Externally applied pressure activates pancreatic stellate cells through the generation of intracellular reactive oxygen species

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Asaumi H, Watanabe S, Taguchi M, Tashiro M, Otsuki M. Externally applied pressure activates pancreatic stellate cells through the generation of intracellular reactive oxygen species. Am J Physiol Gastrointest Liver Physiol 293: G972–G978, 2007. First published August 30, 2007; doi:10.1152/ajpgi.00018.2007.—Local tissue pressure is higher in chronic pancreatitis than in the normal pancreas. We reported recently that pressure application induces synthesis of extracellular matrix (ECM) and cytokines in pancreatic stellate cells (PSCs) that and epigallocatechin gallate (EGCG), a potent antioxidant, inhibits the transformation of PSCs from quiescent to activated phenotype and ethanol-induced synthesis of ECM and cytokines in PSCs. These results suggest that oxidative stress and reactive oxygen species (ROS) are important in PSC activation. The aim of this study was to clarify the effects of ROS on activation and functions of pressure-stimulated PSCs. We used freshly isolated rat PSCs and culture-activated PSCs. Pressure was applied on rat cultured PSCs by adding compressed helium gas into a pressure-loading apparatus. PSCs were cultured with or without antioxidants (EGCG and N-acetyl cysteine) under normal or elevated pressure. Externally applied high pressure (80 mmHg) resulted in a gradual decrease of superoxide dismutase activity in PSCs and increased intracellular ROS generation as early as 30 s, reaching a peak level at 1 h. Antioxidants significantly inhibited ROS generation. Pressure increased the expression levels of α-smooth muscle actin, αI(I)-procollagen, and TGF-β1 in PSCs. EGCG suppressed these alterations, abolished pressure-induced phosphorylation of p38 MAPK, and suppressed pressure-induced PSC transformation to activated phenotype. Our results indicated that ROS is a key player in pressure-induced PSC activation and ECM synthesis. Antioxidants could be potentially effective against the development of pancreatic fibrosis in patients with chronic pancreatitis.

PANCREATIC FIBROSIS IS A KEY histopathological feature of chronic pancreatitis (8). The pathogenesis of pancreatic fibrosis has attracted much attention in recent years because the process may be reversible in its early stages (2). Pancreatic stellate cells (PSCs) have been identified as the central effector cells in the fibrogenic process (2, 5). Recent studies have demonstrated that PSCs are activated when exposed to cytokines such as TGF-β (5, 29). Recent studies have focused on the role of oxidative stress in stimulation of PSCs (3, 4, 16). Ethanol, a major etiological factor of pancreatitis, and its metabolite acetaldehyde induce oxidative stress in PSCs and synthesis of extracellular matrix (ECM) through activation of MAPKs (3, 4, 20, 23). The aldehyde end-product of lipid peroxidation, 4-hydroxy-2, 3-nonenal, increases hydrogen peroxide at noncytotoxic levels in PSCs (15). Furthermore, incubation with hydrogen peroxide induces PSC activation (16). We documented recently (4) that green tea polyphenol epigallocatechin gallate (EGCG) has antioxidant activities that inhibit not only ethanol-induced synthesis of ECM and cytokines but also transformation of PSCs from quiescent (nonactivated type) to activated phenotype. These results indicate that oxidative stress and radical oxygen species (ROS) play a role in activation and functions of PSCs.

In chronic pancreatitis, the pancreatic tissue pressure is elevated compared with the normal pancreas (13, 19). Recent studies demonstrated that increased pancreatic intraductal pressure enhanced pancreatic tissue fibrosis in vivo (14, 36). Mechanical forces are important regulators of cell differentiation in a variety of mammalian cells (10) and may contribute to pathological states such as cardiac hypertrophy (26) and atherosclerosis (34). In addition, there is a close relationship between oxidative stress and cardiac hypertrophy or atherosclerosis. Because elevated externally applied pressure induces the synthesis of α-smooth muscle actin (α-SMA), ECM, and TGF-β1 in PSCs (35), any pressure load or mechanical stress could stimulate PSCs during the progression of pancreatic fibrosis.

