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Camostat mesilate attenuates pancreatic fibrosis via inhibition of monocytes and pancreatic stellate cells activity

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Camostat mesilate (CM), an oral protease inhibitor, has been used clinically for the treatment of chronic pancreatitis in Japan. However, the mechanism by which it operates has not been fully understood. Our aim was to evaluate the therapeutic efficacy of CM in the experimental pancreatic fibrosis model induced by dibutyltin dichloride (DBTC), and we also determined the effect of CM on isolated monocytes and panceatic stellate cells (PSCs). *In vivo*, chronic pancreatitis was induced in male Lewis rats by single administration of 7 mg/kg DBTC and a special diet containing 1 mg/g CM was fed to the DBTC + CM-treated group from day 7, while the DBTC-treated group rats were fed a standard diet. At days 0, 7, 14 and 28, the severity of pancreatitis and fibrosis was examined histologically and enzymologically in both groups. *In vitro*, monocytes were isolated from the spleen of a Lewis rat, and activated with lipopolysaccharide stimulation. Thereafter, the effect of CM on monocyte chemoattractant protein-1 (MCP-1) and tumor necrosis factor-α (TNF-α) production from monocytes was examined. Subsequently, cultured rat PSCs were exposed to CM and tested to see whether their proliferation, MCP-1 production and procollagen α1 messenger RNA expression was influenced by CM. *In vivo*, the oral administration of CM inhibited inflammation, cytokines expression and fibrosis in the pancreas. The *in vitro* study revealed that CM inhibited both MCP-1 and TNF-α production from monocytes, and proliferation and MCP-1 production from PSCs. However, procollagen α1 expression in PSCs was not influenced by CM. These results suggest that CM attenuated DBTC-induced rat pancreatic fibrosis via inhibition of monocytes and PSCs activity.
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Chronic pancreatitis, an irreversible inflammatory disease of the pancreas, is characterized by chronic inflammatory cell infiltration, acinar cell degeneration and development of fibrosis, which lead to impairment of pancreatic exocrine and endocrine function. Although many studies were designed to elucidate the pathophysiology of chronic pancreatitis, the pathobiochemical and molecular mechanisms are still unknown. Recently, a rat model of pancreatitis induced by dibutyltin dichloride (DBTC) was reported by Sparmann *et al.*[1] Thereafter we succeeded in creating a DBTC pancreatitis model with some minor improvements, and demonstrated that this model can be considered as an appropriate model of chronic pancreatitis histologically and enzymologically in our previous study.[2] Progressive pancreatic fibrosis accompanied by inflammatory cell infiltration can be observed after a single intravenous administration of DBTC, and a strong expression of monocyte chemoattractant protein-1 (MCP-1), a family of C–C chemokine, is observed after induction of pancreatitis. Therefore we concluded that MCP-1 plays an important role in the development of pancreatic fibrosis in this model. Furthermore, previous reports have suggested that excessive infiltration of monocytes was also immunohistochemically confirmed in pancreatic tissue from chronic pancreatitis patients,[3] and that a strong expression of MCP-1 messenger RNA (mRNA) was shown to exist in centroacinar ducts, endothelia,

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fibroblasts, macrophages and T cells in pancreatic lobuli with mild to moderate chronic pancreatitis.[4]

On the other hand, since pancreatic stellate cells (PSCs) were identified and characterized in 1998,[5,6] increasing evidence has suggested that PSCs play a central role in the development of pancreatic fibrosis. Once PSCs are activated, they transform into a myofibroblastic phenotype and express α-smooth muscle actin (α-SMA). Activated PSCs exist in the fibrotic area of pancreatic tissue of both human chronic pancreatitis and rat experimental model[7] in response to various mediators derived from conventional or recruited cells. Transforming growth factor-β1 (TGF-β1) activates PSCs and accelerates their synthesis of the extracellular matrix, while platelet-derived growth factor (PDGF) significantly stimulates their proliferation.[8] Since activated PSCs synthesize the majority of the extracellular matrix, including collagen types I and III or fibronectin during fibrogenesis of the pancreas,[5,6] inhibition of activation, proliferation or collagen synthesis of PSCs will provide beneficial effects in the treatment of chronic pancreatitis.

At present, a major component of the conservative treatment of chronic pancreatitis centers on management of complications.[9] However, camostat mesilate (CM), an oral protease inhibitor, has been used clinically for the treatment of pancreatitis[10] and reflux esophagitis after gastrectomy[11] in Japan. The pharmacological effect of CM on chronic pancreatitis has been explained by inhibition of serine proteases,[12] especially trypsin, because activation of trypsinogen in the pancreas is considered to be a trigger reaction in the development of pancreatitis.[13] Although the clinical benefits of CM have been proved in hospitals, the mechanism by which it operates has not been fully understood.

The aim of this study was to examine (1) the effect of CM treatment on rat chronic pancreatitis induced by DBTC *in vivo*, (2) the effect of CM on MCP-1 and tumor necrosis factor-α (TNF-α) production by isolated rat monocytes *in vitro* and (3) the effect of CM on proliferation, MCP-1 production and collagen synthesis in cultured rat PSCs *in vitro*.

Materials and methods

Materials

CM was kindly supplied by Ono Pharmaceutical CO. (Osaka, Japan). For animal experiments, CM powder was mixed at 1 mg/g into CE-2 basal diet (Clea, Tokyo, Japan). Reagents were purchased from the following sources: DBTC from Schering AG (Berlin, Germany); Triton X-100 from ICN Pharmaceuticals, K.K. (Tokyo, Japan); hexadecyltrimethylammonium bromide (HTAB), fetal calf serum (FCS), bovine serum albumin (BSA), lipopolysaccharide (LPS; from *Escherichia coli*, 0111:B4), α-chymotrypsin, hyaluronidase, trypsin/EDTA from Sigma Chemical CO. (St Louis, MO, USA); 3,3′,5,5′-tetramethylbenzidine (TMB) from Dojindo Laboratories (Kumamoto, Japan); N,N-dimethylformamide from Wako Pure Chemical Industries, Ltd. (Osaka, Japan); Ficoll-Paque Plus from Amersham Biosciences Corp. (Piscataway, NJ, USA); RPMI 1640 medium, McCoy's 5A medium, Ham's F-12-DMEM medium from Invitrogen Corp. (Carlsbad, CA, USA); collagenase S-1 from Nitta Gelatin Inc. (Osaka, Japan).

Protocol of Animal Experiments

Male Lewis rats (KBT Oriental, Saga, Japan) weighing 190–220 g were used in these experiments. Throughout the study, the rats were maintained in accordance with the guidelines of the Committee on Animal Care of Kyushu University. Chronic pancreatitis was induced by a single intravenous administration of DBTC as described previously.[2] Briefly, DBTC was first dissolved in ethanol (one part) and then mixed with glycerol (two parts) and dimethyl sulfoxide (two parts). The DBTC solution was injected into the right jugular vein with a syringe at a dose of 7 mg/kg body weight. The animals were allowed free access to water and standard laboratory feed and body weight and food intake were monitored. At day 7, five rats were killed and the remaining rats were divided into two groups randomly, a DBTC-induced pancreatitis group (DBTC-treated group) and a DBTC pancreatitis treated with CM group (DBTC + CM-treated group). The protocol of animal experiments is shown in Figure 1. The DBTC + CM-treated group was fed a diet containing CM at 1 mg/g and the DBTC-treated group was fed a standard diet continuously. At days 14 and 28 after administration of DBTC, five rats from each group were anesthetized by intraperitoneal injection of sodium pentobarbital and the abdominal cavities were opened by a midline incision and bled from the inferior vena cava. After the animals were killed, pancreas were quickly removed, freed from fat and lymph nodes and weighed. Thereafter, the tissues were divided

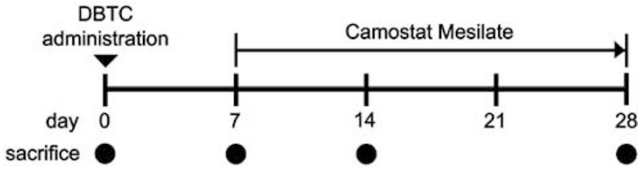

Figure 1 Experimental design. Chronic pancreatitis was induced in male Lewis rats by single administration of 7 mg/kg DBTC at day 0. At day 7, five rats were killed and the remaining rats were divided into two groups randomly, a DBTC-treated group and a DBTC + CM-treated group. The DBTC + CM-treated group was fed a special diet containing CM at 1 mg/g from day 7, while the DBTC-treated group was fed a standard diet continuously. At days 14 and 28, five rats of each group were killed and used for assay. Five normal Lewis rats without DBTC injections were killed and used for control (as on day 0).

into five parts. The first part (head) was fixed in 20% formalin and used for light microscopic and histochemical analyses. The second part (body) was homogenized in 9 vol of 50 mM Tris-HCl buffer (pH 8.0) containing 0.5% Triton X-100 as described previously.[2] The supernatants of homogenates were used for the assay of protein and MCP-1 concentration. The third part (body) was homogenized in 5 ml saline using a Polytron (Kinematica, Luzern, Switzerland) for 1 min. The homogenates were used for the hydroxyproline assay. The fourth part (body) was homogenized in 5 ml/g of 0.5% HTAB buffer (0.5% HTAB in 50 mM phosphate buffer, pH 6.0) in the same manner, plus three freeze–thaw manipulations after sonification (Sonifier; Branson, Danbury, CT, USA). The supernatants of the homogenates were assayed for myeloperoxidase (MPO) activity. The fifth part (tail) was immediately frozen in liquid nitrogen and used for reverse-transcription polymerase chain reaction. Also five normal male Lewis rats of about 200 g weight were killed not having had DBTC injections and were used as control (as on day 0).

