Inflammatory cells regulate p53 and caspases in acute pancreatitis

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1Veterans Affairs Greater Los Angeles Healthcare System and University of California at Los Angeles, Los Angeles, California; 2Division of Gastroenterology and Hepatology, Department of Internal Medicine, Keio University School of Medicine, Tokyo, Japan; 3Division of Gastroenterology, Department of Internal Medicine, Chung-Ang University School of Medicine, Seoul, Korea; and 4Institute of Theoretical and Experimental Biophysics, Russian Academy of Sciences, Pushchino, Moscow Region, Russia

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Nakamura Y, Do JH, Yuan J, Odinokova IV, Mareninova O, Gukovskaya AS, Pandol SJ. Inflammatory cells regulate p53 and caspases in acute pancreatitis. Am J Physiol Gastrointest Liver Physiol 298: G92–G100, 2010. First published October 22, 2009; doi:10.1152/ajpgi.00324.2009.—The inflammatory response during pancreatitis regulates necrotic and apoptotic rates of parenchymal cells. Neutrophil depletion by use of anti-polymorphonuclear serum (anti-PMN) increases apoptosis in experimental pancreatitis but the mechanism has not been determined. Our study was designed to investigate signaling mechanisms in pancreatic parenchymal cells regulating death responses with neutrophil depletion. Rats were neutrophil depleted with anti-PMN treatment. Then cerulein pancreatitis was induced, followed by measurements of apoptosis signaling pathways. There was greater activation of executioner caspases-3 in the pancreas with anti-PMN treatment compared with control. There were no differences between these groups of animals in mitochondrial cytochrome c release or in activities of initiator caspase-8 and -9. However, there was greater activation of caspase-2 with anti-PMN treatment during cerulein pancreatitis. The upstream regulation of caspases-2 includes p53, which was increased; the p53 negative regulator, Mdm2, was decreased by anti-PMN treatment during cerulein pancreatitis. In vitro experiments using isolated pancreatic acinar cells a pharmacological inhibitor of Mdm2 increased caspase-2/3 activities, and an inhibitor of p53 decreased these activities during cholecystokinin-8 treatment. Furthermore, experiments using the AR42J cell line Mdm2 small interfering RNA (siRNA) increased caspase-2/3 activities, and p53 siRNA decreased these activities during cholecystokinin-8 treatment. These results suggest that during acute pancreatitis the inflammatory response inhibits apoptosis. The mechanism of this inhibition involves caspase-2 and its upstream regulation by p53 and Mdm2. Because previous findings indicate that promotion of apoptosis decreases necrosis and severity of pancreatitis, these results suggest that strategies to inhibit Mdm2 or activate p53 will have beneficial effects for treatment of pancreatitis.

IN EXPERIMENTAL MODELS of pancreatitis, cells die through necrosis or apoptosis. Typically, necrosis is characterized by the loss of cell membrane integrity resulting in the release of cell contents and inflammation. Differently, apoptotic cells preserve the integrity of their membrane and apoptosis is associated with minimal inflammation (7, 9). There is a direct correlation between the amount of necrosis in the pancreas and the severity of the pancreatitis. Switching from necrotic to apoptotic cell death decreases the severity of experimental pancreatitis (19, 26, 39). We and others showed that the inflammatory response influences the type of cell death (8, 15, 36, 42). In particular, neutrophil depletion with anti-polymorphonuclear (PMN) serum (34) or with methotrexate (10) increased apoptosis associated with decreased necrosis and the severity of experimental pancreatitis. The mechanisms through which the inflammatory response regulates cell death in pancreatitis remain unknown.

Apoptosis is mediated by caspases, which are a family of cysteine proteases. They act as a cascade and are divided into two types: initiator caspases, e.g., caspase-2, -8, -9, and -10; and executioner caspases, e.g., caspase-3, -6, and -7 (5, 41). There are two major pathways of apoptosis. The intrinsic mitochondrial pathway (cytochrome c/caspase-9 pathway) is activated by cytochrome c released from mitochondria. Cytosolic cytochrome c forms a complex with Apaf-1 and procaspase-9 (called the apoptosome), which, in turn, activates caspase-9 (24). Caspase-9 further activates executioner caspase-3, resulting in apoptosis. Another pathway is the extrinsic pathway (caspase-8 pathway) mediated by receptor-induced activation of caspase-8 and caspase-10, which directly activate caspase-3 or act through causing mitochondrial cytochrome c release (16, 38, 40). We recently published that cytochrome c/caspase-9- and caspase-8-mediated pathways contribute to apoptosis in experimental pancreatitis (3, 13, 14).

