J Gastroenterol 2008; 43:473–483
DOI 10.1007/s00535-008-2179-4

Journal of Gastroenterology

Mitochondrial dysfunction and apoptosis of acinar cells in chronic pancreatitis

Lipi Singh[1], Dapinder K. Bakshi[2], Siddarth Majumdar[2], Sunil Kumar Arora[3], Rakesh Kumar Vasishta[4], and Jai Dev Wig[1]

[1] Department of General Surgery, Postgraduate Institute of Medical Education and Research, Chandigarh 160 012, India
[2] Department of Experimental Medicine & Biotechnology, Postgraduate Institute of Medical Education and Research, Chandigarh, India
[3] Department of Immunopathology, Postgraduate Institute of Medical Education and Research, Chandigarh, India
[4] Department of Histopathology, Postgraduate Institute of Medical Education and Research, Chandigarh, India

Background. The mechanism of acinar cell death in human chronic pancreatitis (CP) remains largely unexplored. Previous studies have demonstrated the role played by apoptosis and necrosis in experimental pancreatitis; however, their relationship with the progression of CP remains unknown. The present study was carried out to elucidate the role and extent of apoptosis in CP tissues with different histopathological scores and to examine the possible apoptotic pathway involved. ***Methods.*** Pancreatic tissues (25 CP patients) that had been histopathologically graded (I–III) and ten normal pancreatic tissue samples were evaluated for apoptosis by DNA fragmentation and an in situ TUNEL assay. The expression of various apoptotic and antiapoptotic markers in the tissues were studied by immunohistochemistry and Western blotting. To elucidate the role of the mitochondria in acinar cell death, the mitochondrial membrane potential ($\Delta\Psi_m$) and ATP levels were determined by flow cytometry and a luminometer. ***Results.*** The presence of DNA fragmentation and apoptotic nuclei in all CP tissues confirmed the presence of apoptosis. The apoptotic index in CP tissue ranged from 0.09% to 0.86% ± 0.02% and was highest in grade II (0.7 ± 0.04%) tissues. Differential upregulation of the apoptotic mediators p53, Bax, cytochrome *c*, and caspase-3 and -9, and downregulation of antiapoptotic Bcl-2, was observed in CP. $\Delta\Psi_m$ on the order of 1.2- to 2.2-fold and ATP depletion in the range of 23%–84% in CP tissues was observed. ***Conclusions.*** Apoptosis plays an important role both in the initial stages and during the progression of CP, as evident in all tissue grades. Increased $\Delta\Psi_m$, loss of ATP, and activation of caspases suggests the involvement of intrinsic pathways.

Received: December 14, 2007 / Accepted: February 25, 2008
Reprint requests to: J. D. Wig

Key words: acinar cells, apoptosis, chronic pancreatitis, mitochondrial dysfunction

Introduction

Chronic pancreatitis (CP) is a continuing inflammatory disease of the pancreas characterized by the irreversible destruction of exocrine parenchyma, and fibrosis followed by destruction of the endocrine parenchyma in the later stage.[1] The incidence of CP ranges from 1.6 to 23 cases per 100 000 population per year worldwide.[2] A gradual rise in the incidence of CP has been observed in some countries, including India, which may be attributed to increased alcohol consumption and earlier diagnosis. The prevalence of CP is very high in India (114–200/100 000 population),[3] and the most common cause is idiopathic pancreatitis, although the prevalence is known to increase remarkably with increasing cumulative alcohol consumption.[4,5]

Inflammation and acinar cell death are hallmarks of both human and experimental pancreatitis.[6,7] Although significant progress has been achieved over the past decade in understanding the inflammatory response,[8] mechanisms behind the acinar cell death in pancreatitis remain largely unexplored. Many previous studies have demonstrated the roles of apoptosis and necrosis in various models of pancreatitis, but the connecting link and their relation to the progression of CP is not clear. Recent studies have highlighted the role of apoptosis in acinar cell loss during CP in murine/rodent models, which showed the involvement of apoptosis in CP and expression of factors such as Fas and Fas ligand.[9,10] A copper-deficient diet, ethionine administration, and pancreatic duct ligation can all lead to pancreatic acinar cell apoptosis, resulting in pancreatic atrophy.[11–13] In a model more relevant to human CP, acinar cell atrophy occurred in rodents fed ethanol and a low-protein diet.[14]

Such studies have established that apoptosis is involved very early in the etiology and progression of CP.[10]

The work by Bateman et al.[15] on human CP also demonstrated loss of pancreatic acinar cells and an increased apoptotic index, but their investigation was based mainly on the role of cell cycle-associated molecules and inflammatory cells. However, data on the extent of apoptosis with disease progression and possible apoptotic pathways involved in human CP are lacking. Hence, the present study aimed to quantify apoptosis in CP tissues of different histopathological grade (I–III) via expression of various proapoptotic and antiapoptotic markers. We also explored the involvement of intracellular pathways by assessing caspase activation, mitochondrial dysfunction via mitochondrial membrane potential transition (Ψ_m), and ATP loss in acinar cells isolated from CP patients.

Materials and methods

Subjects

This prospective study included 25 patients with CP of relatively advanced stage who underwent surgical resection of pancreatic tissue in the Department of General Surgery, Postgraduate Institute of Medical Education and Research, Chandigarh, India, by Frey's procedure.[1] Autopsy specimens ($n = 10$) from patients with no prior pancreatic pathology or history were collected within 2 h of death to serve as normal pancreatic tissue controls.[16] The clinical diagnosis of CP was based on characteristic signs and symptoms supported by characteristic sonography and computed tomography findings. The final diagnosis was confirmed by routine histopathological assessment, and then the pancreatic tissues were histopathologically graded into grades I, II, or III.[17] The study received approval from the Institute Review Board, and informed consent was obtained from all patients as prescribed by the institutional ethics committee in accordance with the institute's guidelines.

Evaluation of apoptosis in CP tissues

DNA fragmentation assay

DNA extraction was carried out with a RNA/DNA extraction kit (QIAGEN, Hilden, Germany). The DNA pellet was resuspended in 20 µl of Tris-EDTA (Tris-Ethylene Diamine Tetraacetic Acid) buffer and allowed to dissolve at 4°C overnight. Finally, DNA was analyzed in 1.8% agarose gel via electrophoresis[18] along with a 100-base pair DNA marker (Roche, Mannheim, Germany). The gel was stained with ethidium bromide (0.5 µg/ml within the gel) and examined under UV light for the presence of nucleosomal DNA fragments, whose size was compared to that of the DNA marker.

