Induction of Apoptosis by Crambene Protects Mice against Acute Pancreatitis via Anti-Inflammatory Pathways

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Apoptosis is a teleologically beneficial form of cell death in acute pancreatitis. Our previous work has demonstrated that induction of pancreatic acinar cell apoptosis by crambene protects mice against acute pancreatitis. However, little is known about how the induction of apoptosis reduces the severity of acute pancreatitis. Because the clearance of apoptotic cells might suppress inflammation and critically regulate immune responses, we postulate that clearance of apoptotic cells stimulates an anti-inflammatory response, which has a protective action against acute pancreatitis. To test this hypothesis, induction of apoptosis in acute pancreatitis *in vivo* and co-cultures of peritoneal resident macrophages with apoptotic acinar cells *in vitro* were used as experimental systems, testing expression of phagocytic receptors and levels of inflammatory mediators. Moreover, neutralizing anti-interleukin (IL)-10 monoclonal antibody (2.5 mg/kg) was used before the induction of apoptosis in acute pancreatitis, testing whether the protection from apoptosis induction would be removed. Our study showed that clearance of apoptotic acinar cells, which may occur essentially through the CD36-positive macrophage, stimulates the release of anti-inflammatory mediators like IL-10. IL-10 plays an important role in crambene-induced protection in acute pancreatitis. Thus, induction of pancreatic acinar cell apoptosis by crambene protects mice against acute pancreatitis via induction of anti-inflammatory pathways. (*Am J Pathol* 2007; 170:1521–1534; DOI: 10.2353/ajpath.2007.061149)

Acute pancreatitis (AP) is a common disorder with potentially devastating consequences.^{1–4} Several recent stud-

ies have shown that the severity of AP is inversely related to the extent of acinar cell apoptosis.^{5–7} Moreover, it has been demonstrated that induction of apoptosis reduces the severity of experimental pancreatitis, whereas inhibition of apoptosis worsens the disease.^{8,9} These studies suggested that apoptosis is a teleologically beneficial form of cell death in AP. However, the mechanisms by which introduction of apoptosis protects against AP are not yet clear.

Phagocytosis of apoptotic bodies is one of the characteristic morphological and biochemical features of apoptosis.¹⁰ A series of receptors presenting on the surface of phagocytes have been identified to mediate the recognition and ingestion of apoptotic cells, such as phosphatidylserine receptor (PSR), scavenger receptor (CD36), and vitronectin receptor (integrin $\alpha v \beta 3$), etc.^{10,11} Besides clearance of apoptotic cells, phagocytosis regulates immune responses by up-regulating anti-inflammatory mediators with or without down-regulating proinflammatory cytokines.¹² For instance, the release of transforming growth factor (TGF)- $\beta 1$ by macrophages may be mediated by PSR during the clearance of apoptotic cells,^{13,14} whereas interleukin (IL)-10 is secreted by macrophages engaging with apoptotic cells via annexin I-dependent mechanisms.¹⁵ It is therefore reasonable to postulate that following apoptosis of pancreatic acinar cells, phagocytosis triggers an anti-inflammatory response, which in turn protects against AP.

Although relatively few methods of inducing pancreatic acinar cell apoptosis have been identified,^{8,16–19} crambene (1-cyano-2-hydroxy-3-butene; CHB), a stable nitrile hydrolysis product of the glucosinolate epi-progoitrin found in many cruciferous vegetables,²⁰ has been shown to induce extensive apoptosis in pancreatic acinar cells

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and to protect mice against experimental AP.⁸ The current study aimed to investigate the role of phagocytic receptors and the anti-inflammatory effect of phagocytosis in protecting mice against AP by crambene.

Materials and Methods

All experimental procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication, 1996). Male Swiss mice (18 to 23 g) were used. Crambene was isolated from autolyzed crambe meal using immiscible solvent extraction followed by high-performance liquid chromatography.¹⁸ Caerulein was obtained from Bachem (Bubendorf, Switzerland). All other reagents were of the highest purity commercially available.

In Vivo Experiments

Crambene Administration

Animals were randomly assigned to receive either saline or crambene at one dose of 70 mg/kg given via tail vein injection in a volume of 0.2 ml.⁸ After receiving crambene for 0, 12, 18, 24, 36, 48, 72, and 96 hours, samples of pancreas were collected for IL-10 and TGF- β 1 assay.

Induction of AP

For the time course of caerulein administration studies, mice were randomly assigned to receive either saline or CHB as described. After 12 hours, animals were given hourly injections of caerulein (Cae; 50 μ g/kg) for 0, 3, 6, or 10 hours.