The tumor suppressor protein p53 has a central role in coordinating cellular responses such as apoptosis, cell cycle arrest, senescence, and DNA repair (21, 22). The function and processing of p53 is tightly controlled by a specific ubiquitin ligase called Mdm2 that mediates the degradation of p53 (4, 27). Recently a novel pathway of p53-mediated apoptosis was discovered (44). It involves formation of PIDDsome, which is a complex formed between PIDD (p53-induced protein with death domain), RAIDD (RIP-associated ICH-1/CED-3 homologous protein with death domain), and procaspase-2 (30). PIDDsome formation results in caspase-2 activation (43, 45), which stimulates cytochrome c release (23) and also directly activates executioner caspases (18, 33), leading to apoptosis.

In the present study we show that a Mdm2/p53/caspase-2 pathway is activated and works together with the cytochrome c/caspase-9 pathway to enhance caspase-3 activity and apoptosis in pancreatitis. We further show that neutrophils inhibit apoptosis in pancreatitis by inhibiting the p53 and caspase-2 by upregulating Mdm2.

MATERIALS AND METHODS

Experimental pancreatitis. Cerulein pancreatitis was induced in 200-g Sprague-Dawley male rats by four hourly intraperitoneal injec-
tions of 50 μg/kg body wt cerulein (American Peptide, Sunnyvale, CA). Control animals were injected with the same volume of physiological saline. For neutrophil depletion, rats were injected with rabbit anti-PMN serum (1.5 ml/kg body wt) in the tail vein 6 h before cerulein injection, and control rats received the same amount of normal rabbit serum (Accurate Chemical & Scientific, Westbury, NY). One hour after the last cerulein injection, rats were euthanized by CO2-induced asphyxiation, and the blood and pancreas were harvested for measurements. All animal care protocols were approved by the Laboratory Animal Users Committee at Veterans Affairs Greater Los Angeles Healthcare System.

**Blood examinations.** Blood neutrophil counts were determined by Coulter-LH-750 (Beckman Coulter, Brea, CA) combined with manual counting by hematologists, who were not informed of the study design. The mean ± SE neutrophil counts were 23 ± 7 μl⁻¹ in animals treated with anti-PMN serum and 701 ± 133 μl⁻¹ in those treated with control serum (n = 5). The numbers of lymphocytes and monocytes were also decreased after treatment with anti-PMN serum (2.3 ± 0.4 × 10⁶ vs. 6.1 ± 0.5 × 10⁶ and 26 ± 12 vs. 201 ± 73 μl⁻¹, respectively). The level of serum lipase was measured in a Hitachi 707 analyzer (Antech Diagnostics, Irvine, CA), and the mean of lipase in cerulein pancreatitis was decreased after treatment with anti-PMN serum (2,393 ± 308 vs. 3,529 ± 535 IU, respectively).

**Fig. 1.** Neutrophil depletion did not affect cytochrome c release, mitochondrial depolarization, and the levels of Bcl-2 proteins in cerulein pancreatitis. A–C: rats received an injection of anti-polymorphonuclear (anti-PMN) serum or control serum and then were subjected to 4 injections of cerulein (CR) or saline. A: pancreas was fractionated and the levels of cytochrome c were measured in mitochondrial and cytosolic fractions with Western blot. Blots were reprobed with Cox IV used as a loading control. B: levels of Bcl-2 and Bcl-xL were measured in whole pancreatic tissue homogenates with Western blot. Blots were reprobed for ERK1/2 used as a loading control. C: pancreatic mitochondria were loaded with membrane potential-sensitive dye, tetramethylrhodamine methyl ester (TMRM), and subjected to 1.3 fractionated and the level of Bax was measured in mitochondrial fraction with Western blot. Blots were reprobed for Cox IV to show equal protein loading of mitochondrial fractions. There was no Cox IV in cytosolic fractions, confirming quality of fractionation. The intensities of cytochrome c bands were quantified by densitometry, and ratios of cytosol- to mitochondria-cytochrome c intensities were presented. Open bars, control serum; solid bars, anti-PMN serum. Results are expressed as means ± SE (n = 3). *P < 0.05 compared with the same conditions without cerulein.