In situ cell death detection

Tissue sections were obtained from all 25 CP patients, and apoptotic cells were detected by an in situ cell death detection kit (Roche) as per the manufacturer's instructions. Briefly, 4- to 5-µm-thick sections cut from formalin-fixed, paraffin-embedded tissues were mounted on poly-L-lysine-coated slides. Sections were dewaxed by heating at 60°C, followed by serial passages through xylene and a graded alcohol series (100%, 95%, and 70%). Then, they were incubated with proteinase-K (0.5 mg/ml in 10 mM Tris/HCl, pH 7.4) for 30 min at 37°C. After being washed with phosphate-buffered saline (PBS) three times, sections were incubated with blocking solution (0.03% H_2O_2 + methanol) for 20 min at room temperature. The sections were rinsed with PBS and incubated with a TdT-mediated dUTP nick-end labeling (TUNEL) reaction mixture containing (1) terminal deoxynucleotidyl transferase (TdT) enzyme and (2) fluorescein-labeled dUTP for 60 min at room temperature. After another washing, sections were incubated with anti-fluorescein antibody conjugated with peroxidase enzyme by incubating the cells for 30 min at room temperature, washed, and then visualized, using 3,3′-diaminoBenzidine tetrahydrochloride (DAB) as the substrate (Sigma, St. Louis, MO, USA). The sections were lightly counterstained with hematoxylin and mounted with DPX (Sigma, USA). The final positive reaction product was identified by brown-stained nuclei (apoptotic nuclei) and counted with a Leica image analyzer aided by Quantimet 600 software (Cambridge, England). At least four fields were examined in each case, and the number of apoptotic cells per 1000 acinar cells was determined and designated as the apoptotic index.

Analysis of expression of proapoptotic and antiapoptotic markers (p53, Bax, cytochrome c, caspase 9, caspase 3, and Bcl-2)

Immunohistochemistry

Immunohistochemistry (IHC) of tissue sections from CP patients was performed a previously described.[17] In all cases, the negative control was devoid of primary antibody, and a reactive lymph node served as an internal positive control.

Scoring of immunostaining

IHC was scored by the scoring system of Miyamoto et al.[19] IHC was first scored separately based on the number of positive cells and on the overall immunoreactivity intensity of the positive cells, and then the percentage positivity was multiplied by the intensity score to obtain the final score. Positive cells were analyzed in at least five randomly selected fields in each case, and

Table 1. Antibodies used in IHC and Western blotting for elucidation of expression of apoptotic and antiapoptotic molecules

Antigen	Antibody clone	IHC (μg/ml)	Western blot (μg/ml)	Source
p53	MAb p53 clone1801	2 (1:100)	0.5 (1:400)	Santa Cruz Biotechnology, Santa Cruz, CA, USA
Bax	MAb Bax clone B-9	2 (1:100)	0.4 (1:500)	Santa Cruz Biotechnology
Bcl-2	MAb Bcl-2 clone 100	2 (1:100)	0.5 (1:400)	Santa Cruz Biotechnology
Cytochrome *c*	MAb cyt *c* clone 7H8	2 (1:100)	0.8 (1:250)	Santa Cruz Biotechnology
Caspase 9	Polyclonal	1.25 (1:200)	0.25 (1:1000)	BD Pharmingen, San Diego, CA, USA
Caspase 3	MAb caspase 3 clone E-8	2 (1:100)	0.4 (1:500)	Santa Cruz Biotechnology

IHC, immunohistochemistry; MAb, monoclonal antibody

the number of positive cells per 1000 acinar cells was determined with the aid of a light microscope (Olympus Bx 51, Tokyo, Japan).

Western blotting

Tissue lysates (from both CP patients and normal controls) were centrifuged (11 200 *g*, 20 min) to remove debris, and then the protein content was estimated by the bicinchoninic acid method.[20] Proteins (60 μg per sample) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and identified by Western blotting.[21] Blots were developed with 0.05% DAB in PBS containing H_2O_2 (1 μl/ml) for 30 min in dark at 37°C. The reaction was stopped by washing the strips with water. The appropriate protein markers (Biomol, Hamburg, Germany) were run in parallel. The expression of apoptotic and antiapoptotic markers was detected by using primary antibodies (Table 1). The protein content loaded in each lane was quantified in relation to anti-human actin monoclonal antibody. The band intensities in the immunoblot were quantified by densitometry using Scion Image software (Scion, Frederick, MD, USA).

Measurement of caspase 9 and caspase 3 activation

The frozen tissue was homogenized at 4°C in chilled lysis buffer, then the lysate was centrifuged at 25 200 *g* for 30 min at 4°C and subsequently assessed for caspase 9 and caspase 3 activities. The proteolytic reaction was carried at 37°C in 25 mM HEPES buffer (pH 7.2), 10% sucrose, 0.1% CHAPS, 10 mM dithiothreitol, 800 μg cytosolic protein, and 20 μM specific fluorogenic substrate for caspase 9 (LEHD-AMC, 5 mM) and caspase 3 (DEVD-AFC, 1 mM). Specific caspase 9 and caspase 3 inhibitors (LEHD-CHO and DEVD-CHO, 50 μM each) were added, and the fluorescence was read with a spectrofluorometer (Varian, Houten, the Netherlands) with caspase 9 excitation at 380 nm and emission at 460 nm and caspase 3 excitation at 400 nm and emission at 505 nm. Fluorescence was calibrated by using the standard curve for 7-amino-4-methyl coumarin (AMC)/7-amino-4-trifluoromethyl coumarin (AFC). Caspase activity was calculated as follows: Caspase activity = $\Delta F/h \times 1/$calibration curve slope, where $\Delta F/h$ = difference in fluorescence between normal control and CP tissue lysates.

Assessment of mitochondrial membrane potential (Ψ_m)

Isolation and culture of dispersed pancreatic acinar cells

The dispersed pancreatic acinar cells were prepared from CP and normal (approximately 2 mm) tissues obtained from patients and autopsy specimens by the collagenase method.[22,23] In brief, 1×10^6 cells/2 ml 199 M/well were cultured in six-well plates with 50 nM 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6(3)$), and a fluorochrome dye was added for 30 min.[24–26] The cells were then washed with sterile PBS to remove excess fluorochrome, and the concentration of retained $DiOC_6(3)$ was read by using flow cytometry with excitation at 488 nm and emission at 500 nm.

ATP measurement

Tissue was homogenized in trichloroacetic acid (TCA; 6%) for 1 min and centrifuged at 11 200 *g* for 5 min at 4°C. TCA in the supernatant was neutralized and diluted to a final concentration of 0.1% with Tris-acetate buffer (pH 7.75).[27–29] ATP was measured in tissue lysate with a commercially available luciferin–luciferase bioluminescence kit (Promega Enliten, Madison, WI, USA) according to the manufacturer's instructions. The readings were taken using a 10-s integration period with a monolight luminometer. Background luminescence was subtracted, and the amount of ATP in the sample was calculated from the standard curve of an ATP concentration series.

Statistical analysis

Data were expressed as means ± SD. Data were analyzed by the Kruskal-Wallis test followed by Mann-Whitney *U* test, Pearson's correlation coefficient, and Spearman's correlation coefficient, with SPSS for Windows ver. 15 software, and the results were considered significant at $P \leq 0.05$.

Results

Clinical data and histopathological assessment

The patients' clinical features and their histopathological evaluations were reported in detail previously.[17] The mean age of the CP patients was 35 ± 10 years with a male to female ratio of 7:1, while that of the control donors was 36 ± 6 years, with a male to female ratio of 4:1. CP was associated with long history of excessive alcohol consumption in 44% of the patients, was idiopathic in 20%, and of unspecified etiology in 36%. On histopathology, tissues of 84% of the patients showed mild to moderate inflammation with inter- and intralobular or diffuse distribution of mononuclear and polymorphonuclear cells with a partial or total loss of parenchyma and 50% fibrosis. In the remaining 16%, inflammation was minimal, with interlobular distribution of mononuclear cells, less fibrosis (10%–15%), and no loss of pancreatic parenchyma. The histopathological grade of tissues of the CP patients studied was grade I, 16%, grade II, 64%, and grade III, 20%.

