Characterization of Bile Salt-induced Apoptosis in Colon Cancer Cell Lines

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ABSTRACT

Bile salts have been shown to be involved in the etiology of colorectal cancer. Although there is a large body of evidence for bile salts as a cocarcinogen in azoxymethane-induced colorectal cancer, bile salt-induced apoptosis of colorectal cancer cells has not yet been studied in detail. Therefore, we investigated the effects of different bile salts on apoptosis and apoptotic signaling in colon cancer cell lines.

Incubation of colorectal cancer cell lines with physiological concentrations of deoxycholic acid led to a dramatic induction of apoptosis. Caspase cleavage and caspase activation occurred as early as 30 min after the addition of deoxycholate. Caspase-2 (Ich-1, Nedd2), caspase-3 (CPP-32, YAMA, Apopain), caspase-7 (Mch-3, ICE-LAP-3), and caspase-8 (FLICE, Mach-1, Mch5) are activated in HT-29, whereas caspase-1 (ICE) remained intact. Caspase activation and cellular apoptosis induced by bile salts were reversed by broad spectrum and selective caspase inhibitors. As opposed to hepatocyte death mediated by bile acids, CD95 was not involved in deoxycholate-induced apoptosis. The cytoprotective effect of ursodeoxycholic acid in hepatocytes or other tumor cell lines, which is mediated by inhibiting the mitochondrial permeability transition, was not observed in colon cancer cell lines as well. This points to distinct intracellular functions of ursodeoxycholic acid in different cancer cell types.

Here we describe the specificity of bile salt-induced apoptosis in colon cancer cell lines. Differences from hepatocytes are shown. Bile acid-specific caspase activation is part of the apoptotic pathway induced by bile salts in colon cancer cell lines. Furthermore, a lack of cytoprotective function of ursodeoxycholic acid in these cells is demonstrated. Our data raise questions as to the role of bile salts in colorectal carcinogenesis.

INTRODUCTION

Bile salts have been suggested to be involved in the pathogenesis of colorectal cancer, which is supported by epidemiological data, mutagenetic studies, signaling studies, and investigations on the effect of bile salts in carcinogen-treated rats (1, 2). Several studies proposed that in colorectal cancer cell lines, hydrophobic secondary bile salts such as DC(e) exert their cytotoxic effects by mechanisms resulting in the fundamental biological process termed programmed cell death or apoptosis (3, 4). Decreased susceptibility of goblet cells from “healthy” colonic areas in patients with colorectal cancer and large villous adenomas to bile salt-induced apoptosis was correlated with increased risk for colon cancer (4, 5). Present knowledge about the mechanisms by which bile salts cause cellular injury results mostly from studies performed on hepatocytes, whereas the molecular mechanisms involved in the apoptotic process in colon cancer cell lines have not been studied.

The observation of hepatocyte mitochondrial swelling and impaired state III respiration in bile duct-ligated rats prompted further biochemical characterization of the molecular pathways (6), such as disruption of the mitochondrial respiratory chain with reversible ATP depletion (7) and mitochondrial permeability transition (8). Activation of an intramitochondrial calpain-like protease reportedly coincides with the mitochondrial permeability transition attributable to toxic bile salts in hepatocytes (7). Inhibitors of cystein proteases but not serin proteases reversed this effect (9). It was hypothesized that this protease might regulate mitochondrial permeability transition by processing proteins forming the mitochondrial permeability transition pore or proteins regulating the function of this pore (10). Direct activation of the protein kinases C δ and ε by glycochenodeoxycholate positively correlates with bile salt-induced apoptosis and inhibitor studies with protein kinase C antagonists, such as chelerythrine, suggested that protein kinase C might play a role in apoptotic signaling of bile salts in hepatocytes (11).

In a recent report, direct oligomerization of the Fas receptor (CD95/Apo-1) has been suggested as the primary causative mechanism of bile salt-mediated hepatocyte apoptosis. This oligomerization led to the activation of the caspase cascade (caspase: cysteiny1 aspartic acid protease) starting from caspase-8 (FLICE), which is the first caspase activated in Fas-mediated apoptosis (12, 13). Caspases are responsible for morphological and biochemical changes occurring in cells undergoing apoptotic cell death. The caspases differ in their capacity to induce the caspase cascade necessary for these changes. The ICE-like caspases (e.g., caspase-1 or interleukin-1 converting enzyme) rather fulfill proinflammatory tasks, whereas other caspases are clearly proapoptotic, e.g., caspase-3 (CPP-32, Yama, Apopain). Caspases exhibit selectivity in their function attributable to their substrate specificity. An increasing number of intracellular caspase substrates has been identified, and it is believed that the induced destruction of these substrates causes the apoptotic phenotype in cells (14).