Assay

Protein content was determined by the method described by Lowry *et al.*[14] MPO activity was assessed by H_2O_2-dependent oxidation of 3,3′,5,5′-TMB as described previously.[15] Briefly, the reaction mixture for MPO consisted of 50 μl of each homogenate, 1.6 mM TMB, 0.3 mM H_2O_2, 80 mM sodium phosphate buffer (pH 5.4), 8% *N*,*N*-dimethylformamide and 40% PBS in a total volume of 500 μl. After the reaction mixture was incubated at 37°C for 3 min, 1750 μl of 200 mM sodium acetate buffer (pH 3.0) was added, and absorbance at 655 nm was measured by spectrophotometry (U-3000; Hitachi, Tokyo, Japan). Results are expressed as a relative activity and normalized to the average of control samples. Hydroxyproline was measured by a modified method according to Weidenbach *et al.*[16] MCP-1 in serum and in pancreatic tissue were assayed using the commercial rat MCP-1 enzyme-linked immunosorbant assay (ELISA) kit (Immuno-Biological Laboratories, Gunma, Japan) according to the manufacturer's instructions.

Immunostaining for ED-1 and α-SMA

In preparation for immunostaining, paraffin sections of the pancreas were rehydrated and washed twice in PBS for 5 min. Sections were incubated with 0.3% H_2O_2 for 30 min to block endogenous peroxidases and washed. To prevent nonspecific binding of antibodies, sections were incubated with 10% normal goat serum for 30 min at room temperature. For ED-1 immunostaining, sections were then incubated with monoclonal mouse anti-rat ED-1 IgG (Serotec, Hokkaido, Japan) diluted to 1:200 in PBS in a humid chamber at 4°C overnight. After further washes, sections were incubated with Histofine Simple Stain PO (Nichirei, Tokyo, Japan), which is a labeled polymer prepared by combining amino-acid polymers with peroxidase and goat anti-mouse Ig, which are reduced to Fab′ for 30 min at room temperature. After three washes in PBS, chromogen/substrate reagent was applied according to the manufacturer's instructions. Sections were then counterstained in hematoxylin and mounted in nonaqueous mounting solution. For α-SMA staining, paraffin sections of the pancreas were prepared as described above and then incubated with mouse monoclonal anti-α-SMA antibody (Dako, Kyoto, Japan) diluted to 1:100 at 4°C overnight. Sections were incubated with biotinylated rabbit anti-mouse immunoglobulin antibody (Dako, Kyoto, Japan) diluted to 1:500 for 30 min followed by peroxidase-conjugated streptavidin diluted to 1:500 for 30 min. Finally, color was developed by incubating the sections for 5 min with diaminobenzidine, counterstained in hematoxylin and mounted in a similar manner.

Reverse-Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from pancreatic tissue by the guanidium thiocyanate–phenol method as described previously.[17] Extracted RNA was quantified by spectrophotometry, and 1% agarose–formaldehyde gel electrophoresis confirmed its integrity. For RT-PCR, 1 μg of total RNA was reverse-transcribed into complementary DNA (cDNA) using Rever Tra Ace-α (Toyobo, Osaka, Japan) according to the manufacturer's instructions. To examine α-SMA, procollagen α1 and TGF-β1 expression in PSCs, total RNA was extracted from cultured rat PSCs using the commercial RNA extraction reagent ISOGEN (Nippon Gene, Tokyo, Japan). First-strand cDNA was synthesized from 5 μg of total RNA using the Superscript First-strand Synthesis System for RT-PCR (Invitrogen Corp., Carlsbad, CA, USA). For PCR amplification, the following oligonucleotide primers were used:

MCP-1 sense 5′-ACTATGCAGGTCTCTGTCACG-3′,
antisense 5′-AAGTGCTTGAGGTGGTTGTGG-3′ (417 bp);
TGF-β1 sense 5′-CTTCAGCTCCACAGAGAAGAA CTGC-3′,
antisense 5′-CACGATCATGTTGGACAACTGCTCC-3′ (289 bp);
PDGF-BB sense 5′-GAAGCCAGTCTTCAAGAAGGC CAC-3′,
antisense 5′-AACGGTCACCCGAGTTTGAGGTGT-3′ (148 bp);
IL-1β sense 5′-GTGTGATGTTCCCATTAGACAGC-3′,
antisense 5′-TGAGAGGTGCTGATGTACCAGTT-3′ (383 bp);
IL-6 sense 5′-CCTTCCCTACTTCACAAGTC-3′,
antisense 5′-GGATGGTCTTGGTCCTTAGC-3′ (501 bp);

GAPDH sense 5′-TCCATGACAACTTTGGCATCGTGG-3′,
antisense 5′-GTTGCTGTTGAAGTCACAGGAGAC-3′ (377 bp).

Oligonucleotide-specific conditions were as follows: MCP-1, 30 cycles, Ta 58°C; TGF-β1, 32 cycles, Ta 56°C; PDGF-BB, 34 cycles, Ta 62°C; IL-1β, 32 cycles, Ta 59°C; IL-6, 32 cycles, Ta 56°C; GAPDH, 30 cycles, Ta 60°C. For all reactions, final extension was performed for 5 min. And for PCR amplification for PSC, the following primers were used:

α-SMA sense 5′-TGTGCTGGACTCTGGAGATG-3′,
antisense 5′-GATCACCTGCCCATCAGG-3′ (292 bp);
procollagen α1 sense 5′-ACAGCACGCTTGTGGAT-3′,
antisense 5′-GTCTTCAAGCAAGAGGACCA-3′ (469 bp);
TGF-β sense 5′-CGGACTACTACGCCAAAGAA-3′,
antisense 5′-TCAAAAGACAGCCACTCAGG-3′ (295 bp);
GAPDH sense 5′-GGAGGCCATGTAGGCCATGAGGTC-3′,
antisense 5′-GGAGGCCATGTAGGCCATGAGGTC-3′ (295 bp).

Oligonucleotide-specific conditions were as follows: α-SMA, 20 cycles, Ta 62°C; procollagen α1, 25 cycles, Ta 60°C; TGF-β, 30 cycles, Ta 60°C; GAPDH, 25 cycles, Ta 60°C. The PCR products were electrophoresed on 1.5% agarose gels, stained with ethidium bromide, and then photographed under UV illumination. The intensity of the bands was analyzed using the image analysis software NIH image version 1.62 (National Institutes of Health, Bethesda, MD, USA).

Isolation of Rat Monocytes and Production of Conditioned Media

Mononuclear cells were isolated from the spleen of a normal male Lewis rat by a modification of the method described previously.[18] Normal male Lewis rats weighing 200–250 g were killed and the spleen was excised. After having been washed three times in PBS, the spleen was minced in PBS (pH 7.2) containing 2 mM EDTA and 0.5% BSA. Thereafter, minced spleen was filtered through 50-μm nylon mesh and centrifuged for 10 min at 300 *g* at 4°C. After the cell pellet was resuspended in PBS with 2 mM EDTA and 0.5% BSA, the cell suspension was layered on top of Ficoll-Paque Plus density gradient and centrifuged for 30 min at 3000 rpm at 4°C. Mononuclear cells were collected from a hazy band just above the interface of the Ficoll solution and the aqueous buffer. The isolated cells were washed twice and resuspended in RPMI 1640 containing 10% FCS. The cells were counted and the viability was assessed by trypan blue. After cell density was adjusted to 1×10^6 mononuclear cells/ml, the cells were seeded in 96-well plates and maintained in RPMI 1640 with 10% FCS at 37°C in a humidified 5% CO_2 environment for 2 h.

Monocytes mainly composed of macrophages adhered during this incubation period. After 2 h, nonadherent cells were removed and adhered monocytes were washed with serum-free RPMI 1640 three times. Thereafter the cells were exposed to LPS and CM dissolved in RPMI 1640 with 10% FCS. Finally, each well contained 300 μl culture medium, and the concentration of LPS was 10 or 100 ng/ml, and CM was 0, 50, 500 μM or 2 mM. After incubation for 24 h at 37°C, conditioned media were collected and centrifuged for 10 min at 3000 rpm to remove cell debris. The media were assayed for MCP-1 and TNF-α using the rat MCP-1 ELISA kit (Immuno-Biological Laboratories Co. LTD, Gunma, Japan) and the rat TNF-α ELISA kit (Biosource, Camarillo, CA, USA). The operation for each condition was performed in quadruplicate culture wells.

PSC Isolation and Culture

PSCs were isolated from normal male Lewis rats weighing 250–350 g by a modification of the method described previously.[8] Briefly, after the animal was killed the pancreas was rapidly removed and freed from fat, vessels and lymph nodes. Thereafter, the pancreas was washed twice in PBS and McCoy's 5A medium (6 ml) containing 200 U collagenase S-1 was injected into the parenchyma of the pancreas with a 27-gauge needle. The injected pancreas was shaken in a 25 ml Erlenmeyer flask for 20 min at 37°C after having added 6 ml freshly prepared McCoy's 5A medium with 200 U collagenase S-1. Excess medium was withdrawn and 6 ml McCoy's 5A medium containing 2 mM EDTA was added followed by incubation with shaking for another 3 min at 37°C three times. After washed three times in McCoy's 5A medium, a second digestion was performed using 6 ml McCoy's 5A medium with 200 U collagenase S-1, 2 mg α-chymotrypsin, 5 mg hyaluronidase and 20 μl DNase for 20 min at 37°C. Dispersion was accomplished by up-and-down suction through cannulas with decreasing diameters. The digested tissue was filtered through 100-μm nylon mesh and the filtrate was placed on top of 12 ml McCoy's 5A medium with 24 mg BSA and centrifuged for 5 min at 380 *g*. Thereafter, supernatant was removed, and the cell pellet was resuspended in Ham's F-12-DMEM (1:1, vol/vol) with 10% FCS. This cell suspension was layered on top of a Percoll-McCoy's 5A medium (7.5:2.5, vol/vol) density gradient and centrifuged for another 5 min at 180 *g*. Once centrifuged, cells were collected from the top of the gradient, washed, suspended in Ham's F-12-DMEM with 10% FCS and seeded in 10 cm dishes. Freshly isolated PSCs showed the fat-storing phenotype with fat droplets located in the perinuclear region. Within 4–8 days in primary culture, PSCs lost their

fat droplets and developed cytoplasmic extensions. After passage, >95% of the cells showed positive staining for α-SMA.

