ORIGINAL ARTICLE

Tumor Protein p53-Induced Nuclear Protein 1 (*TP53INP1*) in Spontaneous Chronic Pancreatitis in the WBN/Kob Rat: Drug Effects on Its Expression in the Pancreas

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ABSTRACT

Context The tumor protein p53-induced nuclear protein 1 (*TP53INP1*) gene was found using DNA microarray technology as an overexpressed gene in acute pancreatitis. However, expression of *TP53INP1* in chronic pancreatitis has not been previously reported.

Objective This study investigated *TP53INP1* gene expression and its relationship with *p53* and apoptosis in spontaneous chronic pancreatitis in the Wistar-Bonn/Kobori rat.

Methods Ninety four-week-old male Wistar-Bonn/Kobori rats were fed a special breeding diet until sacrifice. Camostat mesilate (n=30) or a herbal medicine (Saiko-keishi-to; n=30) were mixed with the diet, while the other 30 rats were untreated. The rats were sacrificed every 4 weeks for 20 weeks, and the pancreas was examined. In addition, 6 four-week-old male Wistar-Bonn/Kobori rats were sacrificed and studied as starting reference. Finally, Wistar rats (n=36) were studied as controls.

Main outcome measure *TP53INP1* mRNA expression was determined by reverse transcription-polymerase chain reaction using semi-quantitative analysis, direct sequencing and *in situ* hybridization.

Results *TP53INP1* mRNA was strongly expressed at 12 weeks when chronic pancreatitis developed, with a second peak at 20 weeks. The expression kinetics of *TP53INP1* mRNA paralleled acinar cell apoptosis assessed by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling. The *p53* mRNA expression showed a single peak at 12 weeks. *In situ* hybridization revealed that *TP53INP1* mRNA was expressed mainly in acinar cells. Therapeutic drugs such as camostat mesilate and a herbal medicine Saiko-keishi-to suppressed the *TP53INP1* mRNA expression. *TP53INP1* mRNA induction in acinar cells was confirmed with *in vitro* experiments using an arginine-induced rat pancreatic acinar AR4-2J cell injury model.

Conclusions *TP53INP1* expression may reflect the acute-phase response and apoptosis of acinar cells in the course of chronic pancreatitis.

INTRODUCTION

Chronic pancreatitis remains a major health care problem worldwide. Chronic pancreatitis refers to a syndrome of destructive,

inflammatory conditions which encompasses the many sequela of long-standing pancreatic injury. Clinical diagnosis currently depends on identifying defined clinical, functional, morphologic, and histologic features which characterize the final common pathologic pathway of a variety of pancreatic disorders [1]. Histopathologically, chronic pancreatitis is characterized by inflammation, fibrosis, acinar degeneration, and ductular proliferation. The pathogenesis of chronic pancreatitis is still unclear, although pancreatic juice stasis and pancreatic ischemia are considered to be involved.

The tumor protein p53-induced nuclear protein 1 (*TP53INP1*) gene was originally cloned as an overexpressed pancreatic gene induced by cellular stress during acute pancreatitis [2]. *TP53INP1* is a nuclear factor, and is rapidly and strongly induced by various types of stress such as ultraviolet, mutagenic stress, ethanol, heat shock, and oxidative stress [2]. Thus, the *TP53INP1* gene is an important element of cellular stress response. Since we have been studying the expression of pancreatitis-associated proteins in a spontaneous chronic pancreatitis model, the Wistar-Bonn/Kobori (WBN/Kob) rat [3, 4, 5, 6], the above mentioned characteristics of the *TP53INP1* gene prompted us to elucidate its expression kinetics in this model. It is almost impossible to examine pancreatic histology serially in humans. In order to elucidate the mechanism of chronic pancreatitis, several experimental models of chronic pancreatitis have been reported. The WBN/Kob rat is a model of spontaneous chronic pancreatitis without any need for drugs or types of stress [7]. This rat model develops chronic pancreatitis at 24 weeks and becomes diabetic at 48 weeks when fed an ordinary diet. One can promote the development of pancreatitis by feeding the rats a protein-rich, fat-rich diet MB-3 [3]. In that case, the onset of chronic pancreatitis is seen at 12 weeks. In this model, we have revealed the expression kinetics of various genes such as those for pancreatitis-associated protein (*PAP*), *p8*, clusterin, cytokines, chemokines and apoptosis-related factors [3, 4, 5, 6]. Our previous reports suggest that a "cascade" of various cytokines/chemokines and apoptosis-related factors are involved in the onset and progression of chronic pancreatitis. However, the key factor which triggers the development of chronic pancreatitis is yet to be identified. *TP53INP1* [8] is a novel gene which was previously called *SIP* [2, 9] or *p53DINP1* [10]. *TP53INP1* was originally found as one of the genes overexpressed in acute pancreatitis after the analysis of a DNA microarray [2]. *TP53INP1* is strongly induced in acinar cells during acute pancreatitis in the mouse, and is also overexpressed in response to various types of stress *in vitro* [2]. The pancreas is the only organ where *TP53INP1* gene expression is markedly increased during acute pancreatitis. Moreover, *TP53INP1* gene expression is wild-type *p53*-dependent [9]. There is a functional *p53*-response element within the promoter region of the *TP53INP1* gene, and *TP53INP1* mRNA expression is activated in cells expressing wild-type *p53* in response to various types of stress [9]. As suggested above, *TP53INP1* promotes cellular apoptosis [2]. Acinar cell apoptosis is involved in acinar destruction/degeneration in the course of chronic pancreatitis [5, 11, 12, 13]. Therefore, examining *TP53INP1* gene expression will contribute to a better understanding of the molecular mechanisms of the onset and progression of chronic pancreatitis.

The aim of this study was to characterize *TP53INP1* gene expression and to clarify its relationship with *p53* and apoptosis in spontaneous chronic pancreatitis in the WBN/Kob rat.

MATERIALS AND METHODS

Animals

Four-week-old male WBN/Kob (n=96) and Wistar (n=36) rats were purchased from SLC Japan, Inc. (Hamamatsu, Japan) and kept at 23 ˚C under a 12 h light-dark cycle and allowed free access to water and food.

