resulted in greater decrease in circulating aldosterone levels than the either agent alone in the RESOLVD study (McKelvie et al., 1999). To our knowledge, no reports are available regarding the role

of aldosterone in chronic pancreatitis. We hypothesized that protection might be related to its decrease, but contrary to expectation, the combination therapy did not cause further depress of serum aldosterone levels compared with single lisinopril therapy, suggesting that aldosterone may not be involved in the pathogenesis of pancreatic fibrosis.

There are sporadic reports demonstrating that ACEIs caused acute pancreatitis in human (Madsen JS et al., 1995, Muchnick et al., 1999). Although we were able to reduce the effective dose of lisinopril by combined administration with candesartan, caution may be necessary when lisinopril is used for treating chronic pancreatitis in human.

In conclusion, the combination therapy with lisinopril and candesartan may synergistically alleviate chronic pancreatic inflammation and fibrosis in male WBN/Kob rats by suppressing upregulation of TNF-α, PDGF-R ß, and TGF-ß1 mRNA, resulting in the prevention of pancreatic stellate cell activation and pancreatic fibrosis. We propose that combination therapy with an ACEI and an ARB may allow reduction of effective doses so that treatment of chronic pancreatitis with such agents will become possible. Further studies in this area are clearly warranted.

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Legends for Figure

Figure 1

Gross appearance of pancreas tissue in the (A) untreated, (B) lisinopril, (C) candesartan, and (D) combination groups.

(A), (B), and (C) Note the scattered brown and dark red foci.

(D) The pancreas has an intact appearance.

Figure 2

Representative histologic appearance of pancreas tissue in the (A) untreated, (B) lisinopril, (C) candesartan, (D) combination groups, and (E) Wistar rat (H&E staining. the object lens of x10, intermittent lens of x4, and eyepiece lens of x10). Scale bar in the figure 2E indicates 100 μm.

(A) Massive infiltration of neutrophils, lymphocytes, and plasma cells, with disappearance of acinar cells and replacement with fibrous tissue are evident. This sample was scored 1 for hemorrhage, 2 for inflammatory cell infiltration, interstitial edema, and acinar cell necrosis, and 3 for fibrosis.

(B)(C) Severe inflammatory changes observed in the lisinopril and candesartan groups. (B) was scored 2 for all histologic parameters and (C) was scored 1 for acinar cell necrosis and 2 for inflammatory cell infiltration, interstitial edema, hemorrhage, and fibrosis.

(D) Focal lymphocyte infiltration with slight bleeding is observed in the combination group. This sample was scored 1 for interstitial edema, acinar cell necrosis, and fibrosis and 2 for inflammatory cell infiltration and hemorrhage.

(E) No alteration is observed.

Figure 3

Expression of TNF-α, PDGF-B, PDGF-R ß, and TGF-ß1 mRNAs analyzed by RT-PCR. Findings are for untreated, lisinopril, candesartan, and combination groups and 20-week-old male Wistar rats. The ß-actin is included in each pancreas tissue as an internal control.

Figure 4

Representative histologic appearance of the pancreas tissue in the (A) untreated, (B) low combination, (C) medium combination, and (D) combination groups (H&E staining. the object lens of x10, intermittent lens of x4, and eyepiece lens of x10). Scale bar in the figure 4D indicates 100 μm.

(A) Severe inflammation is apparent, with lymphocyte infiltration, disappearance of acini, bleeding, and replacement with fibrous tissue. This sample was scored 2 for infiltration of inflammatory cells, interstitial edema, acinar cell necrosis, and hemorrhage, and 3 for fibrosis.

(B) Severe inflammation, characterized by lymphocyte infiltration, bleeding, and fibrosis is focally observed. This sample was scored 1 for acinar cell necrosis, 2 for infiltration of inflammatory cells, interstitial edema, and hemorrhage, and 3 for fibrosis.

(C) Inflammatory changes are reduced and limited to small foci of lymphocyte infiltration and bleeding without severe fibrosis or disappearance of acini. This sample was scored 1 for all histologic parameters.

(D) Inflammatory foci are not obvious but some fibrous tissue is evident between acinar

cells. This sample was scored 0 for interstitial edema, acinar cell necrosis, and hemorrhage and 1 for infiltration of inflammatory cells and fibrosis.