For IL-10-neutralizing studies, animals were divided randomly into five groups: 1) control group, mice were given hourly intraperitoneal injections of saline for 10 hours; 2) caerulein group (Cae), mice received 10 hourly intraperitoneal injections of caerulein (50 μ g/kg)^{21,22}; 3) crambene + caerulein group (CHB + Cae), 12 hours after crambene administration as described, mice were injected with caerulein as in group 2; 4) anti-IL-10 + caerulein group [monoclonal antibody (mAb) + Cae], mice received anti-mouse IL-10 monoclonal antibody (R&D Systems, Inc.) intraperitoneally at one dose of 2.5 mg/kg, suitable for *in vivo* blocking^{21,22}; 12 hours afterward, mice were injected with caerulein as in group 2; and 5) anti-IL-10 + crambene + caerulein group (mAb + CHB + Cae), mice received crambene together with IL-10 antibody as described in groups 3 and 4, respectively; 12 hours afterward, mice were injected with caerulein as in group 2.

One hour after the final caerulein injection in all experiments, animals were euthanized by an intraperitoneal injection of a lethal dose of pentobarbital. Blood and pancreatic tissue were rapidly harvested for the studies as described below.

Evaluation of Pancreatitis Severity

The severity of pancreatic injury induced by caerulein was evaluated by plasma amylase, pancreatic edema, and myeloperoxidase (MPO). Plasma amylase activity was measured as described.^{3,8,23} Pancreatic water content and MPO activity were determined as previously described.^{21,22,24}

Morphological Examination

Ten randomly chosen microscopic fields were examined for each pancreatic sample, and the extent of acinar cell injury/necrosis was expressed as the percentage of the total acinar tissue that was occupied by areas meeting the criteria for injury/necrosis as previously described.^{21,22,25,26} Those criteria were defined as either 1) the presence of acinar cell ghosts or 2) vacuolization and swelling of acinar cells, both of which had been associated with an inflammatory reaction.

IL-10, TGF- β 1, Monocyte Chemoattractant Protein-1 (MCP-1), IL-1 β , and Tumor Necrosis Factor- α (TNF- α) Assays

Pancreatic homogenates were assayed for IL-10, TGF- β 1, IL-1 β , TNF- α , and MCP-1 using a sandwich enzyme-linked immunosorbent assay according to the manufacturer's instructions (Duoset kit; R&D Systems, Minneapolis, MN).

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from pancreas with TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The primers were synthesized by Prologo (Singapore). The sequences of CD36 was 5'-ATGACGTGGCAAAGAACAGCAGC-3' (sense) and 5'-GCAACAAACATCACCCTCCAATCC-3' (antisense). The reaction mixture was first heated to 95°C for 3 minutes and followed by 35 cycles of amplifications, consisting of 95°C for 30 seconds, 68°C for 30 seconds, and 72°C for 30 seconds. PCR amplification was performed in MyCycler (Bio-Rad, Hercules, CA). PCR products were analyzed on 1.5% (w/v) agarose gels containing 0.5 μ g/ml ethidium bromide.

Western Blot

Individual pancreata from mice were homogenized in ice-cold lysis buffer containing a cocktail of protease inhibitors. After removing nuclei and cell debris, proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (50 μ g/lane) and electrophoretically transferred to nitrocellulose membranes. Nonspecific binding was blocked by 5% nonfat dry milk. The blots were then incubated overnight with goat anti-mouse CD36 polyclonal antibody (R&D Systems) at 1:2000 dilution, followed by a donkey anti-goat horseradish peroxi-

Figure 1. Time-course study of crambene treatment on the severity of caerulein-induced AP. Mice ($n = 12$ in each group) were given hourly injections of Cae for 0, 3, 6, or 10 hours with or without 12-hour CHB prophylactic treatment. One hour after the last caerulein injection, mice were sacrificed by an intraperitoneal injection of a lethal dose of pentobarbitone. Plasma amylase activity (**A**), pancreatic water content (**B**), and pancreatic MPO activity (**C**) were determined as described in Materials and Methods. Results shown are the means $\pm$ SE of three independent experiments. $^{\#}P < 0.01$ when treated animals were compared with control animals; $^*P < 0.01$ when compared with caerulein-treated group.

dase-conjugated secondary antibody (R&D Systems) at 1:1000 dilution in the buffer containing 2.5% nonfat dry milk. The blots were developed for visualization using enhanced chemiluminescence detection kit (Pierce, Rockford, IL).

Terminal Deoxynucleotidyltransferase-Mediated dUTP-Biotin Nick-End Labeling

The terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick-end labeling reaction was performed with the ApopTag Plus Fluorescein *In situ* Apoptosis Detection kit (Chemicon International, Temecula, CA) in accordance with the manufacturer's protocol. Images were

Figure 2. RT-PCR analysis of phagocytic receptors. Mice ($n = 12$ in each group) were given hourly injections of Cae for 0, 3, 6, or 10 hours with or without 12-hour CHB prophylactic treatment. One hour after the last caerulein injection, mice were sacrificed by an intraperitoneal injection of a lethal dose of pentobarbitone, and mRNA levels of phagocytic receptor mRNA levels were examined as described in Materials and Methods. **Aa:** Gel photographs showing the RT-PCR analysis of PSR and integrin $\beta 3$ mRNA from 10-hour caerulein-treated (Cae) mice with or without 12-hour CHB prophylactic treatment. Mouse 18S rRNA was amplified as a loading control. **Ab:** Quantitation of RT-PCR analysis. **Ba:** Gel photographs showing the RT-PCR analysis of CD36. Mouse 18S rRNA was amplified as a loading control. **Bb:** Quantitation of RT-PCR analysis. $^*P < 0.01$ when compared with caerulein-treated group.

acquired on a Carl Zeiss fluorescence microscope (Carl Zeiss Inc., Thornwood, NY) using band-pass filters designed to detect fluorescein isothiocyanate (FITC).