Experimental Design

Ninety WBN/Kob rats were fed for 20 weeks with a protein-rich, fat-rich pellet-diet, MB-3 (Funabashi Farm, Funabashi, Japan), which contains 28.1% protein, 6% fat, and 48.8% carbohydrate [3] and were divided into 3 groups:

- 30 were given camostat mesilate (Ono Pharmaceutical Co., Ltd., Osaka, Japan) at a dose of 10 mg/100 g body weight, mixed with the MB-3 diet (Camostat mesilate group);

- 30 were given a herbal medicine, Saiko-keishi-to (TJ-10, Tsumura and Co., Ltd, Tokyo, Japan), at a dose of 80 mg/100 g body weight, mixed with the MB-3 diet (TJ-10 treated group);

- the remaining 30 WBN/Kob rats were fed the MB-3 diet without these drugs for 20 weeks (Untreated group).

The animals of each group were sacrificed every 4 weeks under anesthesia with diethyl ether. Therefore, the number of camostat mesilate treated rats, TJ-10 treated rats, and untreated rats was 6 at every time point (8, 12, 16, 20 and 24 week-old).

The remaining 6 WBN/Kob rats were sacrificed and used as a starting reference (4-week-old group) for the other 3 WBN/Kob rat groups (camostat mesilate-treated, TJ-10-treated, and untreated).

Finally, 36 Wistar rats were also studied as controls: 6 were sacrificed at the start (4-weeks old), while the other 30 were fed with an ordinary pellet diet, MM-3 (20.9% protein, 4.4% fat, 53.5% carbohydrate) and were sacrificed at sub-groups of 6 at 4-week time intervals for 20 weeks (from 8 to 24-weeks old).

The pancreases were quickly removed, and part of the pancreatic tissue was stored at -80 °C. The remaining parts were fixed in 4% paraformaldehyde at 4 °C overnight, embedded in paraffin and cut into 3 μm-thick sections for histopathologic examination.

Cell Culture and Treatment

Rat pancreatic acinar AR4-2J cells were purchased from the Dai-Nippon Pharmaceutical Co., Ltd. (Osaka, Japan) and were maintained in F12K medium supplemented with 10% fetal calf serum, penicillin, and kanamycin at 37 °C in a 5% CO_2, 95% air atmosphere. L-arginine (Nacalai Tesque, Inc., Kyoto, Japan) at a concentration of 5 mg/mL was dissolved in the culture medium with pH adjustment. We harvested the cells at 0, 2, 4, 6, 24, 48 and 72 hours after the addition of arginine into the culture media. The experiments were repeated six times.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from pancreatic tissue using an Ultraspec-II ™ RNA extraction kit (Biotex Laboratories, Inc., Houston, Texas, USA), and from AR4-2J cells using a Simple Nucleic Acid Prep (SNAP) RNA extraction kit (Invitrogen BV, The Netherlands). RNA concentrations were determined by spectrophotometry. RT was performed using a PowerScript™ Reverse Transcriptase kit. (Clontech Laboratories, Inc., Palo Alto, CA, USA). First strand cDNA was synthesized from 8 μg of total RNA at 65 °C for 5 min after RT, the reverse transcriptase was inactivated by incubating at 42 °C for 60 min and the reaction was terminated by heating at 70 °C for 25 min. Then 30 cycles of amplification were performed on a DNA thermal cycler (Perkin Elmer Cetus, Inc., Norwalk, Connecticut, USA) as follows: *TP53INP1* primers were denatured at 94 °C for 50 sec, annealed at 60 °C for 45 sec, and polymerized at 72 °C for 40 sec. Then the extension was done at 72 °C for 10 min. The *p53* primers were denatured at 94 °C for 20 sec, annealed at 60 °C for 40 sec, and polymerized at 72 °C for 50 sec for 30 cycles, then extended at 72 °C for 10 min. The

sequence information for the rat *TP53INP1* primers was from GenBank with the accession number BF564888 (http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nucleotide&val=11674618). The primers were chosen from the exon 5. *TP53INP1* mRNA was specifically amplified with the following primers: sense, 5'-TGAACACATTTGCCTTGTGAA-3' and antisense, 5'-GGCAAAGTGCTGTGCTGTT-3'. The size of the fragment was 289 base pairs (bp). The *p53* primer pairs were sense: 5'-GGATGCCCGTGCTGCCGAGGAG-3' and antisense: 5'-AGTGAAGGGACTAGCATTGTC-3', producing a fragment of 243 bp. The *GAPDH* of 452 bp was used as an internal control. Eight-microgram aliquots of the RT-PCR products were subjected to electrophoresis on a 2% agarose gel and visualized with SYBR Gold (Molecular Probes, Inc., Eugene, Oregon, USA) at a 10,000× dilution in dimethylsulfoxide, and exposed to ultraviolet 312 nm light.

Semiquantitative RT-PCR Analysis

The gene expression of *TP53INP1* and *p53* was semi-quantitatively analyzed with an image analyzer (ATTO Densitograph ver. 3.02, ATTO, Inc., Tokyo, Japan). The relative expression intensity was calculated according to the following formula: *TP53INP1* or *p53* mRNA in a sample/*GAPDH* mRNA in a sample.

Direct Sequencing

The PCR products were centrifuged at least three times using SUPPER-02 cup. Then, they were stained under the conditions of denaturation at 94 ℃ for 50 sec and annealing at 60 ℃ for 4 min, 20 cycles, using a BigDye™ Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems Japan, Tokyo, Japan). Then the samples were denatured with template suppression reagent at 95 ℃ for 2 min to remove the primers. The sequence was determined using the automated DNA sequencing system. (ABI PRISM 310,

Applied Biosystems, Inc., Foster City, CA, USA).