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Table 1. Primers used for RT-PCR in pancreas extracts

TNF-α	
sense	5'-TTCTGTCTACTGAACTTCGGGGTGATCGGTCC-3'
anti-sense	5'-GTATGAAGTGGCAAATCGGCTGACGGTGTGGG-3'
PDGF-B	
sense	5'-CGAGCACCTTTGTTCTGACA-3'
anti-sense	5'-CCATGGTCATTCACACTCAC-3'
PDGF-Rß	
sense	5'-GTCGAGTCGGAAAGCTCATC-3'
anti-sense	5'-GGTCCAGTCCTCTGTGAAGC-3'
TGF-ß1	
sense	5'-GCGGACTACTACGCCAAAGA-3'
anti-sense	5'-TGGTTGTAGAGGGCAAGGAC-3'
ß-actin	
sense	5'-TGGCCTCACTGTCCACCTTC-3'
anti-sense	5'-CGAATGGCTGACCATTCAGA-3'

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Table 2. Effects of lisinopril, candesartan, and combination of lisinopril and candesartan in drinking water on body weight, pancreas weight, pancreas myeloperoxidase (MPO) activity, and pancreas hydroxyproline content in 20-week-old male WBN/Kob rats

	Body Weight (g)	Pancreas Weight (g/kg)	Pancreas MPO Activity (Unit/g)	Pancreas Hydroxyproline Content (μg/kg)
Untreated (n=6)	372.8 ± 5.5	1.40 ± 0.25	6.30 ± 1.90	709.2 ± 290.9
Lisinopril (n=5)	350.6 ± 18.8	2.30 ± 0.32	5.79 ± 2.18	427.2 ± 96.2
Candesartan (n=5)	387.0 ± 29.8	1.78 ± 0.24	3.12 ± 0.51	578.0 ± 144.3
Combination of Lisinopril and Candesartan (n=7)	320.4 ± 16.0**	2.80 ± 0.34++	1.68 ± 0.55##	177.3 ± 90.0 ff,&

NOTE. Statistical analyses were performed with one-way analysis of variance, followed by multiple comparisons using the least significance difference method.

++: Statistically different from the corresponding data in the untreated and candesartan groups by p< 0.01.

****: Statistically different from the corresponding data in the untreated, lisinopril, and candesartan groups by p< 0.01.**

##: Statistically different from the corresponding data in the untreated and lisinopril groups by p< 0.01.

ff: Statistically different from the corresponding data in the untreated group by p< 0.01.

&: Statistically different from the corresponding data in the lisinopril and candesartan groups by p< 0.01.

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Table 3. Effects of lisinopril, candesartan, and combination of lisinopril and candesartan in drinking water on histologic scores, relative fibrosis area, and relative area of α-smooth muscle actin positive cells of the pancreas in 20-week-old male WBN/Kob rats

	Histologic Score &					Relative Fibrosis Area (%)	Relative Area of α-Smooth MuscleActin Positive Cells (%)
	Inflammatory Cell Infiltration	Interstitial Edema	Acinar Cell Necrosis	Hemorrhage	Fibrosis		
Untreated (n=6)	2.2 ± 0.4	2.3 ± 0.5	1.7 ± 0.8	1.8 ± 0.8	2.7 ± 0.5		
Lisinopril (n=5)	2.2 ± 0.4##	2.4 ± 0.5###	1.4 ± 0.5	2.0 ± 0.0#	2.6 ± 0.5###	113.0 ± 44.4	96.5 ± 55.6
Candesartan (n=5)	1.8 ± 0.4#	2.0 ± 0.7##	1.2 ± 0.4	1.8 ± 0.1	2.2 ± 0.4##	117.1 ± 26.1#	195.5 ± 79.8#
Combination of Lisinopril and Candesartan (n=7)	0.6 ± 0.9**	0.2 ± 0.4***	0.4 ± 0.5*	0.6 ± 0.9*	0.6 ± 0.5***	55.6 ± 19.0	56.8 ± 35.4

NOTE. &: Scores for each factor are averages and SDs for the grade, none, mild, moderate, and severe (0-3).

Statistical analyses were perfomed with Mann-Whitney's U-test.

*****, **, * : Statistically different from the corresponding data in the untreated group by p<0.001, 0.01, and 0.05, respectively.**

###, ##, #: Statistically different from the corresponding data in the combinaiton group by p<0.001, 0.01 and 0.05, respectively.

