Inhibition of acinar apoptosis occurs during acute pancreatitis in the human homologue ΔF508 cystic fibrosis mouse

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Submitted 16 February 2010; accepted in final form 28 May 2010

DiMagno MJ, Lee SH, Owyang C, Zhou SY. Inhibition of acinar apoptosis occurs during acute pancreatitis in the human homologue ΔF508 cystic fibrosis mouse. Am J Physiol Gastrointest Liver Physiol 299: G400–G412, 2010. First published June 3, 2010; doi:10.1152/ajpgi.00061.2010.—Previously, we found that the University of North Carolina cystic fibrosis (UNC-CF) mouse had more severe experimental acute pancreatitis (AP) than wild-type (WT) mice characterized by exuberant pancreatic inflammation and impaired acinar apoptosis. Because exon 10 CFTR gene mutations exhibit different phenotypes in tissues such as the mouse lung, we tested the hypothesis that ΔF508-CF mice also develop severe AP associated with an antiapoptotic acinar phenotype, which requires indirect effects of the extracellular milieu. We used cerulein hyperstimulation models of AP. More severe pancreatitis occurred in cerulein-injected ΔF508-CF vs. WT mice based on histological severity (P < 0.01) and greater neutrophil sequestration [P < 0.001; confirmed by myeloperoxidase activity (P < 0.005)]. In dispersed acini cerulein-evoked necrosis was greater in ΔF508-CF acini compared with WT (P < 0.05) and in WT acini pretreated with CFTIRh-172 compared with vehicle (P < 0.05). Cerulein-injected ΔF508-CF vs. WT mice had less apoptosis based on poly(ADP-ribose) polymerase (PARP) cleavage (P < 0.005), absent DNA laddering, and reduced terminal deoxynucleotidyltransferase biotin-dUTP nick end labeling (TUNEL) staining (P < 0.005). Unexpectedly, caspase-3 activation was greater in ΔF508-CF vs. WT acini at baseline (P < 0.05) and during AP (P < 0.0001). Downstream, ΔF508-CF pancreas overexpressed the X-linked inhibitor of apoptosis compared with WT (P < 0.005). In summary, the ΔF508-CF mutation, similar to the UNC-CF “null” mutation, causes severe AP characterized by an exuberant inflammatory response and impaired acinar apoptosis. Enhanced acinar necrosis in ΔF508-CF occurs independently of extracellular milieu and correlates with loss of CFTR-CI conductance. Although both exon 10 models of CF inhibit acinar apoptosis execution, the ΔF508-CF mouse differs by increasing apoptosis signaling. Impaired transduction of increased apoptosis signaling in ΔF508-CF acini may be biologically relevant to the pathogenesis of AP associated with CFTR mutations.

Experimental pancreatitis; inflammation

IN HUMANS CFTR gene mutations associate with recurrent acute pancreatitis (RAP), primarily in patients with exocrine pancreatic sufficiency. Previously, to investigate this association, we found that the University of North Carolina cystic fibrosis (UNC-CF) mouse developed more severe experimental acute pancreatitis (AP) with exuberant pancreatic inflammation and impaired pancreatic acinar apoptosis (15). Phenotypically, the UNC-CF pancreas also has mild age-dependent morphological changes (20), reduced in vivo function in response to secretagogues (15), and constitutive activation of inflammatory mediators (15, 34).

These findings and others (25, 28, 32) indicate that pancreatic acinar cell injury during AP is generally a mixture of apoptosis and necrosis (25, 28, 32) and a shift from acinar apoptotic to necrotic death during AP associates with more severe inflammation and cell injury (15, 25, 28, 32, 57, 67). The balance between apoptotic and necrotic cell death is significant because pancreatic necrosis correlates with organ failure and severe human AP (13). Furthermore, according to the sentinel AP event (SAPE) hypothesis (68), an initial (sentinel) attack of AP coupled with an exaggerated early proinflammatory response may predispose to RAP and chronic pancreatitis (CP) (16), particularly with continuous exposure to risk factors (e.g., alcohol, genetic, oxidative stress). Exposure to ethanol exemplifies these relationships. Ethanol upregulates proinflammatory molecules in rats (56), predisposing to enhanced pancreatic acinar necrosis in experimental (23, 78) and clinical (58) studies, and serves as a major risk factor for AP and CP. Although human pancreatitis associated with CFTR gene mutations typically is not severe (7, 19, 36), the SAPE hypothesis is supported by the RAP pattern of pancreatitis (9, 26) rather than single acute attacks of AP (62) and the similarity between human pancreatitis associated with CFTR gene mutations and the RAP of early-onset idiopathic CP (10, 12, 70).

It remains controversial whether CFTR mutations impair (2, 6, 15, 27, 31, 37, 38, 50, 83) or increase (33, 35, 65, 75) cellular apoptosis in response to cell injury. That apoptosis execution machinery is impaired in models of CF is supported by in vivo studies (6, 15), including ours in UNC-CF mice (15) and those performed by Cannon et al. (6) in respiratory epithelial cells from ΔF508-CF mice.

The “null” mutant UNC-CF and the human homologue ΔF508-CF mouse models have unrelated mutations of exon 10 of the CFTR gene, which may cause different cellular responses during pancreatitis. The “null” mutant UNC-CF mouse model has complete replacement of exon 10 (by a double neomycin cassette), which results in a chain-termination stop codon at amino acid residue 489. In contrast, the human homologue ΔF508-CF mouse model has a three-base pair deletion (CTT) in exon 10, which results in the loss of a phenylalanine residue at a position corresponding to human position 508 and the disruption of normal cellular trafficking of the mutant protein (85). While both the UNC-CF and ΔF508-CF mouse models impart useful biological information, we reason that the ΔF508-CF model has potentially more relevance to human disease because it has the same genetic...
defect found in the majority of patients with CF (1) and the specific ΔF508-CF defect permits treatment aimed at restoring some CFTR-CI conductance by increasing cellular trafficking of the mutant protein and increasing apical function.