***In situ* Hybridization for *TP53INP1* mRNA**

The rat *TP53INP1* cRNA probes were transcribed from the 574-bp *Not*I-*Eco*RI rat cDNA fragment (GenBank accession number: BF564888. http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nucleotide&val=11674618) inserted into the plasmid vector pT7T3D-Pac (ResGen™, Invitrogen Corporation, Carlsbad, CA, USA). The templates were linearized with *Eco*RI for antisense or with *Not*I for sense cRNA synthesis *in vitro* by T7 RNA polymerase. The cRNA probes were labeled with digoxigenin-uridine 5'-triphosphate (dUTP) (Roche Molecular Biochemicals, Inc., Alameda, CA, USA). *In situ* hybridization was performed on 5 μm-thick, formalin-fixed, paraffin-embedded tissue sections. The sections were deparaffinized, hydrated, and permeabilized with 0.3% Triton X-100 in phosphate-buffered saline (PBS) for 15 min, and with 20 μg/mL proteinase K for 30 min at 37 ℃. Then the slides were fixed with 4% paraformaldehyde in PBS and passed through 0.1 M triethanolamine containing 1/400 (vol/vol) acetic anhydrase. The slides were prehybridized in 50% formamide, 4×SSC buffer for 1 h at 68 ℃, and were hybridized overnight at 68 ℃ in a humidified chamber with the 5 ng/mL digoxigenin-labeled antisense cRNA probe in a hybridization buffer (40% formamide, 10% dextran sulfate, 1× Denhardt's solution, 4×SSC, 10 mM dithiothreitol, 1 mg/mL yeast tRNA, and 1 mg/mL denatured herring sperm DNA). As negative control experiments, the sections were either treated with RNase A prior to prehybridization, or hybridized with the sense cRNA probe. Post-hybridization washes were performed with 50% formamide in 2×SSC for 15 min at 52 ℃, then in 2×SSC, and 1×SSC at 37 ℃. Unbound probe was digested with RNase A (20 μg/mL) in 10 mM Tris HCl (pH 8.0), 0.5M NaCl, and 1 mM EDTA for 30 min at 37 ℃ and then washed in 0.1×SSC. The

hybridization signal was detected according to the instructions of the tyramid signal amplification system for in situ hybridization kit (DAKO Cytomation, Carpinteria, CA, USA). The sections were incubated in diaminobenzidine for 2 to 10 min for color reaction.

Detection of Acinar Cell Apoptosis

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) on light microscopy was performed as reported previously [5]. Quantification of acinar cell apoptosis was conducted as follows: TUNEL-positive acinar cell nuclei were counted in fields (100×magnification) chosen at random, and the percentage of the number of labeled nuclei per 1,000 acinar cell nuclei was expressed as the apoptotic index (%).

Immunohistochemistry for p53 Protein

The p53 protein was immunolocalized using a DAKO LSAB kit (DAKO-Japan, Kyoto, Japan). After deparaffinization and blocking, the sections were incubated with the primary antibody (rabbit polyclonal antibody against rat p53, dilution 1:200, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) overnight, then incubated sequentially with biotinylated goat anti-rabbit immunoglobulin and peroxidase-labeled streptavidin at room temperature for 20 min. The primary antibody reacts with both wild-type and mutated p53. Finally, the sections were incubated with diaminobenzidine as a chromogen for up to 2 min. The cell nuclei were lightly counterstained with Mayer's hematoxylin. Negative control experiments were performed by substituting normal rabbit IgG for the primary antibody.

ETHICS

All animals received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of
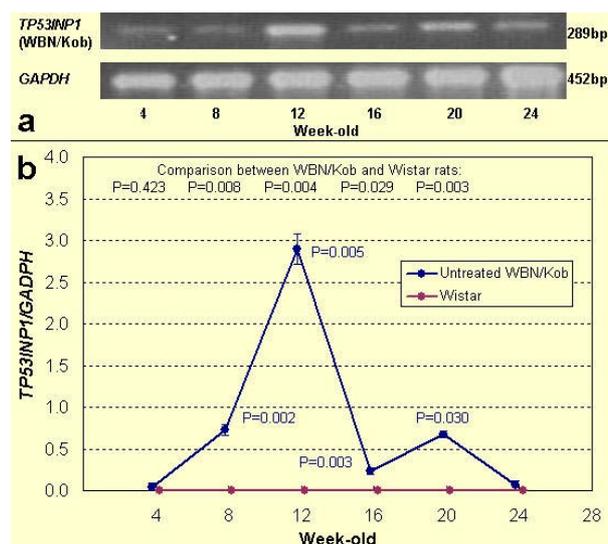
Figure 1. Expression of *TP53INP1* mRNA in WBN/Kob rats (RT-PCR). **a.** *TP53INP1* mRNA of 289 bp was expressed from 4 to 24 weeks. *TP53INP1* expression was strongest at 12 weeks. The *GAPDH* mRNA of 452 bp as an internal control was expressed in all pancreatic tissue. **b.** Serial changes of *TP53INP1* mRNA expression as determined by semiquantitative analysis.
Each point represents the mean±SEM of 6 observations.
P values vs. previous time interval in untreated WBN/Kob rats.

Sciences (NIH publication 86-23 revised 1985). All experimental protocols were approved by the Committee for the Care and Use of Laboratory Animals of Kanazawa University.

STATISTICS

Experimental results were expressed as the mean±SEM. Data were analyzed by means of the Mann-Whitney U test and the linear regression. The Statview 4.5 software (Abacus Concepts Inc., Berkeley, CA, USA) was used for statistical analysis. Two tailed P values less than 0.05 were considered as statistically significant.

RESULTS

Gene Expression of *TP53INP1* in the Course of Chronic Pancreatitis

TP53INP1 mRNA expression was examined with RT-PCR in the pancreatic tissue of
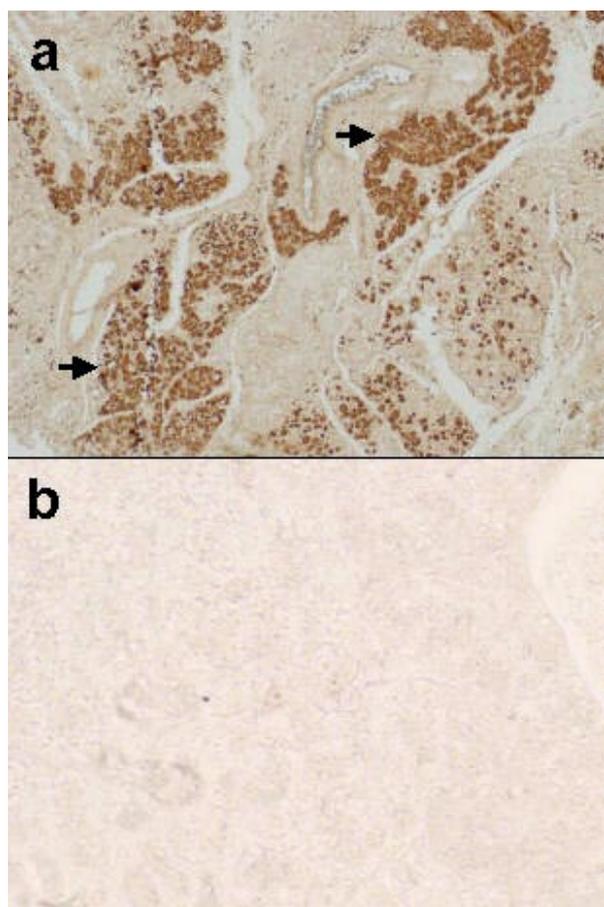
Figure 2. *In situ* hybridization for *TP53INP1* mRNA in the chronic pancreatitis tissue of the WBN/Kob rat at 12 weeks. **a.** The hybridization signal was strong in acinar cells. **b.** No significant signal was observed in the negative control using a sense probe. Original magnification ×40.