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Table 4. Effects of lisinopril, candesartan, and combination of lisinopril and candesartan in drinking water on serum angiotensin-converting enzyme (ACE) activity, plasma angiotensin II levels, and serum aldosterone levels in 20-week-old male WBN/Kob rats

	Serum ACE Activity (IU/L)	Plasma Angiotensin II (pg/mL)	Serum Aldosterone (pg/mL)
Untreated (n=6)	14.7 ± 0.7	198.7 ± 13.4	136.8 ± 19.7
Lisinopril (n=5)	4.5 ± 0.4$^{**,++}$	92.0 ± 21.1**	80.2 ± 11.1^{+}
Candesartan (n=5)	15.4 ± 0.3	175.8 ± 26.9	109.7 ± 10.8
Combination of Lisinopril and Candesartan (n=7)	4.1 ± 0.3$^{**,++}$	106.6 ± 6.4**	91.2 ± 14.4

NOTE. Statistical analyses were performed with one-way analysis of variance, followed by multiple comparisons using the least significance difference method.

***,**: Statistically different from the corresponding data in the untreated group by $p< 0.05$ and 0.01, respectively.**

+, ++: Statistically different from the corresponding data in the candesartan group by $p< 0.05$ and 0.01, respectively.

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Table 5. Relative expression intensity of tumor necrosis factor (TNF)-α, platelet derived growth factor (PDGF)-B, PDGF-receptor (PDGF-R) ß, and transforming growth factor (TGF)-ß1 mRNA of the pancreas in 20-week-old male WBN/Kob and Wistar rats

	TNF-α	PDGF-B	PDGF-R ß	TGF-ß1
WBN/Kob				
Untreated (n=4)	0.83 ± 0.15*	0.77 ± 0.16	1.65 ± 0.15*	0.78 ± 0.08**
Lisinopril (n=4)	0.75 ± 0.15	0.68 ± 0.14	1.40 ± 0.21	0.57 ± 0.05
Candesartan (n=4)	0.63 ± 0.39	0.74 ± 0.18	1.50 ± 0.25	0.60 ± 0.10
Combination of Lisinopril and Candesartan (n=4)	0.40 ± 0.20+	0.72 ± 0.16	0.98 ± 030++	0.26 ± 0.17++
Wistar (n=4)	0.45 ± 0.25	0.84 ± 0.17	1.11 ± 0.22	0.05 ± 0.03

NOTE. Statistical analyses were performed with one-way analysis of variance, followed by multiple comparisons using the least significance difference method.

The relative expression intensity of each band was determined with an image analyzer and semiquantitative analyses relative to ß-actin were performed.

***: Statistically different from the corresponding data in Wistar rats by $p < 0.05$.**

+,++: Statistically different from the corresponding data in the untreated group by $p < 0.05$ and $p < 0.01$, respectively.

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Table 6. Effects of low combination, medium combination, and combination of lisinopril and candesartan in drinking water on body weight, pancreas weight, pancreas myeloperoxidase (MPO) activity, and pancreas hydroxyproline content in 20-week-old male WBN/Kob rats

	Body Weight (g)	Pancreas Weight (g/kg)	Pancreas MPO Activity (Unit/g)	Pancreas Hydroxyproline Content (μg/kg)
Untreated (n=6)	375.8 ± 21.7	1.90 ± 0.41	5.70 ± 1.36	760.8 ± 326.8
Low Combination (n=5)	328.2 ± 15.1**	2.80 ± 0.66**	2.64 ± 0.27**	254.3 ± 76.6**
Medium Combination (n=5)	314.2 ± 15.1**	3.12 ± 0.20**	2.93 ± 1.32**	272.0 ± 77.3**
Combination (n=5)	332.4 ± 14.0**	3.17 ± 0.25**	1.68 ± 0.55**	205.7 ± 79.1**

NOTE. Statistical analyses were performed with one-way analysis of variance, followed by multiple comparisons using the least significance difference method.

****: Statistically different from the corresponding data in the untreated, low combination, and medium combination groups by $p < 0.01$.**

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Table 7. Effects of low and medium combination and combination of lisinopril candesartan in drinking water on histologic scores, relative fibrosis area, and relative area of α-smooth muscle actin positive cells of the pancreas in 20-week-old male WBN/Kob rats

	Histologic Score &					Relative Fibrosis Area (%)	Relative Area of α-Smooth MuscleActin Positive Cells (%)
	Inflammatory Cell Infiltration	Interstitial Edema	Acinar Cell Necrosis	Hemorrhage	Fibrosis		
Untreated (n=6)	2.2 ± 0.4	2.2 ± 0.4	2.4 ± 0.5	2.2 ± 0.8	2.6 ± 0.5		
Low Combination (n=5)	2.0 ± 0.0	1.6 ± 0.5	0.8 ± 0.4**	2.0 ± 0.7	2.6 ± 0.5	40.2 ± 26.8**	59.2 ± 19.1
Medium Combination (n=5)	1.5 ± 0.6	1.3 ± 1.0	1.0 ± 0.8*	1.3 ± 1.0	1.3 ± 0.5*,#	14.6 ± 9.1***	16.5 ± 5.9**
Combination (n=5)	0.9 ± 0.4***,##	0.3 ± 0.5***,#	0.4 ± 0.5***	0.7 ± 0.5*	1.0 ± 0.6**,##	28.1 ± 14.8***	46.9 ± 17.9

NOTE. &: Scores for each factor are averages and SDs for the grade, none, mild, moderate, and severe (0-3).