Separate mutations in exon 10 may impart different phenotypes. For example, studies of genomic responses indicate that mouse respiratory epithelial cells from both CF models had similar constitutive overexpression of genes influencing several regulatory processes (81, 82), but ΔF508-CF mice had “a primary disturbance in expression of genes regulating redox and antioxidant systems” (82). Hence, comparisons between ΔF508-CF and UNC-CF mouse pancreas phenotypes may provide insight into CFTR genotype-phenotype correlations and illustrate how seemingly minor differences in exon 10 mutations of the CFTR gene may exert individual effects on cellular processes.

We tested the hypothesis that the undercharacterized but potentially more clinically relevant human homologue ΔF508-CF mouse develops more severe AP in response to in vivo cerulein hyperstimulation (15) and has an antiapoptotic acinar phenotype. We also performed in vitro studies with dispersed pancreatic acini because CFTR expression localizes primarily to duct cells in the pancreas (43, 86), although expression in acini varies by species (55). Specifically, we investigated in dispersed acini whether the ΔF508-CF acinar phenotype observed during in vivo AP manifests as enhanced or reduced acinar necrosis, requires the influence of the extracellular milieu, and/or correlates directly with loss of CFTR-CI conductance (40, 60).

Finally, we also aimed to clarify controversy about whether CFTR has pro- or antiapoptotic effects.

MATERIALS AND METHODS

Materials. Sulfated ceruline was from Bachem (Torrence, CA). SYBR Green I dye, type I bovine pancreatic trypsin, and dimethyl sulfoxide (DMSO) were from Sigma Chemical (St. Louis, MO). Crescent crude collagenase (lot 107/02) from Crescent Chemical (Islandia, NY), Eagle’s minimal essential amino acids from Gibco (Grand Island, NY), aprotinin, leupeptin, and Complete Mini protease inhibitor cocktail tablets from Boehringer Mannheim (Mannheim, Germany), myeloperoxidase (MPO) from Calbiochem Novabiochem (La Jolla, CA), TaqMan reverse transcription reagents from Applied Biosystems (Foster City, CA), and nitrocellulose membranes from Schleicher & Schuell (Keene, NH). The enhanced chemiluminescence detection system, horseradish peroxidase (HRP)-conjugated secondary antibodies, and X-ray film were from Amersham Pharmacia Biotech (Piscataway, NJ). Tris-HCl precast gels, broad-range prestained SDS-PAGE molecular mass standards, and Bio-Rad Protein Assay were from Bio-Rad Laboratories (Hercules, CA). Rabbit polyclonal antibodies included anti-ERK1/2 (44/42) MAP kinase and anti-cleaved poly(ADP-ribose) polymerase (PARP) from Cell Signaling Technology (Beverly, MA) and anti-caspase-3 antibody from R&D Systems (Minneapolis, MN). Mouse monoclonal anti-X-linked inhibitor of apoptosis (XIAP) was from BD Biosciences Pharmingen (San Diego, CA). Propidium iodide was from Invitrogen (Carlsbad, CA). All other reagents were from Sigma.

Animal care and selection. All experiments were approved by the University of Michigan Committee on Use and Care of Animals. A mouse colony was maintained for the human homologue ΔF508-CF model (B6.129S6-CFTR<sup>tm1Kth</sup>, Jackson Laboratory, Bar Harbor, ME) (85), congenic on a C57BL/6 background. Breeders were fed standard laboratory chow and given water ad libitum, and offspring were fed a liquid elemental diet beginning at 10 days, which has been shown to prevent intestinal obstruction and allow relatively normal growth in CF mutant mice (15, 21). Genotyping was performed as described previously (15, 85). Experiments were performed on homozygous ΔF508-CF and wild-type (WT) mice that were age- and sex-matched offspring 6–12 wk old, weighing 13–30 g.

Induction of acute pancreatitis. The AP experiments focused on comparisons between homozygous ΔF508-CF and WT mice because preliminary data failed to show consistent differences between heterozygous UNC-CF and WT mice (15) and the risk of pancreatitis in humans with atypical CF applies most convincingly to compound heterozygotes rather than to simple heterozygotes (10, 11, 70). In vivo AP was studied by euthanizing mice 1 h after the last of 12-hourly intraperitoneal injections of the secretagogue cerulein (50 μg/kg body wt) in 0.9% NaCl (pH 7.4), a time point that correlates with the full-blown inflammatory phase of AP (15). Control groups received an equal volume of 0.9% NaCl.

Serum and tissue preparation and morphology. Mice were euthanized by CO<sub>2</sub> asphyxiation, mixed arteriovenous blood was collected from the decapitated body and centrifuged at 4°C, and serum was stored at −70°C. The pancreas was removed and separated into parts. One part was used to quantitate tissue water content, determined by the difference in tissue weight before and after desiccation (95°C, 48 h) and expressed as relative water content in percentage (15). Pancreas samples were also prepared for Western blotting, real-time PCR, and DNA studies (described below). For morphological studies pancreas was rapidly removed, fixed overnight in 4% neutral phosphate-buffered paraformaldehyde (pH 7.4), embedded in paraffin, sectioned in 3-μm slices, stained with hematoxylin and eosin (H & E), and examined by light microscopy. Tissue sections were coded to mask the experimental group, and multiple, randomly chosen microscopic fields were graded with a 0 (absent) to 4 (severe) scale modified for edema, inflammation, and acinar cell injury/death, as described by Rongione et al. (15, 64).

Tissue myeloperoxidase assay. Pancreatic tissue homogenates were assayed for MPO activity as described previously and calculated as milliunits per milligram of tissue weight (15).

