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Original Article

Effect of Taurine on Acinar Cell Apoptosis and Pancreatic Fibrosis in Dibutyltin Dichloride-induced Chronic Pancreatitis

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The relationship between pancreatic fibrosis and apoptosis of pancreatic acinar cells has not been fully elucidated. We reported that taurine had an anti-fibrotic effect in a dibutyltin dichloride (DBTC)-chronic pancreatitis model. However, the effect of taurine on apoptosis of pancreatic acinar cells is still unclear. Therefore, we examined apoptosis in DBTC-chronic pancreatitis and in the AR42J pancreatic acinar cell line with/without taurine. Pancreatic fibrosis was induced by a single administration of DBTC. Rats were fed a taurine-containing diet or a normal diet and were sacrificed at day 5. The AR42J pancreatic acinar cell line was incubated with/without DBTC with taurine chloramines. Apoptosis was determined by using terminal deoxynucleotidyl transferase-mediated dUTP-digoxigenin nick end labeling (TUNEL) assay. The expression of Bad and Bcl-2 proteins in the AR42J cells lysates was detected by Western blot analysis. The apoptotic index of pancreatic acinar cells in DBTC-administered rats was significantly increased. Taurine treatment inhibited pancreatic fibrosis and apoptosis of acinar cells induced by DBTC. The number of TUNEL-positive cells in the AR42J pancreatic acinar cell lines was significantly increased by the addition of DBTC. Incubation with taurine chloramines ameliorated these changes. In conclusion, taurine inhibits apoptosis of pancreatic acinar cells and pancreatitis in experimental chronic pancreatitis.

Key words: apoptosis, chronic pancreatitis, pancreatic acinar cells, taurine

Chronic pancreatitis is characterized by irreversible fibrosis and destruction of acinar cells in the pancreas. The development of chronic pancreatitis has been thought to be a necrosis-fibrosis sequence [1]. Recently, it has been increasingly recognized that cellular apoptosis is related to fibrosis in chronic pancreatitis [2, 3]. However, the relationship between pancreatic fibrosis and apoptosis of pancreatic acinar cells has not been fully elucidated.

Taurine was reported to have anti-fibrotic effects in several organs, including the liver [4] and lung [5]. We also reported that taurine had an anti-fibrotic effect in dibutyltin dichloride (DBTC)-induced rat pancreatic fibrosis [6]. However, the effect of taurine on apoptosis of pancreatic acinar cells is still unclear. Therefore, we examined apoptosis in DBTC-chronic pancreatitis and in the AR42J pancreatic acinar cell line with/without taurine.

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Materials and Methods

Animals. The animals used in this study were 8-week-old male Wistar rats (weight 200–250 g) purchased from Japan SLC, Inc. (Hamamatsu, Japan). The rats were provided free access to food and water and were kept in cages in a temperature- and humidity-controlled room with a 12-h dark-light cycle before and during the experiment. All the experiments were performed in accordance with the Okayama University Animal Care Guidelines.

Cell culture. AR42J cells were obtained from DS Pharma Biomedical Co., Ltd. (Osaka, Japan) and were cultured in F12K medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS); the cells were maintained at 37℃ in a humidified atmosphere of 5% CO_2/95% air.

In vivo experimental design. Experimental chronic pancreatitis was induced using the protocol described previously [6–9]. Briefly, DBTC (Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan; 4.5 mg/kg body weight) was first dissolved in 100% ethanol (Nacalai Tesque, Inc., Kyoto, Japan) and mixed with glycerol (Nacalai Tesque, Inc.), and then the rats were divided into the following groups: DB rats, which received a single injection of DBTC (4.5 mg/kg) into the tail vein and were fed a normal diet; DT rats, which received a single injection of DBTC (4.5 mg/kg) and were fed a diet (Oriental Yeast Co., Ltd., Tokyo, Japan) containing 10% taurine (Sigma-Aldrich, St. Louis, MO, USA) beginning 6 days before the DBTC injection and continuing for 5 days after the injection; and C rats, which received a solvent injection (0.1 ml) and were fed a normal diet. Each group (DB, DT, C rats) consisted of 6 rats that were sacrificed at 5 days after injection, and an additional 6 rats of the DB group (defined as DB28 rats) were sacrificed at 28 days after the DBTC injection (Fig. 1).

