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Apoptosis is a major mechanism of deoxycholate-induced gastric mucosal cell death

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Redlak, Maria J., Miranda S. Dennis, and Thomas A. Miller. Apoptosis is a major mechanism of deoxycholate-induced gastric mucosal cell death. *Am J Physiol Gastrointest Liver Physiol* 285: G870–G879, 2003. First published June 4, 2003; 10.1152/ajpgi.00330.2002.—This study was undertaken to determine whether necrosis or apoptosis was the predominant mechanism responsible for gastric mucosal cellular death using the cell line known as AGS cells. Cells were exposed to various concentrations of deoxycholate (DC; 50–500 μM) for periods ranging from 30 min to 24 h. Lactic dehydrogenase (LDH) activity was used as a marker for necrotic cell death, whereas apoptosis was characterized by 4',6-diamidino-2 phenylindole staining, DNA gel electrophoresis, terminal deoxynucleotidyl transferase dUTP nick-end labeling assay and DNA-histone-associated complex formation. When cells were bathed in Hank's balanced salt solution, DC-induced necrosis was the predominant mechanism of cell death. In contrast, when cells were bathed in Ham's F-12 solution (a more physiologically relevant medium), no evidence of cytotoxicity (by LDH assay) was discernible when cells were exposed to DC (50–300 μM) for periods as long as 8 h; instead, clear evidence of apoptosis was noted that was time and dose dependent. When cells were exposed for 24 h to these DC concentrations, cytotoxicity was also present, indicating necrosis as well. Furthermore, acidification of the ambient environment also evoked a necrotic response when exposed to DC. We demonstrated that apoptosis induced by DC shows early activation of caspase-3 that is dependent on both receptor and mitochondrial pathways. Our results indicate that physiological concentrations of DC (50–300 μM) primarily induce cellular death through an apoptotic process. Only after prolonged exposure to DC or acidification of the bathing solution does necrosis also occur.

necrosis; caspases; cell culture; prostaglandins

THE POTENTIAL TOXIC EFFECT of bile salts on gastric mucosal cells is well established both clinically and experimentally (6, 8, 13, 30). In fact, duodenogastric reflux is thought to play a major role in the pathogenesis of human gastric ulceration, and bile seems to be a key participant in this process (8, 30). In previous studies (20, 21) using a human gastric mucosal cell line, known as AGS cells, we have shown that deoxycholate (DC), in concentrations ranging from 200 to 500 μM , consistently injures gastric cells, ultimately leading to irre-

versible damage and death if exposure is prolonged. The clinical relevance of these findings is obvious when it is realized that DC concentrations approaching 370 μM occur in stomach remnants after distal gastric resection (7).

The precise mechanism of cellular death induced by DC and other bile salts remains controversial. A necrotic pathway has been implicated in many studies evaluating effects of DC on gastric cells (9, 20, 21), but the possibility of apoptosis has also been proposed (26). In hepatocytes, apoptosis appears to be the major mechanism of cell death in response to DC exposure (16–18). This study was undertaken to clarify the relative roles of apoptosis vs. necrosis in mediating cellular death induced by DC in gastric mucosal cells and to define the conditions under which a particular pathway is more likely to supervene. AGS cells were used to accomplish these goals as we have employed in our studies previously.

MATERIALS AND METHODS

Gastric Epithelial Cell Culture

A human gastric epithelial cell line (AGS), purchased from American Type Culture Collection (Manassas, VA), was used in these experiments. The cells were seeded in 75-cm² culture flasks (Corning) and maintained in Ham's F-12 (Cellgrow) culture medium supplemented with 10% fetal bovine serum, 100 $\mu\text{g}/\text{ml}$ penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, and 0.25 $\mu\text{g}/\text{ml}$ amphotericin B. The cells were grown at 37°C in a humidified atmosphere of 5% CO₂. Experiments were performed in 3.5-cm² dishes or in 96-well tissue culture plates (Costar) at 80–90% confluence. Before DC treatment, medium was aspirated and cells were washed with PBS. Experiments were performed under two different conditions: 1) in Ham's F-12 (without serum, growth arrested) or 2) in HBSS (Cellgrow) plus 10 mM HEPES consisting of (in mM) 137 NaCl, 5.7 NaHCO₃, 5.3 KCl, 1.26 CaCl₂, and 0.8 MgSO₄. The cells were treated with DC at a dose range of 50–500 μM .

Chemicals and Reagents

The prostaglandin analog 16,16 dimethyl PGE₂ (Sigma; dmPGE₂) was maintained at –20°C as a stock solution of 1 mg/ml in ethanol. Deoxycholic acid (the sodium salt; Sigma), 10 mM stock solution in PBS, was incubated in water bath at 37°C for 20–30 min before each use. Other reagents and

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chemicals used in our studies included 4',6-diamidino-2 phenylindole (DAPI; Roche), propidium iodide (PI; Molecular Probes), caspase-3 inhibitor IV (Ac-DMQD-CHO, Calbiochem), caspase-6 inhibitor I (Z-VEID-FMK, Calbiochem), and caspase-9 inhibitor I (Z-LEHD-FMK, Calbiochem).

Measurement of Cellular Injury (Cellular Cytotoxicity)

Cellular injury was quantitated by measuring lactate dehydrogenase (LDH) release (5). LDH content was determined by using an LDH-Cytotoxicity Assay Kit (BioVision), which is based on a coupled enzymatic reaction that results in the conversion of a tetrazolium salt into a red formazan product. The increase in the amount of formazan produced in culture supernatant directly correlates with the increase in the number of lysed cells. The formazan was quantified spectrophotometrically by measuring its absorbance at 500 nm. Cytotoxicity in experimental samples measured as %LDH release was compared with cells treated with 1% Triton X-100.

Isolation and Measurement of Fragmented DNA

After treatment, cells were harvested and genomic DNA was isolated using a Suicide-Track DNA ladder isolation kit (Oncogene). Fragmented DNA was resolved on a 1.5% agarose-1x Tris-Acetate-EDTA, visualized by ethidium bromide staining and examined under a ultraviolet light. For detection of histone-associated DNA fragments, a Cell Death Detection ELISA kit (Roche Molecular Biochemicals) was used according to the manufacturer's instructions adapted for suspension cultures (12).

Determination of Apoptotic Cells

Cellular DNA was stained with PI or DAPI and examined under fluorescence microscopy. Apoptotic cells were examined by changes in their nuclear morphology, including shrinkage, condensation and margination, and fragmentation of chromatin. Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP deoxyuridine triphosphate nick-end-labeling (TUNEL) assay was used to determine the yield of apoptotic cells (ApoAlert DNA Fragmentation Assay kit, Clontech), according to the manufacturer's recommendations (10). In brief, TUNEL staining was performed in formaldehyde-fixed AGS cells permeabilized with 0.2% Triton X-100. Cells were incubated with TdT buffer containing fluorescein-dUTP and TdT enzyme at 37°C for 1 h in a humid chamber. Incorporation of fluorescein-dUTP at the free 3'-hydroxyl ends of fragmented DNA was terminated by washing with 2× SSC (0.3 M NaCl, 30 mM sodium citrate). The number of TUNEL-positive cells, expressed as percentage of total cells, was counted using a fluorescence microscope (Nikon) equipped with FITC filters.