In vitro model of cerulein-induced acinar cell injury. Pancreatic acini were dispersed by collagenase digestion of pancreases (14, 17), suspended in 3-mM HEPES buffer (pH 7.48) with Eagle’s minimal essential amino acids and 1 mg/ml BSA, and equilibrated with 100% O<sub>2</sub>. Viability of acini was >95% based on Trypan blue exclusion. Cerulein-evoked acinar cell injury (17, 66) was performed by preincubating dispersed pancreatic acini 30 min in HEPES-Ringer buffer at 37°C and then incubating acini with 100 nM ceruline for 1, 2, or 3 h.

Cellular membrane injury was determined by measuring propidium iodide (PI) intercalation into DNA, a marker of necrosis (66). Dispersed acini were incubated with PI (50 μg/ml) 15 min before the end of the incubation time point and then washed immediately three times with HEPES-Ringer, sonicated on ice (three 5-s intervals), and assayed fluorometrically with excitation and emission wavelengths of 536 nm and 617 nm, respectively. Total potential PI intercalation was quantitated from sonicated samples that were exposed for 15 min to PI.

CFTR inhibition. The potent, specific thiazolidinedione CFTR inhibitor CFTRinh-172 (40, 60) was kindly provided as a gift from Dr. Alan S. Verkman (University of California, San Francisco, CA). Dispersed pancreatic acini were preincubated 30 min with 5 μM CFTRinh-172, a dose that provides potent and specific inhibition of CFTR in vitro (40, 60). DMSO, the vehicle for CFTRinh-172, was used as a control (0.01% in HEPES buffer).

Caspase-3 activation. We immunohistochemically labeled cleaved caspase-3, an indicator of caspase-3 activation, in deparaffinized and rehydrated tissue sections (5 μm), as previously described (15). Preparations were viewed by fluorescence microscopy with an LSM 510 Laser Scanning Confocal Microscope mounted on a Zeiss Axiovert 100M inverted microscope. For each sample, 5 images of 1,012 × 1,012 pixels were taken by a digital camera and processed with image analysis software. Quantitation of cleaved caspase-3

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staining was graded from 0 to 4 per field (×63 magnification): 0 (no cells), 1 (1–2 cells), 2 (3–5 cells), 3 (>10%), 4 (>25%).

Terminal apoptosis markers cleaved-PARP and DNA fragmentation. We performed Western blotting as described previously to measure whole pancreas lysate expression of the 89-kDa cleaved catalytic domain of endogenous PARP (15). Second, we isolated whole pancreas DNA, separated DNA fragments electrophoretically, and identified DNA fragmentation based on the appearance of a ladder pattern, as previously described (15).

TUNEL staining to confirm DNA fragmentation. Paraffin embedded pancreas tissue sections (5 μm) were warmed 30 min (64°C), deparaffinized, and rehydrated. Terminal deoxynucleotidyltransferase biotin-dUTP nick end labeling (TUNEL) labeling of nuclei was performed by using the APO-BrdU TUNEL Assay Kit (A-23210; Molecular Probes, Eugene, OR) according to the manufacturer’s protocol with a 1:20 dilution of the bromodeoxyuridine (BrdU) antibody. Tissue sections were sealed with aqueous mounting medium with antiﬁnding agents (Gel/Mount, Biomedica, Foster City, CA). As a negative control, no TUNEL staining occurred when the BrdU antibody was omitted. Preparations were viewed by fluorescence microscopy with an LSM 510 Laser Scanning Confocal Microscope mounted on a Zeiss Axiovert 100M inverted microscope. For each sample, 5 images of 1.012 × 1.012 pixels were taken by a digital camera and processed with image analysis software. Data are expressed as TUNEL-positive nuclei per field (×63 magnification).

SDS-PAGE and Western blotting. As described previously (15), protein lysates were prepared, mixed with SDS stop solution (final concentrations 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.05% bromphenol blue, and 5% 2-mercaptoethanol), and boiled for 5 min. Thirty micrograms of protein was electrophoresed by SDS-PAGE, transferred to nitrocellulose membranes, incubated with the respective primary antibody and thereafter the corresponding secondary antibody conjugated to HRP, and immunodetected with the ECL reagent. Quantitation of Western blots was performed with Multi-Analyst software (Bio-Rad Laboratories).

Total RNA isolation, reverse transcription, and quantitative real-time PCR. As described in detail elsewhere (15), total RNA was isolated and purified from whole pancreas tissue and reverse transcribed to cDNA, specific gene products were amplified by quantitative real-time PCR, and data were analyzed with an I-Cycler IQ Detection system (Bio-Rad Laboratories). The speciﬁcity of the products (in distinction from primer-dimers) was determined by monitoring the melt curve, obtained in increments of 0.5°C every 10-s interval from 55°C to 96°C. As a negative control, real-time PCR runs included the primers alone (and PCR cocktail) without cDNA. Finally, primer pairs were also validated with a real-time PCR run, and expressed as fold change compared with WT, which was assigned the value 1.

Table 1. Primers used for quantitative real-time PCR of mouse pancreas RNA

<table>
<thead>
<tr>
<th>Name</th>
<th>Accession No.</th>
<th>Orientation</th>
<th>Primer Sequence (5’ to 3’)</th>
</tr>
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<tbody>
<tr>
<td>RB 23 kDa</td>
<td>NM 009438</td>
<td>Sense</td>
<td>GGG CCA TGG TGG CCA A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Antisense</td>
<td>TGG TGA TGC CTT CAG AGC GTA C</td>
</tr>
<tr>
<td>XIAP</td>
<td>NM 009688</td>
<td>Sense</td>
<td>GAG GAG GGC TCA CCG ATT G</td>
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<tr>
<td></td>
<td></td>
<td>Antisense</td>
<td>CAC CCT GGG TAG CAC TTA GCA</td>
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Gene sequences were obtained from the GenBank NCBI Sequence Viewer (http://www.ncbi.nlm.nih.gov), and primers for quantitative RT-PCR (Table 1) were designed with Primer Express Primer software (Applied Biosystems) and synthesized by Invitrogen.