Immunohistochemistry and Double Immunofluorescence Labeling

Cryosections were prepared. CD36 was visualized after incubation with a goat anti-mouse primary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 1:50 dilution and then followed by rabbit anti-goat peroxidase-labeled polymer-conjugated secondary antibody (Chemicon) at 1:500 dilution in goat serum. Immuno-stained sections were counterstained with hematoxylin.

Figure 3. Double immunofluorescence labeling of CD36 and F4/80 in pancreatic sections. Pancreatic cryosections from mice with caerulein-induced pancreatitis (10 hourly injections) pretreated with crambene were examined for the expression of CD36 (red) and F4/80 (green) by immunofluorescent microscopy. The merged images (center) indicate whether the two proteins are colocalized in the same cell. Original magnification $\times 1000$. **White arrows** indicate the positive stained cells.

For double labeling, tissue sections were incubated with goat anti-mouse CD36 polyclonal (Santa Cruz Biotechnology) and rabbit anti-activated caspase-3 polyclonal (Sigma-Aldrich, St. Louis, MO) or anti-F4/80 (Santa Cruz Biotechnology) antibodies. Sections were then incubated with appropriate secondary antibodies (Alexa Fluor-488 chicken anti-rabbit IgG for anti-activated caspase-3 or anti-F4/80 and Alexa Fluor-568 rabbit anti-goat IgG for anti-CD36). Images were acquired on a Carl Zeiss fluorescent microscope using band-pass filters designed to detect FITC and rhodamine, respectively.

In Vitro Experiments

Medium

RPMI 1640 medium (Invitrogen) was supplemented with containing 100 U/ml penicillin, 100 μ g/ml streptomycin, and 2 mmol/L glutamine (Invitrogen). Another supplement added to the medium was 10% fetal bovine serum (FBS) (HyClone, Logan, UT), heat-inactivated for 30 minutes at 56°C. The solution is subsequently referred to as 10% FBS.

Isolation of Resident Peritoneal Macrophages

Closed peritoneal lavage was performed on anesthetized mice by injection of 10 ml of ice-cold 10% FBS, followed by gravity drainage through a 1.1-mm diameter needle. The peritoneal lavage was kept on ice until seeding, during which time the cells were washed once and taken up in 4 ml of ice-cold 10% FBS. A 20- μ l sample was taken for counting the total number of cells by hemacytometer.

Peritoneal lavage cells were seeded at 7.5×10^4 in 10% FBS in each 1.8 cm² well of a four-well Lab-Tek glass chamber slide (NUNC A/S, Roskilde, Denmark). Cells were allowed to settle and adhere for 1.5 hours at 37°C in an incubator, after which nonadherent cells were removed by washing with 10% FBS using a standardized protocol of vigorous manual washing via a sterilized Pasteur pipette. FBS (0.9 ml, 10%) was refilled per well, and cells were maintained at 37°C for an additional 1.5 hours. Preparations routinely consisted of >99% mononuclear cells, as verified by Turk's staining. Just before the interaction stage, the cells were again rinsed with 10% FBS using a Pasteur pipette.

Isolation of Pancreatic Acinar Cells and Apoptosis Induction

Pancreata from the same mouse in which isolation of peritoneal macrophages was performed were infused with buffer A (140 mmol/L NaCl, 4.7 mmol/L KCl, 1.13 mmol/L MgCl₂, 1 mmol/L CaCl₂, 10 mmol/L glucose, and 10 mmol/L HEPES, pH 7.2) containing 200 IU/ml collagenase using a 29G syringe, minced with sharp-tip surgical scissors until a fine suspension was achieved, and incubated for 10 minutes at 37°C in a shaking water bath. The digested tissue was passed through buffer A containing 50 mg/ml bovine serum albumin and washed twice with buffer A for further experiments. Cell viability was determined by trypan blue exclusion. Viability of the cells was greater than 95%.