The present study is an extension of our recent study (4) and was designed to elucidate the relationship between pancreatic tissue pressure and ROS in PSCs as well as to evaluate the role of ROS or antioxidants on pressure-induced activation and synthesis of ECM and cytokine. We also explored the effects of two antioxidants on pressure-induced pancreatic fibrosis in vitro.

MATERIALS AND METHODS

Materials. Chemicals were obtained from the following sources: Dulbecco’s modified Eagle’s medium (DMEM), penicillin, and streptomycin from Gibco Life Technologies (Grand Island, NY); anti-phospho-p38 MAPK antibody and anti-p38 MAPK antibody from Cell Signaling Technology (Beverly, MA); p38 MAPK inhibitor SB-203580 from Calbiochem (La Jolla, CA); antibodies to α-SMA, EGCG, and N-acetyl cysteine (NAC) from Sigma-Aldrich (St. Louis, MO), aminophenyl fluorescein (APF) and hydroxyphenyl fluorescein (HPF) from Daiichi Pure Chemicals (Tokyo, Japan); and superoxide dismutase (SOD) assay kit WST from Dojindo (Kumamoto, Japan). Multiwell plates and cell-culture flasks were purchased from Iwaki Glass (Funabashi, Japan).

Isolation and culture of PSCs. The study protocols were approved by the Animal Care Committee at the University of Occupational and Environmental Health, Japan, and all procedures related to animal care and euthanasia followed the guidelines set by the American Care and Euthanasia Committee of the American Veterinary Medical Association. All procedures were conducted in accordance with the guidelines set by the American Association for the Accreditation of Laboratory Animal Care.

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Veterinary Medical Association. Rat PSCs were isolated as described in DMEM containing 10% FBS, 4 mM glutamine, and antibiotics above the interface of the Nycodenz solution and the aqueous buffer. This band was harvested, and the cells were washed and resuspended in DMEM containing 10% FBS, 4 mM glutamine, and antibiotics (penicillin (100 U/ml) and streptomycin (100 μg/ml)). All experiments were performed by using cells between passages 2 and 3 except for those using freshly isolated PSCs; we incubated PSCs in serum-free medium for 24 h before the addition of experimental reagents or pressure loading.

Pressure-loading apparatus. We used the previously described pressure-loading apparatus (35). The apparatus consisted of a resealable steel chamber with inlet and outlet ports (Miwa, Osaka, Japan). The inlet port was connected through a tube to a reservoir of compressed helium, whereas the exit port was connected through a tube to a sphygmomanometer and an air-release valve. Compressed helium gas was pumped into the chamber to raise the internal pressure. During the delivery of helium gas into the apparatus, no prepakced room air was released, so that the partial pressure of the gases originally contained in the chamber such as oxygen, nitrogen, and carbon dioxide were kept constant, consistent with Boyle’s and Charles’s laws, as described previously (35). The plates and the flasks used for experiments were placed on a warm plate (37°C) inside the chamber. The partial pressure of oxygen, temperature, and pH of the incubation medium in the plates remained constant throughout the experiments.

Application of pressure on PSCs and treatment of PSCs with antioxidants. PSCs were plated at equal seeding densities into uncoated plastic wells in DMEM with 10% FBS and were incubated for 24 h. The cells were then serum starved for 24 h by incubation in DMEM containing 0.1% FBS, then exposed to pressure for various periods of time. We chose 80 mmHg of pressure as the stimulant, based on our previous study (35). To determine whether the presence of the antioxidants alters the response of PSCs to a pressure load, the PSCs were cultured under normal or high pressure with or without EGCG or NAC. We chose 25 μM (final concentration) EGCG or 5 μM (final concentration) NAC as the antioxidants on the basis of previous studies (4, 16) and lactate dehydrogenase release assay (data not shown).

Measurement of SOD activity. SOD, the primary enzyme in the defensive system against oxidized stress, acts as the catalyze to induce dismutate superoxide into hydrogen peroxide and is elicited to reduce the effect of superoxide radicals (38). To evaluate pressure-induced oxidative stress on PSCs, we measured total SOD activity by a xanthine-based spectrophotometric assay using the SOD assay kit WST. The estimated SOD activity for each sample was normalized to the cellular protein content.