Statistical analyses were perfomed with Mann-Whitney's U-test.

###, ##, #: Statistically different from the corresponding data in the low combination group by p<0.001, 0.01 and 0.05, respectively.

*****, ** , *: Statistically different from the corresponding datat in the the untreated group by p<0.001, 0.01, and 0.05, respectively.**

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Fig. 1A and 1B

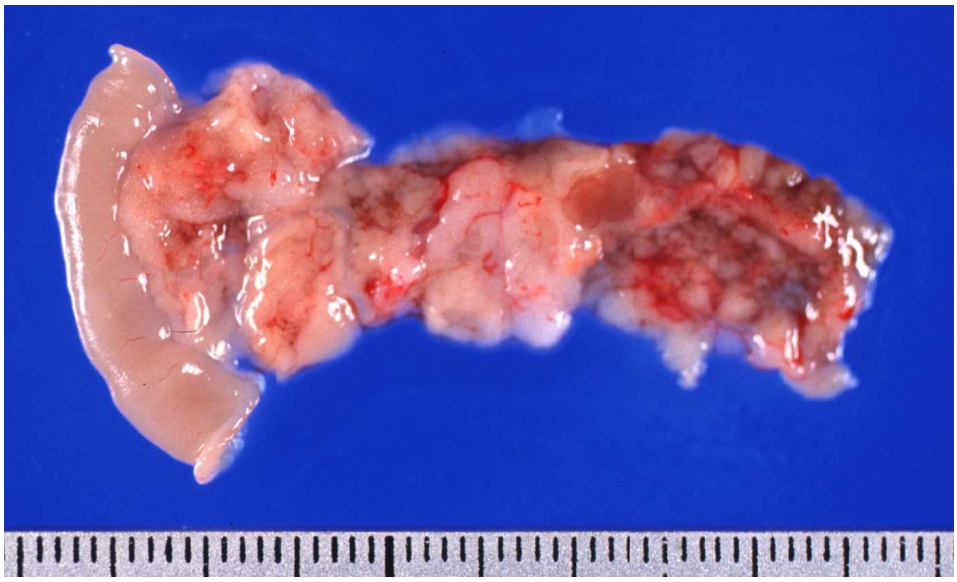

Fig. 1A: Untreated

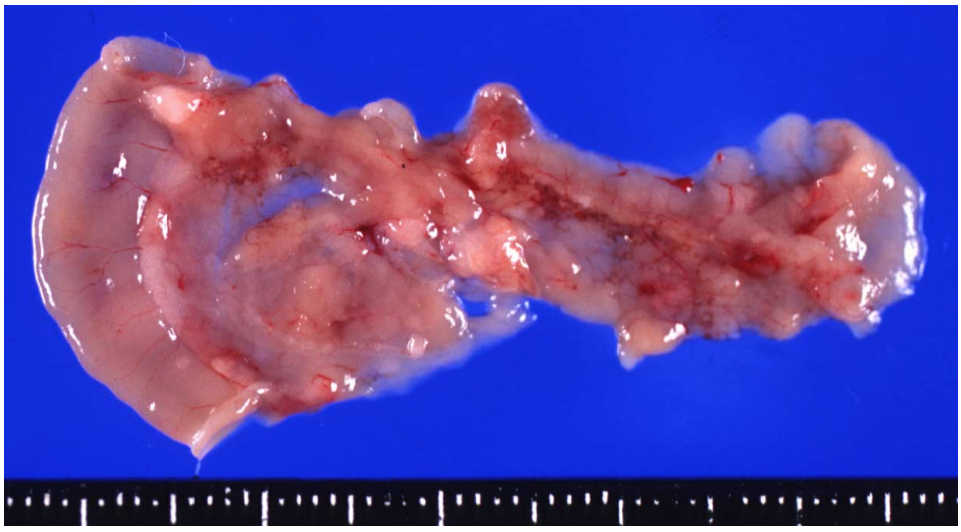

Fig. 1B: Lisinopril

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Fig. 1C and 1D

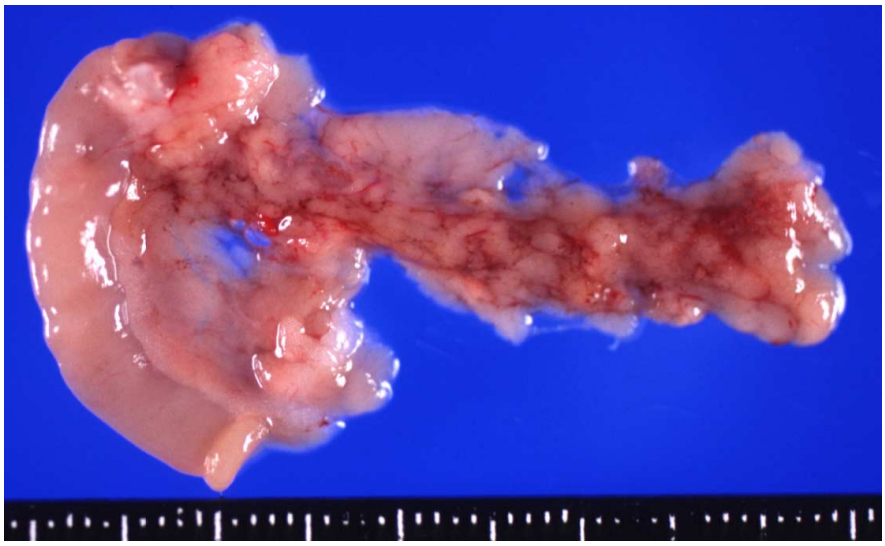