Caspase Activity Assay

Caspase activation was determined from cytosol of AGS cells treated with DC for the indicated times. The catalytic activities of caspase-2, caspase-3, caspase-6, and caspase-8 were measured using the Apo Target Caspase colorimetric kit (Biosource), which is based on spectrophotometric detection of the chromophore p-nitroanilide, after cleavage from the labeled synthetic caspase substrate (39). Briefly, after the respective treatment cells were collected and spun down, then they were washed with ice-cold PBS and lysed (50 mM HEPES, 1 mM DTT, 0.1 mM EDTA, 10% glycerol, and 0.1% CHAPS, pH 7.4), nuclei were removed (12,000 g at 4°C for 15 min), and the supernatants (cytosol extracts) were frozen and stored at -70°C until usage. Proteolytic reaction was carried

out in reaction buffer (50 mM HEPES pH 7.4, 100 mM NaCl, 0.1% CHAPS, 10 mM DTT, 0.1 mM EDTA, 10% glycerol) containing cytosolic protein extract and freshly prepared colorimetric substrate (VDVAD-pNA for caspase-2; DEVD-pNA for caspase-3; VEID-pNA for caspase-6 and IETD-pNA for caspase-8) at 37°C for 1 h. The absorbance at 405 nm was quantified and normalized for protein content. Enzyme activity was expressed as absorbance at 405 nm/mg of protein.

Protein Concentration Assay

Protein concentration was determined by the Bradford method using the Bio-Rad protein assay reagent and BSA as a standard (3).

Statistical Analysis

All data represent at least three independent experiments and are expressed as the means ± SE. Statistical analysis was performed by a paired Student's *t*-test, with significant differences determined at $P < 0.05$. For figure preparation, agarose gels were photographed with Gel DOC 1000 (Bio Rad) and PowerPoint software was used for image creation.

RESULTS

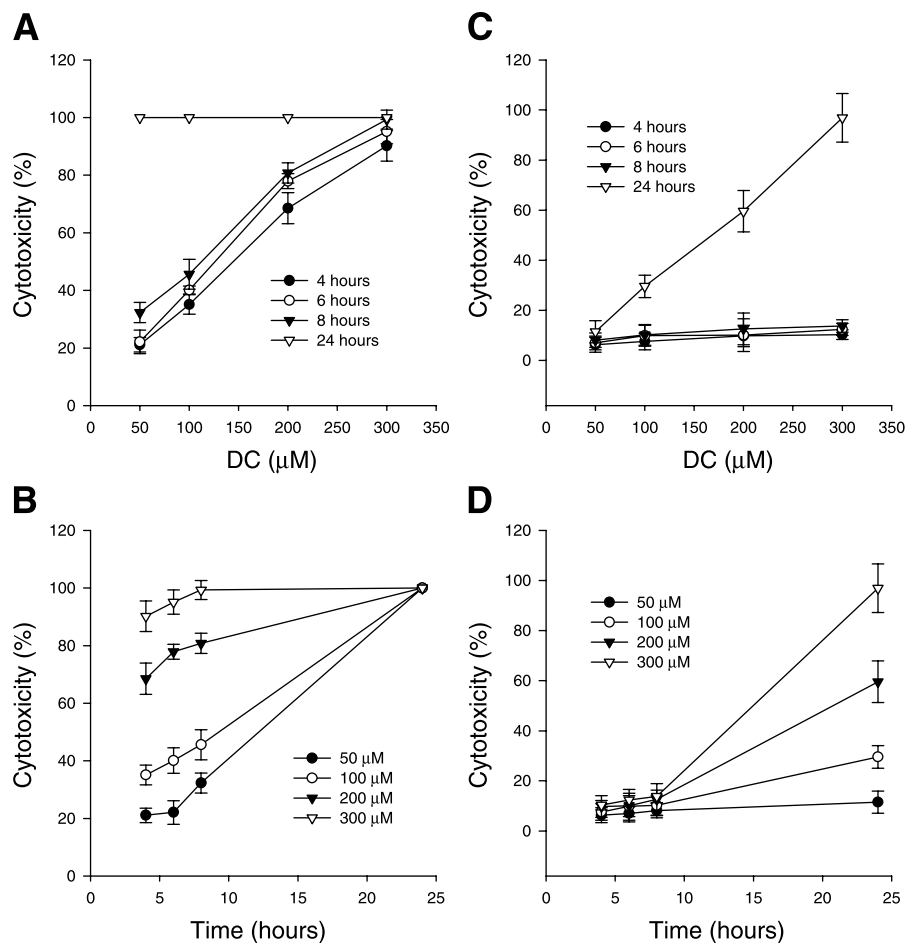
Effect of DC on Viability of AGS Cells

Necrosis as a dominant mechanism of cell death. In this study, we investigated the effect of DC on cytotoxicity of AGS cells in two different media conditions. Incubation of human gastric cells (AGS) with DC in HBSS produced a dose- and time-dependent increase in cell cytotoxicity measured as LDH release. The results presented in Figs. 1, A and B, and 2 show that DC concentrations from 50 to 200 μM had no effect on LDH release for up to 2 h, but longer exposure for DC induced a significant increase in LDH release. The most damaging effect was observed with 300 μM DC, with 20% cytotoxicity demonstrated at 30 min and 90% noted at 4 h of treatment. Such response to DC treatment was observed when AGS cells were incubated with DC in buffered HBSS only. Because the toxicity with 300 μM DC was so profound, higher concentrations of DC were not tested.

Surprisingly, when cells were treated with DC in Ham's F-12 medium, concentrations of DC ranging from 50 to 300 μM demonstrated no evidence of cytotoxicity by the LDH assay for cellular exposure times as long as 8 h (see Fig. 1, C and D). Only at 24 h of exposure did even high concentrations of DC (i.e., >50 μM) demonstrate some degree of cytotoxic damage.

Apoptosis as a dominant mechanism of cell death. To determine whether apoptosis is activated as a result of DC treatment, cells were treated with 100 and 300 μM DC in Ham's F-12. Cells were harvested for the indicated times, spun, and DNA was isolated from cell pellets and from supernatants. Figure 3, A and B, shows an agarose gel electrophoresis of DNA with the characteristic laddering pattern of fragmented DNA. Each band represents polynucleosomal DNA in its typical multiple 200-bp fragments. As shown in Fig. 3B, 300 μM DC caused a rapid increase (~50%) in the rate of DNA fragmentation over the basal value, which was sustained over a period of 1–12 h. For 100 μM DC (Fig.

Fig. 1. Concentration dependence and time dependence of cytotoxicity of AGS cells treated with deoxycholate (DC) in HBSS (A, B) and in Ham's F-12 (C, D) medium. Cytotoxicity, measured as %lactate dehydrogenase (LDH) release, was compared with cells treated with 1% Triton X-100. Results are expressed as means \pm SE from 4 experiments.



3A), longer time periods (4–12 h) were required to initiate \sim 10–20% apoptosis. When cells were exposed to concentrations of 100–300 μ M DC for 24 h, clear evidence of cytotoxicity (by LDH assay and DNA gel

electrophoresis, Figs. 1, C and D, and 3, A and B, bottom, respectively) was present, indicating necrosis as well.