Biomaging of ROS generation in PSCs. Whereas SOD induces the dismutation of superoxide into oxygen and hydrogen peroxide, decrease in SOD activity may mean increase in superoxide and hydrogen peroxide in pressure-stimulated PSCs. Hydrogen peroxide activates PSC and works as a key factor of collagen synthesis (11, 16). To further confirm that high pressure generates ROS in PSCs, we chose APF as a detector for generated ROS, including superoxide and hydrogen peroxide. APF immediately reacts with ROS and is resistant to light-induced autooxidation (31). Furthermore, to examine whether high pressure generates highly reactive oxygen species (hROS) such as hydroxyl radical in PSCs, we use HPF as a detector for generated hROS (31).

PSCs were seeded onto glass-bottomed dishes and were mixed with 10 mM APF or HPF (final concentration) for 10 min before pressure load. After the stimulation, fluorescence images were acquired with a confocal laser-scanning unit coupled to an Axiovert 135 inverted microscope with a Plan-Apochromat lens and a ×40 objective (Carl Zeiss). The excitation wavelength was 485 nm, and the emission was filtered with a 515- to 565-nm barrier filter.

Measurement of intracellularly generated ROS. PSCs were seeded in 200 μl of DMEM containing 10 mM APF or HPF (final concentration) at a density of 1 × 10^5 cells/well onto a 96-well plate and were incubated for 10 min before pressure loading. After stimulation, the net generated ROS was measured as described previously (30) on the basis of HPF in a Cytofluor plate reader (Fluoroskan Ascent FL; Thermo Fisher Scientific, Waltham, MA) with an excitation wavelength of 485 nm and an emission wavelength of 520 nm.

RNA isolation and quantitative RT-PCR. We then assessed the effect of pressure and EGCG on the expression of fibrict factors, α(I)-procollagen, TGF-β1, and α-SMA. Total RNA was isolated with Isogen (Nippon Gene, Tokyo, Japan). Reverse transcription was performed with the TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA). The reactions were incubated at 25°C for 10 min and then at 42°C for 30 min followed by 5-min incubation at 95°C to denature the secondary RNA structure. No genomic DNA contamination or pseudogenes were detected by PCR without the reverse transcription step on the use of total RNA. The oligonucleotide primer and TDF probe was designed to anneal to the gene of interest between two PCR primers (TaqMan Assays-on-Demand; Applied Biosystems). PCR was programmed with 40 cycles of 15 s at 95°C and 60 s at 62°C. Quantitative PCR reactions were carried out in the ABI PRISM 7000 sequence-detection system (Applied Biosystems) by using the SYBR Green PCR Core Reagents kit (PE Biosystems, Warrington, UK) following the recommendation of the manufacturer. Negative controls (cDNA-free solutions) were included in each reaction. GAPDH was used as an internal control. For quantification of the PCR products, samples were expressed relative to the PCR product to GAPDH.

Western blotting. To assess the effect of pressure and EGCG on PSC, we determined the quantity of α-SMA production. Western blotting was performed as described previously (4). Cell lysate was isolated by using a commercially available kit (Cellytic-M, Sigma, Tokyo, Japan). Samples were then centrifuged (2,200 × g, 10 min), and supernatants were harvested for measurement of protein concentration by using the Bradford method (6). Supernatants were prepared for one-dimensional SDS-PAGE. Proteins (2 μg per lane) were then separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane (Hybond-P; Amersham Biosciences, Little Chalfont, UK). The membranes were blocked with 5% fat-free dry milk for 1 h in PBS (pH 7.4) and then incubated with α-SMA antibody at a 1:5,000 dilution in PBS (pH 7.4) containing 0.05% Triton X-100 for 1 h at room temperature. After being washed, the membranes were incubated with appropriate IgG antibody conjugated with horseradish peroxidase in PBS for 1 h at room temperature, and antibody binding was detected by an enhanced chemiluminescence detection system (ECL Plus; Amersham Biosciences). The gels were exposed to X-ray films (Scientific Bio-Imaging Film; Kodak). Western blot images were determined by scanning bands using a GT-8500 scanner (Seiko Epson, Tokyo, Japan), and the results were analyzed and quantified by using NIH Image (National Institutes of Health, Bethesda, MD).