Fig. 1C: Candesartan

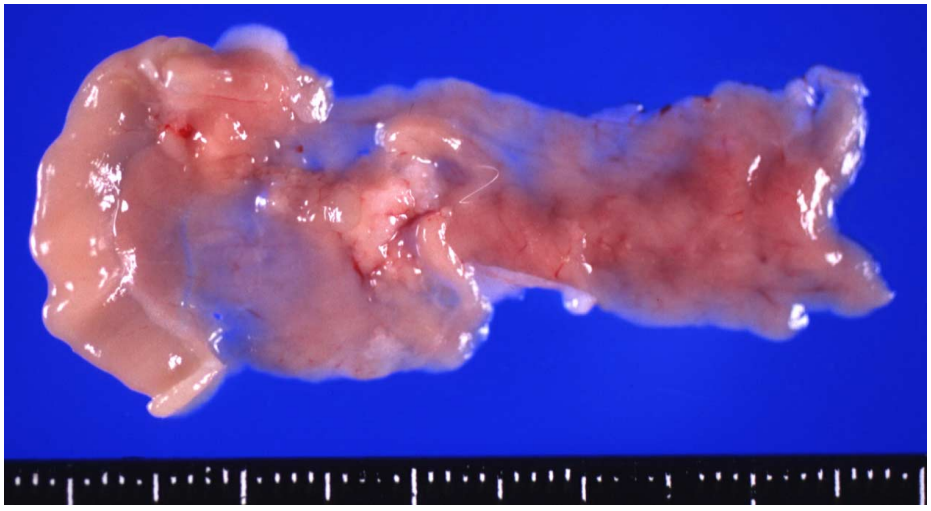

Fig. 1D: Combination

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Fig. 2A, 2B, 2C, 2D, and 2E

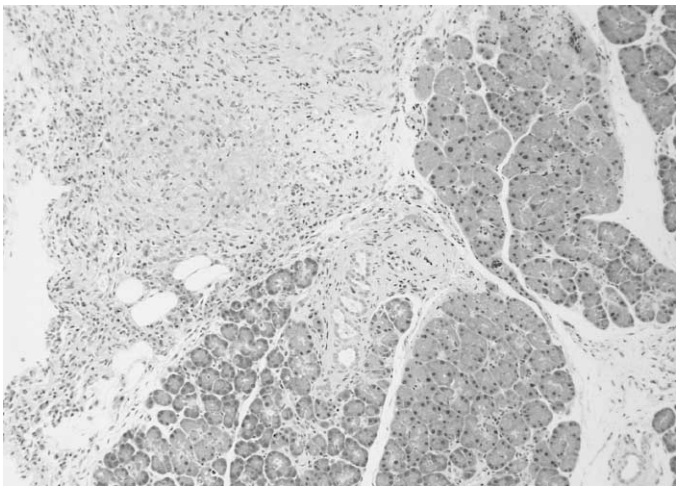

Fig. 2A: Untreated

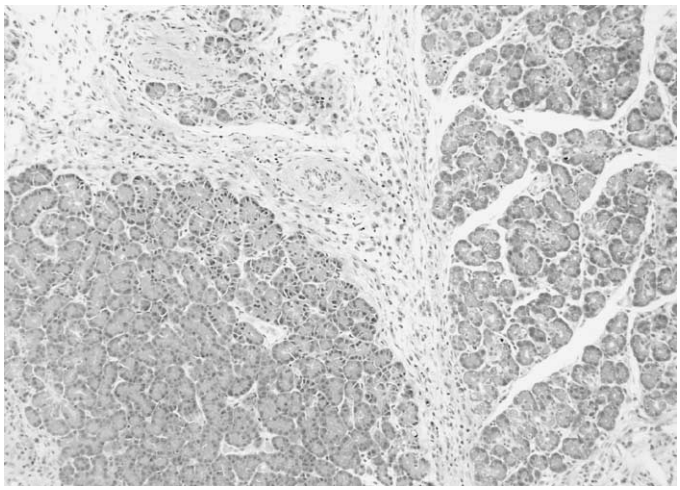

Fig. 2B: Lisinopril