Evaluation of apoptosis in CP tissues

DNA fragmentation assay

The extracted nucleosomal DNA from both CP and control tissues were visualized in a 1.8% gel via electrophoresis. The electrophoretic separation of the fragments yielded a ladder-like pattern in all CP tissues, but no such DNA ladder was observed in normal pancreatic tissues (Fig. 1A).

In situ cell death detection: TUNEL assay

The extent of apoptosis in normal and CP tissues was determined by in situ TUNEL staining (Fig. 1B–F), which showed the presence of brown apoptotic nuclei in CP tissues under a light microscope. The number of apoptotic nuclei was counted, and the apoptotic index calculated. The apoptotic index was higher in CP tissues (0.09%–0.86%; median, 0.02%) than in normal pancreatic tissue (0.02 ± 0.01%). The maximum apoptotic index was found in grade II CP tissues (0.7 ± 0.04%, $P \leq 0.001$), followed by grade III (0.3 ± 0.06%, $P \leq 0.001$) and grade I (0.1 ± 0.02%, $P \leq 0.001$) tissues (Fig. 1F).

Expression of apoptotic and antiapoptotic markers

p53 protein expression

Immunohistochemistry revealed that p53 was overexpressed in CP patients compared with in normal tissues. CP tissues showed strong nuclear but diffused cytoplasmic p53 positivity in acinar cells. Grade I tissue stained faintly positive for p53 protein, whereas grade II and III tissues showed strong to moderate positivity (Fig. 2Aa–d and 2B, panel I). To quantify the differences in protein levels, Western blotting of tissue lysates obtained from CP and normal tissues was performed. Incubation with monoclonal antibody revealed a strong 53-kDa protein band in all CP tissues, whereas a very faint band was present in normal pancreatic tissues. Densitometric scanning of the bands revealed upregulation of p53 expression: twofold in grade I, ninefold in grade II, and eightfold in grade III CP tissues, compared with normal pancreas (Fig. 3Aa and Ba′).

Bax protein expression

IHC revealed weak positivity for Bax protein in grade I CP tissues, whereas cytoplasm of grade II and III tissues showed strong to moderate positivity (Fig. 2Ae–h and B, panel II). Normal pancreatic acinar cell cytoplasm stained faintly positive for Bax. A ~21-kDa protein band of strong intensity was observed in CP tissue lysates. Further, densitometric scanning of the bands revealed upregulation of Bax expression by onefold in grade I, eightfold in grade II, and sixfold in grade III CP tissues (Fig. 3Ab and Bb′).

Bcl-2 protein expression

Bcl-2 protein expression was downregulated in acinar and ductal cells of CP tissues compared with in normal pancreatic tissue sections. Grade I tissues showed weak positivity for Bcl-2, whereas grade II and III tissues showed very faint positivity in acinar cells (Fig. 2Ai–l and B, panel III). Western blot analysis demonstrated a ~26-kDa protein band of similar intensity in normal and grade I CP tissues, whereas grade II and III CP tissues showed twofold downregulation (Fig. 3Ac and Bc′).

Cytochrome c expression

IHC demonstrated the overexpression of cytochrome *c* in cytoplasm of acinar and ductal cells of CP tissues. Grade I tissue showed faint positivity, whereas grade II and III tissues showed strong to moderate positivity for cytochrome *c* in acinar cells (Fig. 4Aa–d and B, panel I). Western blot showed a ~10-kDa protein band of strong intensity in CP tissue lysates, and densitometric scanning demonstrated upregulation of cytochrome *c* protein: twofold in grade I, ninefold in grade II, and eightfold in grade III, compared with in normal pancreas (Fig. 3Ad and Bd′).

Caspase 9 protein expression

IHC revealed cytoplasmic localization of caspase 9 protein, mainly in acinar cells, of CP tissues, whereas normal cells showed faint positivity. Grade I CP tissues showed weak positivity, whereas grade II and III tissues showed strong to moderate positivity (Fig. 4Ae–h and