Activated PSCs synthesize ECM protein, including collagens (2, 3, 32). To evaluate the effect of ethanol and EGCG on the production of α(I)-procollagen, we conducted Western blotting by using a type I collagen antibody that recognizes α(I)-procollagen chain, the mature α(I)-collagen chain, and the heterotrimer of type I collagen, but it does not recognize α(I)-collagen chain and does not cross-react with other collagen (33).

Because p38 MAPK is known to regulate α-SMA and type I procollagen protein production in PSCs (21), we examined the effect of pressure and EGCG on phosphorylation of p38 MAPK by Western blotting.

Treatment of freshly isolated PSCs. To analyze the effects of pressure and EGCG on the transformation of freshly isolated (quiescent) PSCs to their activated (myofibroblast-like) phenotype, freshly isolated cells were stimulated with or without pressure load for 30 s or...
1 h after preincubation in DMEM, with or without EGCG (25 μM), NAC (5 μM), or SB-203580 (25 μM), a p38 MAPK inhibitor. After 5-day incubation in DMEM with or without EGCG, NAC, or SB-203580, morphological changes in PSC were identified by comparing images obtained before and after each treatment, which were photographed with an Axiovert 135 TV microscope (Carl Zeiss).

Statistical analysis. Data are presented as means ± SD. All data are representative of at least three experiments. Data from three or more groups were compared by using ANOVA. Differences with a P value of <0.05 were considered statistically significant.

RESULTS

Pressure reduces SOD activity in PSCs. Application of 80 mmHg pressure for 1 h reduced SOD activity to 86.4% of control, but the activity recovered to 93% of control at 2 h after withdrawal of pressure (Fig. 1). These results indicate that the applied pressure induces reversible oxidative stress and ROS in PSCs. Whereas SOD is known as the catalyst that dismutates superoxide into hydrogen peroxide and oxide, pressure induced ROS to be superoxide, and hydrogen peroxide is catalyzed by SOD.

Pressure induces ROS generation in cultured PSCs. Activated PSCs cultured with HPF but without any other treatment showed low levels of fluorescence in the cells (Fig. 2A). However, after 30 s, pressure loading resulted in the appearance of fluorescence in the cells (Fig. 2B), and the intensity of the fluorescence was much stronger and sustained for longer period of time after 1-h pressure application (Fig. 2C). NAC (Fig. 2D) and EGCG (Fig. 2E) suppressed pressure-induced fluorescence. These changes in fluorescent intensity were similar in the cell bodies of all PSCs. Activated PSCs cultured with APF showed the same changes of fluorescence (data not shown). These results indicate that antioxidants inhibit pressure-induced ROS generation in activated PSCs.

We quantified the fluorescent intensity of APF or HPF in pressure-stimulated PSCs. One-hour pressure loading significantly increased ROS generation up to about four times the control level (Fig. 3). NAC suppressed pressure-induced ROS generation in PSCs to ~1.5 times the control level, whereas EGCG produced a more profound inhibition of pressure-induced ROS generation (Fig. 3). These results indicate that EGCG is a better antioxidant than NAC in pressure-induced PSCs. In the following experiments, we examined the effects of antioxidant on cell functions in PSCs subjected to pressure loads.

EGCG inhibits pressure-induced synthesis of ECM, cytokines and, α-SMA in PSCs. We examined the effects of pressure and EGCG on the expression of fibrotic factors, type I procollagen, TGF-β1, and α-SMA. The applied pressure significantly increased the gene expression of these fibrotic factors (Fig. 4A) and protein production of type I procollagen (Fig. 4B) and α-SMA (Fig. 4C) in PSCs. Furthermore, EGCG inhibited pressure-induced gene expression and protein production of these factors; the levels of gene expression of type

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**Fig. 1.** Effects of pressure load on total superoxide dismutase (SOD) activity in pancreatic stellate cells (PSCs). PSCs were subjected to 80 mmHg pressure for indicated time interval (up to 60 min). Total SOD activity in PSCs was quantified by SOD assay as described in MATERIALS AND METHODS. Data are means ± SD (n = 4). *P < 0.05 vs. control.