Because the morphological changes in the cells undergoing apoptosis show characteristic nuclear condensation and formation of apoptotic bodies, we used two DNA-binding dyes to examine nuclei. PI- and DAPI-stained cells examined under the fluorescent light indicated fragmented nuclei compared with unfragmented ones in untreated cells (data not shown). Another indicator of apoptosis, internucleosomal cleavage of DNA, was determined and quantified by counting cells stained by the TUNEL method as described in MATERIALS AND METHODS. We found a concentration-dependent increase in TUNEL-positive cells exposed to DC (Fig. 4). Concentrations ranging from 100 to 300 μ M caused significant increases in the percentage of apoptotic cells, from 4.5 ± 2.3 to 45.2 ± 3.6 , respectively. Findings with 400–500 μ M DC were not different from those with 300 μ M DC.

We also assessed histone-associated DNA using an ELISA assay. In the early stage of apoptosis, the endogenous endonucleases cleave double-stranded DNA at the most accessible internucleosomal linker regions, generating mono- and oligonucleosomes (4, 29). In contrast, the DNA of the nucleosomes is tightly complexed with the core histones H2A, H2B, H3, and H4 and

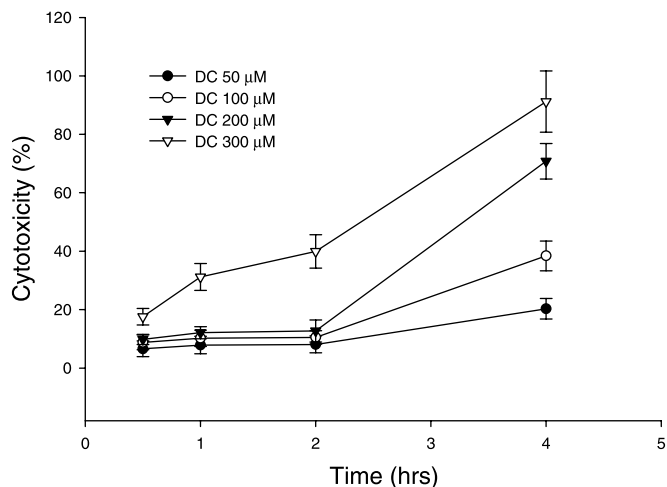


Fig. 2. Effect of different concentrations of DC on cell cytotoxicity. AGS cells were exposed to DC (50, 100, 200, 300 μ M) in buffered HBSS for the indicated times. The cytotoxicity, measured as %LDH release in culture supernatant, was compared with cells treated with 1% Triton X-100. Results are expressed as means \pm SE from 4 separate experiments.

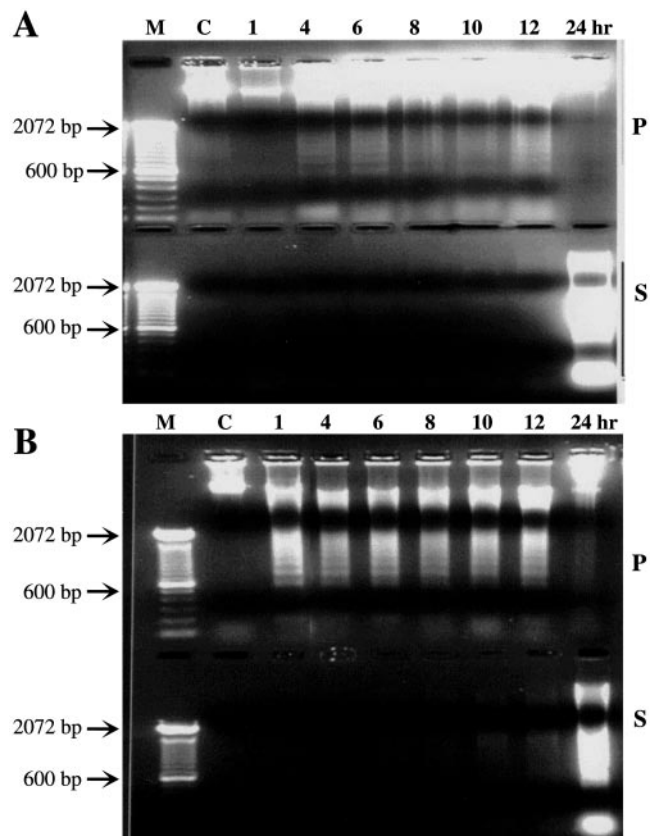


Fig. 3. Apoptotic DNA fragmentation in AGS cells treated with DC. Time course of DNA fragmentation induced by 100 (A) and 300 μ M (B) DC. Cells were treated with DC for the indicated times, harvested, and pelleted. DNA was extracted from cell pellets (P, top) and from supernatants (S, bottom), and it was separated on a 1.5% agarose gel and visualized by ethidium bromide staining. M, molecular mass marker; C, control DNA from untreated cells. Results are representative of 3 separate experiments.

therefore is protected from cleavage by endonucleases (4, 29). ELISA analysis showed time- and concentration-dependent responses in the enrichment of histone-associated DNA fragments (see Fig. 5, A and B). A concentration of 100 μ M DC mediated a small but detectable and proportional increase in histone-DNA formation over a period of 8 h. However, a higher DC concentration (300 μ M) induced a rapid and 50-fold increase in histone-DNA formation, compared with control levels, after 1–3 h of DC-induced apoptosis. Longer times of exposure to 300 μ M DC showed decreases in histone-DNA formation, which indicates the stage when nucleosomal histones and DNA are no longer tightly complexed.

Acidification and apoptosis. An additional series of experiments was conducted to determine the effect of acidification on apoptosis. Studies were performed in identical fashion to those described except that the pH of the cellular bathing solution was altered by adding HCl. A range of pHs were evaluated from pH 3 to 7.4. Control studies assessed the effect of pH change alone, whereas experimental studies evaluated the combination of acid and DC. Apoptosis in these studies was determined by DNA laddering.

Acidification by itself had no effects on AGS cells. No evidence of DNA laddering was noted at either 1 or 4 h regardless of the pH range tested (i.e., pH 3–6, and 7.4; data not shown). When DC (300 μ M) was added to the bathing solution, the resultant response was pH dependent. At neutral pH (i.e., 7.4), DC initiated DNA laddering at both 1 and 4 h, with the response being more pronounced at the latter time period. With acidification, evidence of DNA laddering could no longer be demonstrated. In fact, with prolonged acidification (4 h), DC in acid medium at pH 4 actually induced clear evidence of necrosis in contrast to apoptosis when the pH was 7.4. At 1 h of acidification at pH 4, this necrotic response was present but not near as pronounced as at 4 h. These results indicate that acidification shifts the pattern of cell death from apoptosis to necrosis when AGS cells are exposed to DC. These findings are shown in Fig. 6.