Histological examination. The entire pancreas was obtained at laparotomy under ether anesthesia as described previously [10]. The tissues were stained with hematoxylin-eosin and with Masson's trichrome. The findings of the microscopic analysis were evaluated by 2 pathologists with expertise in pancreatic pathology who ware blinded to the experimental procedure performed on the specimen under observation.

Evaluation of fibrosis. Pancreatic fibrosis was quantitatively evaluated using image analysis software as described previously [10]. We defined a pancreatic fibrosis area for all pancreas tissue samples based on the percentage of fibrotic area and determined four grades of fibrosis as follows: control, 0–3%; mild, 4–15%; moderate, 16–40%; severe, 41–%.

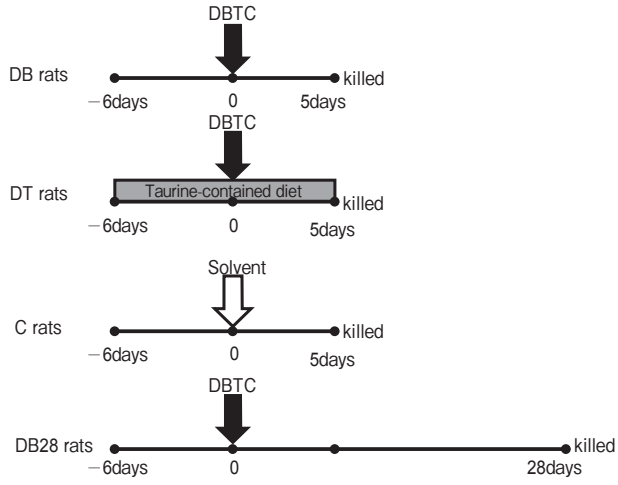

Fig. 1 Flowchart of the experimental design *in vivo*. The black arrows indicate when a single injection of 4.5 mg/kg body weight dibutyltin dichloride (DBTC) was administered into the tail vein, and the white arrow indicates when a solvent injection was administered.

In situ apoptosis detection. Terminal deoxynucleotidyl transferase-mediated dUTP (2'-deoxyuridine 5'-triphosphate)-digoxigenin nick end labeling (TUNEL) assay was performed on paraffin tissue sections or cultured cells on chamber slides using a DeadEnd Colorimetric TUNEL System (Promega, Madison, WI, USA) according to the manufacturer's instructions.

The average number of apoptotic acinar cells was counted by determining the number of TUNEL-positive cells in 10 randomly selected 40 × fields using an optical microscope (Bx51; Olympus, Tokyo, Japan), and the number of apoptotic acinar cells per 1000 acinar cells was determined and designated as the apoptotic index.

In vitro experimental design. After incubation of the AR42J cells, the medium was changed to a serum-free medium, and the AR42J cells were plated in 6-well plates. The cells were divided into the DB group, in which the cells were incubated with DBTC (final concentration, 0.1 μM), the DT group, in which

the cells were incubated with DBTC and taurine chloramines (TauCl; 0.8 mM), and the C group, in which the cells were left untreated for 72 h. TauCl was prepared according to the method described previously [6].

Western blot analysis. The AR42J cells were lysed in RIPA (Radio-Immunoprecipitation Assay) buffer (Sigma-Aldrich), and the cell lysates were centrifuged (12,000 × g, 20 min) to remove debris. The proteins were mixed in an equal volume of Laemmli sample buffer (Bio-Rad, Hercules, CA, USA) containing 2-mercaptoethanol (Sigma-Aldrich; final concentration, 1%).

Equal amounts of protein from each sample were denatured at 100℃ for 5 min and then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred onto nitrocellulose membranes (Amersham, Buckinghamshire, UK). These membranes were blocked by incubating them in Blocking One solution (Nacalai Tesque, Inc.), and then they were incubated with primary antibodies against Bad (BD Biosciences, Franklin Lakes, NJ, USA), Bcl-2 (BD Biosciences), and beta-actin (Abcam plc.) separately at 4℃ overnight. The membranes were washed in Tris-buffered saline containing Tween 20 (TBS-T: 2mM Tris-HCl, pH7.4; 50 mM NaCl; and 0.1% Tween 20) and incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h at room temperature. After washing, immunodetection was performed using an enhanced chemiluminescence kit (Amersham plc.). Signals were visualized by autoradiography, and quantitative analysis of the bands was performed using a Basic Quantifier system (Bio Image Systems, Inc., Jackson, MI, USA).