**Fig. 2.** Fluorescence microscopic images of highly reactive oxygen species (hROS) in PSCs. PSCs were preincubated with 10 mM hydroxyphenyl fluorescein (HPF) as a detector for generated intracellular hROS for 1 h. PSCs were incubated without treatment (control, A), subjected to pressure load for 30 s (B) or 1 h (C), or treated with antioxidant N-acetyl cysteine (NAC; D) or epigallocatechin gallate (EGCG; E) in presence of pressure load for 1 h. Cells were washed with DMEM, and fluorescence of HPF in cells was observed. Original magnification, ×400 objective.
Because p38 MAPK is known to regulate the expression of α-SMA production and α1(I)-procollagen in PSCs (21), and because we demonstrated previously that applied pressure significantly increased the levels of p38 MAPK phosphorylation after 2-min stimulation (35), we examined the effect of pressure and EGCG on phosphorylation of p38 MAPK after 2-min stimulation. Pressure significantly increased phosphorylation of p38 MAPK, whereas EGCG suppressed the levels of pressure-induced phosphorylation of p38 MAPK to the control levels (Fig. 5).

**Pressure induces morphological changes in freshly isolated PSCs via intracellular ROS generation.** Because it is known that p38 MAPK also regulates the transformation of PSCs from quiescent to myofibroblast-like phenotype (21), we examined the effects of pressure on such transformation. Freshly isolated PSCs cultured in serum-free medium remained in a quiescent phenotype for 5 days (Fig. 6A), whereas pressure-stimulated PSCs (1 h) transformed to myofibroblast-like cells (Fig. 6B) after 5-day incubation. PSCs cultured in the presence of 25 μM EGCG were small and round in shape, and most cells contained lipid droplets (Fig. 6C). However, PSCs were of the activated phenotype 48 h after withdrawal of EGCG (Fig. 6F). On the other hand, PSCs cultured with 5 μM NAC contained lipid droplets and had short processes with larger bodies compared with those cultured with EGCG (Fig. 6D). Furthermore, SB-203580, a p38 MAPK inhibitor, inhibited pressure-induced activation of freshly isolated PSCs (Fig. 6E). However, 48 h after withdrawal of SB-203580, PSCs were of the activated phenotype, similar to those noted after withdrawal of EGCG (data not shown).

We also examined the effect of high tissue pressure on intracellular ROS (hROS) in freshly isolated PSCs. Low-level HPF fluorescence was noted in untreated PSCs (Fig. 7A). A 30-s pressure-load produced weak fluorescence (data not shown). One-hour pressure application induced intracellular hROS production (Fig. 7B), and EGCG suppressed such pressure-induced fluorescence (Fig. 7C). These changes in fluorescence intensity were observed in the cell membranes of all PSCs. On the other hand, the p38 MAPK inhibitor SB-203580 did not inhibit pressure-induced hROS generation (Fig. 7D), suggesting that elevated tissue pressure changes the cell phenotype of freshly isolated PSCs to activated phenotype through the ROS-p38 MAPK pathway and that the antioxidant EGCG inhibits such pressure-induced transformation by inhibiting ROS generation.

**DISCUSSION**

The main finding of the present study was that pressure load generates intracellular ROS in PSCs. Our results also revealed that the applied pressure induced reversible and gradual depletion of SOD in PSCs and the release of intracellular ROS at 30-s and 1-h stimulation in PSCs. We reported previously that pressure application significantly increased p38 MAPK phosphorylation in PSCs (35), which reached peak levels at 2 min, and that pressure significantly increased type I procollagen synthesis and collagen production (35). In our study, the antioxidant EGCG significantly suppressed pressure-induced intracellular ROS generation at 30 s and phosphorylation of p38 MAPK at 2 min. These results suggest that the intracellularly generated ROS in pressure-stimulated PSCs act upstream of p38 MAPK. ROS can act as intracellular mediators or second messengers to regulate various signal-transduction pathways and functional responses of target cells (7, 9). A recent study described the ROS-dependent p38 MAPK pathway as crucial for the responses to immune stress (22). Our results also showed EGCG-induced suppression of the pressure-induced increase in gene expression and protein production of type I procollagen in PSCs. In this study, we showed that increased pressure induces ECM synthesis through a ROS-dependent p38 MAPK pathway in PSCs.
nism is similar to that described in hepatic stellate cells, in which platelet-derived growth factor induces cell proliferation through an intracellular ROS-dependent p38 MAPK pathway (1).