In the present study, we characterized the kinetics of bile salt-induced apoptosis in colon cancer cell lines. The specificity of death induction by toxic bile salts is demonstrated. Activation of the caspase cascade and its role in bile salt-mediated apoptosis is described. Differences to bile acid-induced hepatocyte death are identified.

MATERIALS AND METHODS

Cell Lines. Caco-2, HT-29, SW480, and SW620 colon cancer cell lines were obtained from American Type Culture Collection (Rockwell, MA). Cells were cultured in DMEM plus 10% FCS, 1% penicillin/streptomycin, 1% non-essential amino acids, and 1% sodium pyruvate (Biochrom, Berlin, Germany). All cell lines were cultured under standard tissue culture conditions.

Induction of Apoptosis. The respective cell lines were seeded in six-well plates. Cells grew to subconfluence. For the induction of apoptosis, new media containing the respective amounts of the water-soluble sodium salts of DC (Boehringer, Mannheim, Germany), CDC, GC, GDC, C (Sigma, Deisenhofen, Germany), and UD (Calbiochem, Bad Soden, Germany) were added. The bile salts were sonicated for 30 min at 50°C prior to usage. Two μg/ml of the mouse monoclonal anti-Fas (CD95/Apo-1) antibody CH-11 (Upstate Biotechnology, Munich, Germany) were added where necessary. The broad spectrum tripeptide caspase inhibitor z-VAD-fmk and the caspase-1 inhibitor YVAD-...
CHO (100 μM; both Bachem, Heidelberg, Germany) and UDC (500 μM) were added 60 min prior to incubation with bile salts at the indicated concentrations.

**DNA Fragmentation.** Low molecular weight DNA was extracted using a hypotonic lysis buffer consisting of 50 mM Tris, 10 mM EDTA, and 0.5% n-lauroylsarcosine supplemented with 20 μg/ml RNaseA for 1 h at 37°C. Four hundred μg/ml of proteinase K was added, and the solution was incubated at 65°C for 2 h on a shaker. DNA was precipitated in ice-cold 100% ethanol, 2.5 mM ammonium acetate and 1 mM magnesium chloride overnight. After centrifugation for 30 min at 15,000 rpm, the DNA pellet was resuspended in 30 μl of deionized water. Standard loading buffer (1 μl) was added, and samples were run on a 1% agarose gel containing 0.1% ethidium bromide in TAE buffer (Tris 40 mM, sodium acetate 20 mM, and 1 mM EDTA, pH 8.0).

**Flow Cytometry.** Apoptotic cells from the medium supernatant and non-apoptotic adherent cells were collected and pelleted at 2,000 units/min. Pellets were washed in PBS, and DNA propidium iodide staining was performed using the CycleTest Plus DNA Reagent kit according to the manufacturer’s recommendations (Becton Dickinson, San Jose, CA). The samples were analyzed using an an EPICS XL-MCL (Coulter Immunotech, Hamburg, Germany) flow cytometer. Quantitation of the sub-G1 area was performed with the WinMDI Program (J. Trotter).

CD95 surface expression was detected with the anti-CD95 antibody UB2 (PharMingen, San Diego, CA). Adherent cells were collected and pelleted at 2,000 rpm. The resulting pellet was resuspended in ice-cold PBS/2% FCS and incubated at 4°C for 30 min. The cells were again pelleted, resuspended in 200 μl of PBS/2% FCS and stained with 10 μl of the mouse anti-CD95-FITC antibody at 4°C for 1 h prior to flow cytometric analysis.

**Western Blotting.** Cells were lysed in a buffer containing 4 mM HEPES, 320 mM sucrose, 1 mM EDTA, 0.1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 1.5 mM pepstatin, 0.7 mM aprotinin, and 0.1% CHAPS on ice for 20 min. After sonication for 10 s, protein extracts were centrifuged for 20 min at 15,000 units/min. The protein concentration in the supernatant was determined using the BCA test (Sigma). Cytosolic extracts were loaded and run on 15% SDS-PAGE gels under reducing conditions. After electrophoresis, protein was transferred to a polyvinylidene difluoride membrane. Membranes were blocked with 5% nonfat milk in PBS and probed with 1:3000 dilutions of the monoclonal mouse anti-human antibodies anti-caspase-1 (Upstate Biotechnology, Lake Placid, NY), anti-caspase-2, anti-caspase-8 (PharMingen, San Diego, CA), anti-caspase-3, and anti-caspase-7 (Transduction Laboratories/Dianova, Hamburg, Germany), and anti-MAPK-1 (Santa Cruz Biotechnology, Santa Cruz, CA) in 5% nonfat milk/PBS. Immunoblots were incubated with 1:5000 diluted horseradish peroxidase-conjugated anti-mouse IgG (Dianova) using the Enhanced Chemiluminescence Plus (ECL Plus) Western blotting detection system kit (Amersham Pharmacia Biotech Europe, Freiburg, Germany) and exposed to Hyperfilm (Amersham Pharmacia Biotech Europe, Freiburg, Germany).