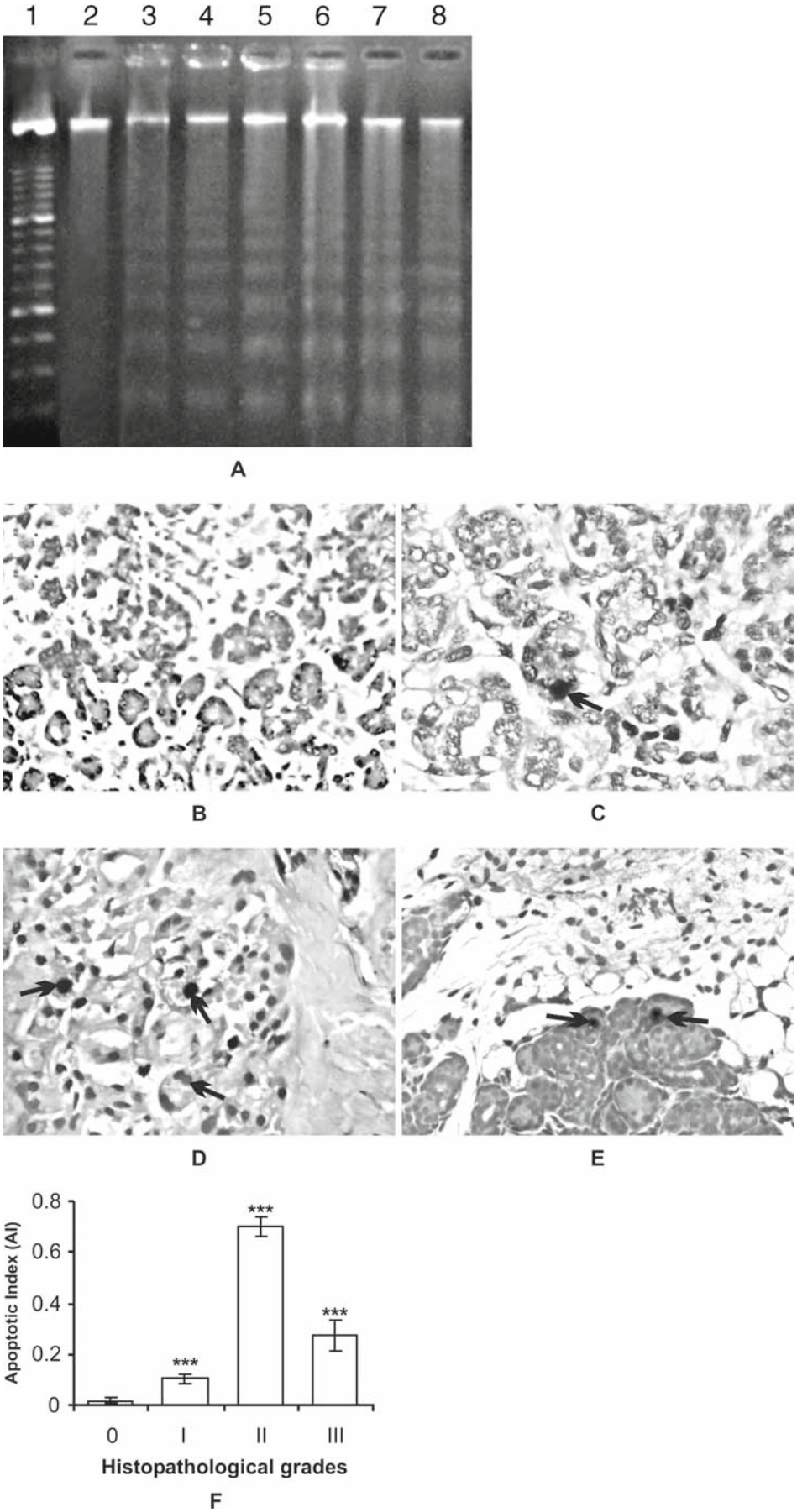

Fig. 1A–F. DNA fragmentation, in situ TdT-mediated dUTP nick-end labeling (TUNEL) staining, and apoptotic index. **A** 1.8% agarose gel electrophoresis of isolated DNA from pancreatic tissue: *lane 1*, 100-bp DNA ladder; *lane 2*, normal pancreas showing intact DNA; *lanes 3–8*, DNA ladder formation in graded chronic pancreatitis (CP) tissues. **B–E** Cell death detection by in situ TUNEL staining showing normal (**B**) and graded (grades I–III) CP tissues. *Arrows* point to apoptotic cells (**C–E**). **F** Histogram showing apoptotic index in normal (*0*) and graded (*I–III*) CP tissues. Data shown are expressed as means ± SD. Statistical analysis by Kruskal-Wallis test followed by the Mann-Whitney *U* test. ***$P < 0.001$; original magnification ×800

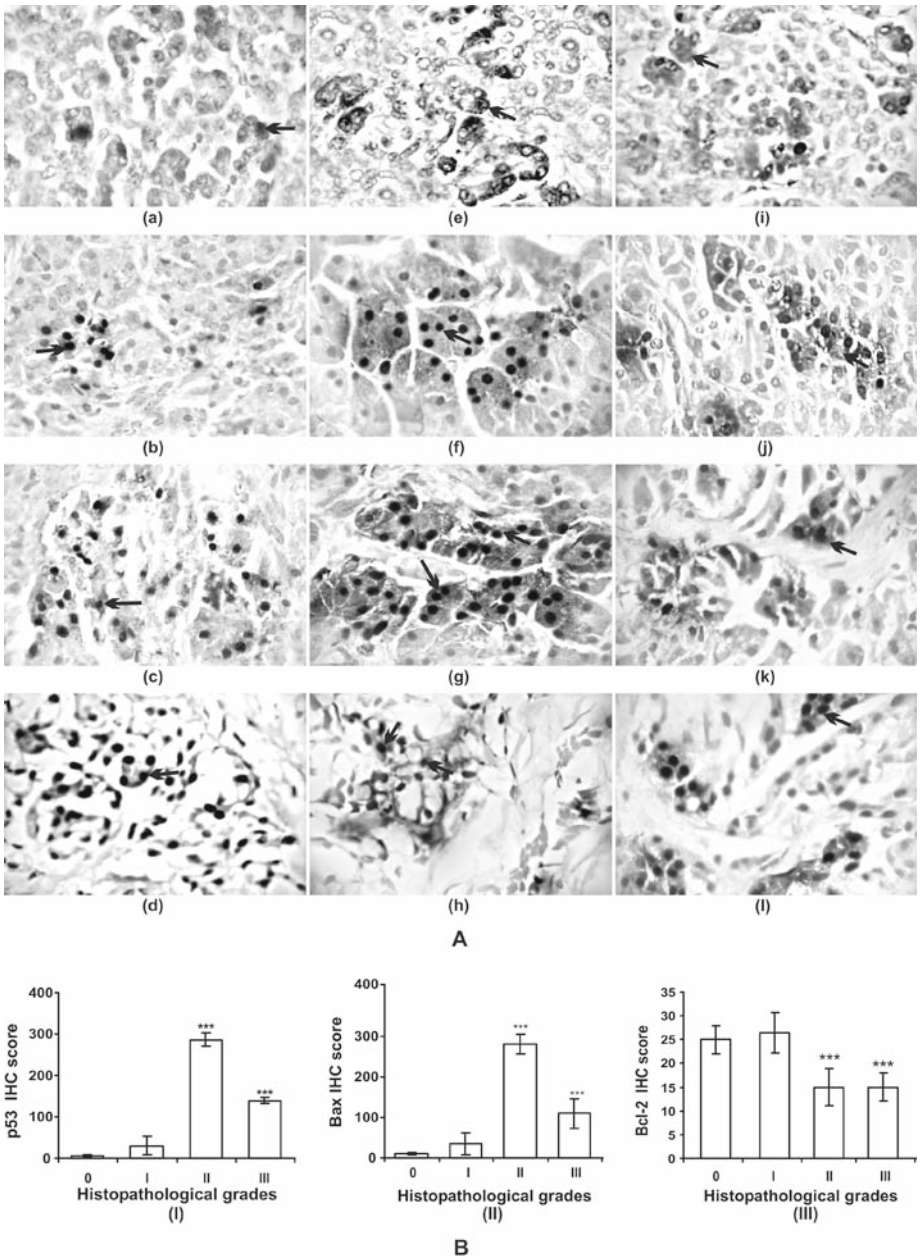

Fig. 2. A Immunohistochemistry (*IHC*) of p53, Bax, and Bcl-2 protein expression in pancreatic tissue sections. *a–d* p53 expression in normal tissue (*a*) and graded (*I–III*) CP tissues showing cytoplasmic and nuclear localization (*b, c, d*). *e–h* Bax expression in normal tissue (*e*) and graded (I–III) CP tissues showing cytoplasmic and perinuclear localization (*f, g, h*). *i–l* Bcl-2 expression in normal tissue (*i*) and graded (I–III) CP tissues showing cytoplasmic localization (*j, k, l*). *Arrows* show the positivity for immunostaining of respective apoptotic and anti-apoptotic markers in acinar cells. **B** Histograms showing p53 (*I*); Bax (*II*), and Bcl-2 (*III*) expression in normal and graded CP tissues. Data shown are expressed as means ± SD. Statistical analysis by Kruskal-Wallis test followed by the Mann-Whitney *U* test. ***$P < 0.001$; original magnification ×800

B, panel II). Western blot analysis demonstrated overexpression of activated caspase 9 (~37 kDa band) in grade II tissue, compared with in normal pancreas (Fig. 3Ae).

Caspase 3 protein expression

Caspase 3 was upregulated in acinar cells of CP tissues, while normal tissues showed very faint positivity for caspase 3. Grade I CP tissues showed weak positivity, whereas grade II and III tissues showed strong to moderate positivity for caspase 3 in acinar cells (Fig. 4Ai–l and B, panel III). Western blot analysis showed overexpression of activated caspase 3 (17 and 20 kDa bands) in grade II tissue, compared with in normal pancreas (Fig. 3Af).