The prepared acini were distributed into microcentrifuge tubes (around 1×10^5 to 1×10^6 pancreatic acinar cells per tube) containing buffer A using a micropipette. Crambene was added into these tubes with the varying working

Figure 4. Detection of apoptosis after 12-hour crambene administration. Pancreatic sections were analyzed using terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick-end labeling (TUNEL). Mice were given 10 hourly Cae injections with or without CHB treatment, which was administered 12 hours before the first caerulein injection. **A:** control; **B:** Cae; **C:** CHB + Cae (original magnification, $\times 200$). **D:** Number of apoptotic cells in 10 fields counted at $\times 200$ magnification. Values are mean (SEM) ($n = 6$ per group). * $P < 0.05$ versus control. **White arrows** indicate the positive stained cells.

concentration of 2 mmol/L. Acini were incubated with or without crambene at 37°C in a shaker water bath for 3 hours. After 3 hours of incubation, the cells were washed three times in buffer A and taken up in 1 ml of 10% FBS. Samples of 20 μ l were taken for analysis of apoptosis. Apoptosis was determined by annexin V-FITC/propidium iodide staining detection as previous described.²⁷

Macrophage and Apoptotic Acinar Cell Interaction

Freshly washed apoptotic acinar cells (0.9 ml) were gently added into wells containing macrophages from the same mouse, after which the whole-chamber slides were placed in a 37°C incubator. Cells were allowed to interact for 1.5 hours. Afterward, culture supernatant

Figure 5. Double immunofluorescence labeling of CD36 and activated caspase-3 in pancreatic sections. Pancreatic cryosections from mice with caerulein-induced pancreatitis (10 hourly injections) pretreated with crambene were examined for the expression of CD36 (red) and activated caspase-3 (green) by immunofluorescent microscopy. The merged images (center) indicate that CD36-stained cells coincide with activated caspase-3-marked cell. Original magnification, $\times 1000$. **White arrows** indicate the positively stained cells.

Figure 6. CD36 detection on surface of macrophages, which phagocytized apoptotic acinar cells. **A–C:** Mouse resident peritoneal cells were isolated and maintained in RPMI 1640 medium; after vigorous manual washing, the remaining cells underwent the following staining. **A:** Turk's stain (nuclear stain) shows nuclear morphology of macrophage; original magnification, $\times 400$. **B** and **C:** Double immunofluorescence labeling of F4/80 (**B**, green) and CD36 (**C**, red) on macrophages. The images indicate the isolated resident peritoneal cells were F4/80-positive but CD36-negative; original magnification, $\times 1000$. **D–F:** Mouse resident peritoneal cells were isolated and co-cultured with pancreatic acinar cells at an early stage of apoptosis induced by crambene for 2 hours; after vigorous manual washing, the remaining cells were undergoing the following staining. **D:** Turk's stain shows that macrophages ingested apoptotic bodies. **Black arrows** indicate cases of phagocytosis; original magnification, $\times 400$. **E** and **F:** Double fluorescence labeling of Hoechst 33342 stain (bis-benzimide) (**E**, blue) and CD36 (**F**, red). Hoechst 33342 staining shows that macrophages ingested apoptotic cells as evidenced by condensed nuclei, indicated by a **white arrow**. CD36 staining shows that macrophages ingested apoptotic acinar cells, which are indeed CD36-positive, indicated by a **white arrow**. Original magnification, $\times 1000$.

was collected for the IL-10 assay as described above. The remaining nonphagocytosed acinar cells were removed by washing with 10% FBS using a standardized protocol of vigorous manual washing via a sterilized Pasteur pipette.

Double Immunofluorescence Labeling

After taking off the chamber structure part with a scalpel, slides were fixed with 3.7% formaldehyde for 10 minutes followed by extensive washing with PBS. Slides were then blocked with 2% FBS for 45 minutes.

Areas for macrophage seeding alone were incubated with rabbit anti-mouse F4/80 antibodies (Santa Cruz Biotechnology) at 1:100 dilution in 2% FBS and goat anti-mouse CD36 polyclonal antibodies (Santa Cruz Biotechnology) at 1:200 dilution in 2% FBS. Slides were washed and then incubated with the appropriate secondary antibodies (Alexa Fluor-488 chicken anti-rabbit IgG for anti-activated anti-F4/80 and Alexa Fluor-568 rabbit anti-goat IgG for anti-CD36, all at 1:200 dilution in 2% FBS) for 30 minutes at room temperature. Slides were allowed to air dry for at least 15 minutes and were mounted with fluorescent mounting medium (Immersol 518F; Zeiss, Wetzlar, Germany). Images were acquired on a Carl Zeiss fluorescent microscope using band-pass filters designed to detect FITC and rhodamine.

Areas for macrophage seeding with pancreatic acinar cells were incubated with goat anti-mouse CD36

polyclonal antibody (Santa Cruz Biotechnology) at 1:200 dilution in 2% FBS followed by incubation with Alexa Fluor-568 rabbit anti-goat IgG for anti-CD36 at 1:200 dilution in 2% FBS for 30 minutes at room temperature. Following washing, slides were then incubated with 10 $\mu\text{g/ml}$ Hoechst dye for 10 minutes according to Bonifacino et al.²⁸ After washing, slides were allowed to air dry for at least 15 minutes and mounted with fluorescent mounting medium (Zeiss). Images acquired on a Carl Zeiss fluorescent microscope using band-pass filters designed to detect 4,6-diamidino-2-phenylindole and rhodamine.