untreated WBN/Kob rats from 4 to 24 weeks. As shown in Figure 1, a single band of 289 bp for *TP53INP1* mRNA was obtained on agarose gel. *TP53INP1* mRNA was expressed from 4 weeks to 24 weeks. Identification of these bands was confirmed by direct sequencing, in which the sequence of *TP53INP1* mRNA was able to be read up to 250 nucleotides (data not shown). The sequences matched well with those listed in the Gene Database. The semiquantitative analysis (Figure 1, blue line) showed that *TP53INP1* mRNA expression began at 4 weeks and it was significantly different from the previous one at each time period: it progressively increased until 12 weeks, it significantly decreased after 16 weeks, it showed a second significant increase at 20

weeks and then significantly decreased at 24 weeks. The relative intensity of *TP53INP1* mRNA expression at 12 weeks was significantly strong, compared with all the other time periods (P=0.003, P=0.005, P=0.003, P=0.009, and P=0.002 vs. 4, 8, 16, 20, and 24 weeks, respectively). Therefore, *TP53INP1* mRNA expression reached its peak at 12 weeks and presented a second peak, although smaller, at 20 weeks. The pancreatic tissue of normal Wistar rats showed no expression of *TP53INP1* mRNA in any time period (Figure 1, violet line). Significant differences between untreated WBN/Kob and Wistar rats were observed in animals from 8 to 20 weeks old.

In situ Hybridization of *TP53INP1* mRNA

Figure 2a shows that the hybridization signal was strong in the acinar cells. There was no signal in duct or islet cells. No significant signal was observed in negative control experiments using either the sense cRNA probe (Figure 2b) or tissue sections pretreated with RNase A.
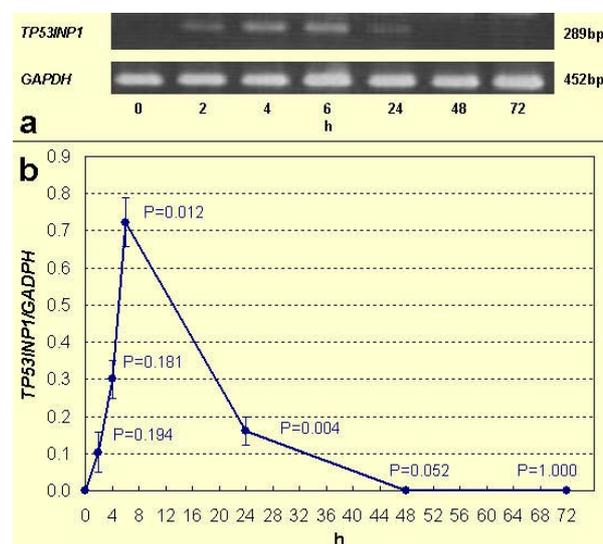
Figure 3. *TP53INP1* mRNA was expressed in arginine-treated AR4-2J cells. **a.** A single band of 289 bp was seen on agarose gel from 2 to 24 h after the addition of arginine into the culture medium. **b.** In the semiquantitative analysis, *TP53INP1* mRNA was induced after 2 h, reached its peak at 6 h, then gradually decreased and disappeared at 48 hr.
Each point represents the mean±SEM of 6 observations. P values vs. previous time interval.
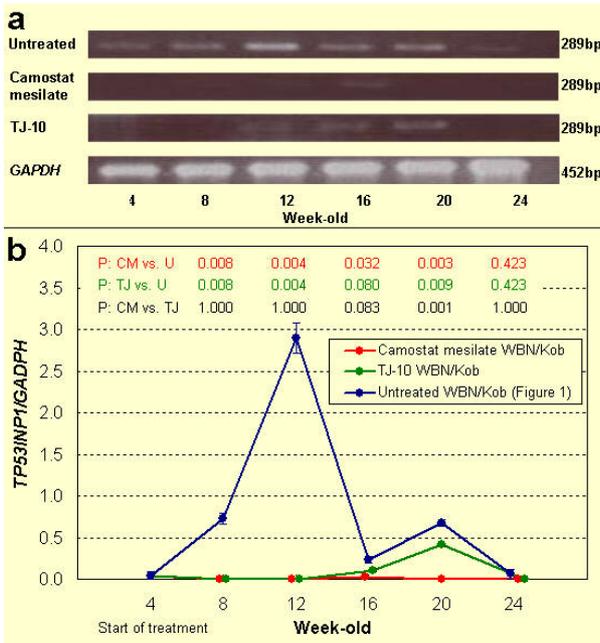
Fig.4. The effect of camostat mesilate (CA) and TJ-10 (TJ) on *TP53INP1* mRNA expression in the WBN/Kob rat. **a.** A single band of 289 bp was faintly seen in the camostat mesilate and TJ-10-treated groups. The *GAPDH* mRNA of 452 bp as an internal control was expressed in all pancreatic tissue. **b.** *TP53INP1* mRNA levels were reduced at all ages, especially at 12 weeks, in camostat mesilate (CM) and TJ-10 (TJ) treated WBN/Kob rats compared with the untreated group (U: these data are also reported in Figure 1).
Each point represents the mean±SEM of 6 observations.
P values between pairs of different groups.

TP53INP1 mRNA Expression in Arginine-Treated Pancreatic Acinar AR4-2J Cells *in vitro*

In order to confirm that *TP53INP1* mRNA is expressed in acinar cells stimulated by oxidative stress, we examined *TP53INP1* mRNA expression in arginine-treated rat acinar AR4-2J cells. RT-PCR revealed that *TP53INP1* mRNA was expressed in AR4-2J cells from 2 to 24 hours after the addition of arginine. Semiquantitative analysis showed that *TP53INP1* mRNA began to be expressed at 2 h, peaked at 6 h (P=0.012 vs. 4 h), then gradually decreased at 24 h (P=0.004 vs. 6 h) and disappeared at 48 and 72 h (Figure 3). The expression level was significantly higher at 6 h than those at other time points (P=0.008, P=0.035, P=0.012; P=0.004, P=0.008, and P=0.008 vs. 0, 2, 4, 24, 48, and 72 h, respectively).