Effect of DC on Caspase Activity

Caspases that are involved in the execution of apoptosis exist in living cells as inactive zymogens that become activated through intracellular caspase cascades. There are two caspase cascades (1, 14). One is initiated by the activation of cell-surface death receptors, such as Fas and tissue necrosis factor, leading to caspase-8 activation, which, in turn, cleaves and activates downstream caspases such as caspase-3, -6, and -7. In this pathway, mitochondrial signaling seems to be unnecessary but may contribute to apoptosis at a later time as an amplification mechanism (24, 34, 43). The second general apoptotic pathway involves cytochrome *c* release from mitochondria, which promotes the activation of caspase-9 through APAF-1 (1, 14, 23, 25, 33, 37, 45). Recent observations suggest that bile salt-induced apoptosis in hepatocytes is mediated by the Fas-receptor signaling pathway and caspase-8 ac-

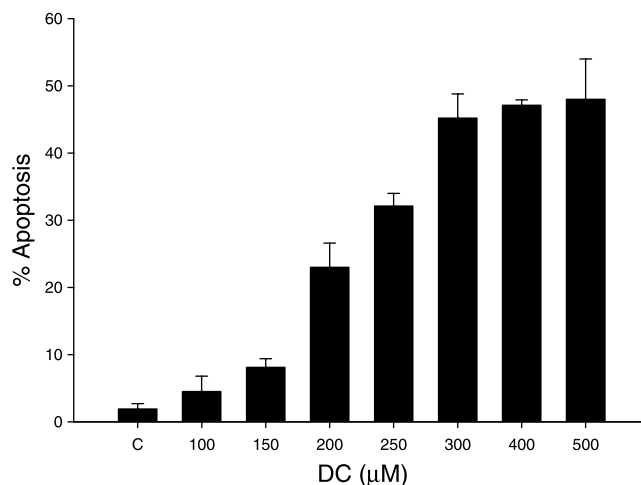


Fig. 4. Concentration dependence of DC-induced apoptosis. AGS cells were incubated for 4 h with different concentrations of DC from 100 to 500 μ M. The number of apoptotic cells [terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) positive] was expressed as %total cells. Results shown are means \pm SE of 4 experiments.

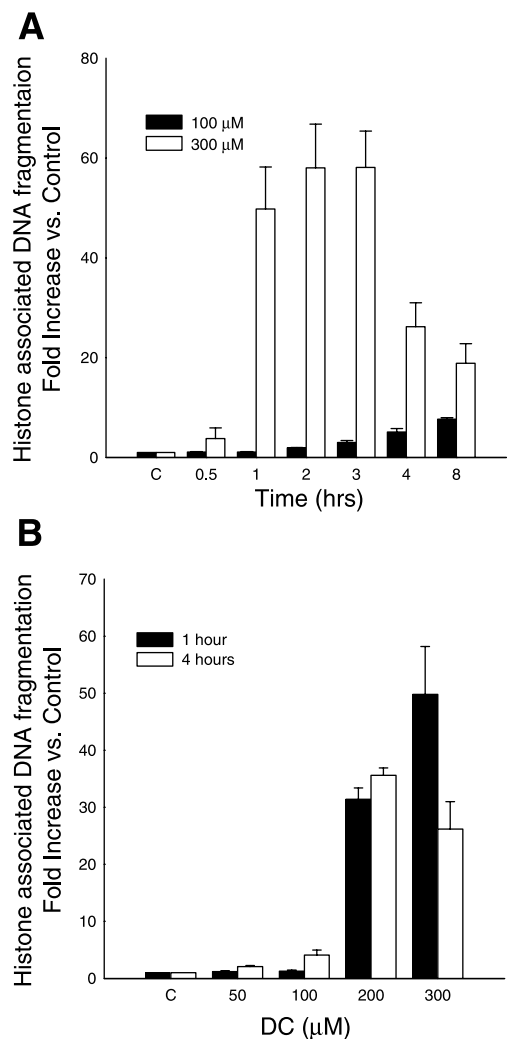


Fig. 5. Time course (A) and concentration dependence (B) of DC-induced DNA-histone complex formation. Cells were treated with 50, 100, 200, and 300 μ M DC for indicated times, harvested, and lysed. DNA-histone-associated complexes were isolated and assayed by the ELISA method. Results are presented as means \pm SE from 3 experiments.

tivation (11). There is also evidence that bile salts can directly mediate mitochondrial permeability transition in isolated mitochondria from hepatocytes (2, 32) and from the HT-29 cell line (36). Enhanced activation of caspase-8 during apoptosis after mitochondrial dysfunction has been reported in specific cell types (36). In addition, release of the zymogen form of caspase-2 from mitochondria and caspase-2 expression is reported in HT-29 colon carcinoma cells treated with DC (36, 40). Recent findings also suggest that caspase-2 may be a key initiator of the mitochondrial pathway of apoptosis (23). We therefore analyzed the activation of caspase-2, -3, -6, and -8 in the cytoplasm of DC-treated AGS cells by colorimetric assays. Caspase-3 activity was tested by its ability to cleave synthetic peptide substrate DEVD-pNA and was noted to be detectable within 15 min after the start of DC treatment, with maximum activity at 1 h (see Fig. 7). Interestingly, we also found caspase-2, -6, and -8 activation. Caspase-2 cleaved syn-

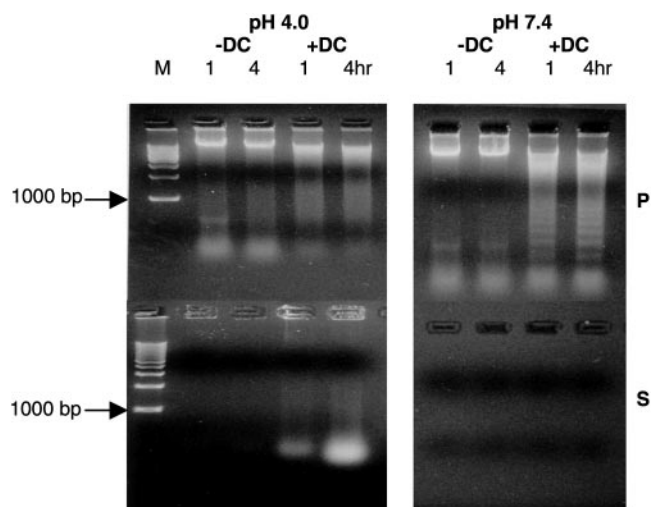


Fig. 6. Effect of media acidification on DC-induced DNA fragmentation. AGS cells were treated for 1 and 4 h with 300 μ M DC in acid medium at pH 4 (left) or in neutral medium at pH 7.4 (right). Cells were harvested, and DNA was extracted from cell pellets (P) and from supernatants (S), separated on 1.5% agarose gel, and visualized by ethidium bromide staining. The presence of nucleic acids in supernatants from DC-treated cells in pH 4 indicates early disturbance of membrane cell integrity, which is characteristic of necrotic death. Results are representative of 3 separate experiments.

thetic substrate VDVAD-pNA, and a sustained increase of activity was detectable over a period of 2 h. The same pattern of enzyme activation was present for caspase-6 and -8, which cleaved VEID-pNA and IETD-pNA synthetic peptide substrate, respectively (see Fig. 7).