Analysis of Data

Statistical analysis of the data was accomplished using one-way analysis of variance (analysis of variance). The data reported in this article represent mean $\pm$ SEM from multiple determinations in three or more different experiments. Differences in the observed results were considered significant if $P < 0.05$.

Results

Effect of Crambene Treatment on the Severity of Caerulein-Induced AP as a Function of Time

As shown in Figure 1, caerulein treatment results in a time-related increase in severity of AP. Prophylactic ad-

Figure 7. Time course study of crambene treatment on levels of proinflammatory mediators in the caerulein-induced AP. Mice ($n = 12$ in each group) were given hourly injections of Cae for 0, 3, 6, or 10 hours, respectively, with or without 12-hour CHB prophylactic treatment. One hour after the last caerulein injection, mice were sacrificed by an intraperitoneal injection of a lethal dose of pentobarbitone, and pancreatic levels of MCP-1 (A), TNF- α (B), and IL-1 β (C) were determined as described in Materials and Methods. Results shown are the means $\pm$ SE of three independent experiments. * $P < 0.01$ when treated animals were compared with control animals; # $P < 0.01$ when compared with caerulein-treated group.

ministration of crambene attenuates the severity of AP prominently in mice given 10 hourly caerulein injections. However, the protective effect of crambene in terms of hyperamylasemia attenuation starts in mice receiving six hourly caerulein injections, ie, 18 hours after crambene treatment. Because the clearance of apoptotic cells occurs from 18 hours after crambene administration as suggested by our previous work,⁸ the current data indicate that the hyperamylasemia attenuation effect of crambene is temporally coincident with clearance of apoptotic cells. In other words, phagocytosis may stimulate a protective effect on AP.

Figure 8. Effect of crambene treatment on anti-inflammatory mediators levels in pancreas. Mice ($n = 10$ in each group) were given crambene via tail vein injection, and 0, 12, 18, 24, 36, 48, 72, or 96 hours after receiving crambene, samples of pancreas were collected for IL-10 and TGF- β assay. **A:** Levels of IL-10 in pancreas. **B:** Levels of TGF- β in pancreas. ** $P < 0.01$ when crambene-treated animals were compared with placebo-treated animals. * $P < 0.05$ when crambene-treated animals were compared with placebo-treated animals.

RT-PCR Analysis of Phagocytic Receptors in AP

To display the phagocytosis in AP *in vivo*, we investigated pancreatic expression of key phagocytic receptors such as PSR, integrin $\alpha\beta3$, and CD36 under conditions that result in AP. The mRNA levels of these molecules were all detectable in normal pancreas. However, both PSR and integrin $\alpha\beta3$ mRNA in caerulein-treated groups were lower than in their respective control groups, with no significant change in mice pretreated with crambene (Figure 2A). On the contrary, pancreatic CD36 mRNA showed a time-dependent elevation above the control. Moreover, pretreatment with crambene significantly up-

Figure 9. Effect of co-cultures of early apoptotic acinar cells with peritoneal macrophages on IL-10 production. Mouse primary peritoneal macrophages (MΦ) were cultured with or without the apoptotic pancreatic acinar cells (Apo) in a chamber slide for 2 hours. Supernatants of cultures were collected for IL-10 assay. * $P < 0.01$ when IL-10 levels in supernatants harvested from co-cultures of macrophage and apoptotic acinar cells were compared with those from macrophage culture alone.

Figure 10. Effects of neutralizing anti-IL-10 antibody and crambene pretreatment on the severity of caerulein-induced AP. Mice ($n = 5$ in each group) were given 10 hourly injections of Cae ($50 \mu\text{g/kg}$ i.p.). Anti-IL-10 Ab ($50 \mu\text{g}$ per mouse, i.p.) was administered to mice either with or without CHB 12 hours before the first caerulein injection. One hour after the last caerulein injection, mice were sacrificed by an intraperitoneal injection of a lethal dose of pentobarbitone, and plasma amylase activity (A), pancreatic water content (B), pancreatic MPO activity (C), pancreatic acinar cell injury/necrosis (D), and pancreatic MCP-1 levels (E) were determined as described in Materials and Methods. Results shown are the means $\pm$ SE of three independent experiments. * $P < 0.01$ when treated animals were compared with control animals; # $P < 0.01$ when compared with the caerulein-treated group; \$ $P < 0.01$ when compared with the Ab+CHB+Cae group.

regulated mRNA levels of CD36 in mice receiving six and 10 hourly caerulein injections compared with mice treated with caerulein alone (Figure 2); ie, the increase of pancreatic CD36 mRNA was temporally coincident with phagocytosis. Therefore, CD36 is likely to be the primary scavenger receptor involved in crambene-mediated phagocytosis in mouse AP.

Clearance of Pancreatic Acinar Cell Apoptosis via CD36-Positive Macrophages

To identify the cells that express CD36, double immunofluorescence labeling was performed in pancreatic sec-

tions from mice pretreated with crambene followed by 10 hourly injections of caerulein, using antibodies against CD36 and F4/80. F4/80 is a well-known macrophage-specific marker present on the surface of most macrophages.^{29,30} As indicated in the top panel of Figure 3, CD36-stained cells are co-localized with F4/80-marked cells. However, not all F4/80-positive cells were labeled with CD36 (Figure 3, bottom panel). These data suggest that macrophages in the pancreas are the major cells responsible for the clearance of apoptotic pancreatic acinar cells.