Effect of Therapeutic Drugs on the Expression of *TP53INP1* mRNA

TP53INP1 mRNA expression was significantly suppressed by the oral administration of camostat mesilate and TJ-10. As shown in Figure 4, *TP53INP1* mRNA was only faintly expressed at 16 weeks in the camostat mesilate-treated group (red line), and it was detected only at 16 and 20 weeks in the TJ-10 group (green line). In semiquantitative analysis, the relative intensity of *TP53INP1* mRNA expression was significantly suppressed in the camostat mesilate group at 8, 12, 16, and 20 weeks (P=0.008, P=0.004, P=0.032, and P=0.003, respectively), and it was also significantly suppressed in the TJ-10-treated group at 8, 12 and 20 weeks (P=0.008, P=0.004, and
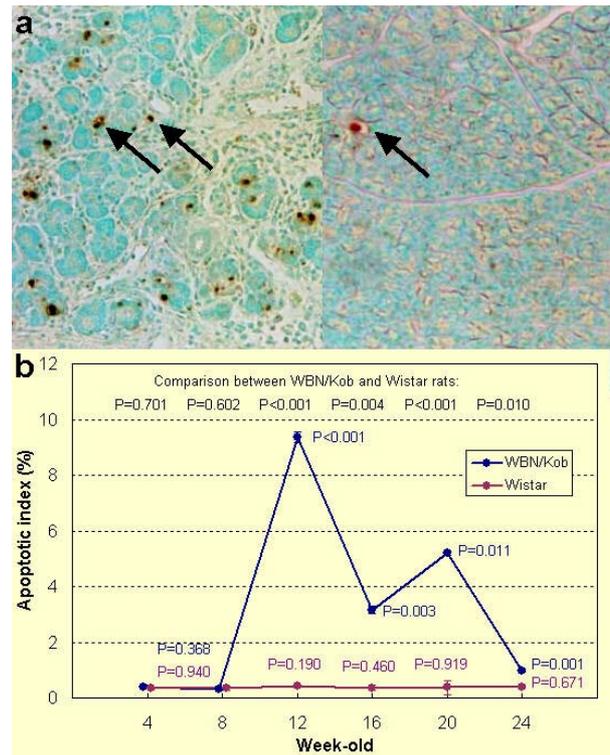
Figure 5. a. The TUNEL-positive acinar cells are abundant in the pancreas of the WBN/Kob rat at 12 weeks (left), but there are only a few TUNEL-positive acinar cells in the pancreas of the Wistar rat at 12 weeks (right). **b.** Time course of apoptotic index in the pancreases of the WBN/Kob rat. The apoptotic index showed two peaks at 12 and 20 weeks.
Each point represents the mean±SEM of 6 observations.
P values vs. previous time interval.
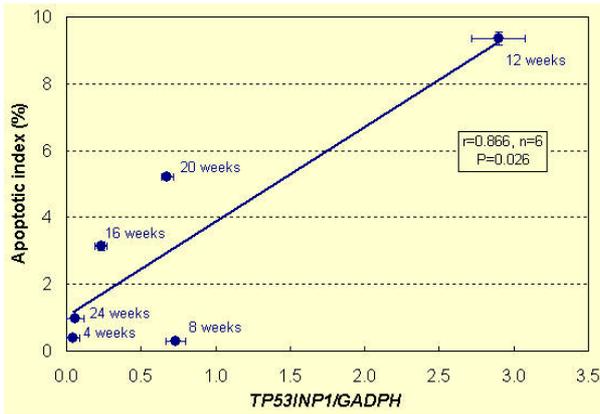
Figure 6. Significant relationship between apoptotic index (data from Figure 5) and expression of *TP53INP1* mRNA (data from Figure 1).

P=0.009, respectively), compared to that in the untreated WBN/Kob rats (Figure 4). Finally, the comparison between the two treated groups showed that the suppression of *TP53INP1* mRNA expression resulted significantly higher in the camostat mesilate group than in the TJ-10 group at 20 weeks (P=0.001).

The Relationship between *TP53INP1* Expression and Acinar Cell Apoptosis

TUNEL-positive acinar cells were found in the inflammatory regions of the pancreas of WBN/Kob rats (Figure 5a). Figure 5b shows the semi-quantitative analysis (blue line). The apoptotic index was significantly different than the previous time interval at 12, 16, 20, and 24 weeks. Two peaks were observed (12 and 20 weeks) and they were significantly different when compared to all other weeks (12 weeks: P<0.001, P<0.001, P=0.003, P<0.001, and P<0.001 vs. 4, 8, 16, 20, and 24 weeks, respectively; 20 weeks: P<0.001, P<0.001, P<0.001, P=0.011, and P=0.001 vs. 4, 8, 12, 16, and 24 weeks, respectively). In Wistar rats, TUNEL-positive labeling was found in only a few pancreatic acinar cells without any significant variation among weeks.

The kinetics of *TP53INP1* mRNA expression paralleled the apoptotic index in WBN/Kob rats (Figure 6).

Expression of *p53* mRNA in the Pancreas of WBN/Kob Rats *in vivo* and Arginine-Treated Pancreatic Acinar AR4-2J Cells *in vitro*