**Caspase Activity Assay.** Caspase activation was determined from cytosol of HT-29 cells treated with bile acids for the indicated times. The colorimetric activity assays were performed with a commercially available caspase assay kit (Biomol Research Laboratory, Plymouth Meeting, PA) according to the manufacturer’s recommendations. In brief, after the respective treatment, cells were collected and briefly spun down. Cells were lysed [50 mM HEPES (pH 7.4), 0.1% CHAPS, 1 mM DTT, and 0.1 mM EDTA], nuclei were removed (10,000 rpm for 10 min), and the cytosolic preparations were quickly frozen and stored at −80°C until usage (maximum, 2 weeks). Equal amounts of cytosolic protein were added to the assay buffer [50 mM HEPES (pH 7.4), 100 mM NaCl, 0.1% CHAPS, 10 mM DTT, 1 mM EDTA, 10% glycerol, and caspase-3 substrate Ac-DEVD-pNA] in 96-well ELISA plates. The absorbance at 405 nm was quantified with an ELISA plate reader.

**Microscopy.** Cells were seeded in petriPerm Culture “hydrophil” dishes (Heraeus, Munich, Germany) and grown to subconfluency. Apoptosis was induced as described above, and the dishes were placed under an inverse fluorescence microscope (Axiovert S 100, Zeiss, Germany) with an incubation unit. Cells were filmed with a cooled CCD camera, and the generated photographs were analyzed using Metamorph Software (Visitron Systems, Puchheim, Germany). Morphological criteria used to identify apoptotic cells were membranes blebbing, cell shrinkage, and nuclear condensation as well as formation of apoptotic bodies.

**CD95 RT-PCR.** Total RNA of SW480 and SW620 cells was extracted using the RNeasy kit (Qiagen, Hilden, Germany). Quality and quantity of RNA were analyzed measuring absorption at 260 and 280 nm, respectively. RT-PCR was performed using the RETROscript kit (Ambion, Austin, TX) to generate cDNA with random decamer primers according to the manufacturer’s recommendations. The oligonucleotides used for the PCR amplification were as follows for CD95: sense strand, 5'-GACAAAAGCCCCATTTTTCTCC-3'; and the antisense strand, 5'-ATTATTTGGACCTTTTCCG-3'. To test cDNAs for representation and full-length genes, PCR with a 3' β-actin, 2K clathrin, and glyceraldehyde-3-phosphate dehydrogenase primer set from the Gene Checker kit (Invitrogen, Leek, the Netherlands) was performed according to the manufacturer’s protocol. The PCR for CD95 comprised 30 cycles with denaturing at 94°C for 60 s, annealing at 53°C for 60 s, and extension at 72°C for 120 s. The PCR for the genes from the Gene Checker kit comprised 25 cycles with denaturing at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s. The reactions were performed in a TRIO-Thermoblock (Biorad, Goettingen, Germany). The PCR products were then subjected to 2% agarose gel electrophoresis.

**RESULTS**

The influence of different bile salts on apoptosis after incubation of the HT-29, Caco-2, and SW-480 colon carcinoma cell lines was established. Morphological criteria of apoptosis in SW-480 cells, such as membrane blebbing, cell shrinkage, nuclear condensation, and nuclear fragmentation, were assessed by phase-contrast video microscopy. The unconjugated hydrophobic bile salt DC induced apoptosis in a concentration- and time-dependent manner. Concentrations of 500 μM DC had a strong cytotoxic effect. Morphological changes indicating apoptosis were observed as early as 40 min after the addition of DC (Fig. 1A). DNA laddering, a typical sign of apoptosis, was detectable 1 h after addition of 500 μM DC to HT-29 with increasing intensity over time (Fig. 1B). Flow cytometric analysis of apoptosis again showed a strong time-dependent and concentration-dependent response after DC (Fig. 1C). Concentrations as low as 100 μM DC induced detectable apoptosis after an incubation time of 90 min as well, however, to a much lower degree.