**Isolation and incubation of pancreatic acini.** Isolation of pancreatic acini (acinar cells) from rats was performed by using the collagenase digestion procedure as we described previously (13). Dispersed pancreatic acini were then incubated at 37°C in 199 medium in the presence or absence of 100 nM cholecystokinin-8 (CCK-8, American Peptide, Sunnyvale, CA). Three hours after CCK-8 incubation initiation, pancreatic acini were harvested for measurements. In some experiments acini were pretreated for 30 min before CCK-8 incubation with Mdm2 inhibitor, 1 μM nutlin-3 (47) (Calbiochem, San Diego, CA), or p53 inhibitor, 40 μM pifithrin-α (20) (Bionol, Plymouth Meeting, PA), or caspase-2 inhibitor, 10 μM Z-VDVAD- FMK (12, 32) (Calbiochem).

**Western blot analysis.** Apoptosis-related proteins were analyzed by Western blot analysis as previously described (26). Blotting membranes were incubated with antibodies against Bcl-2, Bcl-xL, Bax, phospho-Mdm2 (Ser166), p44/42 mitogen-activated protein kinase (Erk1/2) (Cell Signaling, Beverly, MA); caspase-2, caspase-3, caspase-8, p53, PIDD, RAIDD (Santa Cruz Biotechnology, Santa Cruz, CA); histone H2B (Abcam, Cambridge, MA); cytochrome c (BD Biosciences, San Diego, CA); Cox IV (Molecular Probes, Eugene, OR); and caspase-9 (Stressgen, San Diego, CA). Catch and release Reversible Immunoprecipitation System was used for immunoprecipitation (IP) technique (Millipore, Billerica, MA). The amount of...
antibody used for immunoprecipitation was calibrated to provide equal amounts of immunoprecipitated protein from each sample. For example, with IP of PIDD, 3 μg antibody was used with 1 mg tissue lysate, giving equal amounts of PIDD immunoprecipitated from each sample. The blots were developed for visualization by enhanced chemiluminescence detection kit (Thermo Fisher Scientific, Rockford, IL). Optical densitometry reading of the Western blot was performed by use of Scion imaging software (Scion, Frederick, MD).

Caspase activities. Caspase activities were measured by fluorogenic assays as previously described (26), using specific substrates for caspase-2 (Ac-VDVAD-AMC), caspase-3 (Ac-DEVD-AMC), caspase-8 (Ac-IETD-AMC), or caspase-9 (Ac-LEHD-AMC). All substrates were from Biomol (Plymouth Meeting, PA). The data were expressed as moles of AMC per milligram of protein per minute.

Nuclear, mitochondrial, and cytosolic fractions. Mitochondrial and cytosolic fractions were obtained as previously described (13), and nuclear protein extracts were prepared by using ActiveMotif Nuclear Extract Kit (Active Motif, Carlsbad, CA) following the manufacturer’s instructions. Intact mitochondria were purified from rat pancreas and membrane potential, and cytochrome c release by various Ca2+ concentrations in mitochondrial suspension was measured as recently described (29).

Immunohistochemical analysis for p53. Pancreatic tissues were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) and 6-μm sections were prepared. The primary antibody, goat polyclonal anti-p53 (diluted 1:50, Santa Cruz Biotechnology), was incubated with the slides at 4°C for overnight, and after washes with PBS the sections were reacted with a secondary antibody, biotinylated anti-goat antibody, at room temperature for 3 h. After being rinsed with phosphate-buffered saline, the slides were immersed in diaminobenzidine solution for 5 min and counterstained for 20 s with Mayer-hematoxylin solution. Sections stained with the secondary antibodies alone were evaluated as negative controls.