Since *TP53INP1* expression is mainly wild-type *p53*-dependent, we also investigated the expression of *p53* mRNA in our model. By RT-PCR, a single band of 243 bp for *p53* mRNA was found in the chronic pancreatitis tissue (Figure 7a). The semi-quantitative analysis (Figure 7b, blue line) showed that the serial changes of *p53* mRNA almost paralleled those of *TP53INP1* mRNA (Figure 1b). The *p53* mRNA expression was induced at 8 weeks, it was strongest at 12 weeks (P=0.013 vs. 8 weeks), it decreased at 16 weeks (P=0.018 vs. 12 weeks) and then disappeared at 20 weeks. The intensity of *p53* mRNA expression was significantly increased at 12 weeks as compared to all other weeks (P=0.008, P=0.013, P=0.018, P=0.014, and P=0.008 vs. 4, 8, 16, 20, and 24 weeks, respectively). The pancreatic tissue of normal Wistar rats showed no expression of *p53*
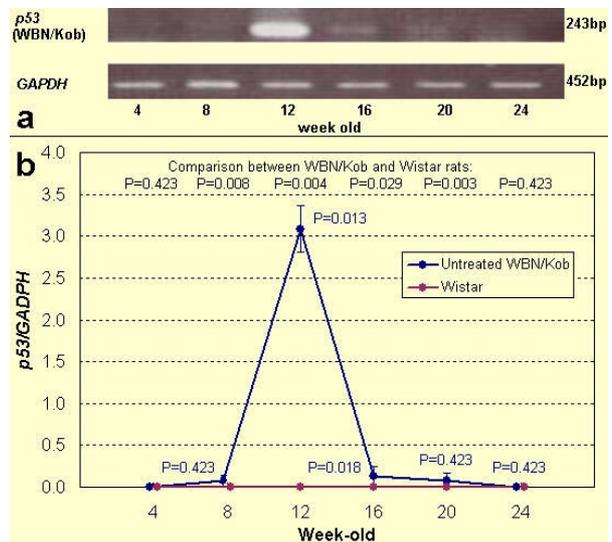
Figure 7. a. A single band of 243 bp of *p53* mRNA was detected from 8 to 20 weeks. The *p53* mRNA expression was also strongest at 12 weeks. The *GAPDH* mRNA of 452 bp as an internal control was expressed in all pancreatic tissue. **b.** Serial changes of *p53* mRNA expression as determined by semi-quantitative analysis.
Each point represents the mean±SEM of 6 observations. P values vs. previous time interval.
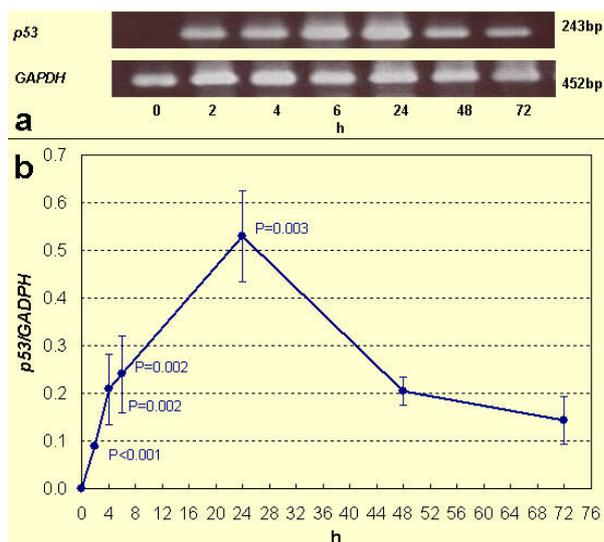
Figure 8. The *p53* mRNA was expressed in arginine-treated AR4-2J cells. **a.** A single band of 243 bp was seen on agarose gel from 2 to 72 hr after the addition of arginine to the culture medium. **b.** In the semiquantitative analysis, *p53* mRNA expression was induced after 2 h, reached its peak at 24 h, then gradually decreased.

Each point represents the mean±SEM of 6 observations.
P values vs. previous time interval.

mRNA (violet line). Significant differences between untreated WBN/Kob and Wistar rats were observed from 8 to 20 week-old animals.

In *in vitro* experiments, *p53* mRNA was induced in arginine-treated AR4-2J cells from 2 to 72 h (Figure 8a). In the semiquantitative time course study (Figure 8b), *p53* mRNA progressively increased until 24 h, then it progressively decreased. As shown, *p53* mRNA was strongly expressed at 24 h and, at this time interval, it was significantly higher when compared to all other time points ($P<0.001$ vs. 0, 2, 48, and 72 h; $P=0.002$ vs. 4 h; $P=0.003$ vs. 6 h).

Direct sequencing showed that the PCR product matched with the expected sequence of rat *p53* (data not shown).

Immunohistochemistry for *p53* Protein

The p53 protein expression in pancreatic tissue was examined by immunohisto-chemistry. Only acinar cells were positive for *p53* in their nuclei in inflammatory regions at 12 weeks (Figure 9a). Duct and stromal cells

were not labeled. We could not identify whether *p53*-positive cells were also positive for *TP53INP1* mRNA. The expression of *p53* was weakly detected at 16 weeks, but it was not observed in the pancreata at other weeks, or in the negative controls (Figure 9b). There was no *p53* expression in the pancreatic tissue of normal Wistar rats at any age (data not shown). In addition, there is no suitable antibody against rat *TP53INP1*; therefore, we can't do immunohistochemical study on the WBN/Kob rat slides.

DISCUSSION

In the present study, we have shown that *TP53INP1* expression was induced prior to and peaked at the onset of chronic pancreatitis (i.e. at 12 weeks), resembling the gene expression of other pancreatitis-associated
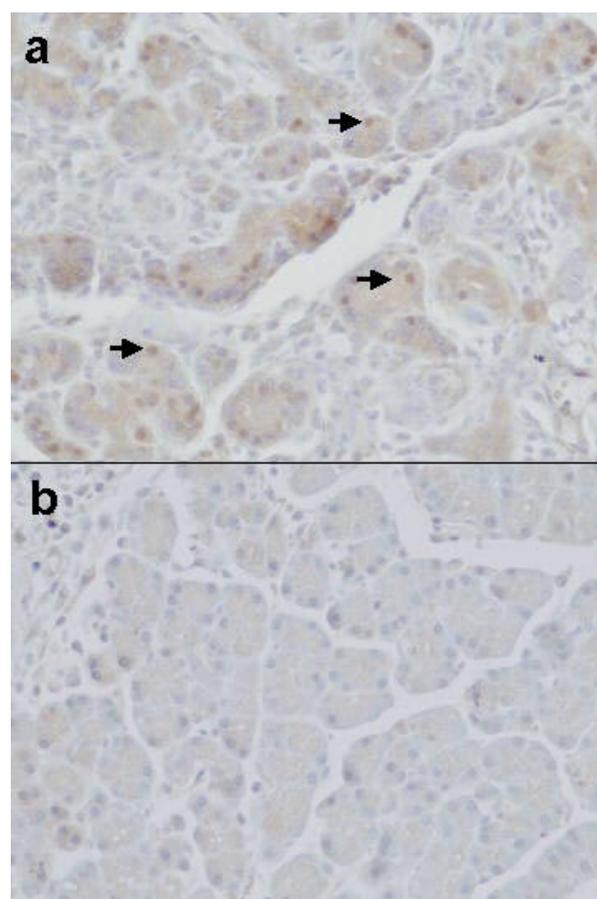
Figure 9. Immunohistochemistry of p53 protein in the pancreas of WBN/Kob rats. **a.** Only acinar cells were positive for *p53* in the nuclei in inflammatory regions. **b.** *p53* was not expressed in the negative control using normal rabbit IgG. Original magnification ×50.