RESULTS

Mouse breeding and genotyping. PCR and gel analysis of mouse tail DNA identiﬁed the genotype of offspring from heterozygous CFTR breeding pairs. The body weight of the ΔF508-CF group averaged 68% (range 42–90%) of the WT group, which is similar to body weights reported for the UNC-CF model versus WT groups (15).

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ΔF508-CF mice exhibit greater pancreatic inflammation during cerulein-induced AP. No morphological evidence of pancreatic injury was present in saline-injected WT (Fig. 1A) and ΔF508-CF (Fig. 1B) groups. In contrast, the cerulein-injected WT (Fig. 1C) and ΔF508-CF (Fig. 1D) groups had evidence of pancreatic injury based on a 0 (absent) to 4 (severe) scale for edema, inﬂammation, and acinar cell injury/death described by Rongione et al. (15, 64). Cerulein-injected ΔF508-CF mice had greater total histological scores than WT mice (Fig. 2A; 6.38 ± 0.5 vs. 4.7 ± 0.3; P < 0.01) due to greater neutrophil inﬁltration (Fig. 2B; P < 0.05) rather than tissue edema (Fig. 2C) and acinar cell injury/death (Fig. 2D). The degree of acinar cell injury/death appeared similar in the two groups (Fig. 2D), similar to our previous observations in UNC-CF versus WT mice (15), but the major type of cell injury/death (e.g., necrosis vs. apoptosis) was qualitatively different and was discerned by quantitating markers of necrosis (demonstrated by in vitro studies summarized below, Fig. 3) and apoptosis (demonstrated in Figs. 4–6).

ΔF508-CF mice have greater pancreatic MPO activity after induction of AP. Nonhistological markers of tissue neutrophil sequestration and edema corroborated the histological findings. Pancreatic MPO activity (Fig. 2E) was signiﬁcantly greater in the ΔF508-CF group compared with the WT group after induction of AP (P < 0.005). The relative pancreatic water content (in %), a marker of edema, was signiﬁcantly greater (P < 0.0005) in the saline-injected ΔF508-CF compared with WT groups (Fig. 2F) but was similar in both cerulein-injected groups.
Ceruline evokes greater necrosis in dispersed ΔF508-CF acini and WT acini pretreated with a specific CFTR inhibitor. Ceruline hyperstimulation (100 nM) evoked a time-dependent increase in percent PI intercalation into pancreatic acinar DNA (Fig. 3), a marker of necrosis (66). Percent PI intercalation was significantly greater in the ΔF508-CF group compared with the WT group after ceruline hyperstimulation for 120 min (Fig. 3A; \( P < 0.0005 \)) and 180 min (\( P < 0.0005 \)). Similarly, PI intercalation was significantly greater in WT acini pretreated with 5 μM CFTRinh-172 vs. vehicle after ceruline hyperstimulation for 180 min (Fig. 3B; \( P < 0.05 \)). From these studies we conclude that ΔF508-CF acini develop greater cerulin-evoked necrosis, this phenotype does not require the influence of the extracellular milieu, and specific inhibition of CFTR-CI secretion recapitulates this phenotype.

ΔF508-CF mice exhibit reduced pancreatic apoptosis during ceruline-induced AP. We examined three sequential apoptosis execution steps in pancreatic acini from ΔF508-CF mice during AP: caspase-3 activation (by immunohistochemical labeling of cleaved caspase-3), cleavage of the caspase-3 substrate PARP into the 89-kDa domain (by Western blotting), and DNA fragmentation (by DNA laddering and TUNEL staining), a hallmark of apoptosis (54). Cleaved caspase-3 staining, appearing as a red granular pattern in the cytoplasm of acinar cells, was absent in the saline-injected WT group (Fig. 4A) but was increased in the saline-injected ΔF508-CF group (Fig. 4B; \( P < 0.05 \)), similar to expression in the ceruline-injected WT group (Fig. 4C; \( P < 0.05 \)). The ceruline-injected ΔF508-CF group (Fig. 4D) had greater caspase-3 activation compared with the ceruline-injected WT group (Fig. 4C; \( P < 0.0001 \)), which is displayed graphically in Fig. 4E. These findings raised the unexpected possibility that ΔF508-CF acini may have increased apoptosis signaling and contrast with the previously reported absent or minimal expression in the UNC-CF mouse acini (15).

Detailed examination of the terminal apoptosis execution steps downstream of caspase-3 indicates that ΔF508-CF acini have impaired apoptosis execution. First, expression of cleaved PARP (Fig. 5, A and B), which ensures that apoptosis is completed and irreversible (54), was significantly lower in ceruline-injected ΔF508-CF group pancreas compared with the WT group (\( P < 0.005 \)). Similarly, oligonucleosomal DNA fragmentation (in contrast to large-scale DNA fragmentation) occurs at short, defined intervals (180–200 bp) and was detected by DNA laddering, a hallmark of apoptosis (54); DNA fragmentation was not detectable in whole pancreases from the ceruline-injected ΔF508-CF group (Fig. 5C, 1st lane) but was detectable in the ceruline-injected WT group (Fig. 5C, 2nd lane). We further localized and quantitated DNA fragmentation by TUNEL labeling of acinar nuclei. Pancreatic acinar TUNEL staining was minimally present in acini from the saline-injected WT and ΔF508-CF groups (Fig. 6, A and B). In response to ceruline injections, acinar TUNEL staining increased significantly in the WT group (Fig. 6C; \( P < 0.0005 \)), but not in the ΔF508-CF group (Fig. 6D), which is displayed graphically in Fig. 6E. Together, these data provide evidence that ΔF508-CF acini have an antia apoptotic phenotype similar to previously described UNC-CF acini (15), with the exception that caspase-3 activation is enhanced in ΔF508-CF acini.