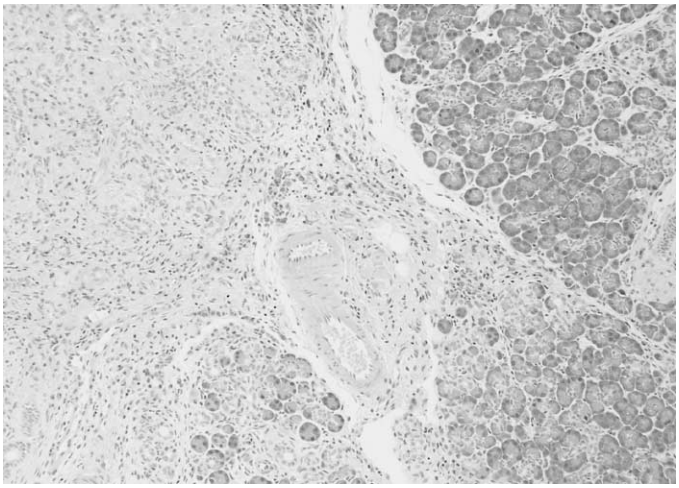

Fig. 2C: Candesartan

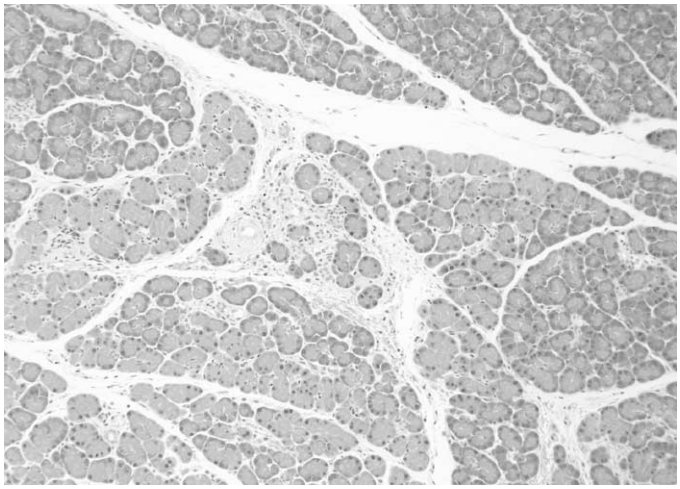

Fig. 2D: Combination

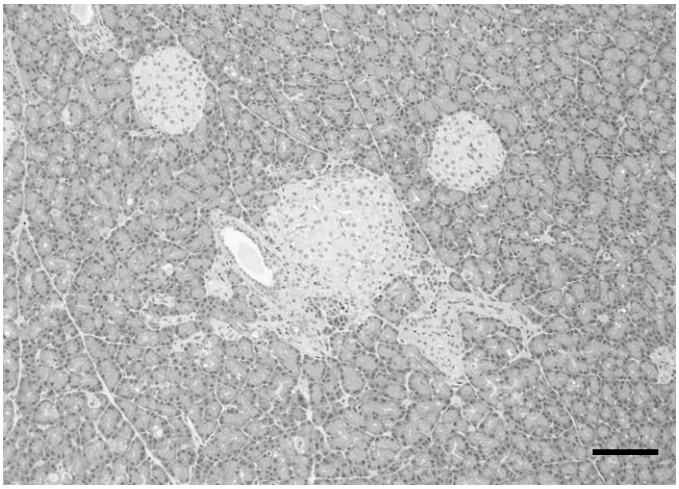

Fig. 2E: Wistar

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Fig.3

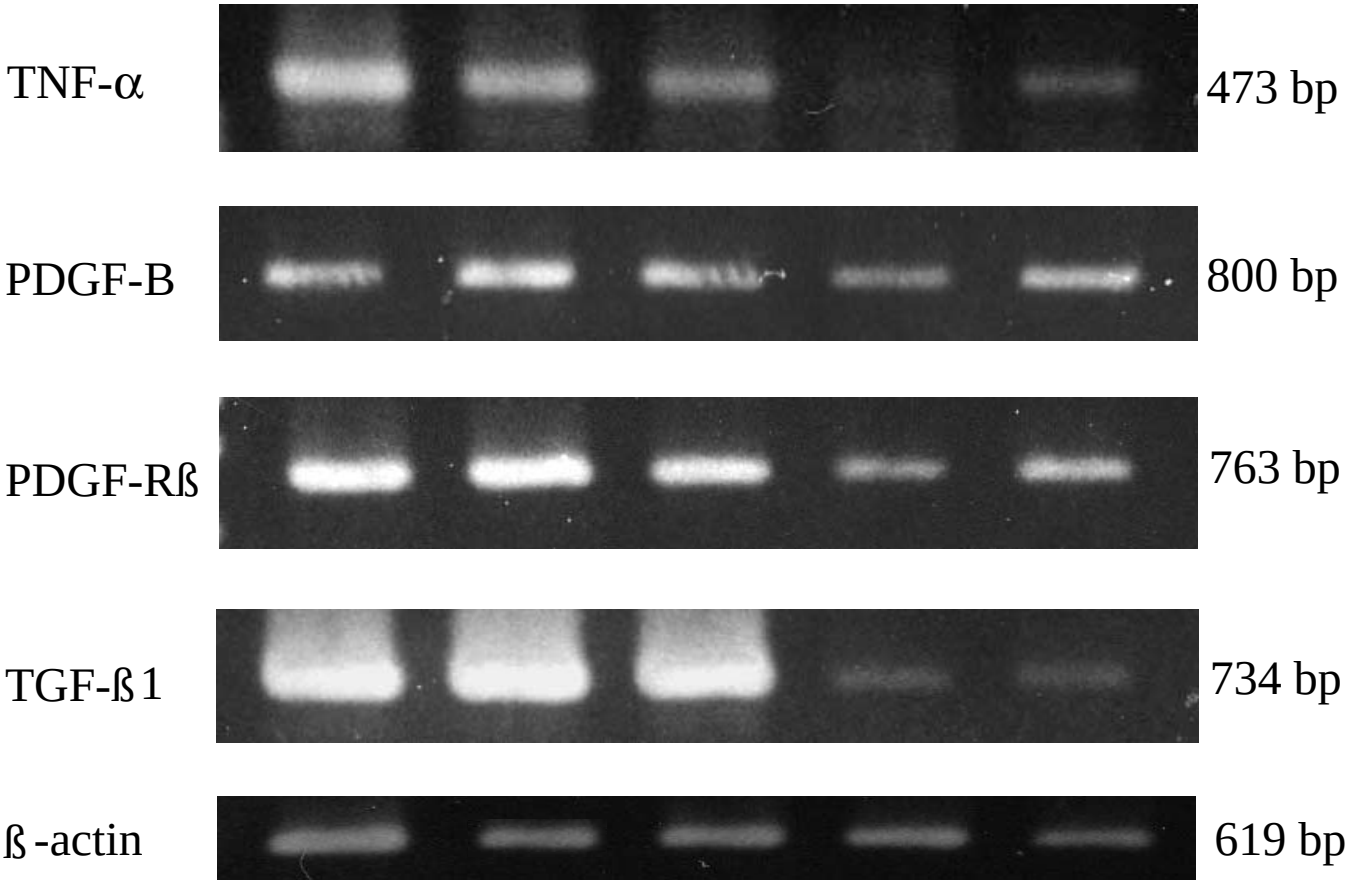