proteins such as *PAP* [3] and *p8* [12]. However, there is a difference between *TP53INP1* and *PAP* (and *p8*); *TP53INP1* expression has a second peak at 20 weeks, whereas *PAP* and *p8* showed a single peak at the onset of chronic pancreatitis (12 weeks). The primers used in the rat experiments were different from those used in the mouse study. The primers we used in this experiment were chosen from the exon 5 of the rat *TP53INP1*, which is common in the two isoforms. Using these primers we get a single band on agarose gel after PCR, whereas the primers chosen from the exon 4 produce two bands due to alternative splicing as reported previously [2]. As for the apoptosis of acinar cells in the WBN/Kob rat model, our previous reports show two peaks, at 12 and 20 weeks, in the time course of the apoptotic index calculated using the TUNEL assay [5]. There are also two peaks at the same time points in the expression kinetics of pro-apoptotic factors such as Fas and Fas ligand [5]. On the other hand, there is only a single peak at 12 weeks in the expression of anti-apoptotic factors such as *PAP* [3], *p8* [12], and *clusterin*. Tomasini *et al.* [2] confirmed that cell death occurred by apoptosis in the *TP53INP1*-transfected cells. Therefore *TP53INP1* does promote cell death, whereas *TP53INP1* is not induced by apoptosis. Since *TP53INP1* is a pro-apoptotic factor, two peaks of its gene expression in the WBN/Kob rat model are consistent with the previous results on pro-apoptotic factors. The first peak of apoptosis would reflect acinar cell responses to stress at the onset of chronic pancreatitis. Around this time point, the histopathology of the pancreas resembles acute edematous pancreatitis, although there is already pancreatic fibrosis. The second peak of apoptosis may be involved in acinar cell remodeling and regeneration. Proliferating cell nuclear antigen (PCNA) was also up-regulated at 16 and 20 weeks in this model [14]. Therefore, it may also reflect acinar cell proliferation. Both pro- and anti-apoptotic factors are simultaneously activated in acute pancreatitis [15]. Acinar cell apoptosis has several biological functions in the course of chronic

pancreatitis. First, during the acute phase of pancreatitis, apoptosis would attenuate the severity of the pancreatitis, preventing the excessive release of harmful pancreatic enzymes. Second, apoptosis is a major process of acinar cell loss in the progression of chronic pancreatitis. Third, acinar cell apoptosis could be part of the structural alteration, or remodeling, towards pancreatic regeneration in the process of inflammation and fibrosis. Therefore, the second peak of acinar cell apoptosis and pro-apoptotic factors including *TP53INP1* could reflect acinar cell loss and acinar remodeling or regeneration during the progression of chronic pancreatitis. As mentioned above, *TP53INP1* expression is *p53*-dependent [9]. The *p53* gene has been extensively studied for its role in mediating cell cycle arrest in the G_1 phase following DNA damage. It also functions in the removal of damaged cells by initiating apoptosis in certain physiological situations. The *p53* gene is activated by a variety of types of stress including DNA damage, oxidative stress, hypoxia, nucleotide depletion and oncogenic transformation. Cell cycle arrest or apoptosis may occur in response to *p53* activation, depending on the intensity of the stress. Wild-type *p53*, but not a mutated form, is a transactivator of *TP53INP1* gene expression. Okamura *et al.* [10] proposed that *p53* might determine the selectivity of targets that are directly involved in apoptosis by inducing expression of *TP53INP1/p53DINP1*, which in turn regulates phosphorylation at Ser46 of the *p53* protein. Maacke *et al.* [16] reported that overexpression of the *p53* protein was detected in nearly 59% of cytological specimens of pancreatic juice from patients with chronic pancreatitis and 67% of patients with pancreatic carcinoma. The *p53* overexpressed in chronic pancreatitis appears to be the wild-type, perhaps resulting from DNA damage due to chronic inflammation. During chronic pancreatitis, acinar cells would die through apoptosis accompanied by wild-type *p53* overexpression. We have shown, for the first time, *p53* mRNA overexpression in chronic pancreatitis of WBN/Kob rats. The expression kinetics of

TP53INP1 mRNA almost paralleled that of *p53* mRNA in the course of chronic pancreatitis, although there was no second peak in *p53* mRNA expression.

The mRNAs of *TP53INP1* and *p53* were also induced in arginine-treated AR4-2J cells *in vitro*. During chronic inflammation, DNA damage is likely to occur due to the increase in free radicals which are known to be potent DNA-damaging agents. In our previous reports [17, 18], oxidative stress markers such as inducible nitric oxide synthase (iNOS) and superoxide dismutase (SOD) are induced at the onset of chronic pancreatitis in the WBN/Kob rat *in vivo* and in arginine-induced AR4-2J cell injury *in vitro*. Our results suggest that *TP53INP1* functions as an apoptosis regulator together with *p53* in the course of chronic pancreatitis.

Camostat mesilate, a low-molecular-weight serine protease inhibitor, increases exocrine pancreatic secretion and pancreatic weight in rats, and reduces amylase release from pancreatic acini [19, 20], suggesting clinical usefulness for chronic pancreatitis. The herbal medicine Saiko-keishi-to (TJ-10) has long been used clinically as an anti-inflammatory, analgesic, and anti-emetic agent. Pharmacological actions of TJ-10 include the adjustment of T-lymphocyte functions by saikosaponins in bupleurium root, and immunomodulatory and anti-inflammatory effects of glycyrrhizin in glycyrrhizae root [21, 22]. In our previous reports, camostat mesilate [23] and TJ-10 [3, 24, 25] inhibited inflammatory cell infiltration, acinar degeneration, and inter- and intralobular fibrosis at 12 and 16 weeks. Suppression of *TP53INP1* mRNA expression by these therapeutic drugs may confirm that *TP53INP1* expression is deeply involved in the onset and progression of chronic pancreatitis in this model.

In conclusion, we have shown that *TP53INP1* mRNA expression is strongly induced in acinar cells at the onset of chronic pancreatitis in WBN/Kob rats. *TP53INP1* may be involved in the pathogenesis of chronic pancreatitis as a pro-apoptotic factor, regulated by *p53*.