Experiments with selected caspase inhibitors have shown that activation of the caspase cascade is functionally involved because inhibition of caspases reversed the proapoptotic effect of DC. The most inhibitory effect was observed with the caspase-6 inhibitor,

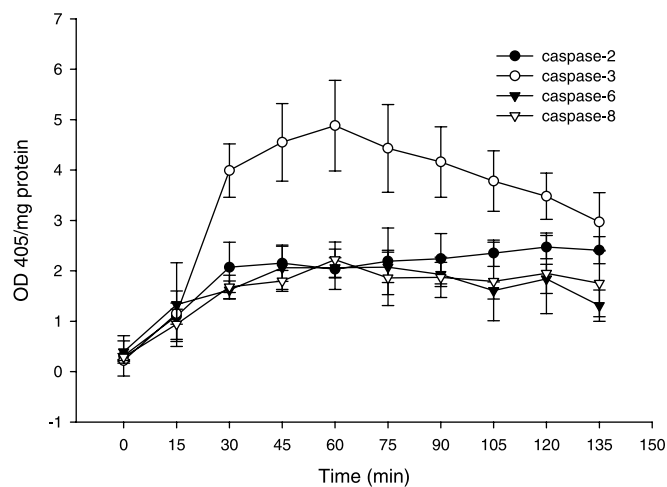


Fig. 7. Caspase-2, caspase-3, caspase-6, and caspase-8 activity from cytosol of DC-treated AGS cells. AGS cells were treated with 300 μ M DC and harvested at indicated times. Cytosol extracts cleaved specific substrates for selected caspases in a colorimetric assay as described in MATERIALS AND METHODS. Results are means \pm SE from 3 experiments. OD, optical density.

which at 100 μM concentration abolished completely DC-induced DNA fragmentation. Caspase-9 and -3 inhibitors were also effective and reversed DC-mediated apoptosis by 70 and 60%, respectively (see Fig. 8). Enhanced activation of caspase-3, measurable just after 15 min of DC treatment, indicates that this effector caspase is involved early in the signaling cascade. On the other hand, inhibition of another effector caspase, namely caspase-6, which completely prevented DC-induced apoptosis, indicates the particular and important role that this caspase also plays in transferring the signal downstream to DNA degradation. Our observations demonstrate that DC-induced apoptosis in the AGS cell line is mediated by both mitochondrial (caspase-2 and -9) and receptor (caspase-8) pathways; however, further study is necessary to elucidate the precise mechanisms of this process.

Effect of PGE₂ on Cytotoxicity and Apoptosis Induced by DC

We also investigated the effect of dmPGE₂ pretreatment on cytotoxicity and apoptosis in cells treated with DC. Several studies have implicated PG as a protective agent against cell injury (19, 20, 22, 44). Our findings demonstrated that the cytotoxic effect of DC, measured as LDH release, is attenuated when cells are pretreated with dmPGE₂. Figure 9 showed that a 30-min pretreatment with 2.6 μM dmPGE₂ decreased the cytotoxic effect of 300 μM DC at 0.5 to 2 h in cells incubated in HBSS/HEPES. However, we did not observe any protective effect of dmPGE₂ against apoptosis induced by DC in Ham's F-12 media (Fig. 10) when using a 2.6 μM concentration or even higher doses of this agent.

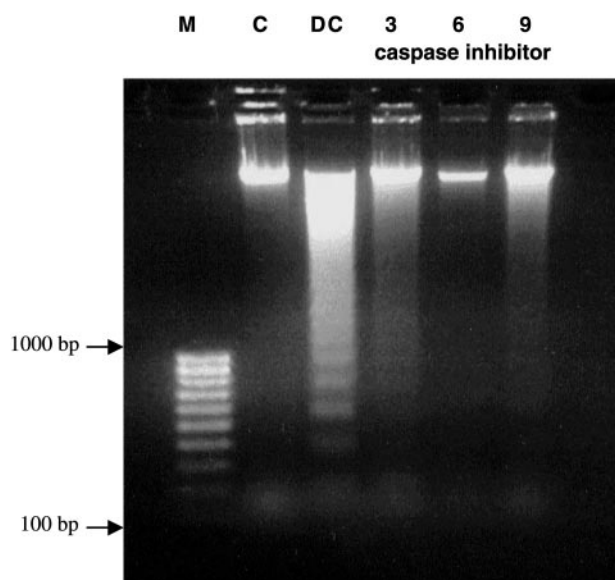


Fig. 8. Effect of caspase inhibitors on DC-induced apoptosis. AGS cells were pretreated with 100 μM of caspase-3 inhibitor (Ac-DMQD-CHO), caspase-6 inhibitor (α -VEID-FMK), and caspase-9 inhibitor (α -LEHD-FMK) for 1 h. Next, cells were treated with 200 μM DC for 1 h. Results are representative of 3 separate experiments.

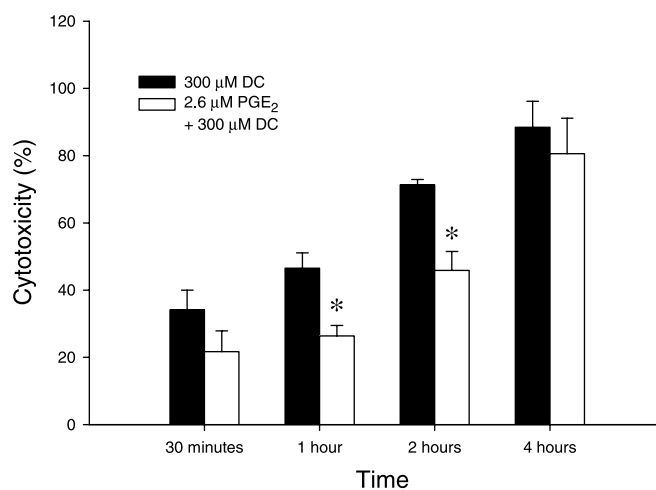


Fig. 9. Effect of PGE₂ on DC-induced cytotoxicity. AGS cells were pretreated with PGE₂ for 30 min, and then DC was added for indicated times. The cytotoxicity, measured as %LDH release in culture supernatant, was compared with cells with 1% Triton X-100. Results are expressed as means \pm SE from 3 experiments. * P < 0.05; statistically different from values with DC alone.

DISCUSSION

The present study was undertaken to determine the relative roles of necrosis vs. apoptosis in mediating cellular death in gastric mucosal cells exposed to DC. Although we had previously shown that DC could induce necrosis in gastric epithelial cells exposed to this damaging agent in a concentration-related fashion (20, 21), we wondered whether the design of our studies potentially obscured an apoptotic response that might have been discernable with lower concentrations of this substance and thereby unraveling a more fundamental mechanism of DC-induced injury and death. This consideration was entertained because various bile acids, including DC, have been noted to induce apoptosis in hepatocytes that generally did not evoke necrosis until

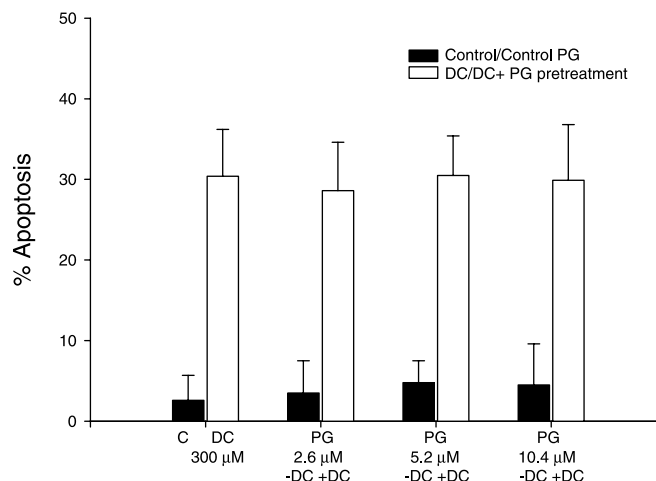


Fig. 10. Effect of prostaglandin (PGE₂) on DC-induced apoptosis. AGS cells were pretreated with PGE₂ (2.6, 5.2, and 10.4 μM) for 30 min, and then DC (300 μM) was added and cells were incubated for 4 h. The number of apoptotic cells (TUNEL positive) was expressed as %total cells. Results shown are means \pm SE of 4 experiments.

much higher concentrations were employed (24, 31). Interestingly, our results demonstrated that both apoptosis and necrosis can occur with DC exposure in gastric cells and that the primary response is, to a large extent, influenced by the culture media in which the cells are bathed.