TGF-β stimulates ECM protein synthesis (2, 3). Recent studies described TGF-β-induced autocrine and paracrine stimulatory loops of ECM protein synthesis in PSCs (32). On the other hand, inhibition of TGF-β action prevented the progress of pancreatic fibrosis in an animal model of pancreatic fibrosis (24). We have shown that pressure increases the production of activated TGF-β1 from cultured PSCs (35). In the present study, EGCG suppressed pressure-induced TGF-β1 gene expression, suggesting that pressure induces TGF-β1 synthesis via intracellular ROS generation. TGF-β induces the accumulation of hydrogen peroxide, which acts as a mediator of TGF-β-elicited upregulation of the α1(I)-procollagen gene in hepatic stellate cells (33). These observations suggest that ROS inhibit the actions of various stimulants and that the use of antioxidants could avert pancreatic fibrosis by inhibiting TGF-β expression.

Our results also demonstrated that pressure load resulted in the transformation of freshly isolated PSCs (quiescent) to a myofibroblast-like phenotype (activated) and that the applied pressure induced the production of ROS in the quiescent type of PSCs. We also demonstrated a ROS-p38 MAPK signaling pathway in pressure-stimulated PSCs and that EGCG inhibited pressure-induced PSC activation and ROS generation. These results indicate that pressure induces PSC activation through ROS generation and that the antioxidant inhibits PSC activation by suppressing pressure-induced ROS generation. This is in agreement with the results of a previous study showing that

Fig. 4. Effects of pressure and EGCG on gene expression of fibrotic factors [α1(I)-procollagen, TGF-β1, and α-smooth muscle actin (α-SMA)] assessed by quantitative RT-PCR (A), and production of α1(I)-procollagen (B) and α-SMA (C) examined by Western blotting in PSCs. PSCs were preincubated with EGCG (25 μM) for 1 h before 60-min pressure loading (80 mmHg). After 24-h incubation, mRNA and protein levels were determined. Data are means ± SD (n = 6). Results are expressed as percentage of controls (no pressure load). *P < 0.05 vs. control (no pressure load); †P < 0.05 vs. pressure (without EGCG).

Fig. 5. Effects of pressure and antioxidant on phosphorylation of p38 MAPK in PSCs. Protein levels of phosphorylated p38 MAPK were determined by Western blotting. PSCs were preincubated with EGCG (25 μM) for 1 h. After 2-min stimulation, protein levels in cells were determined. Data are means ± SD (n = 4). Results are expressed as percentage of controls (no pressure load and absence of EGCG). *P < 0.05 vs. control (no pressure load and absence of EGCG); †P < 0.05 vs. pressure (absence of EGCG).
ethanol and acetaldehyde activated quiescent PSCs (3). Our results indicate a link between PSC activation and pressure-induced ROS signaling and the effectiveness of the antioxidant EGCG against pressure-induced PSC activation. In our study, both EGCG and NAC were effective in inhibiting pressure-induced intracellular ROS generation and PSC activation, and the antioxidative effects of EGCG were more powerful than those of NAC. Because EGCG has both hydrophilic and lipophilic properties, it is possible that EGCG could suppress ROS through the lipid-rich PSC cell membrane. In this regard, we described previously that EGCG suppresses ethanol-induced oxidative stress of both the cytosol and cell membrane in PSCs (4).

Pancreatic intraductal and tissue pressures are elevated in patients with chronic pancreatitis (13, 19). Similar changes are observed also in animal models of chronic pancreatitis (14). There is no trigger of increased tissue pressure in the absence of fibrosis in the pancreas. However, pancreatic tissue pressure may increase once the process of pancreatic fibrosis is initiated. Thereafter, the rise of pancreatic tissue pressure might accelerate the development of pancreatic fibrosis via increased synthesis of ECM and cytokines by PSCs. Thus high pancreatic-tissue pressure is an important factor that may accelerate the development of fibrosis and inflammation in chronic pancreatitis.

In summary, we reported the effects of externally applied pressure on PSC activation. Our results suggest that elevated tissue pressure has a profibrotic effect on PSCs. These observations enhance our understanding of the mechanisms involved in the development of pancreatic fibrosis. Extrapolation of the data clinically suggests that the increased pancreatic tissue pressure noted in chronic pancreatitis might accelerate the development of pancreatic fibrosis.
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