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Fig. 4A, 4B, 4C, and 4D

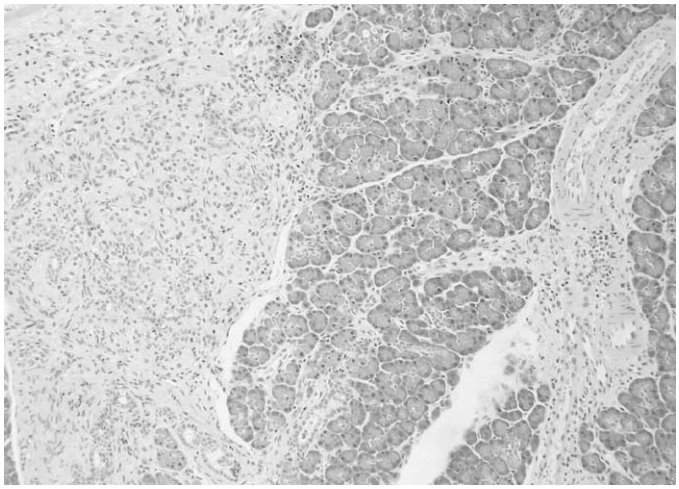

Fig. 4A: Untreated

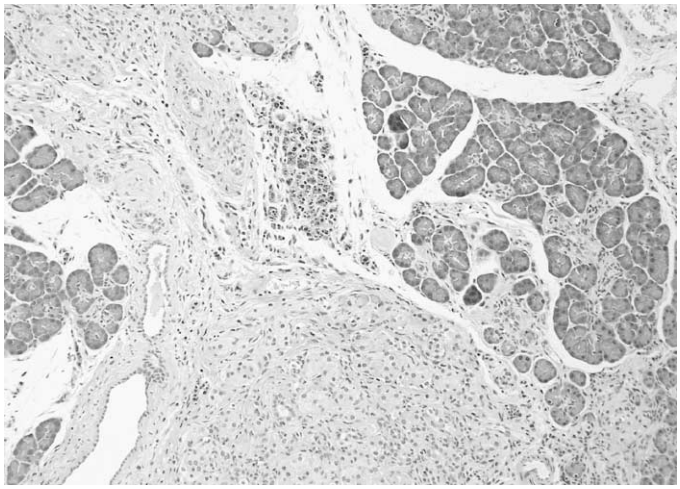

Fig. 4B: Low Combination

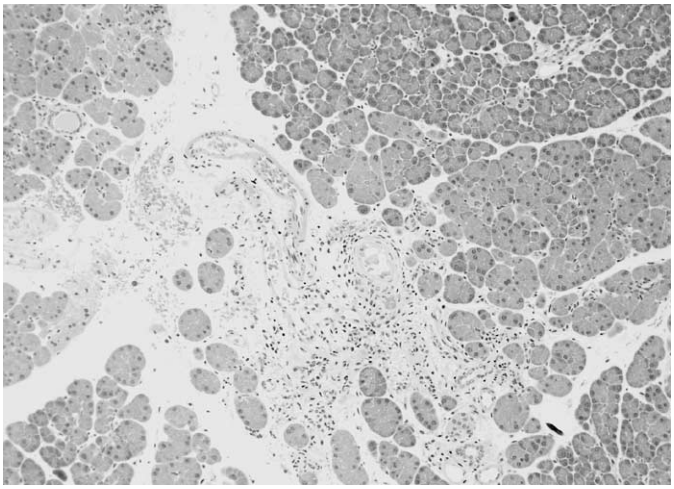

Fig. 4C: Medium Combination

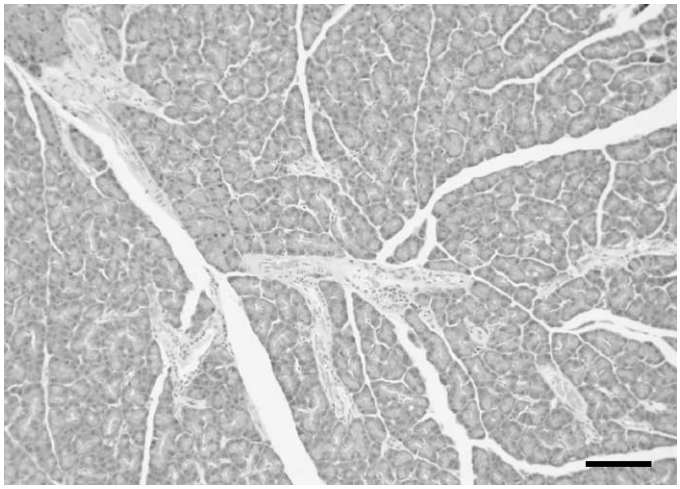

Fig. 4D: Combination