PSCs Proliferation Assay

Cultured rat PSCs (passages three) were adjusted to a density of 2×10^3/well in 96-well plates and incubated with Ham's F-12-DMEM containing 10% FCS for 24 h. Thereafter, the culture medium was changed to a freshly prepared medium containing CM at 0, 0.5, 1.0 or 2.0 mM with 5% FCS. The medium was changed everyday each containing the same amount of CM. At 72 h after incubation, cell proliferation was assessed by MTT assay (CellTiter non-radioactive cell proliferation assay; Promega, Madison, WI, USA) according to the manufacturer's instructions. The assay for each condition was conducted in 10 independent culture wells.

Preparation of Conditioned Media of PSCs for MCP-1 Assay

Cultured rat PSCs (passages three) were seeded in 96-well plates at the density of 1×10^5/well. After the cells were incubated for 6 h in the presence of 0.1% FCS, the cells were stimulated with LPS (0 or 100 ng/ml), and treated with various amounts of CM (0, 0.5, 1.0 or 2.0 mM) at the same time. After PSCs were incubated with reagents for 24 h at 37°C, the media was collected, and MCP-1 levels in the supernatants were measured using the rat MCP-1 ELISA kit.

Analysis of mRNA Expression in PSCs

Cultured rat PSCs (passages five) were seeded in 10 cm dishes with Ham's F-12-DMEM containing 10% FCS and 2.0 mM CM at day 0. The medium was changed daily to a freshly prepared medium containing an equal amount of CM. At days 1, 3 and 5, total RNA was extracted and RT-PCR was performed as described formerly. Before the treatment of CM, total RNA was extracted from untreated PSCs and used for control (shown as day 0).

Analyses of TGF-β Release from PSCs by ELISA, Matrix Metalloproteinases (MMPs) Secretion by Gelatin Zymography and α-SMA Expression by Western Blotting

Cultured rat PSCs (passages three) were seeded equally in 10 cm dishes and incubated for 24 h in serum-free medium containing 0, 0.5, 1.0 or 2.0 mM CM. Thereafter, conditioned media were collected and centrifuged to remove cells and debris. A part of supernatants were assayed for TGF-β using the TGF-β ELISA kit (Biosource, Camarillo, CA, USA). The remaining of supernatants were concentrated using a centrifugal filter device with a molecular weight limit of 30 kDa (Amicon Ultra-15; Millipore corporation, Bedford, MA, USA) at 5000 *g* for 20 min. This technique resulted in a 30-fold concentration of the PSC secretion. Each 12 μl of the concentrated media was assayed for MMPs activity using a commercial gelatin zymography kit (Gelatin Zymo electrophoretic kit; Yagai, Yamagata, Japan) according to the manufacturer's instructions. Cells were homogenated in lysis buffer containing 50 mM Tris-HCl, 2% sodium dodecyl sulfate (SDS) and protease inhibitor cocktail tablet (Complete, Mini; Roche Applied Science, Penzberg, Germany) 1 tablet for 10 ml. After centrifuged for 10 min at 12 000 rpm, supernatants were collected. Each 10 and 20 μg of protein from the supernatants was electrophoresed through 12.5% SDS polyacrylamide gel (Bio-Rad, Hercules, CA, USA) and transfered to a Hybond-P (Amersham Biosciences Corp., Piscataway, NJ, USA). The membrane was incubated with primary antibody against α-SMA (clone1A4; Sigma-aldrich, St Louis, MO, USA) at a dilution of 1:2000 at 4°C overnight. After incubation with HRP-labeled biotin rabbit anti-mouse IgG (Zymed Laboratories Inc., South San Francisco, CA, USA) for 1 h, proteins were visualized using an ECL kit (Amersham Biosciences Corp., Piscataway, NJ, USA).

Statistical Analysis

Differences between experimental goups were evaluated by Student's *t*-test. A *P*-value of less than 0.05 was considered statistically significant.

Results

CM Improved Physical Findings and Pancreatic Exocrine Function of Rat Chronic Pancreatitis Induced by DBTC

Chronic pancreatitis was induced in male Lewis rats by single administration of 7 mg/kg DBTC at day 0, and a special diet containing 1 mg/g CM was fed to the DBTC + CM-treated group from day 7 (Figure 1). Throughout the experiment, body weight and food intake of all rats were monitored (Table 1). During the first 7 days after administration of DBTC, an acute interstitial pancreatitis can be observed histologically.[2] In this acute phase, the rats demonstrated evidence of significant illness including ruffled fur, loss of activity and jaundice in their tails. The food intake and body weight decreased due to acute pancreatitis during this period in both groups. Thereafter, body weight increased gradually following recovery of food intake, and there was no significant difference in body weight between both groups throughout the entire experimental period.

Table 1 Mean body weight, food intake, and pancreas wet weight in each group

Treatment	*Body wt (g)*				*Food intake (g/day)*			*Pancreas wet wt (g)*	
	At day 0	*At day 7*	*At day 14*	*At day 28*	*Day 0–7*	*Day 7–14*	*Day 14–28*	*At day 14*	*At day 28*
Control	195±7.7								
DBTC	205±7.5	189±14.1	191±46.5	271±38.1	6.7±2.1	11.8±2.5	15.8±1.7*	0.78±0.20*	0.81±0.13*
DBTC+CM	202±5.1	190±19.5	224±35.9	297±21.8	7.1±2.8	13.3±2.2	22.0±0.6*	1.46±0.18*	2.49±0.42*

Chronic pancreatitis was induced by a single intravenous administration of 7 mg/kg DBTC at day 0. At day 7, five rats were killed and the remaining rats were divided into two groups randomly, a DBTC-treated group and a DBTC+CM-treated group. From day 7, the DBTC+CM-treated group was fed a special diet containing 1 mg/kg CM, while the DBTC-treated group was fed a standard diet continuously. Body weight and food intake of all rats were recorded daily during experimental period. Thereafter, five rats from each group were killed at days 14 and 28. After killing, pancreas was quickly removed, freed from fat and lymph nodes, and weighed (pancreas wet weight). Five normal male Lewis rats were killed at day 0 for the control group. Each value represents means±s.e. *Significant differences ($P<0.05$) for the DBTC-treated group *vs* the DBTC+CM-treated group.

Although the DBTC+CM-treated group was fed a diet containing 0.1% CM after day 7, there was no significant difference in food intake between both groups until day 14. The pancreas wet weight had not changed in the DBTC-treated group at days 14 and 28. However, it increased markedly in the DBTC+CM-treated group. The ratio of pancreas wet weight (g)/body weight (kg) in each group at the time of killing was calculated to evaluate for a corrected pancreatic volume. As previously reported, the ratio of pancreas wet weight to whole body weight in this model increased until day 3 because of acute inflammation and edema of the pancreas but started to decrease from day 7 representing progression of pancreatic atrophy.[2] In the DBTC-treated group, atrophy of the pancreas progressed throughout the experiment, whereas the weight of pancreas increased notably in the DBTC+CM-treated group (Figure 2a).

To evaluate the pancreatic exocrine function, protein content corrected by pancreatic volume was examined (Figure 2b). In the DBTC-treated group, the intrapancreatic protein content significantly decreased to 60% at day 14 and to 47% at day 28, reflecting marked injury of the pancreatic exocrine gland. In contrast, the protein content improved to 86% of control rats at day 14 and to 95% at day 28 in the DBTC+CM-treated group.

CM Attenuated Inflammation and Microscopic Findings in DBTC Pancreatitis Model

Microscopic findings and MPO activity in pancreatic tissue are shown in Figure 3. After the single intravenous administration of DBTC, extensive infiltration of inflammatory cells, including neutrophilic granulocytes or mononuclear cells, was observed and the exocrine gland of the pancreas was injured notably at day 7 (Figure 3b). Slight interstitial edema still remained at day 7. At day 14, inflammation remained and an extracellular matrix began to deposit mainly from the interlobular space in the DBTC-treated group (Figure 3c). The presence of an elevated number of monocytes and fibroblasts should be noted. Although slight inflammation remained mainly around the biliopancreatic duct at day 14, the infiltration of inflammatory cells and the damage of exocrine glands were attenuated markedly in the DBTC+CM-treated group at same time point (Figure 3d). In the DBTC-treated group, pancreatic fibrosis in intra- and interlobular space progressed to the end of the experiment and remarkable infiltration of mononuclear cells was observed at day 28 (Figure 3e). However, the degeneration and disappearance of acinar cells was notably inhibited and the lobular structure recovered in the DBTC+CM-treated group at day 28 (Figure 3f). Infiltration of monocytes was notably suppressed and the deposition of connective tissue was barely evident in the DBTC+CM-treated group.

MPO activity in the pancreatic tissue was examined to evaluate neutrophils infiltration. In the DBTC-treated group, MPO activity increased markedly at day 7 (23 times more than control) reflecting acute inflammation in the pancreas and decreased gradually after day 7 (nine times at day 14 and seven times at day 28 *vs* control). However, the level of MPO activity was at a lower level in the DBTC+CM-treated group, and there were significant differences between both groups at days 14 and 28 (Figure 3g).