siRNA transfection. The rat pancreatic acinar cell-line, AR42J cells, were obtained from ATCC (Manassas, VA) and cultured as previously described (51). Transfection of AR42J cells with small interfering RNAs (siRNAs) was performed by using NucleofectorTM II (Amaxa, Gaithersburg, MD) according to the manufacturer’s instruction. For siRNA transfections, we used pools (pool of 4 duplexes) provided by Dharmacon (Chicago, IL). The sequences of the four mouse p53 siRNAs (sense strands) were as follows: CCACUAUCCACUACUGUA, CAGUCUAAGCGCCGCAUA, GGCAAGGCUGACCUAAU, and GGAACUCUGUAGUGAGAG. The sequences for Mdm2 siRNAs were as follows: GAUACAGCCUGAGUGAU, GCAGACGCUGUCCAAAUA, GAGGUAAGCUAAGCGA, and GAACAGAGAGAAGAACCU. Nontargeting siRNA pool (pool of 4 duplexes, siCONTROL) from Dharmacon was used as a control. Six hours after p53 siRNA transfection or 24 h after Mdm2 siRNA transfection, cells were harvested for measurements.

Statistical analysis. All data are expressed as means ± SE. The two-tailed nonparametric (unpaired t-test) was used for statistical analysis. Probability values of P < 0.05 were considered statistically significant.

RESULTS

Effects of neutrophils on cytochrome c/caspase-9-mediated pathway of apoptosis in cerulein pancreatitis. Cytochrome c release from mitochondria into cytoplasm is a central event mediating the pathway of apoptosis in cerulein pancreatitis (13). Therefore, we first measured the effect of neutrophil depletion on pancreatic mitochondrial cytochrome c release during cerulein-induced pancreatitis. In agreement with our previous data we found that cerulein pancreatitis stimulated cytochrome c release (Fig. 1A). However, neutrophil depletion...
had no effect on the cerulein-induced cytochrome c release. We also measured the effect of neutrophil depletion on the changes in Bcl-2 proteins, major regulators of cytochrome c release. In agreement with our previous data (39), we found that Bcl-xL, Bcl-2, and Bax were all upregulated in cerulein pancreatitis, but neutrophil depletion had no effect on the expression of these proteins in pancreatitis (Fig. 1, B and C). We next isolated pancreatic mitochondria from control and neutrophil-depleted rats with pancreatitis and measured the effect of exogenous Ca\(^{2+}\)/H\(_{11001}\) on mitochondrial membrane potential and release of cytochrome c. Ca\(^{2+}\) is a major mediator of mitochondrial permeabilization and release of cytochrome c from pancreatic mitochondria. However, the stimulation of cytochrome c from mitochondria by Ca\(^{2+}\) was the same in pancreatic mitochondria from rats with and without neutrophils (Fig. 1, D and E). These combined results indicate that neutrophils do not regulate mitochondrial cytochrome c release in pancreatitis.

We next measured the effect of neutrophil depletion on the activities of caspases-9, -8, and -3 in pancreatitis. Our previous data indicate that cytosolic cytochrome c causes activation of initiator caspase-9, leading to the activation of caspase-3, which executes apoptosis (26). Caspase-8 is activated and partially mediates apoptosis in pancreatitis (3). In agreement with the published results we found that activities of caspases-3, -8, and -9 increased in cerulein pancreatitis (Fig. 2, left). Caspase activation was associated with an increased caspase processing manifest by appearance of active caspase-3, -8, and -9 bands on Western blot (Fig. 2, right). Importantly, although neutrophil depletion had no effect on the activities of caspases-9 and -8, it significantly (by 90%) increased activity of caspase-3 in cerulein pancreatitis. Correspondingly, Western blot showed that neutrophil depletion did not affect processing of caspases-9 and -8 but greatly increased processing of caspase-3, which manifests by an increase in 17-kDa caspase-3 active form in cerulein pancreatitis. The lack of the effect of neutrophil depletion on cytochrome c release (Fig. 1), as well as on the caspases-9 and -8 activities (Fig. 2), indicates that neutrophils had no effect on the mitochondrial pathway of apoptosis. The finding that neutrophil depletion stimulated caspase-3 but didn’t activate the mitochondrial pathway indicated that the neutrophils’ effect on caspase-3 was due to another input.