ΔF508-CF mice exhibit greater pancreatic expression of antia apoptotic XIAP protein but not mRNA during AP. XIAP is a potent member of a family of inhibitors of apoptosis (IAPs) that binds directly to and inhibits initiator and effector caspases, including caspase-3 (Fig. 7, A and B), and thereby

\[ \text{Fig. 1. Effect of ΔF508-CF genotype on pancreatic histology at 12 h during acute pancreatitis (AP) (described in MATERIALS AND METHODS). Hematoxylin and eosin (H & E)-stained sections were examined by light microscopy (×200 magnification). Images are representative of saline-injected wild-type (WT) (A) and ΔF508-CF (B) groups (\( n = 3 \)) and ceruline-injected WT (C) and ΔF508-CF (D) groups (\( n = 7 \)).}

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may function as a gatekeeper to execution of apoptosis by downstream caspases (30, 48, 73). Mouse pancreas overexpressed XIAP protein in the saline-injected (n = 3) and cerulein-injected (n = 7) groups. A: the composite severity score was based on morphological changes graded 0–4 for inflammation (B), edema (C), and acinar cell injury or death (D), as previously described (15). Cerulein-injected ΔF508-CF mice had greater total histological scores than WT mice (A: 6.38 ± 0.5 vs. 4.7 ± 0.3; P < 0.01) because of greater neutrophil infiltration (B; P < 0.05) rather than tissue edema (C) and acinar cell injury/death (D). Quantitation of pancreatic tissue neutrophil infiltration was measured by myeloperoxidase (MPO) activity (E; mU/mg tissue wt; n = 3/group) and edema by relative pancreatic water content in % (F; n = 6/group). Columns represent means and SE. *P < 0.05, **P < 0.01, ***P < 0.005 vs. saline-injected WT; +P < 0.01, ++P < 0.005, +++P < 0.001 vs. cerulein-injected WT.

**Histologic Scoring of Acute Pancreatitis at 12 Hours**

A: Total Score  
B: Inflammation  
C: Edema  
D: Cell Injury  
E: MPO Level  
F: Water Content

Fig. 2. Greater histological and nonhistological parameters of severity in ΔF508-CF vs. WT at 12 h during AP (described in MATERIALS AND METHODS). H & E-stained sections (see Fig. 1) were examined by light microscopy (×200 magnification) for saline-injected (n = 3) and cerulein-injected (n = 7) groups. A: the composite severity score was based on morphological changes graded 0–4 for inflammation (B), edema (C), and acinar cell injury or death (D), as previously described (15). Cerulein-injected ΔF508-CF mice had greater total histological scores than WT mice (A: 6.38 ± 0.5 vs. 4.7 ± 0.3; P < 0.01) because of greater neutrophil infiltration (B; P < 0.05) rather than tissue edema (C) and acinar cell injury/death (D). Quantitation of pancreatic tissue neutrophil infiltration was measured by myeloperoxidase (MPO) activity (E; mU/mg tissue wt; n = 3/group) and edema by relative pancreatic water content in % (F; n = 6/group). Columns represent means and SE. *P < 0.05, **P < 0.01, ***P < 0.005 vs. saline-injected WT; +P < 0.01, ++P < 0.005, +++P < 0.001 vs. cerulein-injected WT.
humans with CF (46, 63, 72, 84) have constitutive upregulation of inflammatory cells in the pancreas (20) and lungs, and overexpression of genes influencing inflammation (81, 82).

Norkina et al. (51) examined a mild UNC-CF mouse AP model and reported that pancreatic neutrophil sequestration was increased at 72 h. In nonpancreatitis studies, lungs from UNC-CF mice both displayed constitutive neutrophil infiltration that correlates with less acinar apoptosis (79). Hence, strategies to reduce the severity of AP by inhibiting neutrophil sequestration, however, may serve as a common pathway to reduce cell (apoptotic and necrotic) death in heterogeneous tissues. Neutrophils are particularly important because they have delayed apoptosis and perpetuate inflammation of multiple inflammatory mediators. Perez et al. (60) provided evidence that CFTR directly influences the inflammatory phenotype by reporting that short-term (5 day) inhibition of CFTR in normally functioning primary airway cells promotes increased IL-8 secretion without causing cell injury. An example of a proinflammatory phenotype predisposing (or sensitizing) to cell injury is the upregulation of pancreatic proinflammatory molecules by ethanol (56), predisposing to enhanced pancreatic acinar necrosis in experimental (23, 78) and clinical (58) studies. In the CF mouse pancreas, it is possible that one or more factors may sensitize to an exuberant inflammatory response (15), including sequelae from the antipapoptotic phenotype (discussed below), cAMP-induced upregulation of genes affecting inflammation and cell stress (34), or a cell stress response involving NF-kB activation (79).

In vitro studies indicate that necrosis is the predominant nonapoptotic form of acinar cell death/injury in ΔF508-CF mice. Dispersed pancreatic acini from ΔF508-CF mice and WT acini pretreated with the selective CFTR inhibitor CFTRinh-172 developed greater cerulein-evoked necrosis (% PI intercalation) compared with WT and vehicle, respectively. These findings indicate that the antipapoptotic, pronecrotic phenotype of ΔF508-CF acini occurs independently of the extracellular milieu and correlates directly with the loss of CFTR-CI conductance, which resembles the antipapoptotic phenotype of CFTRinh-172-treated renal tubules (37, 38). The antiapoptotic and proinflammatory phenotypes may be primary or secondary phenomena but are likely interrelated. Conceptually, apoptotic cells may not trigger an inflammatory response because they safely regulate and package intracellular contents into membrane-bound bodies that are phagocytosed and eliminated, whereas necrotic cells suffer from unregulated rupture of the cell membrane and free release of intracellular contents that evoke an inflammatory response.