CM Prevented Fibrosis and Activation of PSCs *In Vivo*

Azan staining revealed increasing collagen deposition in the pancreas in the DBTC-treated group and peaked at day 28 (Figure 4a). In the DBTC+CM-treated group, fibrosis of the pancreas was markedly inhibited compared to the DBTC-treated group at day 28 (Figure 4b). Immunostaining for α-SMA was performed to identify the activated PSCs. In the DBTC-treated group, it revealed a marked

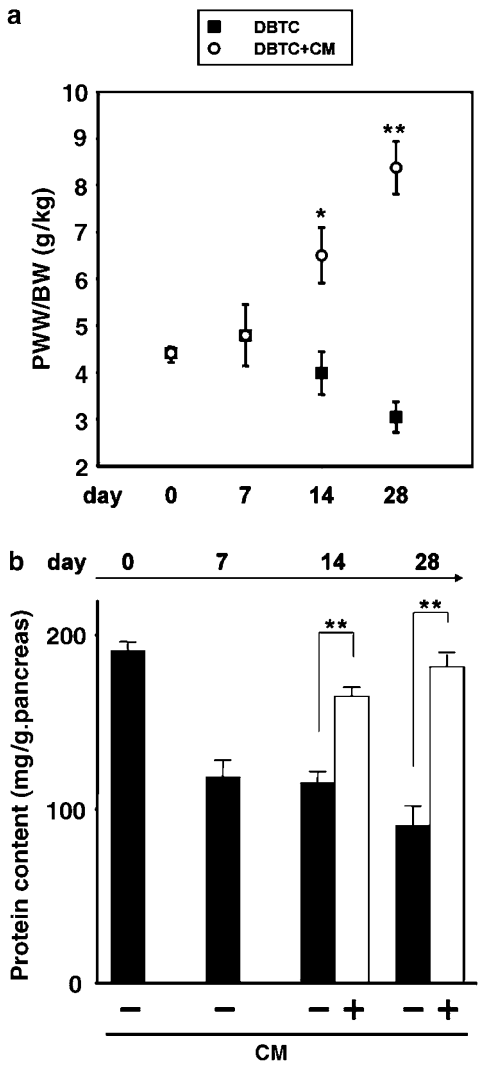

Figure 2 CM prevented pancreatic atrophy and improved pancreatic exocrine function. (**a**) Pancreas wet weight (PWW) was measured after the rats were killed, and the ratio of PWW (g) to whole body weight (BW;kg) was calculated. (**b**) After each rat was killed, the pancreas was removed and homogenized in 50 mM Tris-HCl buffer (pH 8.0) containing 0.5% Triton X-100. Thereafter protein content in homogenized pancreatic tissue was measured. Values are expressed as means ± s.e. ($n=5$). Significant differences: $^{*}P<0.05$, $^{**}P<0.01$, DBTC + CM-treated group *vs* DBTC-treated group.

proliferation of α-SMA-positive cells in the fibrous area of the pancreas at day 28 (Figure 4c). However, there were few α-SMA-positive cells in the DBTC + CM-treated group at day 28 except for vascular smooth muscle cells (Figure 4d). For quantitative analysis of collagen deposition, the hydroxyproline content in homogenate of the pancreas was examined (Figure 4e). Hydroxyproline content in the pancreas increased significantly in the DBTC-treated group at day 14 and showed further increase at day 28. However, increase of hydroxyproline content was notably suppressed in the DBTC + CM-treated group.

CM Inhibited Monocytes Infiltration and MCP-1 Expression in Serum and in Pancreatic Tissue *In Vivo*

Immunostaining for ED-1 revealed marked infiltration of monocytes/macrophages in the pancreas in the DBTC-treated group at day 28 (Figure 5a). However, the number of cells stained positively for ED-1 was much lower in the DBTC + CM-treated group (Figure 5b).

MCP-1 protein expression proved to be enhanced in serum and in pancreatic tissue during chronic pancreatitis induced by DBTC, and we previously demonstrated that MCP-1 plays a role in fibrogenesis in this pancreatitis model.[2] Therefore, the MCP-1 protein concentration in serum and in homogenates of pancreatic tissue was examined with ELISA. MCP-1 protein in serum and in pancreatic tissue markedly increased at day 7 and a high concentration of MCP-1 continued throughout the experimental period in the DBTC-treated group. However, the enhancement of MCP-1 expression was suppressed significantly in the DBTC + CM-treated group, and there were significant differences between both groups at days 14 and 28 (Figure 5c, d).

To investigate mRNA expression of cytokines, total RNA was extracted from pancreatic tissue and RT-PCR was performed. The results of RT-PCR showed that strong mRNA expression of MCP-1 in pancreatic tissue peaked at day 7. In the DBTC-treated group, strong expression of MCP-1 continued to day 28, whereas MCP-1 expression was suppressed in the DBTC + CM-treated group at day 14 and 28. PDGF and TGF-β were upregulated at a later period than MCP-1, and PDGF peaked at day 14 while TGF-β peaked at day 28. The expression of PDGF and TGF-β was also suppressed in the DBTC + CM-treated group compared to the DBTC-treated group (Figure 6a). Densitometry analysis showed MCP-1, IL-1β, IL-6, TGF-β and PDGF mRNA was significantly suppressed in DBTC + CM-treated group at days 14 and 28 (Figure 6b).

CM Inhibited MCP-1 and TNF-α Production in Cultured Monocytes *In Vitro*

To examine the direct effect of CM on inflammatory cells, isolated monocytes were stimulated with LPS at a dose of 10 and 100 ng/ml, and treated with CM at a dose of 0, 50, 500 μM and 2 mM. After 24 h, conditioned media were collected and assayed for MCP-1 and TNF-α concentration. Stimulation of even 10 ng/ml LPS induced MCP-1 and TNF-α production in monocytes. Moreover, greater production of both mediators was observed by 100 ng/ml LPS stimulation (Figure 7a, b). Addition of CM in culture medium attenuated MCP-1 production induced by LPS in a dose-dependent manner (Figure 7a). Similarly, TNF-α production in monocytes was suppressed by CM in a dose-dependent manner (Figure 7b).

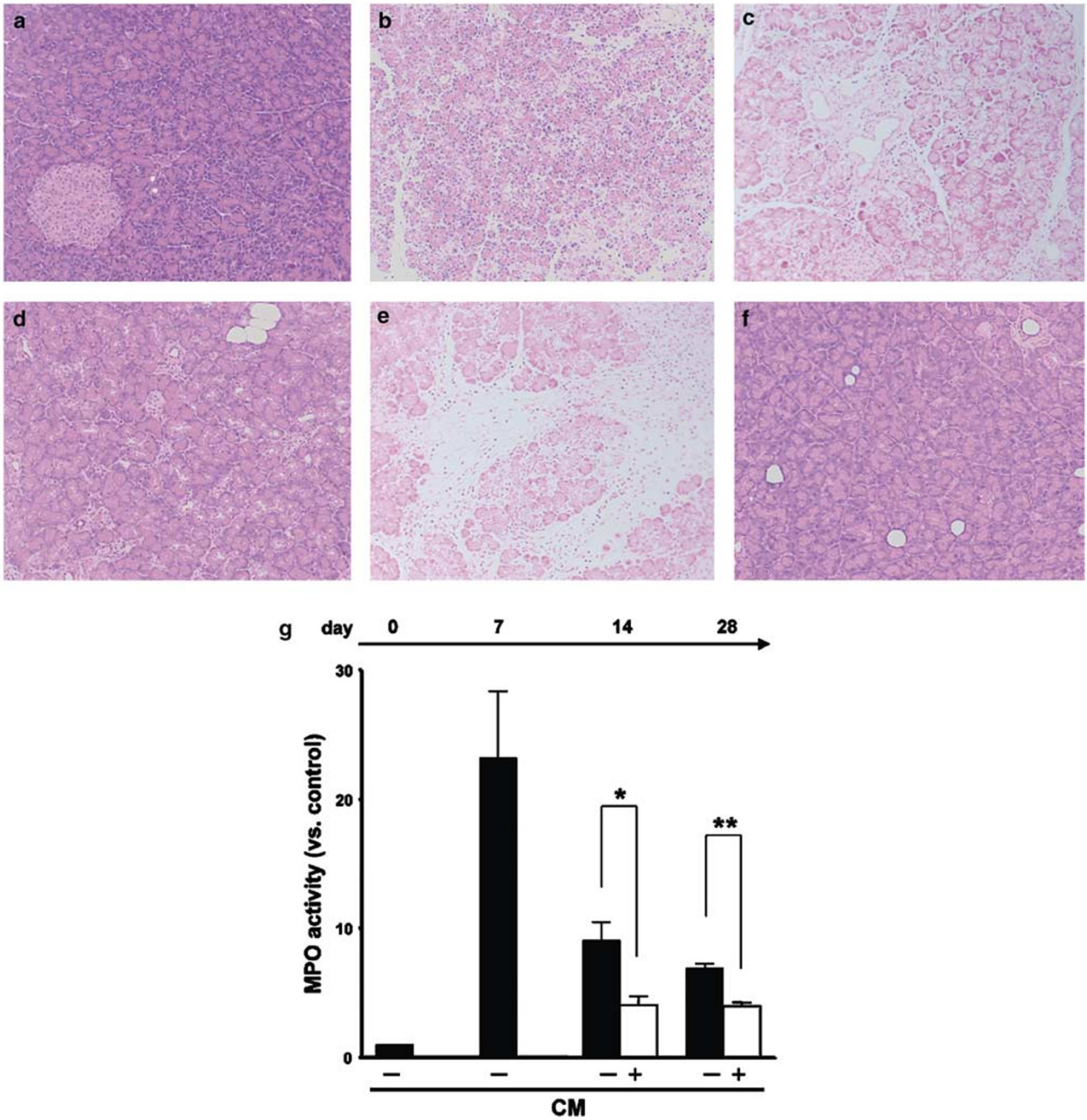

Figure 3 Oral administration of CM inhibited chronic inflammation and pancreatic fibrosis induced by DBTC. Photomicrographs of representative H&E-stained pancreas sections at indicated points of time (original magnification ×100). (**a**) control, (**b**) DBTC-treated group at day 7, (**c**) DBTC-treated group at day 14, (**d**) DBTC+CM-treated group at day 14, (**e**) DBTC-treated group at day 28, (**f**) DBTC+CM-treated group at day 28. (**g**) MPO activity was measured to evaluate neutrophils infiltration. Pancreas was homogenized in HTAB buffer, and MPO activity in homogenized samples was assessed by H_2O_2-dependent oxidation of TMB. Results are expressed as relative activity and normalized to control samples. Values are expressed as means±s.e. ($n=5$). Significant differences: $^*P<0.05$, $^{**}P<0.01$.