In previous studies, we grew our cells in Ham's F-12 media and then conducted our experimental studies in HBSS. With this approach, we found that low concentrations of DC (i.e., $<200 \mu\text{M}$) were generally nontoxic until cells had been exposed to such concentrations for extended periods (20, 21). In contrast, higher concentrations could evoke significant damage within 20–30 min and often irreversibly killed the cells in a short period of time thereafter. Employing LDH release as a marker of necrotic damage, we were able to validate these earlier findings in this study, and we noted that concentrations of DC $<200 \mu\text{M}$ were generally non-damaging until such cells were exposed to this chemical insult for ≥ 4 h. If a higher concentration of DC (namely $300 \mu\text{M}$) was used to challenge our cells, significant damage was noted as early as 30 min, which became consistently more pronounced over time so that by 4 h, $\sim 90\%$ of the cells had died a necrotic death.

Unexpectedly, a different pattern emerged if cells were not only grown in Ham's F-12 media but continually maintained in this environment for our experiments. Under these conditions, a concentration of DC as high as $300 \mu\text{M}$ elicited no evidence of cellular toxicity, as measured by LDH release, even after continuous exposure for 8 h. After 24 h, this concentration evoked considerable cellular death by necrosis, but a lower concentration such as $100 \mu\text{M}$ DC, which induced necrosis in $\sim 30\%$ of cells within 4 h when maintained in Hank's solution, only elicited a 20% necrotic response at 24 h in Ham's F-12 media. Of further note, clear evidence of apoptosis could be demonstrated in cells exposed to DC in Ham's F-12 media that was both concentration and time related. Thus, at 4 h of incubation in this media, an apoptotic response was evoked in fewer than 10% of cells if the concentration of DC was $150 \mu\text{M}$ or less. With a concentration of $200 \mu\text{M}$, the apoptotic response involved 25% of cells. At higher concentrations, such as 400 and $500 \mu\text{M}$, this response reached a level of $\sim 50\%$.

To dissect out the events leading to apoptosis, we employed indexes of this type of cellular death that measured both early and late-term responses. Early markers that we employed included measurement of caspase activity and histone-associated DNA fragmentation. Depending on the concentration of DC used to damage gastric cells, caspase activation could be identified in <1 h after exposing cells to DC. For low concentrations of DC, such as 50 and $100 \mu\text{M}$ DC, no caspase activity was noted by 60 min following DC challenge. In contrast, caspase-3 activation was profoundly enhanced in cells exposed to 200 or $300 \mu\text{M}$ DC in this time frame (data not shown). In fact, in cells exposed to $300 \mu\text{M}$ DC, activation of caspase-3 was evident as early as 15 min after exposing cells to this damaging agent and reached its highest level by 60

min. Interestingly, caspase-2, caspase-6, and caspase-8 activation also occurred as briskly but did not achieve the levels observed with caspase-3. Because caspase-2 and caspase-8 are upstream initiators of caspase-3 and caspase-6 activation via both a receptor-mediated (i.e., caspase-8) and mitochondrial (i.e., caspase-2) mechanism, involvement of these caspases in bile-salt-induced apoptosis indicates that the processes through which DC mediates its apoptotic effects presumably employ both pathways.

Histone-associated DNA fragmentation was also markedly increased at 1 h following exposure to $300 \mu\text{M}$ DC. This effect remained sustained for at least 3 h and then slowly decreased. In agreement with our caspase data, no histone-associated DNA fragmentation was discernable with concentrations of DC in the range of 50 – $100 \mu\text{M}$ DC at either 1 or 4 h. Similar to $300 \mu\text{M}$ DC, however, $200 \mu\text{M}$ DC also elicited significant histone-associated DNA fragmentation as early as 1 h. Time course findings suggested that caspase activation precedes the DNA fragmentation. Furthermore, the fact that caspase inhibitors (such as caspase-3, caspase-6, and caspase-9) can significantly prevent the formation of DNA fragmentation lends support to the contention that caspase activation is essential for the subsequent effects on DNA. Moreover, whereas blockades of caspases-3 and -6 are not surprising in their ability to prevent DNA fragmentation because they are both downstream effector caspases, the fact that caspase-9 inhibition is also effective suggests that the mitochondrion may also play a role because this caspase is an upstream initiator that orchestrates effects induced by this intracellular organelle. On the basis of these findings, a schematic representation of the possible pathways through which DC may trigger apoptosis in AGS cells is shown in Fig. 11.

In addition to these early markers of apoptosis, two late markers were also measured, namely DNA ladder-

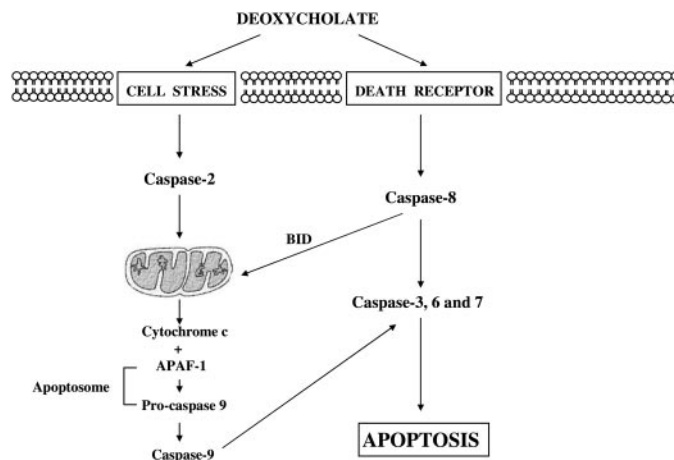


Fig. 11. Proposed pathways by which DC may induce apoptosis. Although most evidence suggests that activation of a death receptor is the primary pathway through which apoptosis occurs, induction of caspase-2 activation through cell stress, independent of the death-receptor pathway, may also play a key role.

ing and the TUNEL assay. TUNEL positivity was not consistently evident until 4 h of exposure to DC and was clearly concentration related as observed with the earlier markers. Thus low concentrations of DC, such as 100 and 150 μM , showed mild degrees of apoptosis by the TUNEL assay, whereas concentrations of 200 μM or greater demonstrated more pronounced effects. In concentrations of DC approaching 300 μM or greater, $\sim 50\%$ of cells were observed to be apoptotic by this assay. The results for DNA laddering were similar. Cells exposed to 100 μM DC demonstrated no consistent evidence of laddering until 4 h of exposure. In contrast, cells exposed to 300 μM DC had clear evidence of DNA laddering as early as 1 h following exposure to this damaging agent.