Measurement of caspase 9 and caspase 3 enzyme activation

Caspase 9 activity measured quantitatively in whole CP tissue lysate by fluorogenic assay reached a maximum in grade II and III tissues (i.e., 4 nmol/mg compared with 0.4 nmol/mg in grade I CP tissues; Fig. 5A). Similarly, maximum caspase 3 activity was found in grade II and III CP tissues (i.e., 5 nmol/mg, compared with 0.9 nmol/mg in grade I tissue; Fig. 5B). Normal pancre-

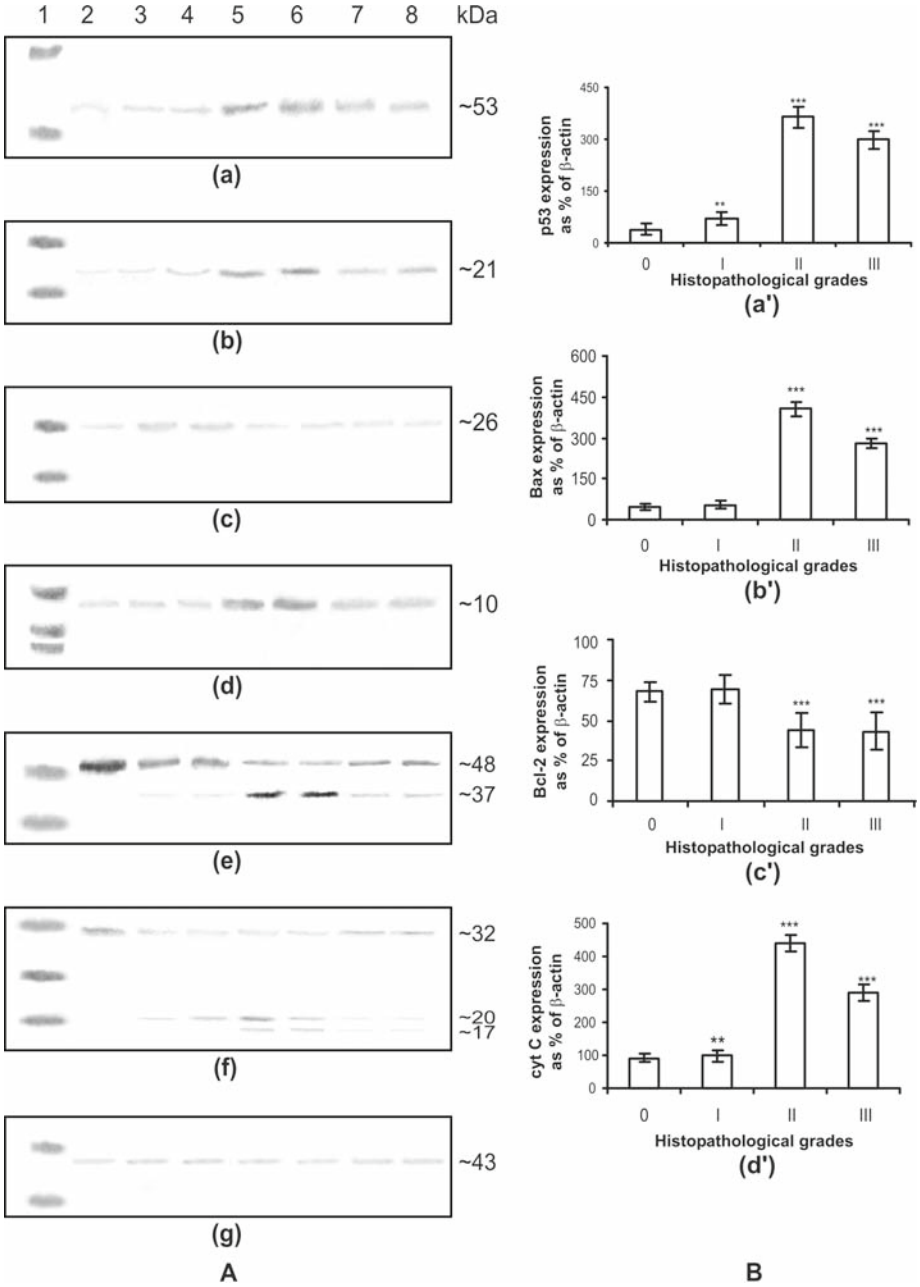

Fig. 3. A Western blot analysis in pancreatic tissue lysates exhibiting protein expression of p53 (*a*), Bax (*b*), Bcl-2 (*c*), cytochrome *c* (*d*), caspase 9 (*e*), caspase 3 (*f*), and β-actin (*g*) in normal and graded CP tissues: *lane 1*, protein molecular weight marker; *lane 2*, normal tissue; *lanes 3–8:* CP tissues of grade I (*lane 3, 4*); grade II (*lane 5, 6*) and grade III (*lane 7, 8*). **B** Histograms showing p53 (*a′*); Bax (*b′*); Bcl-2 (*c′*); and cytochrome *c* (*d′*) protein expression as % age of β-actin in graded CP tissues. Data shown are expressed as means ± SD. Statistical analysis by Kruskal-Wallis test followed by the Mann-Whitney *U* test. ***$P < 0.001$; **$P = 0.010$

atic tissues showed basal levels of caspase 9 and caspase 3 enzyme activity (0.2 and 0.4 nmol/mg, respectively). Caspase 9 and 3 inhibitors blocked proteolytic activity of caspase 9 and caspase 3, to a minimum level and to close to the basal level, respectively.

Assessment of mitochondrial membrane potential

Mitochondrial membrane potential (Ψ_m), measured by mean fluorescent intensity using the fluorochrome dye $DiOC_6(3)$, was significantly decreased in acinar cells isolated from CP tissues compared with those isolated from normal pancreas (Fig. 5C). A 1.2-fold decrease of Ψ_m was observed in grade I, and 2.2-fold decreases in grade II and III, CP tissues (Fig. 5D). Retention of dye by control cells was considered to be 100%.

ATP levels

The ATP concentration was measured and the percentage loss of ATP was calculated from the ATP curve. ATP depletion of 23% in grade I, 42% in grade II, and 84% in grade III CP tissues was observed, compared with the normal pancreas (Fig. 5E).