**Effects of neutrophils on caspase-2-mediated pathway of apoptosis in cerulein pancreatitis.** Figure 3 showed that neutrophil depletion greatly (by ~70%) enhanced caspase-2 ac-

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**Fig. 3.** Neutrophil depletion enhanced caspase-2 activity and increased PIDD and PIDD-RAIDD complex formation in cerulein pancreatitis. Rats received an injection of anti-PMN or control serum and then were subjected to 4 injections of cerulein or saline. A: caspase-2 activity in pancreatic tissues was measured by fluorogenic assay with the substrate specific for caspase-2 (Z-VDVAD-AMC). The level of caspase proform and active form were measured with Western blot. Blots were reprobed for ERK1/2 used as a loading control. B: levels of PIDD and RAIDD were measured in whole pancreatic tissue homogenates with Western blot. Blots were reprobed for ERK1/2 used as a loading control. The intensities of PIDD and RAIDD bands were quantified by densitometry and normalized to those in the absence of cerulein and anti-PMN. Open bars, PIDD; solid bars, RAIDD. C: PIDD was immunoprecipitated from whole pancreatic tissue homogenates from rats treated with and without anti-PMN serum and with and without cerulein. The levels of RAIDD and PIDD in immunoprecipitates (IP) were measured with Western blot. The intensities of RAIDD and PIDD bands were quantified by densitometry. The results are expressed as means ± SE (n = 3). *P < 0.05 compared with the same conditions without cerulein. #P < 0.05 compared with rats treated with cerulein and without anti-PMN. Blots are representative of 2 independent experiments.
tivity and increased procaspase-2 processing in cerulein pancreatitis. As we discussed above caspase-2 activation in death responses is mediated through PIDDosome, a complex composed of PIDD, RAIDD, and procaspase-2. We found that PIDD was increased in cerulein pancreatitis and neutrophil depletion further increased PIDD level during cerulein pancreatitis. Coimmunoprecipitation experiments also show that neutrophil depletion increased PIDD-RAIDD complex formation in cerulein pancreatitis (Fig. 3C). In this immunoprecipitation experiment we adjusted the amount of antibody to PIDD to immunoprecipitate the same amount of PIDD from each sample. These findings suggest that cerulein pancreatitis and neutrophil depletion increase PIDDosome formation through upregulation of PIDD expression as well as increased complexing with RAIDD.

Because PIDD expression is controlled by p53 (25), we next measured the effect of neutrophil depletion on the levels of p53 and its negative regulator, Mdm2. We found that cerulein pancreatitis increased both p53 and Mdm2 levels in control rats. Neutrophil depletion further increased p53 but decreased Mdm2 level in cerulein pancreatitis (Fig. 4A). Thus Mdm2 downregulation could be a mechanism for p53 upregulation by neutrophil depletion. Furthermore, neutrophil depletion affected not only p53 level but also its subcellular localization in acinar cells in cerulein pancreatitis. Immunohistochemical staining showed diffuse p53 cytosolic staining in pancreatic acinar cells during cerulein pancreatitis. Differently, neutrophil depletion increased nuclear p53 staining in cerulein pancreatitis (Fig. 4B). Similarly, Western blot showed that neutrophil depletion increased p53 in nuclear fraction but didn’t change cytosolic p53 level (Fig. 4C). These differences are important because functions of cytosolic and nuclear p53 are different. Cytosolic p53 possesses transcription-independent activities, such as increasing mitochondrial membrane permeabilization and inhibiting autophagy, but nuclear p53 is characterized as a transcription factor and transactivates a number of genes, including PIDD (11).

These data demonstrate that a Mdm2/p53/caspase-2 mediated pathway regulates caspase-3 in apoptosis in cerulein pancreatitis. They further indicate that this mediated pathway is regulated by neutrophils.

Role of p53 in caspase activation in vitro model of cerulein pancreatitis. We further evaluated the role of p53 in caspase activation in in vitro pancreatitis by using pharmacological and molecular approaches. These experiments were performed on acinar cells stimulated with supramaximal dose of CCK-8 (in vitro model of cerulein-induced pancreatitis) and we used both isolated rat pancreatic acinar cells and the AR42J cell line. To

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![Fig. 4](http://ajpgi.physiology.org/)