We previously reported that the prostaglandin dmPGE_2 could prevent DC-induced gastric damage in cells in which a necrotic mechanism of death occurred (20, 21). We confirmed this finding in the present study. Thus cells exposed to 300 μM DC in Hank's buffered salt solution demonstrated a time-related necrotic response with $>30\%$ of cells undergoing necrosis by 30 min and $>85\%$ of cells experiencing necrotic death by 4 h. Pretreatment with PGE_2 significantly attenuated the magnitude of this necrotic response during the first 2 h of DC exposure. By 4 h, this efficacy was overcome. Interestingly, in cells exposed to this same concentration of DC and maintained in Ham's culture media in which apoptosis occurred, pretreatment with dmPGE_2 was ineffective in attenuating the apoptotic response (see Fig. 10). This being the case, such findings suggest that PGE_2 can prevent the necrotic pathway of death but apparently has no effect on the apoptotic pathway. The mechanism(s) responsible for this difference in action remains to be elucidated.

As shown in Fig. 6, an ambient pH of 4.0 altered the method of cell death in response to DC exposure to one of necrosis in contrast to an apoptotic pathway at pH 7.4. There are several possible explanations for this finding. First, it is well known that when gastric cells are bathed with luminal acid, some of this acid back-diffuses into these cells. Normally, this is not a problem because intracellular buffering mechanisms adequately neutralize this influx and thereby maintain the intracellular pH. In the presence of bile, however, this back-diffusion is substantially increased, making it more difficult for the cell to maintain acid-base balance. Second, bile is also known to inhibit active transport mechanisms within gastric cells under acidic conditions. This effect would further make it difficult to neutralize influxing hydrogen ions. Third, the generation of an "alkaline tide," which is present in an acid-secreting gastric epithelium and assists in maintaining an acid-base balance in the gastric epithelium when challenged by a toxic substance such as bile, is absent under resting conditions and would clearly be absent in our model because it contains only gastric surface mucous cells. These mechanisms have been previously reviewed (27, 28, 42) and almost certainly are responsible, at least in part, for the differences in cell death induced by DC that we observed under acidic and

nonacidic ambient environments. Whether other mechanisms are also operational will require further elucidation. Thus, whereas DC clearly induces apoptosis in gastric mucosal cells, this process can revert to a necrotic pathway of cell death under acidic conditions. Consistent with our findings, ammonia-induced apoptosis in gastric mucosal cells has also been shown to be pH dependent and to optimally occur in a neutral environment (41).

Finally, why DC initiates necrosis when cells are maintained in Hank's balanced salt solution but seems to be more resistant to injury and undergo primarily an apoptotic mechanism of death in Ham's medium remains uncertain. Because Ham's solution is primarily an amino acid-rich media, whereas Hank's solution is primarily a balanced salt solution, it is possible that the presumed growth-enhancing capabilities of Ham's solution enable cells to be more resistant to a noxious insult than what occurs under the other experimental conditions. Glutamine is a particularly important amino acid in Ham's solution and has been shown to be an essential nutrient in maintaining the health of gastrointestinal cells (15, 38). Thus it seemed likely that the rich amino acid mixture comprising Ham's solution, and particularly glutamine, provided a more healthy environment for cells, making it a more appropriate medium to study potentially injurious processes than the Hank's solution, which has been commonly employed in such research. To test the potential role that glutamine might play in protecting cells exposed to DC from following a necrotic pathway toward cell death, we added glutamine to Hank's buffer before challenging cells with DC when studied in this solution. Such glutamine addition did not prevent the induction of the necrotic pathway of cell death (data not shown). This being the case, further study is obviously needed to dissect out the parameters responsible for the different results that we observed with DC when cells were maintained in these differing culture media.

In conclusion, we have shown that DC can induce both necrosis and apoptosis in gastric epithelial cells and that the predominant mechanism of cell death is dependent upon the environment in which the cells are cultured. Because Ham's culture media more likely simulates conditions that would exist under *in vivo* circumstances, we believe that apoptosis is the major mechanism by which gastric cells die when exposed to damaging concentrations of DC. When cells are housed in an environment less likely to exist under normal living conditions, such as Hank's balanced salt solution, they become less resistant to the potential toxic effects of DC and die a necrotic death.