To investigate the locational relationship between phagocytic cells and apoptotic cells, we used terminal

Figure 11. Morphological changes in mouse pancreas of AP with or without prophylactic treatment with anti-IL-10 antibody/crambene. **A:** Control; no pancreatitis. **B:** Caerulein-induced AP with pretreatment of anti-IL-10 and crambene. **C:** Caerulein-induced AP with pretreatment of crambene. **D:** Caerulein-induced AP. **E:** Caerulein-induced AP with pretreatment of anti-IL-10. Changes in AP include various degrees of pancreatic edema, neutrophil infiltration, and acinar cell injury/necrosis. An **asterisk** indicates acinar cell injury/necrosis.

deoxynucleotidyltransferase-mediated dUTP-biotin nick-end labeling staining as well as double immunofluorescent staining. As shown in Figure 4, prophylactic administration of crambene results in a significant increase of *in situ* labeling of cleaved DNA, an indicator of apoptosis. In Figure 5, merged, activated caspase-3-labeled cells (left) were observed close to CD36-

stained cells (right). This indicates that macrophages act as the phagocytic cells, acting through the surface receptor CD36.

This conclusion was supported by our *in vitro* findings. We have previously shown that treatment of 2 mmol/L crambene in isolated pancreatic acinar cells for 3 hours induces the apoptosis of pancreatic acinar cells.²⁷ In the current

study, we co-cultured the apoptotic acinar cells, which were induced by the treatment of crambene as described above, with isolated primary peritoneal macrophage from the same mouse in a chamber slide for 2 hours. After rigorous washing and immunofluorescent staining, an increased CD36 immunoactivity was found on the membrane of macrophages, which ingested the apoptotic body of pancreatic acinar cell (Figure 6F). Therefore, CD36-positive macrophages seem to play an important role in phagocytosis of apoptotic cell of pancreatic acini.

Effect of Crambene Treatment on Levels of Pancreatic Inflammatory Mediators As a Function of Time

To determine the possible anti-inflammatory characters of crambene-mediated phagocytosis during AP, we investigated the effect of crambene treatment on pancreatic levels of proinflammatory mediators such as MCP-1, IL-1 β , and TNF- α in AP. As shown in Figure 7, levels of pancreatic MCP-1 were time-dependently attenuated by prophylactic administration of crambene. Pancreatic TNF- α and IL-1 β levels were significantly attenuated by crambene in mice receiving three and six hourly caerulein injections, respectively. Nevertheless, this generalized attenuation of proinflammatory mediators was not temporally coincident with clearance of apoptotic cells.

Besides proinflammatory cytokines, levels of pancreatic anti-inflammatory cytokines such as IL-10 and TGF- β 1 were studied from 12 to 96 hours after intravenous administration of crambene at a single dose of 70 mg/kg. Levels of pancreatic IL-10 (Figure 8A) were significantly increased 30 hours (from 18 to 48 hours) after crambene administration, whereas TGF- β 1 (Figure 8B) was only increased significantly at 12 hours compared with untreated control groups. This suggested crambene-mediated phagocytosis stimulates an anti-inflammatory response, in which IL-10 in particular may play an important role.

Effect of Co-Cultures of Early Apoptotic Acinar Cells with Peritoneal Macrophages on IL-10 Production

To clarify whether the production of IL-10 results from phagocytosis of apoptotic acinar cells, levels of IL-10 were determined in supernatants from co-cultures of apoptotic acinar cells and resident peritoneal macrophages. Figure 9 shows a significant increase of IL-10 levels in supernatants that were harvested from co-cultures of macrophages and apoptotic acinar cells compared with IL-10 levels in those from macrophage culture alone. We also analyzed the production of IL-10 level in pancreatic acinar cells after treatment with crambene for 3 hours to exclude the possibility that apoptotic cell may itself produce IL-10. However, the levels of IL-10 are too low to be detected. Therefore, the significant increase of IL-10 levels in supernatant from co-cultures is likely due to the interaction between macrophages and apoptotic

acinar cells. This indicates that phagocytosis of apoptotic acinar cells seems to play an important role in IL-10 production.