Statistics. Results were expressed as the means ± standard error (SE). Statistical analysis was performed by the paired Student *t*-test or the Mann-Whitney *U* test (JMP, SAS Institute Inc., Cary, NC, USA). $P < 0.05$ was considered to be statistically significant.

Results

Pancreatic fibrosis with loss of acinar cells was observed 5 to 28 days after a single administration of DBTC in rats (Fig. 2). In DBTC-administered rats, that is, DB rats and DB28 rats, the % fibrotic area was significantly increased in 5 days and 28 days, respectively, compared with controls (C rats). The apoptosis index in the pancreas was significantly increased at 5 days and normalized at 28 days after

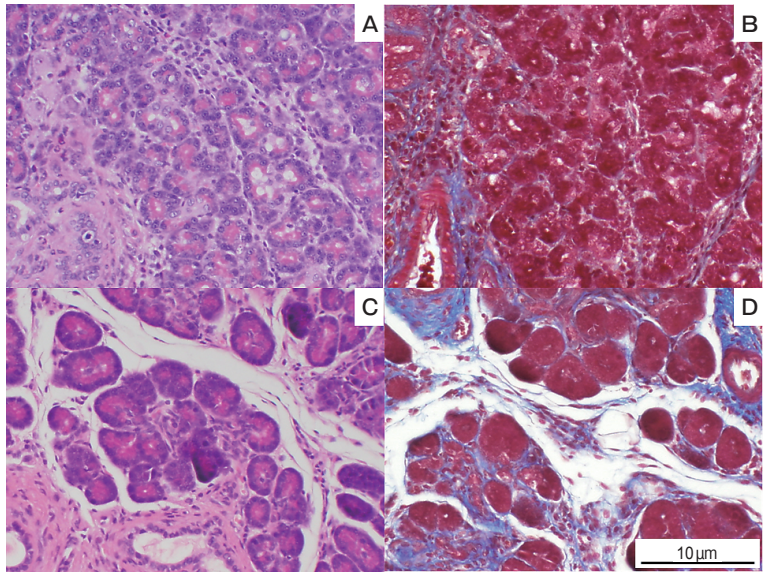

Fig. 2 **A**, **B**: Histological studies of the rat pancreas at 5 days after DBTC injection with hematoxylin and eosin [HE] stain and Masson stain. **C**, **D**: Histological studies of the rat pancreas at 28 days after DBTC injection with HE stain and Masson stain. These samples showed inflammatory cells and a widespread area of pancreas replaced by connective tissue, and interlobular and intralobular pancreatic fibrosis was formed remarkably in 28 days compared with that at 5 days.

administration (Table 1). As for the severity of fibrosis, moderate (2.77 ± 1.31) or severe (4.27 ± 1.31) fibrosis was accompanied by a significant (0.12 ± 0.03) or mild (0.48 ± 1.31) increase of the apoptotic index in comparison with controls, respectively (Fig. 3).

Taurine treatment inhibited pancreatic fibrosis (Fig. 4-C) and the decrease of the apoptotic index in DBTC-administered rats (Fig. 4-D). In AR42J cells, incubation with DBTC significantly increased Bad protein and significantly decreased Bcl-2 protein (Fig. 5). In addition, incubation with taurine chloramines ameliorated these changes, while taurine had no effect.

Discussion

Apoptosis in the pancreas is suggested to be related to pancreatic fibrosis, because a significant increase of apoptotic cells in the pancreas was observed in moderate or severe fibrosis induced by DBTC in our studies. In human chronic pancreatitis, apoptosis was reported to play an important role in disease progression [11] and acinar cell loss [2]. Apoptosis was reportedly increased in angiotensin II type I receptor antagonist-induced rat pancreatic fibrosis [12].

Table 1 Time course of pancreatic fibrosis and apoptosis of pancreas

	% fibrotic area (%)	Apoptotic index ($/10^3$PAC)
Control	1.75 ± 0.83	0.13 ± 0.03
5 days	$36.65 \pm 6.91^*$	$4.09 \pm 1.13^*$
28 days	$29.78 \pm 4.69^*$	$1.61 \pm 0.61^{\#}$

% fibrotic area and apoptotic index were expressed as mean ± SE. $^*p < 0.05$ versus control. $^{\#}p < 0.05$ versus 5 days.