Neutrophil depletion increased p53 and decreased Mdm2 in cerulein pancreatitis. A–C: rats received an injection of anti-PMN or control serum and then were subjected to 4 injections of cerulein or saline. A: levels of p53 and Mdm2 were measured in whole pancreatic tissue homogenates with Western blot. Blots were reprobed for ERK1/2 used as a loading control. The intensities of p53 and Mdm2 bands were quantified by densitometry and normalized to those with cerulein and without anti-PMN. Open bars, p53; solid bars, Mdm2. B: immunohistochemical staining of p53 was measured on pancreatic tissue sections from rats treated with control serum and saline (a), anti-PMN serum and saline (b), control serum and cerulein (c), or anti-PMN serum and cerulein (d). No staining was observed with secondary antibody only. The values represent the percentage of positively stained nuclei vs. total nuclei (5–10 fields for each pancreas for 3 animals). Arrows indicate positively stained nuclei. C: pancreas was fractionated and the levels of p53 were measured in nuclear and cytosolic fractions with Western blot. Histone H2B served as a loading control for nuclear and ERK1/2 for cytosolic fraction. There was no histone H2B in cytosolic fractions, confirming quality of fractionation. The intensities of p53 bands were quantified by densitometry and normalized to those with cerulein and without anti-PMN. Open bars, cytosolic fraction; solid bars, nuclear fraction. The results are expressed as means ± SE (n = 3). *P < 0.05 compared with the same conditions without cerulein. #P < 0.05 compared with rats treated with cerulein and without anti-PMN. Blots are representative of 2 independent experiments.

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modulate p53 activity we applied the pharmacological Mdm2 inhibitor, nutlin-3 (1 μM), as well as the p53 inhibitor, pifithrin-α (40 μM). Similarly to what we observed in vivo cerulein pancreatitis, CCK-8 increased the levels of both p53 and Mdm2 in isolated pancreatic acinar cells (Fig. 5A). Importantly, nutlin-3 significantly increased p53 levels, indicating that p53 is negatively regulated by Mdm2 in pancreatic acinar cells. In agreement with the data on the in vivo model, CCK-8 increased activities of both caspase-2 and -3 in the acinar cells (Fig. 5). Inhibition of p53 with pifithrin-α inhibited, whereas activation of p53 with nutlin-3 stimulated caspase-2/-3 activities supporting the role of p53 in caspase-2/-3 activations. Furthermore, caspase-3 activity in in vitro model of pancreatitis was suppressed by the specific caspase-2 inhibitor, Z-VADVAD-FMK (10 μM). These results support a hypothesis that caspase-2 mediates p53-induced caspase-3 activation in pancreatitis.

We also evaluated the role of p53 in caspase activation by molecular approaches using AR42J cells. Knocking down p53 with siRNA transfection inhibited activities of both caspases-2 and -3 in AR42J cells (Fig. 6). Correspondingly, Mdm2 siRNA transfection increased activities of these caspases. Furthermore, decreasing Mdm2 expression increased p53 levels in AR42J cells (Fig. 6D). Thus both pharmacological and molecular approaches indicated that p53 leads to activation of initiator caspase-2, which, in turn, leads to the further activation of executioner caspase-3 in pancreatitis. The data also show that in acinar cells in pancreatitis p53 is negatively regulated by Mdm2. Furthermore, the expression of PIDD was increased by Mdm2 siRNA transfection (data not shown) as would be expected because of the role of p53 in regulating transcription of PIDD.

**DISCUSSION**

The study presents several novel findings on the mechanisms of apoptosis in experimental pancreatitis and its regulation by the inflammatory response. We and others have shown that apoptosis in experimental pancreatitis is mediated by the classical intrinsic mitochondrial pathway involving cytochrome c release followed by the activation of the initiator caspase-9, leading to the activation of the executioner caspase-3 (26). Here we present evidence indicating that, in addition to the classical mitochondrial pathway, a Mdm2/p53/caspase-2-mediated pathway also contributes to apoptosis in cerulein pancreatitis and that neutrophils decrease apoptosis in acinar cells in cerulein pancreatitis through this pathway. We also show that neutrophils do not affect the mitochondrial pathway of apoptosis in cerulein pancreatitis. Our results suggest that neutrophils limit apoptosis and this promotes necrosis in acinar cells during pancreatitis by suppressing p53, which leads to inhibition of caspase-2 activation. The suppression of p53 is mediated by upregulation of Mdm2, an ubiquitin ligase for p53 promoting its degradation. The inhibitor of Mdm2, nutlin-3, prevented this suppression of p53, resulting in increased caspase activation. This result in combination with previous reports showing that increased apoptosis is associated with less severe pancreatitis suggests that ubiquitin ligase inhibitors including nutlin-3 and others being identified (6) will have a beneficial effect in pancreatitis by stabilizing p53. Figure 7 illustrates the effect of inflammatory cells on pathways of apoptosis in cerulein pancreatitis and shows the novel pathway mediated by Mdm2/p53/caspase-2 in addition to the mitochondrial pathways.