Effect of Pretreatment with Neutralizing Anti-IL-10 Antibody and Crambene on the Severity of Caerulein-Induced AP

To establish further the role of phagocytosis-stimulated IL-10 production in the protective effect of crambene during AP, we used a neutralizing antibody against IL-10 to investigate whether the protection of crambene would be removed. Figure 10 shows that prophylactic administration of anti-IL-10 antibody together with crambene results in a significant increase of the magnitude of hyperamylasemia, pancreatic edema, pancreatic MPO, pancreatic MCP-1 levels, and the histomorphological changes (ie, acinar cell injury/necrosis as well as pancreatic edema) compared with the control mice treated with intraperitoneal normal saline. However, pretreatment with anti-IL-10 antibody alone 12 hours before caerulein

Figure 12. Examination of expression of CD36 in mouse pancreas of AP with or without prophylactic treatment with anti-IL-10 antibody/crambene. Mice ($n = 5$ in each group) were given 10 hourly injections of Cae (50 μ g/kg i.p.). Anti-IL-10 Ab (50 μ g per mouse, i.p.) was administered to mice either with or without crambene 12 hours before the first caerulein injection. One hour after the last caerulein injection, mice were sacrificed by an intraperitoneal injection of a lethal dose of pentobarbitone; mRNA levels and protein levels (**B**) of CD36 expression were examined as described in Materials and Methods. **A:** Quantitation and representative gel photographs of RT-PCR analysis. 18S rRNA was used as a loading control. **B:** Quantitation and representative photograph of Western blot analysis. HPRT protein was used as a loading control.

administration had no effect on the progression of pancreatic injury compared with that in mice treated with caerulein only (Figures 10 and 11). These results demonstrated that administration of anti-IL-10 antibody together with crambene 12 hours before caerulein injection significantly reversed all of the crambene-mediated protection against AP.

mRNA And Protein Expression of CD36 in Caerulein-Induced AP with Pretreatment of Neutralizing Anti-IL-10 Antibody and Crambene

To investigate the relationship between the release of pancreatic IL-10 and phagocytosis in AP, we did RT-PCR, Western blotting, and immunohistochemistry to examine both mRNA and protein expression of CD36 in AP (Figures 12 and 13). Pretreatment of both anti-IL-10 and crambene together shows the similar magnitude of increase in levels of CD36 as in crambene + caerulein group. However, pretreatment of anti-IL-10 alone 12 hours before caerulein injection shows no affect on the alterations of CD36 compared with animals treated with caerulein alone (Figure 12, A and B). As shown in Figure 13, immunostaining of CD36 expression is very low in the normal pancreas. However, the expression of CD36 is significantly up-regulated in pancreatic sections from mice given the prophylactic treatment of crambene or anti-IL-10 + crambene. Pancreatic sections from mice that received either caerulein alone or anti-IL-10 + caerulein show low expression of CD36. These results suggest that release of IL-10 is downstream of phagocytosis.

Discussion

Apoptosis is a teleologically beneficial form of cell death in AP. However, the exact mechanism by which the induction of apoptosis protecting against progression toward AP is still not clear. This study shows that crambene induces phagocytosis of apoptotic acinar cells by CD36-positive macrophages during AP. The anti-inflammatory effect of the phagocytosis is mediated by the production of pancreatic anti-inflammatory mediators like IL-10. Besides the anti-inflammatory response of phagocytosis, suppressing levels of pancreatic proinflammatory mediators also contributes to crambene protection against mouse AP.

It is well known that the final phase of apoptosis *in vivo* is swift, with rapid phagocytosis of intact "unwanted" cells by resident tissue macrophages.^{10,11} Our data also suggest that macrophages in pancreas are the major means of clearance of apoptotic pancreatic acinar cells (Figure 3). However, the large complexity of pattern recognition receptors depends on the means of induction of cell death, cell type of both apoptotic cells and phagocytes, and the surrounding microenvironment.^{10,11} For instance, PSR has been recently reported to be expressed on pancreatic cells other than macrophages in human chronic pancreatitis, in particular, in regions with high apoptotic activity.³¹ Integrin $\alpha v \beta 3$ is a molecule that

was observed to be involved in phagocytotic clearance during retinal detachment in the mouse.³² Expression of CD36 has been found to be expressed in rat AP.³³ In the current study, the increase of CD36 expression has always been observed in mice with crambene prophylactic administration compared with mice without that treatment (Figures 2, 12, and 13). Our *in vitro* data also show that CD36-positive macrophages take up the apoptotic acinar cells (Figure 6). Therefore, it is likely that CD36-positive macrophages are mainly involved in crambene-mediated phagocytotic clearance in mouse AP *in vivo*.

A convincing body of evidence shows that phagocytes not only phagocytose apoptotic cells, but they also respond to apoptotic cell death by suppressing inflammation.¹² It has been suggested the recognition of phosphatidylserine-positive apoptotic cells by macrophages through a PSR may play an important signaling role in the release of TGF- $\beta 1$ during the clearance of apoptotic cells.^{13,14} Phosphatidylserine-PSR bridging protein TSP1 and other phosphatidylserine-nonspecific binding receptors like CD36 and the vitronectin receptor also have been reported to be able to suppress monocyte/macrophage inflammatory responses by stimulating TGF- $\beta 1$ release.^{34,35} Annexin I, a newly defined endogenous ligand for tethering and internalization of apoptotic cells, can stimulate IL-10 production and inhibit nitric oxide synthesis.¹⁵ The present study demonstrates that inducing production of IL-10 by crambene is temporally coincident with clearance of apoptotic cells and that the release of the IL-10 is downstream of phagocytosis (Figures 8A, 12, and 13). Moreover, our *in vitro* data also support that phagocytosis of apoptotic acinar cells indeed stimulates the production of IL-10 (Figure 9). Therefore, phagocytosis of apoptotic acinar cells seems to play a major role in production of IL-10, an anti-inflammatory mediator. Furthermore, when IL-10 production by crambene-mediated phagocytosis was blocked, the protective effects of crambene against AP were significantly impaired (Figures 10 and 11). These results signify the important role of IL-10 in the anti-inflammatory effect of crambene-induced phagocytosis during AP.