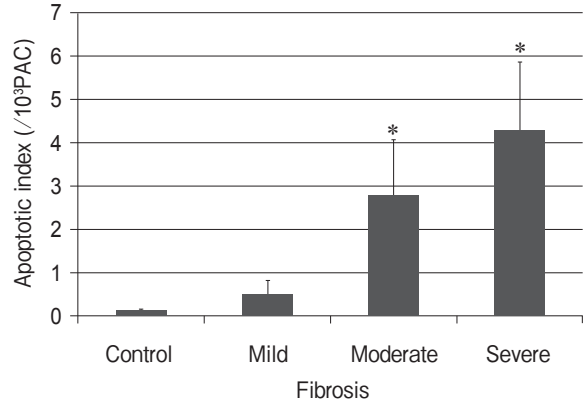

Fig. 3 Relationship between the apoptotic index and the severity of pancreatic fibrosis. The apoptotic index was expressed as the mean ± SE. $^*p < 0.05$ versus the control.

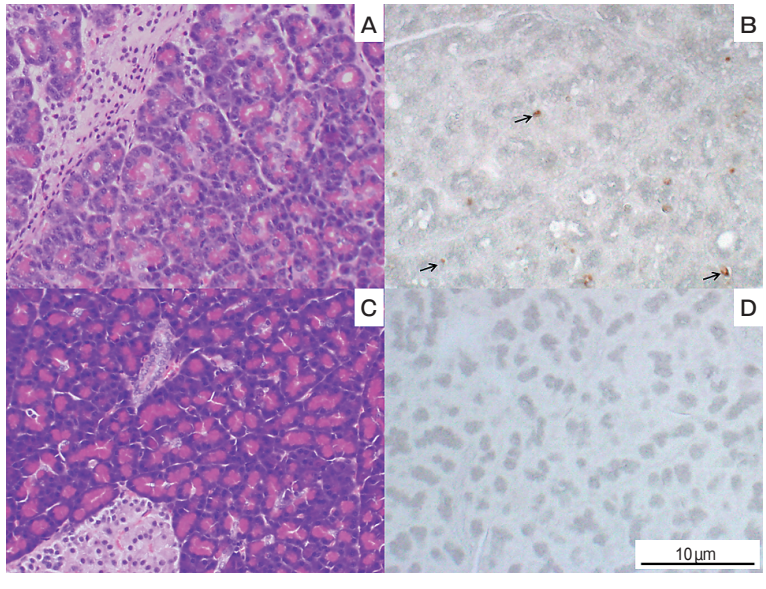

Fig. 4 **A**, **B**: Histological studies of the rat pancreas at 5 days after DBTC injection with HE stain (**A**) and TUNEL stain (**B**). The arrows indicate apoptotic cells. **C**, **D**: Histological studies of taurine-administered rats with HE stain (**C**) and TUNEL stain (**D**). These samples showed inflammatory cells and interlobular and intralobular pancreatic fibrosis (**A**) and TUNEL-positive cells (**B**) in DBTC-administered rats. Taurine treatment inhibited pancreas fibrosis (**C**) and decreased TUNEL-positive cells (**D**).