CM Inhibited Proliferation and MCP-1 Production of PSCs

Since PSCs were reported to be activated in the course of pancreatitis and play an important role through production of extracellular matrix,[5,6] we investigated the effect of CM on PSCs.

To examine the proliferation of PSCs, cultured PSCs were incubated for 72 h with various concentrations of CM, and MTT assay was performed. The results showed that CM significantly suppressed the proliferation of PSCs to 86% at 0.5 mM, 77% at 1.0 mM and 63% at 2.0 mM compared to 0 mM CM, respectively (Figure 8a). PSCs were also reported to

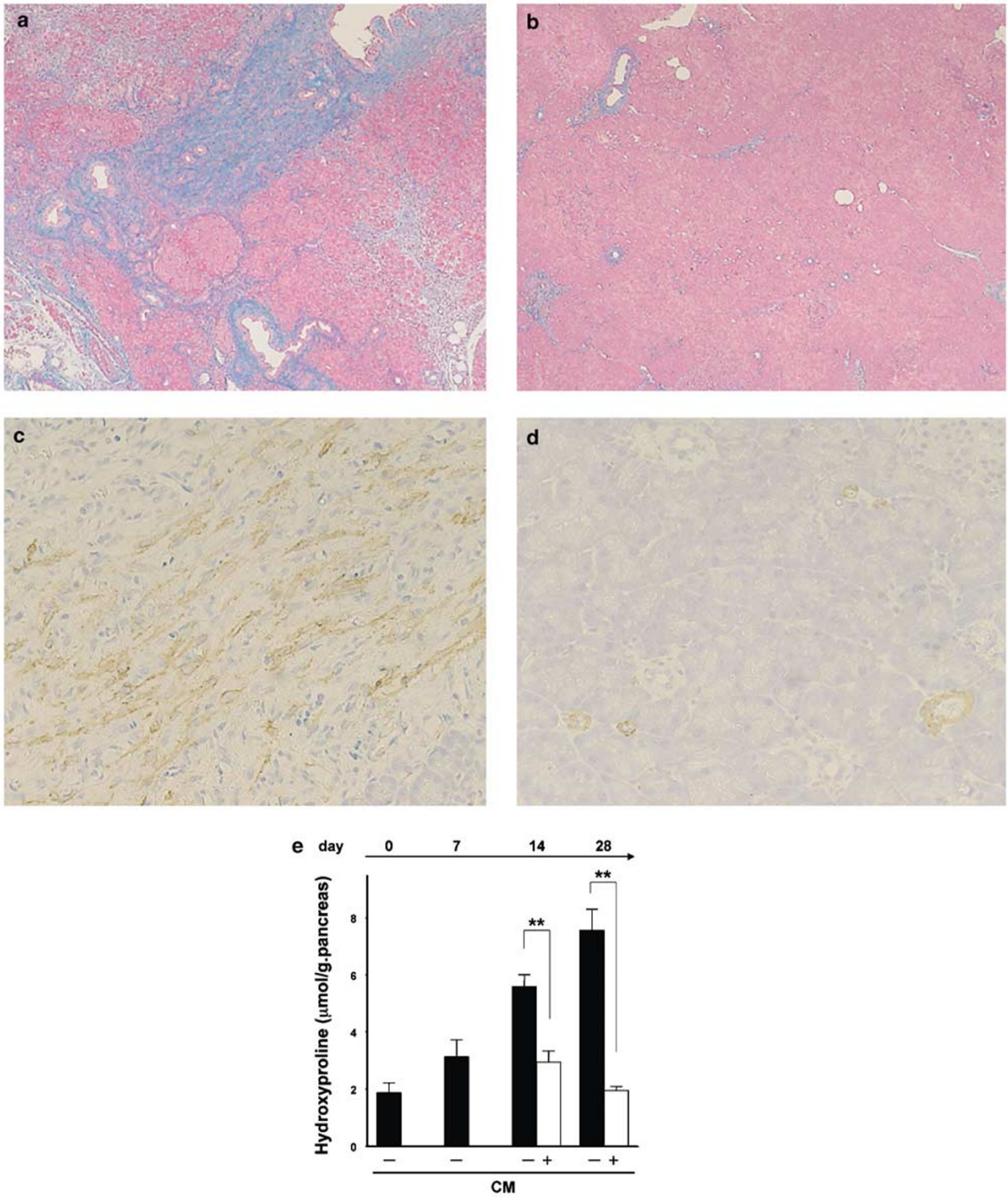

Figure 4 CM inhibited the development of pancreatic fibrosis and PSCs activation in the pancreas induced by DBTC. Azan staining shows collagen deposition in the pancreas (original magnification ×40): (**a**) DBTC-treated group at day 28, (**b**) DBTC + CM-treated group at day 28. Immunostaining for α-SMA was performed to reveal the activated PSCs (original magnification ×400): (**c**) DBTC-treated group at day 28, (**d**) DBTC + CM-treated group at day 28. (**e**) To determine the fibrosis in the pancreas, hydroxyproline content in homogenized pancreatic tissue was examined. Values are expressed as means ± s.e. ($n = 5$). Significant differences: $^{**}P < 0.01$.

produce MCP-1 itself in response to the stimulation of inflammatory cytokines.[19] To examine the direct effect of CM on MCP-1 production in PSCs, PSCs were stimulated by LPS and simultaneously treated with various concentrations of CM. When PSCs were stimulated with 100 ng/ml LPS, MCP-1 was

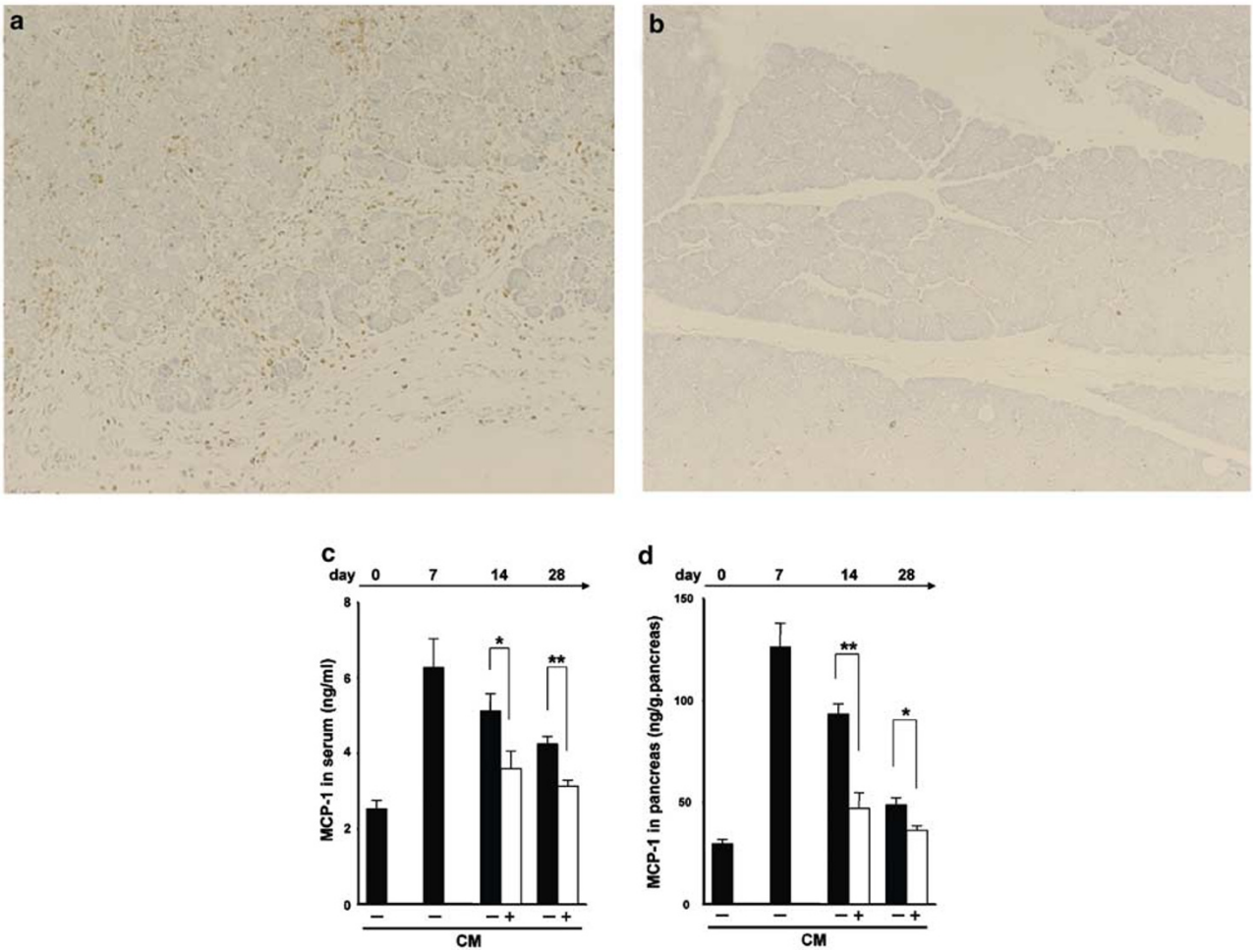

Figure 5 CM suppressed monocytes infiltration and inhibited MCP-1 expression both in serum and in pancreatic tissue. Immunostaining for ED-1 shows infiltrated monocytes into the pancreas (original magnification ×40). (**a**) DBTC-treated group at day 28, (**b**) DBTC + CM-treated group at day 28. (**c**) Serum samples were collected from the inferior vena cava when each rat was killed. MCP-1 concentration in serum was examined by ELISA. (**d**) Pancreatic tissues were homogenized in 9 vol of 50 mM Tris-HCl buffer, and MCP-1 protein concentration in homogenized pancreas was examined in a similar manner. Values are expressed as means ± s.e. ($n = 5$). Significant differences: $^{*}P < 0.05$, $^{**}P < 0.01$.

produced up to 3344 ng/ml in conditioned media. However, the production of MCP-1 was inhibited in the presence of CM in a dose-dependent manner and suppressed to 79% at 0.5 mM CM, 63% at 1.0 mM CM, and 39% at 2.0 mM CM (Figure 8b).