Further studies are required to determine the mechanisms through which neutrophils regulate Mdm2. Mdm2 is regulated
by phosphorylation, and the kinases implicated in Mdm2 phosphorylation and function are DNA-PK (DNA-dependent protein kinase), ATM (ataxia telangiectasis mutated gene), Akt, p38, and Cdk (cyclin-dependent kinase) (2). Cerulein pancreatitis is reported to stimulate the activities of p38 (48) and Akt (35), and both these kinases are known to be activated by cytokines that increase during pancreatitis. Thus one or more of these pancreatitis-associated signaling systems could be responsible for the observed changes in Mdm2 and its effect on the subsequent mitochondrial-independent pathways regulating caspase-3 activation and apoptosis. Of note, in our experiments inflammatory cells in addition to neutrophils were decreased so that the regulation may be a result of products generated by any one or combination of these inflammatory cell types.

We showed that neutrophil depletion increased nuclear p53 in cerulein pancreatitis. Nuclear p53 affects transcriptional regulation of genes involving both classical extrinsic and intrinsic pathways (17), and recent reports indicate that caspase-2 activation is associated with nuclear p53 upregulation (44, 46). The expression of PIDD is dependent on p53 transcriptional regulation and it forms a complex with RAIDD to a PIDDosome, the core of the caspase-2 activating complex (30, 50). These events lead to activation of executioner caspases and apoptosis. Recently, PIDD has been shown to process procaspase-2 without RAIDD by making a different complex with DNA-PKcs (catalytic subunit of DNA-PK) (37). Thus our results of PIDD upregulation by cerulein and neutrophil depletion show their potential role in activation of

![Fig. 6. Effects of small interfering RNA (siRNA) knockdowns of p53 and Mdm2 on caspase-2 and -3 activities in in vitro model of pancreatitis. AR42J cells transfected with p53 (+) and control siRNA (−) (A–C) or with Mdm2 (+) and control siRNA (−) (D–F) were treated with 100 nM CCK-8 for 3 h. A and D: transfection efficiency. The level of p53 and Mdm-2 were measured with Western blot. Blots were reprobed for GAPDH used as a loading control. B, C, E, and F: caspase-2 and -3 activities were measured in AR42J cell lysates by fluorogenic assays with the substrates specific for caspase-2 (Z-VDVAD-AMC) and caspase-3 (Z-DEVD-AMC). The results are expressed as means ± SE (n = 3). *P < 0.05 compared with the same conditions without CCK-8. #P < 0.05 compared with cells treated with control siRNA and CCK-8. $P < 0.05 compared with cells treated with control siRNA and without CCK-8. A and D: blots are representative of 2 independent experiments.](http://ajpgi.physiology.org/)

![Fig. 7. Scheme of the signaling mechanism-mediated apoptosis in pancreatic parenchymal cells during pancreatitis. A: 2 pathways mediate apoptosis in pancreatitis. The mitochondrial pathway is initiated by cytochrome c release followed by caspase activation (3). The p53-mediated pathway also stimulates activation of caspases. Inflammatory cells inhibit the p53-mediated pathway but do not affect the mitochondrial pathway. B: detailed scheme of the p53-mediated pathway. Inflammatory cells inhibit this pathway through up-regulating Mdm2, a negative regulator of p53.](http://ajpgi.physiology.org/)
caspase-2 in pancreatic parenchymal cells. Furthermore, PIDD could be one of the therapeutic targets for use to modulate the severity of pancreatitis in future.

We and others showed that severity of pancreatitis correlates with the necrosis rate, whereas apoptosis is associated with a milder disease (19, 26). Furthermore, agents that stimulate apoptosis in pancreatitis decrease necrosis and the severity of the disease (1, 26, 28). In the present study, we found that a Mdm2/p53/caspase-2 pathway plays a significant role in addition to the classical pathways activation of caspase-3 in pancreatitis. Our data suggest that strategies that lead to inhibition of Mdm2 and/or upregulation of p53 can be used to treat pancreatitis.

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