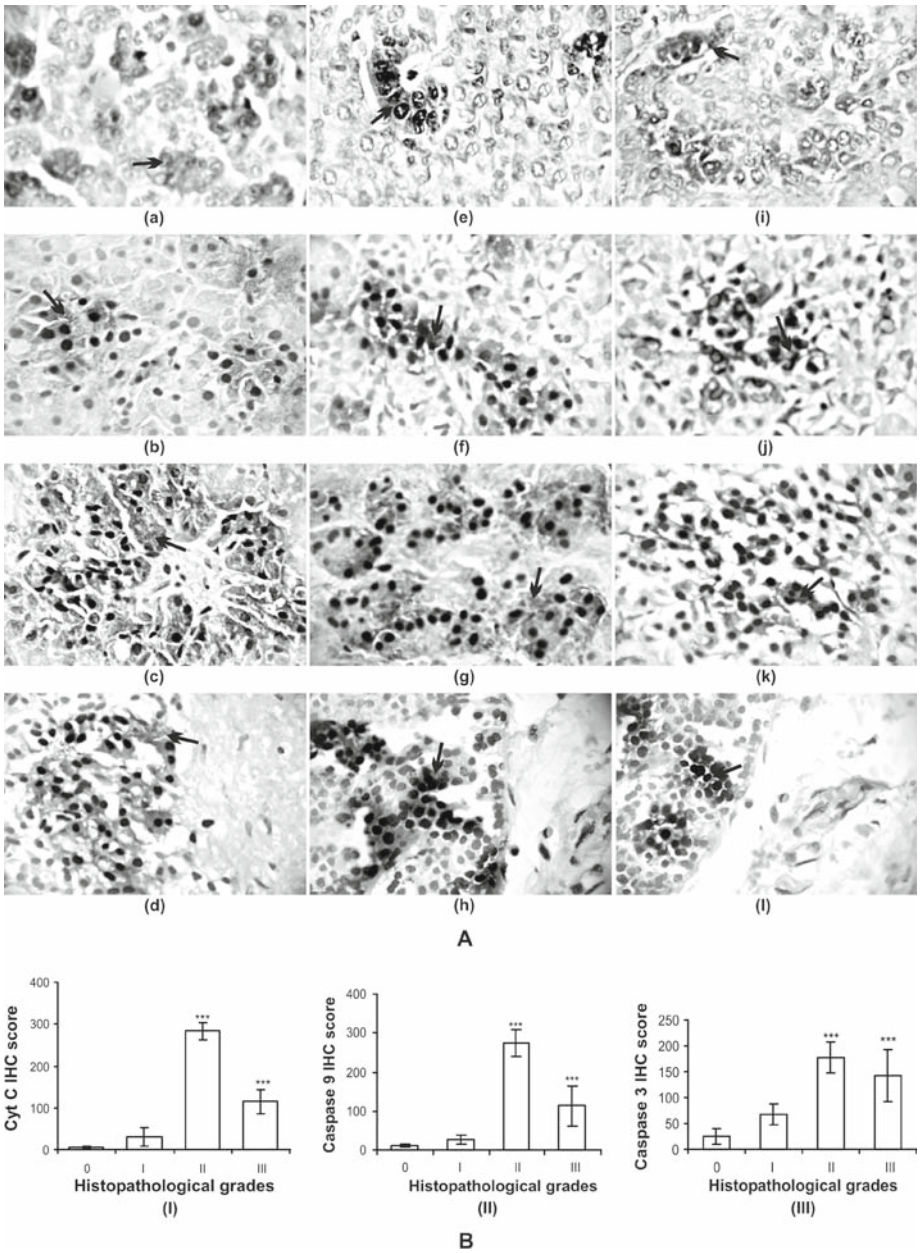

Fig. 4. A Immunohistochemistry of cytochrome (*Cyt*) *c*, caspase 9, and caspase 3 protein expression in pancreatic tissue sections. *a–d* Cytochrome *c* expression in normal tissue (*a*) and graded (I–III) CP tissues showing cytoplasmic localization (*b, c, d*). *e–h* Caspase 9 expression in normal tissue (*e*) and graded (I–III) CP tissues showing cytoplasmic localization (*f, g, h*). *i–l* Caspase 3 expression in normal tissue (*i*) and graded (I–III) CP tissues showing cytoplasmic localization (*j, k, l*). *Arrows* show the positivity for immunostaining of respective apoptotic markers in acinar cells. **B** Histograms showing cytochrome *c* (*I*), caspase 9 (*II*), and caspase 3 (*III*) expression in normal and graded CP tissues. Data shown are expressed as means ± SD. Statistical analysis by Kruskal-Wallis test followed by the Mann-Whitney *U* test. $***P < 0.001$; original magnification ×800

Discussion

Because apoptosis plays a critical physiological role, it is not surprising that deregulation of apoptosis occurs frequently during pathophysiological disturbances, during which the cellular apoptotic machinery may be triggered, leading to rapid and extensive cell death and tissue dysfunction. Several studies in pathophysiological animal models of pancreatitis have documented the role of programmed cell death. Hashimoto et al.[30] reported murine pancreatic acinar apoptosis, determined by TUNEL assay, in relation to glucocorticoid exposure. Similar observations, mainly in murine/rodent models have been reported by other investigators, supporting the role of apoptosis in CP.[9,10] However, the role of apoptosis in the progression of CP and the mechanism behind acinar cell loss in human CP is less well defined. Hence, in the current study, we explored the extent of apoptosis in CP tissues of different histopathological grades and the possible mechanisms involved. Both the DNA fragmentation assay and the estimation of the apoptotic index by TUNEL assay confirmed more apoptosis in CP than in normal cells, supporting earlier observations.[31] The apoptotic index range (0.09–0.86) in