CM does not Effect Collagen Synthesis, α-SMA Expression and MMPs Secretion of PSCs

To examine the effect of CM on activation and collagen synthesis in PSCs, RT-PCR for α-SMA, procollagen α1 and TGF-β was performed. The results of RT-PCR revealed that mRNA expression of α-SMA, procollagen α1 and TGF-β did not change after PSCs were incubated for 5 days with 2.0 mM CM (Figure 9a). To confirm these results, α-SMA expression of PSCs by Western blotting and production of TGF-β was examined. Western blotting for α-SMA showed CM up to 2.0 mM did not suppress the activation of PSCs (Figure 9b). TGF-β release was not influenced by CM, either (data not shown). Furthermore, gelatin zymography was performed to investigate MMPs secretion from PSCs. Gelatin zymography revealed the strong expression of proMMP-2 and weak expression of active MMP-2 in conditioned media. And the results also showed that CM up to 2.0 mM did not effect on gelatinases activity in PSCs (Figure 9c).

Discussion

Up to the present, the efficacy of protease inhibitors for treatment of pancreatitis has been discussed. It has been demonstrated that CM, a synthetic serine protease inhibitor, has the ability to inhibit proteases such as trypsin, kallikrein, thrombin, plasmin, and C1 esterase.[12] CM also has been proven to have a therapeutic effect on some animal models of

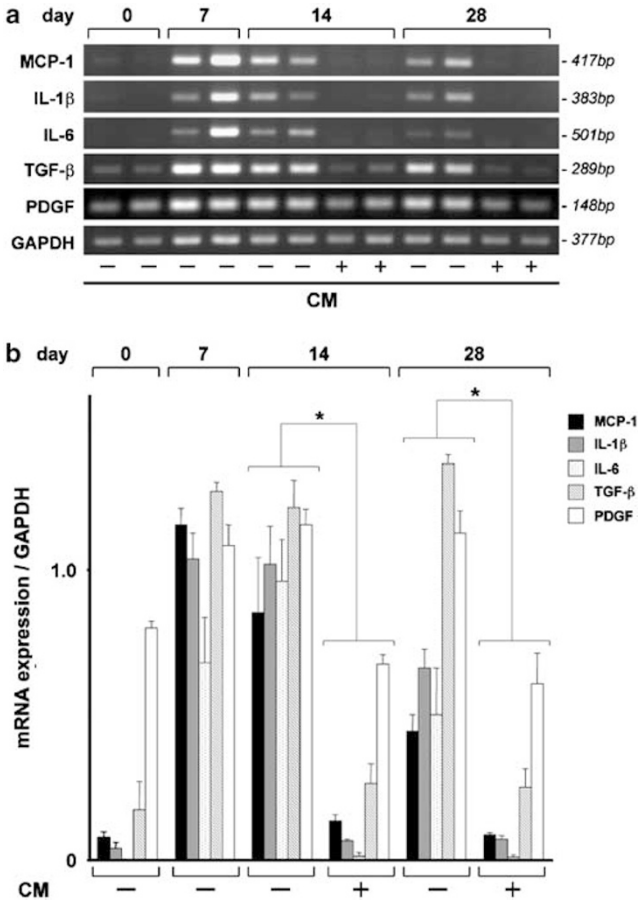

Figure 6 CM suppressed the expression of MCP-1, TGF-β, PDGF, IL-1β and IL-6 in the pancreas. Total RNA was extracted from pancreatic tissues of the rats in each group at indicated time points. RT-PCR was performed to reveal mRNA expression of MCP-1, TGF-β, PDGF, IL-1β and IL-6. (**a**) Representative two results from each group are shown. (**b**) The intensity of the bands was analyzed using the image analysis software, and *Y*-axis indicates relative mRNA expression normalized to GAPDH mRNA. Values are expressed as means $\pm$ s.e. ($n=4$). Significant differences: $^*P<0.05$.

pancreatitis such as spontaneously developed pancreatitis in WBN/Kob rats[20] or cerulein-induced pancreatitis in rats.[21] In addition, it was recently reported that CM prevented rat hepatic fibrosis via inhibition of plasmin-dependent activation of latent TGF-β.[22] However, the pharmacological mechanism of CM in preventing the development of pancreatitis has not been fully documented. In the present study, we demonstrated for the first time that administration of CM attenuates rat chronic pancreatitis

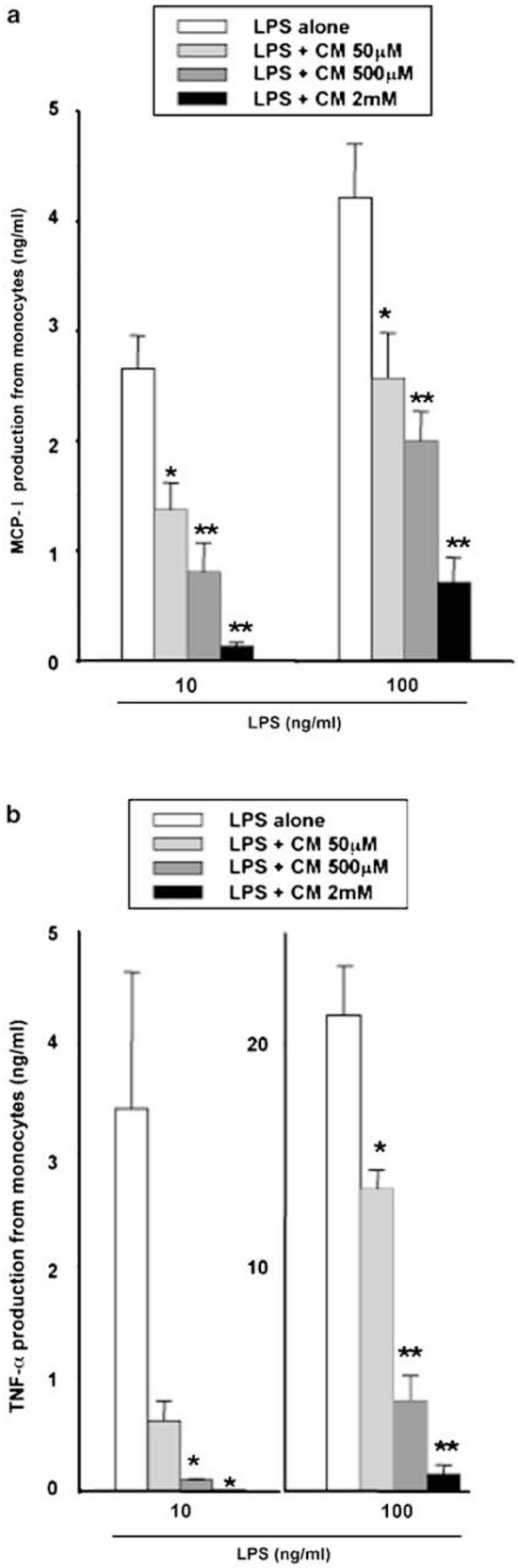

Figure 7 CM inhibited MCP-1 and TNF-α production in activated rat monocytes. Rat monocytes were isolated from the spleen of a normal male Lewis rat. Cultured monocytes were stimulated by 10 or 100 ng/ml LPS for 24 h in the presence of 0, 50, 500 μM or 2 mM CM. Thereafter MCP-1 and TNF-α concentration in conditioned media was examined by ELISA: (**a**) MCP-1 production from monocytes, (**b**) TNF-α production from monocytes. Values are expressed as means $\pm$ s.e. of four independent experiments. Significant differences $^*P<0.05$, $^{**}P<0.01$.

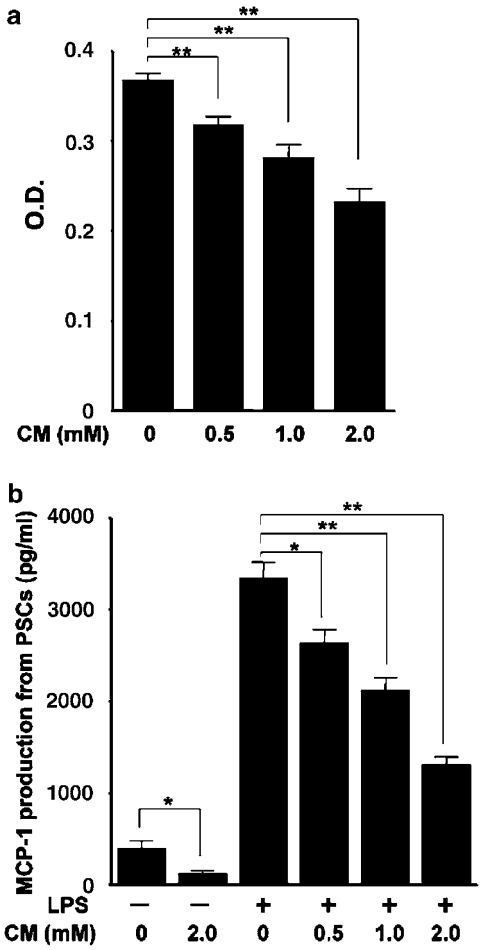

Figure 8 CM inhibited proliferation and MCP-1 production of cultured PSCs. (**a**) Cultured rat PSCs (passages three) were seeded at 2×10^3 cells/well in 96-well plates and incubated for 72 h in the presence of 0, 0.5, 1.0, or 2.0 mM CM. Thereafter, cell proliferation was assessed by MTT assay. The absorbance at 570 nm (O.D.) of the sample is shown. Values are expressed as means ± s.e. ($n = 10$). (**b**) Cultured rat PSCs (passages three) were stimulated with 100 ng/ml LPS and simultaneously treated with CM at 0, 0.5, 1.0, or 2.0 mM. After incubation for 24 h, the conditioned media was collected and MCP-1 levels in the supernatants were measured by ELISA. Values are expressed as means ± s.e. of five independent experiments. Significant differences: $^*P<0.05$, $^{**}P<0.01$.