The inverse association between inflammation and apoptosis is tissue- and perhaps disease dependent. For example, nonpancreatic organ injury/dysfunction in AP is primarily due to apoptotic rather than necrotic epithelial cell death (74) and may be accentuated by administration of an apoptosis inducer. Hence, strategies to reduce the severity of AP by inhibiting necrosis and enhancing apoptosis may require selective targeting of pancreatic rather than nonpancreatic tissues. Treatments targeting inflammatory cells, however, may serve as a common pathway to reduce cell (apoptotic and necrotic) death in heterogeneous tissues. Neutrophils are particularly important because they have delayed apoptosis and perpetuate inflammation in multiple inflammatory conditions (22, 61, 69), including AP (53) and possibly CF (45). Consistent with these observations, preventing pulmonary leukocyte sequestration (with a vascular adhesion molecule-1 inhibitor) reduced apoptosis of pulmonary epithelial and endothelial cells and ameliorated pulmonary inflammation and injury in a murine model of severe AP (5). Similarly, neutrophil depletion reduced the severity of pancreatic and pulmonary injury during a different model of AP (3, 67), but in contrast to the lung, neutrophil depletion reduced acinar necrosis and promoted apoptosis (67). Hence, reducing sequestration of neutrophils (and possibly other leukocytes) potentially could reduce pancreatic and nonpancreatic injury during AP.

Our data in ΔF508-CF mice and our observations in UNC-CF mice (15) support the concept that apoptosis execu-

**DISCUSSION**

The ΔF508-CF model compared with WT developed more severe cerulein-induced in vivo AP characterized by greater neutrophil infiltration that correlates with less acinar apoptosis based on less PARP cleavage and DNA fragmentation. These data are similar to those in UNC-CF mice (15) and other data. Norkina et al. (51) examined a mild UNC-CF mouse AP model by administering only 7- rather than 12-hourly cerulein injections, and reported that pancreatic neutrophil sequestration was elevated at 72 h. In nonpancreatitis studies, lungs from UNC-CF and ΔF508-CF mice both displayed constitutive overexpression of genes influencing inflammation (81, 82). Furthermore, long-lived (9–24 mo old) UNC-CF mice have foci of inflammatory cells in the pancreas (20) and lungs, and other organs and cells from UNC-CF mice (15, 52, 81) and humans with CF (46, 63, 72, 84) have constitutive upregulation of multiple inflammatory mediators. Perez et al. (60) provided evidence that CFTR directly influences the inflammatory phenotype by reporting that short-term (5 day) inhibition of CFTR in normally functioning primary airway cells promotes increased IL-8 secretion without causing cell injury. An example of a proinflammatory phenotype predisposing (or sensitizing) to cell injury is the upregulation of pancreatic proinflammatory molecules by ethanol (56), predisposing to enhanced pancreatic acinar necrosis in experimental (23, 78) and clinical (58) studies. In the CF mouse pancreas, it is possible that one or more factors may sensitize to an exuberant inflammatory response (15), including sequelae from the antipapoptotic phenotype (discussed below), cAMP-induced upregulation of genes affecting inflammation and cell stress (34), or a cell stress response involving NF-kB activation (79).

In vitro studies indicate that necrosis is the predominant nonapoptotic form of acinar cell death/injury in ΔF508-CF mice. Dispersed pancreatic acini from ΔF508-CF mice and WT acini pretreated with the selective CFTR inhibitor CFTRinh-172 developed greater cerulein-evoked necrosis (% PI intercalation) compared with WT and vehicle, respectively. These findings indicate that the antipapoptotic, pronecrotic phenotype of ΔF508-CF acini occurs independently of the extracellular milieu and correlates directly with the loss of CFTR-CI conductance, which resembles the antipapoptotic phenotype of CFTRinh-172-treated renal tubules (37, 38). The antiapoptotic and proinflammatory phenotypes may be primary or secondary phenomena but are likely interrelated. Conceptually, apoptotic cells may not trigger an inflammatory response because they safely regulate and package intracellular contents into membrane-bound bodies that are phagocytosed and eliminated, whereas necrotic cells suffer from unregulated rupture of the cell membrane and free release of intracellular contents that evoke an inflammatory response.

The inverse association between inflammation and apoptosis is tissue- and perhaps disease dependent. For example, nonpancreatic organ injury/dysfunction in AP is primarily due to apoptotic rather than necrotic epithelial cell death (74) and may be accentuated by administration of an apoptosis inducer. Hence, strategies to reduce the severity of AP by inhibiting necrosis and enhancing apoptosis may require selective targeting of pancreatic rather than nonpancreatic tissues. Treatments targeting inflammatory cells, however, may serve as a common pathway to reduce cell (apoptotic and necrotic) death in heterogeneous tissues. Neutrophils are particularly important because they have delayed apoptosis and perpetuate inflammation in multiple inflammatory conditions (22, 61, 69), including AP (53) and possibly CF (45). Consistent with these observations, preventing pulmonary leukocyte sequestration (with a vascular adhesion molecule-1 inhibitor) reduced apoptosis of pulmonary epithelial and endothelial cells and ameliorated pulmonary inflammation and injury in a murine model of severe AP (5). Similarly, neutrophil depletion reduced the severity of pancreatic and pulmonary injury during a different model of AP (3, 67), but in contrast to the lung, neutrophil depletion reduced acinar necrosis and promoted apoptosis (67). Hence, reducing sequestration of neutrophils (and possibly other leukocytes) potentially could reduce pancreatic and nonpancreatic injury during AP.