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DISCLOSURES

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REFERENCES

1. **Ashenazi A and Dixit VM.** Death receptors: signaling and modulation. *Science* 281: 1305–1308, 1998.
2. **Botla R, Spivey JR, Aguilar H, Bronk SF, and Gores GJ.** Ursodeoxycholate (UDCA) inhibits the mitochondrial membrane permeability transition induced by glycochenodeoxycholate: a mechanism of UDCA cytoprotection. *J Pharmacol Exp Ther* 272: 930–938, 1995.
3. **Bradford MM.** A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72: 248–254, 1976.
4. **Burgoyne LA, Hewish DR, and Mobbs J.** Mammalian chromatin substructure studies with the calcium-magnesium endonuclease and two-dimensional polyacrylamide-gel electrophoresis. *Biochem J* 143: 67–72, 1974.
5. **Decker T and Lohmann-Matthes ML.** A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity. *J Immunol Methods* 25: 61–69, 115, 1988.
6. **Dempsey DT, Mercer DW, Deb B, Sauter A, and Ritchie WP.** Adaptive cytoprotection of gastric surface epithelial cells against injury by physiologic concentrations of bile acids. *Surgery* 108: 348–355, 1990.
7. **Domellof L, Reddy BS, and Weisburger JH.** Microflora and deconjugation of bile salts in alkaline reflux after partial gastrectomy. *Am J Surg* 140: 291–295, 1980.
8. **DuPlessis DJ.** Pathogenesis of gastric ulceration. *Lancet* 1: 974–978, 1965.
9. **Dziki AJ, Batzri S, Harmon JW, and Malloy M.** Cellular hypercalcemia is an early event in deoxycholate injury of rabbit gastric mucosal cells. *Am J Physiol Gastrointest Liver Physiol* 269: G287–G296, 1995.
10. **Facchinetti A, Tassarollo L, Mazzocchi M, Kingston R, Collavo D, and Biasi G.** An improved method for the detection of DNA fragmentation. *J Immunol Methods* 136: 1251–1256, 1991.
11. **Faubion WA, Guicciardi GE, Miyoshi H, Bronk SF, Roberts PJ, Sringen PA, Kaufmann SH, and Gores GJ.** Toxic bile salts induce rodent hepatocyte apoptosis via direct activation of Fas. *J Clin Invest* 103: 137–145, 1999.
12. **Fawthrop DY, Boobis AP, and Davies DS.** Mechanisms of cell death. *Arch Toxicol* 65: 437–444, 1991.
13. **Goldner FH and Boyce JW.** Relationship of bile in the stomach to gastritis. *Gastrointest Endosc* 22: 197–199, 1976.
14. **Green DR and Reed JC.** Mitochondria and apoptosis. *Science* 281: 1309–1312, 1998.
15. **Heeneman S and Deutz NEP.** Effects of decreased glutamine supply on gut and liver metabolism in vivo in rats. *Clin Sci (Colch)* 85: 437–444, 1993.
16. **Jones BA and Gores GJ.** Physiology and pathophysiology of apoptosis in epithelial cells of the liver, pancreas, and intestine. *Am J Physiol Gastrointest Liver Physiol* 273: G1174–G1188, 1997.
17. **Jones BA, Stravitz RT, Rao YP, and Gores GJ.** Bile salt-induced apoptosis of hepatocytes involves activation of protein kinase C. *Am J Physiol Gastrointest Liver Physiol* 272: G1109–G1115, 1997.
18. **Kaplowitz N.** Mechanisms of liver cell injury. *J Hepatol* 32, Suppl 1: 39–47, 2000.
19. **Kokoska ER, Smith GS, Deshpande Y, Wolff AB, and Miller TA.** Indomethacin increases susceptibility to injury in human gastric cells independent of PG synthesis inhibition. *Am J Physiol Gastrointest Liver Physiol* 275: G620–G628, 1998.
20. **Kokoska ER, Smith GS, Rieckenberg CL, Deshpande Y, Banan A, and Miller TA.** Adaptive cytoprotection against deoxycholate-induced injury in human gastric cells in vitro. Is there a role for endogenous prostaglandins? *Dig Dis Sci* 43: 806–815, 1998.
21. **Kokoska ER, Smith GS, Wolff AB, Deshpande Y, Rieckenberg CL, Banan A, and Miller TA.** Role of calcium in adaptive cytoprotection and cell injury induced by deoxycholate in human gastric cells. *Am J Physiol Gastrointest Liver Physiol* 275: G322–G330, 1998.
22. **Konda J, Nishisaki H, Nakano O, Matsuda K, Wada K, Nagao M, Matozaki T, and Sakamoto C.** Prostaglandin protects isolated guinea pig chief cells against ethanol injury via an increase in diacylglycerol. *J Clin Invest* 86: 1897–1903, 1990.
23. **Kumar S and Vaux DL.** A cinderella caspase takes center stage. *Science* 297: 1290–1291, 2002.
24. **Kwo P, Patel T, Bronk SF, and Gores GJ.** Nuclear serine protease activity contributes to bile acid-induced apoptosis in hepatocytes. *Am J Physiol Gastrointest Liver Physiol* 268: G613–G621, 1995.
25. **Marchetti P, Castedo M, Susin SA, Zamzami N, Hirsch T, Macho A, Haeflner A, Hirsch F, Geuskens M, and Kroemer G.** Mitochondrial permeability transition is a central coordinating event of apoptosis. *J Exp Med* 184: 1155–1160, 1996.
26. **Miller TA, Redlak MJ, Coy LM, and Taher MM.** *Gastrointestinal Mucosal Repair and Experimental Therapeutics*. Basel: Karger, 2002, p. 69–81.
27. **Miller TA, Smith GS, and Barreto JC.** Gastrointestinal defence: Role of epithelial factors. In: *Immunopharmacology of Epithelial Barriers*, edited by Goldie R. London: Academic, 1994, p. 197–211.
28. **Miller TA and Victor BE.** Gastritis and gastric ulcer. In: *Surgical Treatment of Digestive Disease* (2nd ed.), edited by Moody FG, Carey LC, Jones RS, Kelly JA, Nahrword DL, and Skinner DB. Chicago, IL: Year Book Medical, 1989, p. 174–196.
29. **Oberhammer F, Wilson JW, Dire C, Morris ID, Hickman JA, Wakeling AE, Walker PR, and Sikorska M.** Apoptotic death in epithelial cells: cleavage of DNA to 300 and/or 50 kb fragments prior to or in the absence of internucleosomal fragmentation. *EMBO J* 12: 3679–3684, 1993.
30. **Ritchie WP.** Alkaline reflux gastritis. *Ann Surg* 203: 537–544, 1986.
31. **Roberts LR, Kurosawa H, Bronk SF, Fesmier PJ, Agellon LB, Leung WY, Mao F, and Gores GJ.** Cathepsin B contributes to bile salt-induced apoptosis of rat hepatocytes. *Gastroenterology* 113: 1714–1726, 1997.
32. **Rodrigues CM, Fan G, Wong PY, Kren BT, and Steer CJ.** Ursodeoxycholic acid may inhibit deoxycholic acid-induced apoptosis by modulating mitochondrial transmembrane potential and oxygen species production. *Mol Med* 4: 165–178, 1998.
33. **Rodriguez J and Lazebnik Y.** Caspase-9 and APAF-1 form an active holoenzyme. *Genes Dev* 13: 3179–3184, 1999.
34. **Salvesen GS and Dixit VM.** Caspases: intracellular signaling by proteolysis. *Cell* 91: 443–446, 1997.
35. **Scaffidi C, Fulda S, Srinivasan A, Friesen C, Li F, Tomaselli KJ, Debatin KM, Krammer PH, and Peter ME.** Two CD95 (APO-1/Fas) signaling pathways. *EMBO J* 17: 1675–1687, 1998.
36. **Schlottmann K, Wachs FP, Krieg RC, Kullmann F, Scholmerich J, and Rogler G.** Characterization of bile salt-induced apoptosis in colon cancer cell lines. *Cancer Res* 60: 4270–4276, 2000.
37. **Slee EA, Harte MT, Kluck RM, Wolf BB, Casiano CA, Newmeyer DD, Wang G, Reed HJC, Nicholson DW, Alnemri ES, Green DR, and Martin SJ.** Ordering the cytochrome c-initiated caspase cascade: hierarchical activation of caspases-2, -3, -6, -7, -8, and -10 in a caspase 9-dependent manner. *J Cell Biol* 144: 281–292, 1999.
38. **Souba WW, Klimberg VS, Plumery DA, Salloum RM, Flynn TC, Bland KI, and Copeland EM.** The role of glutamine in maintaining a healthy gut and supporting the metabolic response to injury and infection. *J Surg Res* 48: 383–388, 1990.
39. **Stennicke HR and Salvesen GS.** Biochemical characteristics of caspases-3, -6, -7 and -8. *J Biol Chem* 272: 25719–25723, 1997.
40. **Susin SA, Lorenzo HK, Zamzami N, Marzo I, Brenner C, Larochette N, Prevost MC, Alzari PM, and Kroemer G.** Mitochondrial release of caspase-2 and -9 during the apoptotic process. *J Exp Med* 189: 381–394, 1999.
41. **Suzuki H, Yanaka A, Shibahara T, Matsui H, Nakahara A, Tanaka N, Muto H, Momoi T, and Uchiyama Y.** Ammonia-

- induced apoptosis is accelerated at higher pH in gastric surface mucous cells. *Am J Physiol Gastrointest Liver Physiol* 283: G986–G995, 2002.
42. **Synnerstad I, Johansson M, Nylander O, and Holm L.** Intraluminal acid, and gastric mucosal integrity: the importance of blood-borne bicarbonate. *Am J Physiol Gastrointest Liver Physiol* 280: G121–G129, 2001.
43. **Varcammen D, Brouchaert G, Denecker G, Van De Crean M, Declercq W, Friers W, and Vandenameele P.** Dual signaling of the Fas receptor: initiation of both apoptotic and necrotic cell death pathways. *J Exp Med* 188: 919–930, 1998.
44. **Washo-Stultz D, Crowley-Weber CL, Dvorakova K, Bernstein C, Bernstein H, Kunke K, Waltmire CN, Garewal H, and Rayne CM.** Role of mitochondrial complexes I and II, reactive oxygen species and arachidonic acid metabolism in deoxycholate-induced apoptosis. *Cancer Lett* 177: 129–144, 2002.
45. **Zou H, Li Y, Liu X, and Wang X.** An APAF-1-Cytochrom c multimeric complex is a functional apoptosome that activates procaspase-9. *Biol Chem* 274: 11549–11556, 1999.