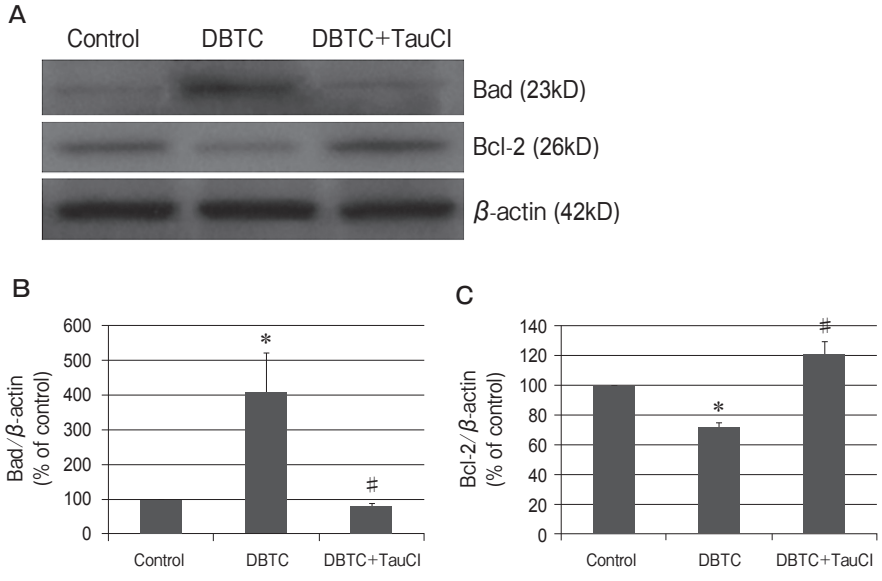

Fig. 5 **A**, Western blot analysis of Bad, Bcl-2 and beta-actin in AR42J cell lysates. Lysates from AR42J cells were incubated with/without DBTC and/or taurine chloramines for 72h; **B**, Western blot analysis of Bad expression. $^{*}p < 0.05$ versus control; **C**, Western blot analysis of Bcl-2 expression. $^{*}p < 0.05$ versus control. $^{\#}p < 0.05$ versus with DBTC.

We thought that apoptosis was induced in acinar cells by the injection of DBTC, because DBTC increased the number of apoptotic cells in the AR42J rat acinar cell line in addition to histological confirmation *in vivo*. Schrader *et al.* [13] reported that chronic pancreatitis patients had an approximately 10-fold increase in apoptotic acinar cells compared with controls. Although the exact relation between pancreatic fibrosis and acinar cell apoptosis is unclear, pancreatic stellate cells (PSCs) have been reported to play an important role in pancreatic fibrosis [14, 15]. As the engulfment of acinar cell by PSCs was reported to inhibit pancreatic fibrogenesis [16], apoptosis of pancreatic acinar cells might control pancreatic fibrosis.

Apoptosis is programmed cell death, and it differs from necrosis by distinct biochemical and morphological alterations [17]. Bcl-2 family proteins play an important role in the apoptotic process. Two classes of regulatory proteins in the Bcl-2 family have opposite effects on apoptosis: the proapoptotic members (Bax, Bak and Bad) promote apoptosis, whereas the antiapoptotic members (Bcl-2 and Bcl-XL) prevent apoptosis [17]. Our results suggest that the administration of DBTC induces an increase of Bad and a decrease of Bcl-2.

In our study, taurine treatment attenuated both apoptosis of pancreatic acinar cells and fibrosis in DBTC rats. Taurine was reported to enhance cellular Bcl-2 content in neonatal rat heart cells [18]. Taurine chloramines are produced abundantly in activated leucocytes from taurine [19]. Since taurine chloramines had an inhibitory effect on acinar cell apoptosis and taurine had no effect in the AR42J acinar cell line, taurine is suggested to be involved in activating leucocytes.

Ribeiro *et al.* [20] reported that taurine improved glucose tolerance and insulin sensitivity in mice, as well as insulin secretion from isolated islets. Chronic pancreatitis often leads to the development of diabetes. Taurine may thus be useful in improving both exocrine and endocrine pancreatic function.

Some limitations apply to this study. We administered taurine to rats at a physiological rather than a pharmacological dose. However, since taurine inhibited apoptosis of pancreatic acinar cells and pancreatic fibrosis in our study, it is possible that taurine could be developed as a medicine to treat chronic pancreatitis in the future. In addition, we used a rat experimental DBTC-chronic pancreatitis model, but this

disease does not match all forms of human chronic pancreatitis, for example, alcohol-induced pancreatitis. Nonetheless, because our findings involved inflammatory cell infiltration, intra- and interlobular fibrosis and atrophy of acinar cells in a DBTC-induced chronic pancreatitis model, our experimental model is useful as a model of chronic pancreatitis. Further studies are needed to test whether fibrosis or apoptosis occurs first in the pancreas in cases of pancreatitis.

In conclusion, taurine inhibits apoptosis of pancreatic acinar cells and pancreatic fibrosis in experimental chronic pancreatitis, suggesting a new candidate for the treatment of chronic pancreatitis.

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