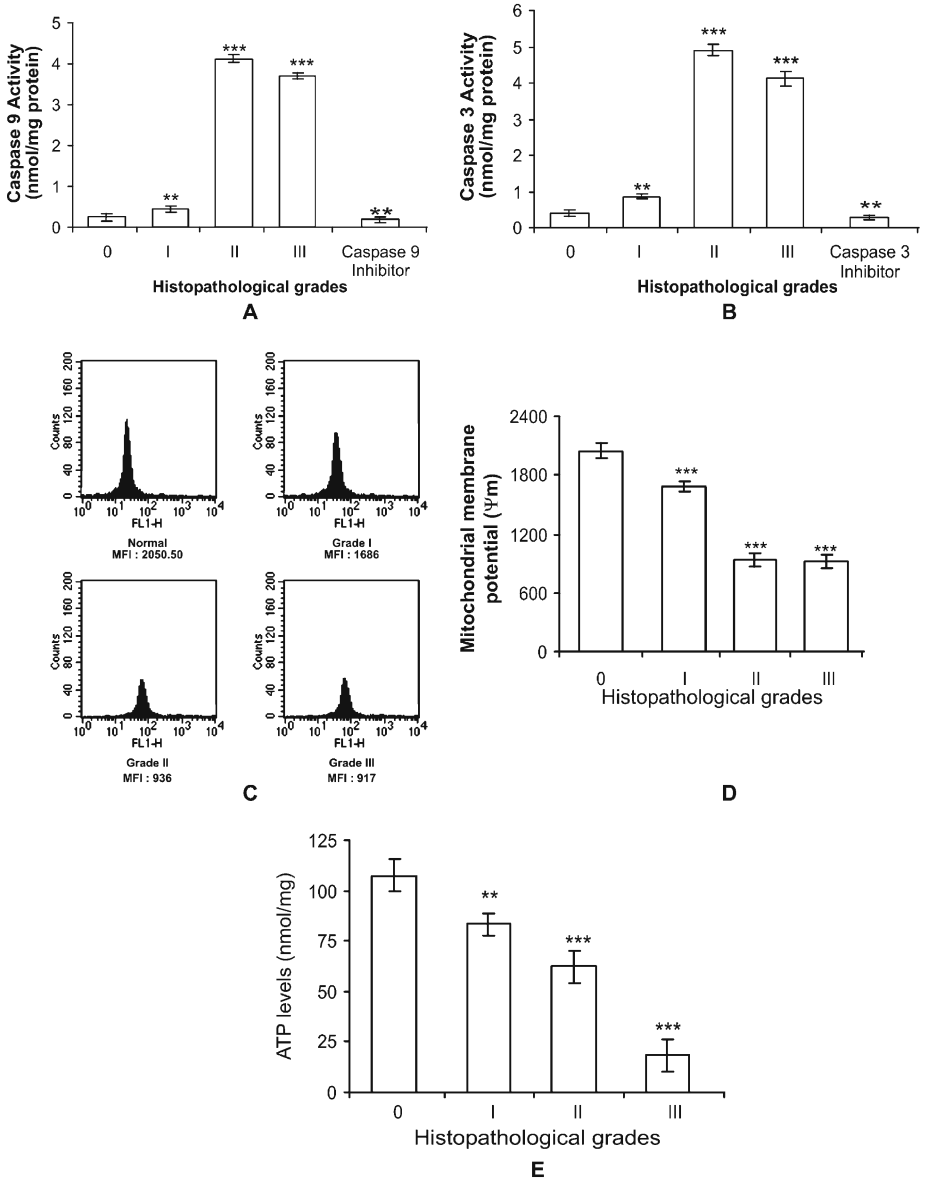

Fig. 5A–E. **A, B** Histograms showing quantitative measurement of caspase 9 (**A**) and caspase 3 (**B**) activity (nmol/mg protein) in graded CP tissues. **C** FACS flow cytometric analysis showing membrane potential Ψ_m in normal and graded CP tissues. **D, E** Histograms showing Ψ_m (**D**) and ATP levels (**E**) (nmol/mg) in normal and graded CP tissues. Data shown are expressed as means ± SD. Statistical analysis by Kruskal-Wallis test followed by the Mann-Whitney *U* test. ***$P < 0.001$; **$P = 0.004$

graded CP tissues was found to be significantly higher than the range of 0.091–0.26 previously reported by Bateman et al.[15] for acinar cells from CP tissues, and the range in normal pancreas (0–0.024).

Various stimuli are known to transmit the death signal via specific sensors to the caspase apoptotic machinery under pathophysiological conditions. In the case of CP, very little is known about the downstream targets and pathway involved during the entire course of disease. This led us to study the expression and the level of various proapoptotic and antiapoptotic markers in tissues with different histopathological grades from CP patients. One such proapoptotic protein is p53, whose expression is known to increase in response to cellular stress, resulting in committed death through apoptosis. Maacke et al.[32] showed overexpression of p53 in 59% of patients with CP and suggested that it reflected DNA damage caused by the disease. The present study also found eight- and ninefold overexpression of p53 in grade II and III tissues, and twofold in grade I CP tissues.

Further, activated p53 is known to induce expression of Bax,[33] another proapoptotic Bcl-2 homolog residing in the cytoplasm or in the mitochondrial membrane, which antagonizes the protective role of antiapoptotic Bcl-2. The Bax:Bcl-2 ratio determines the susceptibility of a cell to apoptosis.[34] Complex interactions between Bcl-2 family molecules (i.e., formation of Bax–Bcl-2 heterodimers or Bax–Bax homodimers) within both normal and CP tissues have been shown.[15] Other

studies have shown that in cells overexpressing Bax, Bax homodimers predominate, causing them to show enhanced susceptibility to apoptotic stimuli, and that in cells overexpressing Bcl-2, Bax–Bcl-2 heterodimers predominate, resulting in the reduced susceptibility of these cells to apoptosis.[35] Hence, in the present investigation, we investigated the expression of both Bax and Bcl-2. We observed a significant increase in Bax expression in CP tissues compared with normal tissues, which was positively correlated with the histopathological grade and the apoptotic index. Bcl-2 expression was downregulated in grade II and III CP tissues compared with normal pancreas, which showed the basal level of Bcl-2 expression. Other investigators have also observed upregulation of Bax and downregulation of Bcl-2 in acinar cells in a murine model of pancreatitis.[15,36]

The mitochondria are known to play a key role in the regulation of apoptosis.[37] The mitochondrial permeability transition (MPT; increased permeability of mitochondrial membranes) is a major cause of cell death in several pathological conditions,[38,39] and overexpression of Bax has also been correlated with both MPT and typical features of apoptosis.[24] Opening of the MPT pores, which is regulated by members of the Bcl-2 family, causes the release of soluble proteins such as cytochrome *c*, which also plays a role in apoptosis.[33] We studied mitochondrial dysfunction by evaluating the mitochondrial membrane potential (Ψ_m) and cytochrome *c* expression in CP tissues of different histopathological grade and in normal tissue, and observed a negative correlation between Ψ_m and both CP grade and the apoptotic index. Increased levels of cytochrome *c* were observed in the cytoplasm of acinar and ductal cells of CP patients, compared with normal tissue, and reached a maximum in grade II CP tissues. The cytochrome *c* level was positively correlated with the CP grade.

The efflux of cytochrome *c* from the mitochondria drives the assembly of a high-molecular-weight caspase-activating complex in the cytoplasm called the mitochondrial apoptosome. Once activated, the caspases generate an intracellular protease cascade leading to cellular demise and exhibiting typical hallmark features of apoptotic cell death.[40] The present study demonstrated the differential expression of caspase 9 and caspase 3 in histopathologically graded CP tissues, and maximum expression occurred in grade II tissue. Previous results of Mareninova et al.[41] showing caspase 3-dependent cell death in pancreatitis leading to degradation of cellular constituents support our findings.[41]

Mitochondrial dysfunction may be mediated by upstream caspases[42] triggered by a variety of stimuli acting directly on the mitochondria, or by an unknown upstream controller, which may be further modulated by the availability of ATP. Thus, we investigated ATP depletion in histopathologically graded CP tissues and found an ATP loss of 23%–84% in CP tissues. We observed a maximum loss of 84% observed in grade III tissues, indicating possible necrosis, as suggested by to Cosen-Binker et al.,[28] who considered a loss of >80% to indicate necrosis. The drastic depletion of ATP in grade III tissue was correlated with inhibition of the ongoing apoptotic pathway and a switch to a strong necrotic mechanism associated with the proinflammatory process.[14]

Our study confirmed active apoptosis in CP, demonstrated or the first time the levels of apoptosis in CP tissues with different histopathological scores, and suggested a possible pathway. The study also showed differential expression of the apoptotic markers p53, BAX, cytochrome *c*, caspase 9, and caspase 3, involved in mitochondrial dysfunction, and ATP loss within acinar cells of the histopathologically graded CP tissues, correlating these findings with disease progression. The identification of mechanisms involved in acinar cell death in pancreatitis is important because it can shed light on possible new therapeutic targets for chronic pancreatitis.