Our data in ΔF508-CF mice and our observations in UNC-CF mice (15) support the concept that apoptosis execu-
tion machinery is dysregulated in models of CF-related pancreatic disease. Furthermore, observations from multiple non-pancreatic studies indicate that resistance to apoptosis occurs in CF (2, 6, 27, 31, 37, 38, 50, 83). This concept remains controversial (33, 35, 65, 75), possibly because the predominant mode of cell death is tissue dependent (74) and because the CFTR-dependent effect on cell death may differ based on cell type, injury model, trigger, and the end points measured. For example, Rottner et al. (65) observed that actinomycin D increased apoptosis in a mutant CFTR pancreatic ductal cell line and also in WT and mutant ductal cell lines pretreated with the CFTR inhibitor CFTRinh-172. The effects of actinomycin D, however, are not likely generalizable; a variety of apoptosis-inducing drugs (aphidicolin, paclitaxel, doxorubicin, puromycin, staurosporin) do not unmask a pro- or antiapoptotic phenotype in ΔF508-CF respiratory epithelial cells (6). Of note, Pseudomonas aeruginosa exposure, frequent in CF pulmonary disease and therefore a more clinically relevant trigger, unmask an antiapoptotic phenotype in respiratory epithelial cells both in vitro and in vivo (6).

Fig. 4. Greater activation of acinar caspase-3 in ΔF508-CF vs. WT whole pancreas tissue sections at 12 h during AP (described in MATERIALS AND METHODS). Staining for cleaved caspase-3, an indicator of caspase-3 activation, was detected by immunofluorescence microscopy (×63 magnification) and appeared as a red granular pattern within the cytoplasm of acinar cells. No staining was detected in negative controls (not shown), in which the primary caspase-3 antibody was omitted. Saline-injected groups had acinar staining of cleaved caspase-3 that was absent in the WT group (A) and modest in the ΔF508-CF group (B; arrow), the latter of which was similar to staining in the cerulein-injected WT group (C; arrow). D: diffuse staining of cleaved caspase-3 was present in the cerulein-injected ΔF508-CF acini. E: quantitation of cleaved caspase-3 staining was graded 0–4 based on % positive (fluorescent) cells per field (see MATERIALS AND METHODS). Columns represent the means and SE of n = 5–8 per group. *P < 0.05, **P < 0.0001 vs. saline-injected WT; +P < 0.0001 vs. cerulein-injected WT.
Important to recognize is that completion of programmed cell death might fail even though apoptosis signaling is increased (2, 41). Furthermore, impaired phagocytosis of apoptotic cells may lead to postapoptotic cell necrosis (76). Therefore, the end points used to measure CFTR-dependent apoptosis may influence data interpretation. For example, based on increased caspase-3 activation an erroneous interpretation of our data is that the H9004F508-CF mutation enhances signaling and execution of apoptosis. Enhanced caspase-3 activation, however, does not equate to exaggerated execution of apoptosis because the transduction of increased apoptosis signaling in H9004F508-CF pancreatic acini is disrupted downstream of caspase-3, based on less PARP cleavage, absence of DNA laddering, and reduced acinar nuclear TUNEL staining. Hence, characterization of cell death in CF models requires careful study design, selection of apoptosis inducers, and measurement of multiple apoptosis markers.

Overexpression of the caspase inhibitor XIAP during AP is a potential explanation for the disruption of apoptosis signaling downstream of caspase-3 activation in H508-CF pancreatic acini. This finding is unique to the H508-CF model because the UNC-CF pancreas has no significant difference in XIAP protein expression compared with WT at baseline or after induction of pancreatitis (DiMagno and colleagues, unpublished results). Also, in Western blotting studies XIAP protein was overexpressed in baseline lung lysates from H9004F508-CF compared with WT lung (1.6-fold increase, *P* < 0.005 vs. saline-injected WT; +*P* < 0.005 vs. cerulein-injected WT). XIAP, one of a family of IAPs, does not prevent caspase-3 activation but rather inhibits catalytic activity by competitively binding to the active site pocket (73). The similar expression of XIAP mRNA among all of our experimental groups, however, indicates that XIAP protein overexpression in H9004F508-CF pancreas is likely due to increased translation (or decreased degradation), possibly involving a ribosome entry site (39). It is not certain that the overexpression of XIAP is biologically significant, but XIAP appears to have a significant, species-dependent, anti-apoptotic effect in experimental AP (42). It is also possible that CFTR-related or -unrelated effects on XIAP or other antiapoptotic molecules might have relevance to pancreatic cancer.

![Fig. 5.](http://ajpgi.physiology.org/)
because XIAP is an important gatekeeper and mediator of acquired resistance to apoptosis in cancer (30, 48); CFTR expression is reduced in 13 of 16 pancreatic cancer cell lines compared with ductal cells and normal pancreas (71); and limited prospective data indicate that CFTR mutation carrier status is associated with a fourfold increased risk of early pancreatic cancer (44). These potential clinical implications warrant further study, including investigation of XIAP as a potential target for therapy in pancreatitis and cancer.

Dysregulation of apoptosis in ΔF508-CF and UNC-CF pancreatic acini might involve a variety of biological derangements that could shift the balance between apoptosis and necrosis, and possibly allow for initiation but failed completion of apoptosis. Although exuberant pancreatic tissue neutrophil sequestration inhibits acinar apoptosis in experimental pancreatitis, this would be expected to reduce rather than increase acinar caspase-3 activation (47). A controversial hypothesis is that defective intracellular acidification contributes to CF disease (29), possibly by deficient apoptosis (2, 50) related to impaired activation of acidic endonucleases and cleavage of DNA (59) or acid sphingomyelinase or ceramidase-dependent apoptosis (50, 83). Other possible antiapoptotic mechanisms include impaired generation of reactive oxygen species coupled to reduced CFTR-dependent glutathione transport (31, 37, 38) and extracellular bicarbonate deficits (pH 7.35–7.40) (8), which have been linked to disruption of postmitochondrial apoptotic events in non-CF studies (18).