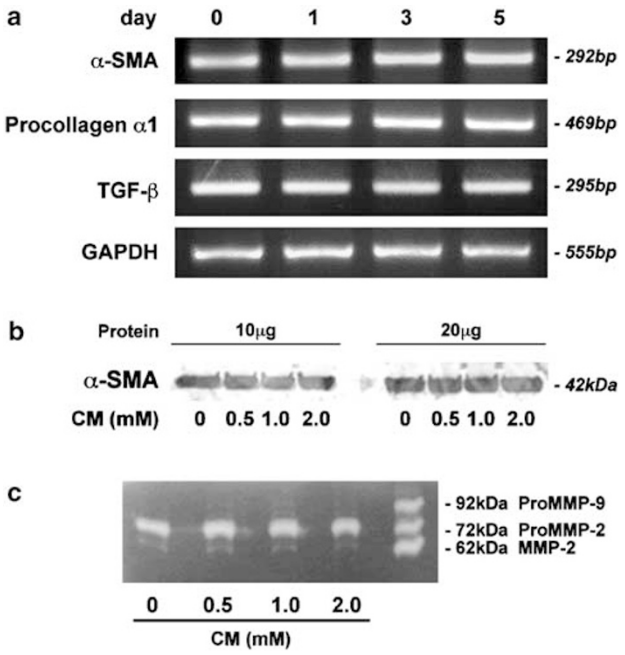

Figure 9 CM does not effect activation, collagen synthesis and MMPs secretion of PSCs. (**a**) PSCs (passages five) were cultured in the presence of 2.0 mM CM for 5 days. Total RNA was extracted from PSCs at days 1, 3 and 5 after addition of 2.0 mM CM. Before the treatment of CM, total RNA was extracted from untreated PSCs and used for control (shown as day 0). RT-PCR was performed to reveal α-SMA, procollagen α1 and TGF-β mRNA expression. (**b**) After PSCs (passages three) were incubated with 0, 0.5, 1.0 or 2.0 mM CM for 24 h in serum-free medium, each 10 and 20 μg protein from cell lysate was assayed for α-SMA protein expression by Western blotting. (**c**) After PSCs (passages three) were incubated with 0, 0.5, 1.0 or 2.0 mM CM for 24 h in serum-free medium, conditioned media was concentrated using a centrifugal filter device and each 12 μl of supernatants was assayed for MMPs activity by gelatin zymography.

induced by DBTC and that CM effects monocytes and PSCs directly and suppresses their activity.

The single intravenous administration of DBTC induces acute interstitial pancreatitis leading to pancreatic fibrosis.[1,2] In the acute phase, 7 days after DBTC administration, pancreatic edema accompanied by granulocytes infiltration and serum elevation of pancreatic enzymes can be observed. Thereafter in the chronic phase, from 7 days after administration of DBTC, pancreatic fibrosis with excessive infiltration of monocytes and the destruction of exocrine pancreas progress. In our preliminary study, we tried to start the oral administration of CM from day 0. However, it resulted in finding no evidence of pancreatitis in rats because CM inhibited acute phase inflammation in the pancreas. From these findings, the current study was designed to focus on chronic phase treatment of the DBTC pancreatitis model by starting CM administration at day 7.

CM was contained in diet at the concentration of 0.1%. Since food intake of CM-treated rats was about 10–20 g/day, the rats in the DBTC + CM-treated group ingested about 40–80 mg CM/kg/day. This amount is about five times as much as a general dose of CM used for chronic pancreatitis patients in Japan. However, no obvious unfavorable effect was observed in CM-treated rats.

CM proved its efficacy in suppressing the progression of chronic pancreatitis *in vivo*. As shown in hematoxylin and eosin (H&E) staining of pancreatic tissue, inflammation and acinar cell degeneration were inhibited in the DBTC + CM-treated group. MPO activity revealed attenuation of neutrophil infiltration, and protein contents in the pancreatic tissue were shown to be preserved. Furthermore, CM inhibited subsequent progressive fibrosis as shown in Azan staining and hydroxyproline content in pancreatic tissue. From these findings, CM is

thought to suppress the development of fibrosis through inhibition of inflammation in the pancreas.

MCP-1 is classified as a C–C subfamily of chemokines and exerts strong chemoattractant in monocytes, macrophages and lymphocytes.[23,24] Overexpression of MCP-1 has been shown in the lesions of several fibrotic diseases including idiopathic pulmonary fibrosis,[25] crescentic glomerulonephritis,[26] systemic sclerosis,[27] and also chronic pancreatitis.[4] It has been also suggested that MCP-1 contributes to fibrogenesis via upregulation of TGF-β.[28–30] In the current study, enhanced expression of MCP-1 mRNA and elevated MCP-1 protein concentration in both serum and pancreatic tissue was inhibited by treatment of CM *in vivo*. As shown in immunostaining for ED-1, infiltration of monocytes/macrophages was significantly attenuated as a result of MCP-1 suppression. These inhibitory effects of CM resulted in suppressed mRNA expression of TGF-β and also PDGF. PDGF was reported to be secreted from activated mononuclear cells and macrophages,[31] and PDGF β-receptor can be upregulated in fibroblasts and blood vessels during wound healing and chronic inflammation.[32] Since it is a well-known fact that TGF-β and PDGF stimulate activation and proliferation of PSCs,[8,33] intrapancreatic downregulation of these growth factors naturally resulted in decreased PSCs activation in the pancreas which is shown by immunostaining for α-SMA.

Since monocytes/macrophages seemed to play an important role in development of the DBTC-induced chronic pancreatitis model, and paracrine stimulation of PSCs via soluble mediators produced by activated mononuclear cells was also reported previously,[34] we investigated the direct effect of CM on isolated monocytes. CM markedly inhibited the production of MCP-1 and TNF-α from cultured rat monocytes in a dose-dependent manner. Since CM is capable of influencing inflammatory cells directly and suppresses the strong monocyte chemotactic factors, CM is thought to suppress inflammation in the course of chronic pancreatitis. Furthermore, recently it was reported that TNF-α, a proinflammatory cytokine, accelerates the proliferation and collagen synthesis of PSCs,[35] and induces MCP-1 production by PSCs.[19] Therefore CM can also inhibit the cell-cell interaction between monocytes and PSCs.

Now, it is widely accepted that PSCs are responsible for the fibrogenesis of pancreas like hepatic stellate cells (HSCs) in the liver. PSCs change their phenotype from quiescent fat-storing cell to highly proliferative myofibroblast-like cell in response to pancreatic injury or inflammation and produce an extracellular matrix during chronic pancreatitis. However, CM significantly inhibited the proliferation and LPS-induced MCP-1 production of PSCs in a dose-dependent manner.

Furthermore, we examined the effect of CM on α-SMA expression, collagen synthesis and MMPs secretion of PSCs. And we showed in this study that CM does not suppress collagen synthesis, activation and MMPs secretion, although CM inhibits proliferation and MCP-1 production of PSCs. These results suggest that CM can suppress a part of PSC's activity through inhibition of some certain specific signaling pathways.

Our findings are summarized as a schematic illustration in Figure 10. Taken together, CM can inhibit the progression of pancreatic fibrosis mainly through suppression of inflammation and PSCs proliferation. However, CM may not attenuate the progressed and established fibrotic lesions since CM does not effect on collagen production and degradation by PSCs.

Considering the molecular mechanism of CM, the intracellular signaling pathway of MCP-1 gives a clue to elucidate. Since MCP-1 expression has proved to be regulated at the transcriptional level, mainly through the activation of nuclear factor-κB (NF-κB) and activator protein-1 (AP-1),[36,37] it is presumed that CM inhibits their activation at any point as its site of action. Gabexate mesilate, also a synthetic serine protease inhibitor and widely used

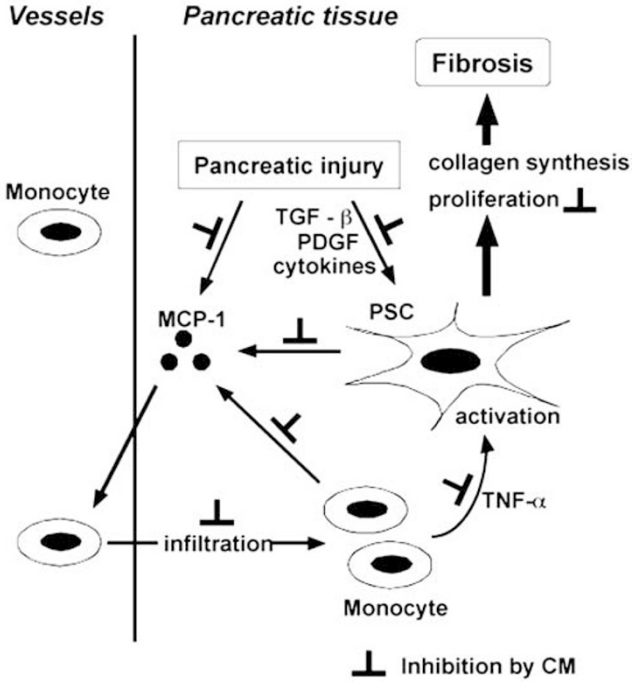

Figure 10 Schematic illustration of development of rat chronic pancreatitis induced by DBTC and inhibitory effect of CM. DBTC induces pancreatic injury due to obstructive pancreatitis. MCP-1, primarily derived from injured conventional cells, induces monocytes infiltration into pancreatic tissue. Monocytes produce MCP-1 and TNF-α after activation in local area of inflammation. MCP-1 induces further inflammation and TNF-α activates PSCs. Once PSCs are activated in response to TGF-β, PDGF or inflammatory cytokines such as TNF-α, they proliferate, produce MCP-1 itself and synthesize extracellular matrix. CM inhibits proliferation and MCP-1 production of activated PSCs. However, the ability of collagen synthesis in PSCs is not influenced by CM.

as an injection for acute pancreatitis and disseminated intravascular coagulation in Japan, was reported to inhibit the binding of NF-κB to DNA-binding elements through preventing inhibitory κBα phosphorylation, in addition to inhibit both the activation of mitogen-activated protein kinase pathways and the binding of AP-1 to DNA-binding elements.[38] Thus it is possible that CM might have a similar action mechanism, although it has not been proved in the current study.