References

1. Stevens T, Conwell DL, Zuccaro G. Pathogenesis of chronic pancreatitis: an evidence-based review of past theories and recent developments. Am J Gastroenterol 2004;99:2256–70.
2. Bornman PC, Beckingham IJ. ABC of diseases of liver, pancreas, and biliary system. Chronic pancreatitis. BMJ 2001;322:660–3.
3. Garg PK, Tandon RK. Survey on chronic pancreatitis in the Asia-Pacific region. J Gastroenterol Hepatol 2004;19:998–1004.
4. Mori M, Hariharan M, Anandakumar M, Tsutsumi M, Ishikawa O, Konishi Y, et al. A case–control study on risk factors for pancreatic diseases in Kerala, India. Hepatogastroenterology 1999; 46:25–30.
5. Chari ST, Mohan V, Jayanthi V, Snehalatha C, Malathi S, Viswanathan M, et al. Comparative study of the clinical profiles of alcoholic chronic pancreatitis and tropical chronic pancreatitis in Tamilnadu, South India. Pancreas 1992;7:52–8.
6. Steinberg W, Tenner S. Acute pancreatitis. N Engl J Med 1994; 330:1198–210.
7. Bhatia M, Brady M, Shokuhi S, Christmas S, Neoptolemos JP, Slavin J. Inflammatory mediators in acute pancreatitis. J Pathol 2000;190:117–25.
8. Norman J. The role of cytokines in the pathogenesis of acute pancreatitis. Am J Surg 1998;175:76–83.
9. Kornmann M, Ishiwata T, Maruyama H, Beger HG, Korc MK. Coexpression of FAS and FAS-ligand in chronic pancreatitis: correlation with apoptosis. Pancreas 2000;20:123–8.
10. Su SB, Motoo Y, Xie M, Sawabu N. Apoptosis in rat spontaneous chronic pancreatitis. Dig Dis Sci 2004;46:166–75.
11. Walker NI, Winterford CM, Williamson RM, Kerr JFR. Ethionine-induced atrophy of rat pancreas involves apoptosis of acinar cells. Pancreas 1993;8:443–9.
12. Kishimoto S, Iwamoto S, Matstani R. Apoptosis of acinar cells in the pancreas of rats fed on a copper-deficient diet. Exp Toxicol Pathol 1994;45:489–95.
13. Watanabe S, Abe K, Anbo Y, Katoh H. Changes in the mouse exocrine pancreas after pancreatic duct ligation a qualitative

and quantitative histological study. Arch Histol Cytol 1995;58: 365–74.
14. Jones BA, Gores GJ. Physiology and pathophysiology of apoptosis in epithelial cells of the liver, pancreas and intestine. Am J Physiol 1997;273:1174–88.
15. Bateman AC, Turner SM, Thomas KS, McCrudden PR, Fine DR, Johnson PA, et al. Apoptosis and proliferation of acinar and islet cells in chronic pancreatitis: evidence for differential cell loss mediating preservation of islet function. Gut 2002;50:542–8.
16. Bhatnagar A, Wig JD, Majumdar S. Expression of activation, adhesion molecules and intracellular cytokines in acute pancreatitis. Immunol Lett 2001;77:133–41.
17. Singh L, Bakshi DK, Majumdar S, Vasishta RK, Arora SK, Wig JD. Expression of interferon-γ inducible protein-10 and its receptor CXCR3 in chronic pancreatitis. Pancreatology 2007;7: 479–90.
18. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual (2nd Ed.). New York: Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1989.
19. Miyamoto Y, Hosotani R, Wada M, Lee JU, Koshiba T, Fujimoto K, et al. Immunohistochemical analysis of Bcl-2, Bax, Bcl-X and Mcl-1 expression in pancreatic cancers. Oncology 1999;56:73–82.
20. Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD. Measurement of protein using bicinchoninic acid. Anal Biochem 1985;150:76–85.
21. Towbin H, Stachelin T, Gordon J. Electrophoresis transfer of protein from polyacrylamide gels to nitrocellulose sheets procedure and some applications. Proc Natl Acad Sci U S A 1979; 76:4350–4.
22. Bruzzone R, Halban PA, Gjinovci A, Trimble ER. A new rapid method for preparation of dispersed pancreatic acini. Biochem J 1985;226:621–4.
23. Singh L, Bakshi DK, Majumdar S, Vasishta RK, Arora Sk, Wig JD. Primary culture of pancreatic (human) acinar cells. Dig Dis Sci 2008 Feb 2.
24. Pastorino JG, Tafani M, Rothman RJ, Marcinkeviciute A, Hoek JB, Farber JL. Functional consequences of the sustained or transient activation by Bax of the mitochondrial permeability transition pore. J Biol Chem 1999;274:31734–9.
25. Liu D, Martino G, Thangaraju M, Sharma M, Halwani F, Shen SH, et al. Caspase-8-mediated intracellular acidification precedes mitochondrial dysfunction in somatostatin-induced apoptosis. J Biol Chem 2000;275:9244–50.
26. Gukovskaya AS, Gukovsky I, Jung Y, Mouria M, Pandol SJ. Cholecystokinin induces caspase activation and mitochondrial dysfunction in pancreatic acinar cells. Roles in cell injury processes of pancreatitis. J Biol Chem 2002;277:22595–604.
27. Fukumura A, Tsutsumi M, Tsuchishima M, Takase S. Correlation between adenosine triphosphate content and apoptosis in liver of rats treated with alcohol. Alcohol Clin Exp Res 2003;27:12S–5S.
28. Cosen-Binker LI, Binker MG, Cosen R, Negri G, Tiscornia O. Relaxin prevents the development of severe acute pancreatitis. World J Gastroenterol 2006;12:1558–68.
29. Fortunato F, Gates LK Jr. Alcohol feeding and lipopolysaccharide injection modulate apoptotic effectors in the rat pancreas in vivo. Pancreas 2000;21:174–80.
30. Hashimoto T, Yamada T, Yokoi T, Sano Ho H, Nakazawa T, Ohara H, et al. Apoptosis of acinar cells is involved in chronic pancreatitis in Wbn/Kob rats: role of glucocorticoid. Pancreas 2000;21:296–304.
31. Kaiser AM, Saluja AK, Sengupta A, Saluja M, Steer ML. Relationship between severity, necrosis, apoptosis in five models of experimental acute pancreatitis. Am J Physiol 1995;269: 1295–304.
32. Maacke H, Kessler A, Schmiegel W, Roeder C, Vogel I, Deppert W, et al. Overexpression of p53 protein during pancreatitis. Br J Cancer 1997;75:1501–4.
33. O'Reilly MA, Staversky RJ, Stripp BR, Finkelstein JN. Exposure to hyperoxia induces p53 expression in mouse lung epithelium. Am J Res Cell Mol Biol 1998;18:43–50.
34. Yang E, Korsmeyer S. Molecular thanatopsis: a discourse on the Bcl-2 family and cell death. Blood 1996;88:386–401.
35. Hengartner MO. The biochemistry of apoptosis. Nature 2000;407: 770–6.
36. Wada M, Doi R, Hosotani R, Lee JU, Fujimoto K, Koshiba T, et al. Expression of Bcl-2 and PCNA in duct cells after pancreatic duct ligation in Rats. Pancreas 1997;15:176–82.
37. Susin SA, Zamzami N, Kroemer G. Mitochondria as regulators of apoptosis: doubt no more. Biochim Biophys Acta 1998;1366: 151–65.
38. White RJ, Reynolds IJ. Mitochondrial depolarization in glutamate-stimulated neurons: an early signal specific to excitotoxin exposure. J Neurosci 1996;16:5688–97.
39. Ichas F, Mazat JP. From calcium signaling to cell death: two conformations for the mitochondrial permeability transition pore switching from low- to high-conductance state. Biochim Biophys Acta 1998;1366:33–50.
40. Wang J, Lenardo MJ. Roles of caspases in apoptosis, development, cytokine maturation revealed by homozygous gene deficiencies. J Cell Sci 2000;113:753–7.
41. Mareninova OA, Sung KF, Hong P, Lugea A, Pandol SJ, Gukovsky I, et al. Cell death in pancreatitis: caspases protect from necrotizing pancreatitis. J Biol Chem 2006;281:3370–1.
42. Li H, Zhu H, Xu CJ, Yuan J. Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell 1998;94:491–501.