Extracellular bicarbonate deficit and/or acidity might give rise to CFTR-dependent apoptosis in pancreatic acini (15). This hypothesis is based on indirect evidence that the extracellular milieu can affect pancreatic phenotype: 1) pancreatic phenotype is coupled tightly to bicarbonate transport (8); 2) pancreatic cancer studies provide evidence that extracellular factors influence apoptosis (77); 3) an experimental model of post-endoscopic retrograde cholangiopancreatography (ERCP) pancreatitis shows that pancreatic duct injection of a low-pH solution activates pH-sensitive ion channels and predisposes to se-

Fig. 6. Less terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) staining in ΔF508-CF vs. WT whole pancreas tissue sections at 12 h during AP (described in MATERIALS AND METHODS). TUNEL staining was detected by immunofluorescence microscopy (all images are at ×63 magnification) and appeared as diffuse, homogenous nuclear staining. No staining was detected in negative controls (not shown), in which the primary TUNEL antibody was omitted. Negligible staining was detected in the saline-injected WT (A) and ΔF508-CF (B) groups. In the cerulein-injected groups, TUNEL staining was strong in the WT group (C; arrows) but significantly less in the ΔF508 group (D; arrows). E: quantitation of TUNEL staining was expressed as positive (fluorescent) cells per field (×63 magnification). Columns represent the means and SE of n = 5 or 6 per group. *P < 0.05, +P < 0.0001 vs. cerulein-injected WT.
vere pancreatitis (49); and 4) low-pH buffer alters the phenotype of dispersed pancreatic acinar cells by inhibiting apical plasma membrane recycling, which causes a secretory blockade (24), and by sensitizing acini to secretagogue-evoked serine protease activation and cell injury (4, 80). In vitro studies we and others (37, 38) have performed, however, indicate that diminished CFTR-CI conductance alone directly impairs apoptosis independent of the extracellular milieu. Further study is required to delineate whether the extracellular environment, specifically a bicarbonate deficit and/or low pH, modulates this CFTR-dependent antiapoptotic acinar phenotype.

In conclusion, our findings support the clinical observation that CFTR gene mutations within exon 10 may predispose to pancreatitis. Furthermore, the human homologue ΔF508-CF mutation, similar to the “null” gene mutation (in the UNC-CF model), causes severe AP characterized by an exuberant inflammatory response and impaired pancreatic acinar apoptosis. Moreover, in vitro studies with dispersed pancreatic acini indicate that the antiapoptotic, pronecrotic phenotype correlates directly with the loss of CFTR-CI conductance and does not require the influence of the extracellular milieu. Although these two CF models (with different CFTR loss of function mutations within exon 10) have a similar end result, the ΔF508 mutation, which has the same genetic defect as most human CF disease (1), dramatically increases apoptosis signaling in mice, which is oppo-

Table 2. XIAP mRNA expression in mouse pancreas by quantitative real-time PCR

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Saline</th>
<th>Cerulein</th>
</tr>
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<tbody>
<tr>
<td>WT</td>
<td>1.0 ± 0.2</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>ΔF508-CF</td>
<td>1.1 ± 0.1</td>
<td>0.9 ± 0.2</td>
</tr>
</tbody>
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Pancreatic mRNA expression of XIAP in ΔF508-CF and wild-type (WT) mice at 12 h during acute pancreatitis (described in MATERIALS AND METHODS). Whole pancreas mRNA was isolated and reverse transcribed, and gene transcripts were quantitated by real-time PCR (see MATERIALS AND METHODS). Data are expressed as mean ± SE fold change (n = 3) vs. saline-injected WT (assigned the value 1). Values were normalized to the RB 23 kDa housekeeping gene.

Fig. 7. A and B: phenotypic comparison of execution apoptotic steps in 2 CF mouse models. During AP at 12 h, pancreatic acinar cells from both the University of North Carolina cystic fibrosis (UNC-CF) model (A) and the ΔF508-CF model (B) show inhibition of the downstream execution apoptosis steps but have individual differences. A: the UNC-CF model shows inhibition of apoptosis at the level of caspase-3 catalysis. In contrast, inhibition of apoptosis in the ΔF508-CF group (B) appears to occur at the level of PARP cleavage because caspase-3 activation is enhanced, possibly due to overexpression of the X-linked inhibitor of apoptosis (XIAP), which inhibits initiator and effector caspases. C and D: XIAP protein is overexpressed in pancreas from ΔF508-CF vs. WT groups at 12 h during AP (described in MATERIALS AND METHODS). C: whole pancreas lysates were subjected to Western blotting studies (described in MATERIALS AND METHODS) to detect changes in XIAP (57 kDa) relative to the loading control protein total ERK (44/42 kDa). D: relative optical densities for protein data were normalized to total ERK values and expressed as % of cerulein-injected WT. Columns represent the means and SE of n/H110054 –9 per group. *P/H110210.05, **P/H110210.005 vs. saline-injected WT; +P < 0.005 vs. cerulein-injected WT.
site to that in UNC-CF mice. That transduction of increased apoptosis signaling in ΔF508-CF acini is impaired from execution caspase-3 to downstream PARP may be biologically relevant in the pathogenesis of AP associated with CFTR mutations and serve as a novel target for potential therapies.

ACKNOWLEDGMENTS

We thank Kwang-Deok Moon for his assistance with Western blotting studies and Hwee-Young Jin for assistance with managing and genotyping the mouse colony.

GRANTS

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-073298 (to M. J. DiMagno) and P30-DK-034933, which supports the Michigan Gastrointestinal Peptide Research Center (to C. Owyang).

DISCLOSURES

M. J. DiMagno received honoraria from Lippincott Williams & Wilkins (Philadelphia, PA) for articles published in Current Opinion in Gastroenterology, honoraria from the British Medical Journal for an article published in BMJ Point of Care, and a consulting fee from MD Evidence (Atlantic City, NJ) for coauthoring a systematic review published in 2009 entitled "Systematic review pancreatic enzyme treatment for malabsorption associated with chronic pancreatitis." Dr. DiMagno also received the drug Pioglitazone from Takeda Pharmaceuticals North America (Deerfield, IL) for use in an NIH-sponsored clinical research trial (2008).

The remaining authors have no financial or other relationship(s) to disclose.

REFERENCES