In summary, administration of CM prevented the progression of pancreatic fibrosis induced by DBTC in rats. The observations that CM inhibited MCP-1 and TNF-α production by cultured monocytes, proliferation of PSCs and MCP-1 production by PSCs *in vitro* proved the existence of the direct effect of CM on immunocompetent cells and PSCs. In addition to the inhibitory effect on trypsinogen activation as previously described, CM has beneficial effects in suppressing chronic inflammation leading to fibrosis in the pancreas. In conclusion, our results support the therapeutic effectiveness of CM for treatment of chronic pancreatitis patients.

Acknowledgements

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References

1 Sparmann G, Merkord J, Jaschke A, *et al.* Pancreatic fibrosis in experimental pancreatitis induced by dibutyltin dichloride. Gastroenterology 1997;112: 1664–1672.
2 Inoue M, Ino Y, Gibo J, *et al.* The role of monocyte chemoattractant protein-1 in experimental chronic pancreatitis model induced by dibutyltin dichloride in rats. Pancreas 2002;25:e64–e70.
3 Emmrich J, Weber I, Nausch M, *et al.* Immunohistochemical characterization of the pancreatic cellular infiltrate in normal pancreas, chronic pancreatitis and pancreatic carcinoma. Digestion 1998;59:192–198.
4 Saurer L, Reber P, Schaffner T, *et al.* Differential expression of chemokines in normal pancreas and in chronic pancreatitis. Gastroenterology 2000;118: 356–367.
5 Apte MV, Haber PS, Applegate TL, *et al.* Periacinar stellate shaped cells in rat pancreas: identification, isolation and culture. Gut 1998;43:128–133.
6 Bachem MG, Schneider E, Gross H, *et al.* Identification, culture, and characterization of pancreas stellate cells in rats and humans. Gastroenterology 1998;115: 421–432.
7 Haber PS, Keogh GW, Apte MV, *et al.* Activation of pancreatic stellate cells in human and experimental pancreatic fibrosis. Am J Pathol 1999;155: 1087–1095.
8 Schneider E, Schmid-Kotsas A, Zhao J, *et al.* Identification of mediators stimulating proliferation and matrix synthesis of rat pancreatic stellate cells. Am J Physiol Cell Physiol 2001;281:C532–C543.
9 Khalid A, Whitcomb DC. Conservative treatment of chronic pancreatitis. Eur J Gastroenterol Hepatol 2002; 14:943–949.
10 Kanoh M, Ibata H, Miyagawa M, *et al.* Clinical effects of camostat in chronic pancreatitis. Biomed Res 1989;10(Suppl 1):145–150.
11 Sasaki I, Suzuki Y, Naito H, *et al.* Effect of camostat in the treatment of reflux esophagitis after gastrectomy: an experimental study in rats and a pilot clinical study. Biomed Res 1989;10(Suppl 1):167–173.
12 Tamura Y, Hirado M, Okamura K, *et al.* Synthetic inhibitors of trypsin, plasmin, kallikrein, thrombin, C1r, and C1 esterase. Biochim Biophys Acta 1977; 484:417–422.
13 Creutzfeldt W, Schmidt H. Aetiology and pathogenesis of pancreatitis (current concepts). Scand J Gastroenterol Suppl 1970;6:47–62.
14 Lowry OH, Rosebrough NJ, Farr AL, *et al.* Protein measurement with the folin phenol reagent. J Biol Chem 1951;193:265–275.
15 Suzuki K, Ota H, Sasagawa S, *et al.* Assay method for myeloperoxidase in human polymorphonuclear leukocytes. Anal Biochem 1983;132:345–352.
16 Weidenbach H, Lerch MM, Turi S, *et al.* Failure of a prolyl 4-hydroxylase inhibitor to alter extracellular matrix deposition during experimental pancreatitis. Digestion 1997;58:50–57.
17 Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidium thiocyanate–phenol–chloroform extraction. Anal Biochem 1987;162: 156–159.
18 Basista MH, Stieffenhofer A, Kim DG, *et al.* Procoagulant activity and tumor necrosis factor in rat hepatic allograft rejection. Hepatology 1991;14: 883–887.
19 Masamune A, Kikuta K, Satoh M, *et al.* Ligands of peroxisome proliferator-activated receptor-γ block activation of pancreatic stellate cells. J Biol Chem 2002;277:141–147.
20 Su SB, Motoo Y, Iovanna JL, *et al.* Effect of camostat mesilate on the expression of pancreatitis-associated protein (PAP), p8, and cytokines in rat spontaneous chronic pancreatitis. Pancreas 2001;23:134–140.
21 Kisfalvi K, Papp M, Friess H, *et al.* Beneficial effects of preventive oral administration of camostate on ceruelein-induced pancreatitis in rats. Dig Dis Sci 1995; 40:546–547.
22 Okuno M, Akita K, Moriwaki H, *et al.* Prevention of rat hepatic fibrosis by the protease inhibitor, camostat mesilate, via reduced generation of active TGF-β. Gastroenterology 2001;120:1784–1800.
23 Valente AJ, Rozek MM, Schwartz CJ, *et al.* Characterization of monocyte chemoattractant protein-1 binding to human monocytes. Biochem Biophys Res Commun 1991;176:309–314.
24 Taub DD, Proost P, Murphy WJ, *et al.* Monocyte chemotactic protein-1 (MCP-1), -2, and -3 are chemotactic for human T lymphocytes. J Clin Invest 1995; 95:1370–1376.

25 Antoniades HN, Neville-Golden J, Galanopoulos T, *et al.* Expression of monocyte chemoattractant protein 1 mRNA in human idiopathic pulmonary fibrosis. Proc Natl Acad Sci USA 1992;89:5371–5375.
26 Liu ZH, Chen SF, Zhou H, *et al.* Glomerular expression of C–C chemokines in different types of human crescentic glomerulonephritis. Nephrol Dial Transplant 2003;18:1526–1534.
27 Distler O, Pap T, Kowal-Bielecka O, *et al.* Overexpression of monocyte chemoattractant protein 1 in systemic sclerosis: role of platelet-derived growth factor and effects on monocyte chemotaxis and collagen synthesis. Arthritis Rheum 2001;44:2665–2678.
28 Gharaee-Kermani M, Denholm EM, Phan SH. Costimulation of fibroblast collagen and transforming growth factor β1 gene expression by monocyte chemoattractant protein-1 via specific receptors. J Biol Chem 1996;271:17779–17784.
29 Wolf G, Jocks T, Zahner G, *et al.* Existence of a regulatory loop between MCP-1 and TGF-β in glomerular immune injury. Am J Physiol Renal Physiol 2002;283:F1075–F1084.
30 Diamond JR, Kees-Folts D, Ding G, *et al.* Macrophage, monocyte chemoattractant peptide-1, and TGF-β1 in experimental hydronephrosis. Am J Physiol 1994;266: F926–F933.
31 Ross R. Platelet derived growth factor. Lancet 1989; 1:1179–1182.
32 Reuterdahl C, Tingstrom A, Terracio L, *et al.* Characterization of platelet-derived growth factor beta-receptor expressing cells in the vasculature of human rheumatoid synovium. Lab Invest 1991;64:321–329.
33 Shek FW, Benyon RC, Walker FM, *et al.* Expression of transforming growth factor-β1 by pancreatic stellate cells and its implications for matrix secretion and turnover in chronic pancreatitis. Am J Pathol 2002; 160:1787–1798.
34 Schmid-Kotsas A, Gross HJ, Menke A, *et al.* Lipopolysaccharide-activated macrophages stimulate the synthesis of collagen type I and C-fibronectin in cultured pancreatic stellate cells. Am J Pathol 1999; 155:1749–1758.
35 Mews P, Phillips P, Fahmy R, *et al.* Pancreatic stellate cells respond to inflammatory cytokines: potential role in chronic pancreatitis. Gut 2002;50:535–541.
36 Ueda A, Ishigatsubo Y, Okubo T, *et al.* Transcriptional regulation of the human monocyte chemoattractant protein-1 gene. Cooperation of two NF-κB sites and NF-κB/Rel subunit specificity. J Biol Chem 1997;272: 31092–31099.
37 Martin T, Cardarelli PM, Parry GC, *et al.* Cytokine induction of monocyte chemoattractant protein-1 gene expression in human endothelial cells depends on the cooperative action of NF-κB and AP-1. Eur J Immunol 1997;27:1091–1097.
38 Yuksel M, Okajima K, Uchiba M, *et al.* Gabexate mesilate, a synthetic protease inhibitor, inhibits lipopolysaccharide-induced tumor necrosis factor-α production by inhibiting activation of both nuclear factor-κB and activator protein-1 in human monocytes. J Pharmacol Exp Ther 2003;305:298–305.