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References

1. Etemad B, Whitcomb DC. Chronic pancreatitis: diagnosis, classification, and new genetic developments. Gastroenterology 2001; 120:682-707. [PMID 11179244]

2. Tomasini R, Samir AA, Vaccaro MI, Pebusque MJ, Dagorn JC, Iovanna JL, Dusetti NJ. Molecular and functional characterization of the stress-induced protein

(SIP) gene and its two transcripts generated by alternative splicing. J Biol Chem 2001; 47:44185-92. [PMID 11557757]

3. Su SB, Motoo Y, Xie MJ, Sakai J, Taga H, Sawabu N. Expression of pancreatitis-associated protein (PAP) in rat spontaneous chronic pancreatitis: effect of herbal medicine Saiko-keishi-to (TJ-10). Pancreas 1999; 19:239-47. [PMID 10505754]

4. Xie MJ, Motoo Y, Su SB, Sawabu N. Expression of tumor-necrosis factor-α, interleukin-6, and interferon-γin spontaneous chronic pancreatitis in the WBN/Kob rat. Pancreas 2001; 22:400-8. [PMID 11345142]

5. Su SB, Motoo Y, Xie MJ, Sawabu N. Apoptosis in the rat spontaneous chronic pancreatitis: role of the Fas and Fas ligand system. Dig Dis Sci 2001; 46:166-75. [PMID 11270781]

6. Su SB, Motoo Y, Xie MJ, Miyazono K, Sawabu N. Expression of transforming growth factor-beta in spontaneous chronic pancreatitis in the WBN/Kob rat. Dig Dis Sci 2000; 45:151-9. [PMID 10695628]

7. Ohashi K, Kim JH, Hara H, Aso R, Akimoto T, Nakama K. WBN/Kob rats. A new spontaneously occurring model of chronic pancreatitis. Int J Pancreatol 1990; 6:231-47. [PMID 1698893]

8. Nowak J, Tomasini R, Mattei MG, Azizi Samir LA, Dagorn JC, Dusetti N, et al. Assignment of tumor protein p53 induced nuclear protein 1 (TP53INP1) gene to human chromosome band 8q22 by in situ hybridization. Cytogenet Genome Res 2002; 92:140. [PMID 12438758]

9. Tomasini R, Samir AA, Pebusque MJ, Calvo EL, Totaro S, Dagorn JC, et al. p53-dependent expression of the stress-induced protein (SIP). Eur J Cell Biol 2002; 81:294-301. [PMID 12067065]

10. Okamura S, Arakawa H, Tanaka T, Nakanishi H, Ng CC, Taya Y, et al. p53DINP1, a p53-inducible gene, regulates p53-dependent apoptosis. Mol Cell 2001; 8:85-94. [PMID 11511362]

11. Yasuda H, Kataoka K, Ichimura H, Mitsuyoshi M, Iida T, Kita M, Imanishi J. Cytokine expression and induction of acinar cell apoptosis after pancreatic duct ligation in mice. J Interferon Cytokine Res 1999; 19:637-44. [PMID 10433365]

12. Motoo Y, Iovanna JL, Mallo GV, Su SB, Xie MJ, Sawabu N. p8 expression is induced in acinar cells during chronic pancreatitis. Dig Dis Sci 2001; 46:1640-6. [PMID 11508662]

13. Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wild-ranging implications in tissue kinetics. Br J Cancer 1972; 26:239-57. [PMID 4561027]

14. Jiang PH, Motoo Y, Vaccaro MI, Iovanna JL, Okada G, Sawabu N. Expression of vacuole membrane protein 1 (VMP1) in spontaneous chronic pancreatitis in the WBN/Kob rat. Pancreas, in press.

15. Gomez G, Lee HM, He Q, Englander EW, Uchida T, Greeley GH Jr. Acute pancreatitis signals activation of apoptosis-associated and survival genes in mice. Exp Biol Med (Maywood) 2001; 226:692-700. [PMID 11444106]

16. Maacke H, Kessler A, Schmiegel W, Roeder C, Vogel I, Deppert W, Kalthoff H. Overexpression of p53 protein during pancreatitis. Br J Cancer 1997; 75:1501-4. [PMID 9166944]

17. Motoo Y, Su SB, Xie MJ, Mouri H, Taga H, Sawabu N. Effect of herbal medicine Keishi-to (TJ-45) and its components on rat pancreatic acinar cell injuries in vivo and in vitro. Pancreatology 2001; 1:102-9. [PMID 12120187]

18. Su SB, Motoo Y, Xie MJ, Mouri H, Asayama K, Sawabu N. Superoxide dismutase is induced during rat pancreatic acinar cell injury. Pancreas 2002; 24:146-52. [PMID 11854619]

19. Motoo Y, Taga K, Su SB, Xie MJ, Sawabu N. Arginine induces apoptosis and gene expression of pancreatitis-associated protein (PAP) in rat pancreatic acinar AR4-2J cells. Pancreas 2000; 20:61-7. [PMID 10630385]

20. Otani T, Ikekita M, Tanuma SI, Sakamoto T, Hashimoto Y, Kuroda A, et al. Subcutaneouas arginine administration causes apoptosis of rat pancreas [Letter]. Pancreas 1997; 14:316-8. [PMID 9094167]

21. Watanabe S, Takeuchi T, Chey WY. Mediation of trypsin inhibitor-induced pancreatic hypersecretion by secretin and cholecystokinin in rats. Gastroenterology 1992; 102:621-8. [PMID 1732130]

22. Otsuki M, Fujii M, Nakamura T, Tani S, Okabayashi Y. Chronic oral administration of synthetic trypsin inhibitor camostat reduces amylase release from isolated rat pancreatic acini. Int J Pancreatol 1995; 18:135-43. [PMID 8530829]

23. Motoo Y, Su SB, Xie MJ, Taga H, Sawabu N. Effect of herbal medicine Saiko-keishi-to (TJ-10) on rat spontaneous chronic pancreatitis: comparison with other herbal medicines. Int J Pancreatol 2000; 27:123-9. [PMID 10862511]

24. Su SB, Motoo Y, Xie MJ, Taga H, Sawabu N. Antifibrotic effect of the herbal medicine Saiko-keishi-to (TJ-10) on chronic pancreatitis in the WBN/Kob rat. Pancreas 2001; 22:8-17. [PMID 11138977]

25. Su SB, Motoo Y, Iovanna JL, Xie MJ, Sawabu N. Effect of camostat mesilate on the expression of pancreatitis-associated protein (PAP), p8, and cytokines in rat spontaneous chronic pancreatitis. Pancreas 2001; 23:134-40. [PMID 11484915]