Not only can phagocytosis suppress the inflammatory response, but it can also suppress the number of cells undergoing apoptosis.³⁶ Unlike necrotic cells, apoptotic cells maintain membrane integrity before phagocytosis. Therefore, leakage of intracellular components (eg, proinflammatory and immunogenic materials) should be prevented actively to avoid inflammatory response.^{36,37} Moreover, apoptotic cells may themselves produce anti-inflammatory factors such as IL-10 and TGF- $\beta 1$. The persistent appearance of apoptotic cells in inflamed tissues could provide a prolonged anti-inflammatory stimulus and help to inhibit long-term tissue damage.³⁶ Our present study shows that production of TGF- $\beta 1$ by crambene happens before phagocytosis (Figure 7B), but the increased levels of TGF- $\beta 1$ are temporally coincident with induction of maximal apoptosis by crambene, as suggested by our previous study.⁸ This indicates that the release of TGF- $\beta 1$ may be due to the apoptosis of pancreatic acinar cells, per se, but not clearance of apoptotic cells.

Besides the release of anti-inflammatory cytokines, we also investigated the effect of crambene treatment on pancreatic levels of proinflammatory mediators such as MCP-1, IL-1 β , and TNF- α in AP. MCP-1 (a CC chemokine) has been regarded as the major chemokine in pancreatitis. Significantly elevated local and systemic concentrations of MCP-1 have been demonstrated in both experimental and clinical AP.^{38–41} IL-1 β and TNF- α are potent proinflammatory cytokines that are predominantly derived from activated macrophages. These two cytokines are involved in the inflammatory cascade subsequent to acinar cell damage, and their levels are elevated on the onset and during the progress of AP.^{1,2,22} In the present study, prophylactic treatment of crambene showed a generalized attenuation of these proinflammatory mediators. Hence, reduced production of proinflammatory mediators such as TNF- α , IL-1 β , and MCP-1 levels is likely as a separate effect of crambene but not directly involved in its phagocytosis-mediated protection against AP.

Caerulein-induced AP in mice results in low levels of apoptosis and high levels of necrosis, whereas caerulein-induced AP in rat results in high levels of apoptosis and low levels of necrosis.⁴² Crambene dose-dependently increases levels of apoptosis above that seen for AP in mice (Figure 4). Further exploration of the role of apoptotic cell clearance in AP in rat, which has a high level of apoptosis to begin with, is clearly required.

Our previous work has demonstrated that crambene does not alter the interaction of caerulein to its receptor on pancreatic acinar cells.⁸ Therefore, it is unlikely that the apoptotic response and the prophylactic effect of crambene are specific to the caerulein model. However, there is potential for further studies in other experimental models of AP, such as L-arginine- or choline-deficient, ethionine-supplemented diet-fed animals, and it would be interesting to see whether the apoptotic response and the prophylactic effect of crambene in those models are similar to those in the caerulein model.

Although clearance of apoptotic cells regulating immune responses have been shown in many experimental systems, to the best of our knowledge, this is the first study to report the anti-inflammatory properties of the clearance of apoptotic cells regulating immune responses in AP. However, the exact mechanisms by which crambene suppresses the production of proinflammatory mediators remain to be understood. Moreover, the signaling pathways in regulating immune responses by the clearance of apoptotic cells merit further study.

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Figure 13. Immunohistochemical examination of CD36 expression in mouse pancreas of AP with/without prophylactic treatment with anti-IL-10 antibody/crambene. Mice ($n = 5$ in each group) were given 10 hourly injections of Cae (50 $\mu\text{g/kg}$ i.p.). Anti-IL-10 Ab (50 μg per mouse, i.p.) was administered to mice either with or without crambene 12 hours before the first caerulein injection. One hour after the last caerulein injection, mice were sacrificed by an intraperitoneal injection of a lethal dose of pentobarbitone, and pancreatic cryosections were prepared as described in Materials and Methods. **A:** Control; no pancreatitis. **B:** Caerulein-induced AP with pretreatment of anti-IL-10 and crambene. **C:** Caerulein-induced AP with pretreatment of crambene. **D:** Caerulein-induced AP. **E:** Caerulein-induced AP with pretreatment of anti-IL-10. Original magnifications, $\times 400$. **F–J** are higher magnifications ($\times 1000$) of **A–E**, respectively. Cells positive for CD36 were full of ingested brown pigment in the cytoplasm as indicated by an **arrow**.

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