To test for specificity of bile salt-induced apoptosis, we used bile salts with differing physicochemical properties. The dihydroxy bile salt CDC exerted the same proapoptotic effect on HT-29 cells as DC, whereas the conjugated bile salt GDC and the trihydroxy bile salt C and its conjugate GC did not exhibit cytotoxicity (Fig. 1D), even at higher concentrations (up to 1 mM; data not shown). Hence, all three apoptosis detection methods point to apoptotic rather than necrotic cell death, and apoptosis was specifically induced by toxic (hydrophobic) bile salts such as DC and CDC, whereas the conjugates of DC as well as the trihydroxy bile salt C and its conjugates do not induce apoptosis in colorectal cancer cell lines. Addition of the protein synthesis inhibitor cycloheximide (50 μg/ml) revealed that DC-induced apoptosis is not protein synthesis dependent (data not shown). The recent observation that bile salt-induced apoptosis in hepatocytes is mediated via the Fas receptor signaling pathway (12) prompted us to compare the kinetics of bile salts and anti-Fas-mediated apoptosis in HT-29, which is a Fas-positive cell line. The anti-Fas-mediated induction of apoptosis in HT-29 requires pretreatment with IFN-γ because of an IFN-γ-mediated increase in the expression of Fas on HT-29 cells (15). In our observations, the apoptotic response in SW480 cells treated with the agonistic anti-Fas (Fas/Apo-1) antibody CH-11 (1 μg/ml) was negligible at time points when almost all DC-treated cells were clearly apoptotic (Fig. 2A). We screened cancer cell lines for CD95 expression. Although the SW480 cell line is highly CD95 positive, the SW620 cell line was CD95 negative in RT-PCR as well as by flow cytometric analysis (Fig. 2B). However, incubation of both cell lines with DC had a comparable rapid proapoptotic effect (Fig. 2C). Together, these data indicate that DC is a potent inducer of apoptosis in colon cancer cells, whereas the
proapoptotic effect of the agonistic anti-Fas antibody CH-11 is negligible at time points when DC caused full-blown apoptosis. These data suggest that DC does not exert its proapoptotic effect by recruiting the CD95 signaling cascade in colon cancer cell lines.

After defining the type of bile salt that can induce apoptosis in colon cancer cell lines and after establishing the kinetics and concentration dependence of DC-mediated apoptosis, signal transduction pathways that are involved in the intracellular transmission of the...
apoptotic stimulus induced by DC were investigated in HT-29 cells. Members of the caspase family of cysteine proteases are activated by most apoptosis inducers. We therefore analyzed the activation of caspases upon DC-mediated apoptosis in HT-29 cells. Caspases intracellularly exist as the zymogen in a proenzyme state. Death stimuli can lead to autoproteolytic cleavage of caspases by cutting off the prodomain and releasing a small and a large subunit, which form heterotetramers representing caspase activation. In Western blots, defined cleavage products occur upon activation of caspases, and subsequently the amount of detectable zymogen decreases over time. Fig. 3A shows typical time courses of caspase cleavage in Western blots. Incubation of HT-29 with 500 μM DC resulted in cleavage of the proapoptotic caspases caspase-2 (Ich-1, Nedd2), caspase-3 (CPP-32, YAMA, Apopain), caspase-7 (Mch-3, ICE-LAP-3), and caspase-8 (FLICE, Mach-1, Mch5). Therefore, all investigated proapoptotic group II caspases (caspase-2, caspase-3, caspase-7) and group III caspases (caspase-8) undergo bile salt-induced cleavage. In contrast, caspase-1 (ICE), a proinflammatory group I caspase, was not cleaved in our experiments (Fig. 3B). A Western blot of MAPK-1 proved that protein amounts of noncaspase substrates remained constant over time after treatment of HT-29 with DC (Fig. 3B).

Fig. 2. CD95 expression does not correlate with DC-mediated apoptosis. A, analysis of SW480 cells treated with the anti-Fas antibody CH-11 (2 μg/ml) for 1 h did not exhibit any morphological signs of apoptosis, when full-blown apoptosis was detectable in DC-treated SW480. B, cell lines were stained with the anti-CD95 antibody UB-2 and analyzed by flow cytometry. Empty graphs, cells stained with control antibody; gray graphs, cells stained with UB-2. Strong CD95 surface expression was detected on SW480, whereas there is no expression on SW620 (left). CD95 receptor RT-PCR from SW480 shows the expected 393-bp CD95 PCR product, whereas the SW620 cell line was negative for CD95 RNA (right). The integrity of the mRNA used for the RT-PCR is shown in the lower figure. Lanes 1, 3, β-actin; Lanes 2, clathrin; Lanes 3, glyceraldehyde-3-phosphate dehydrogenase. C, apoptosis can be induced by DC in SW480 and SW620 cells. The numbers indicate percentage of apoptosis in the sub-G1 area.

Fig. 3. DC induces activation of proapoptotic caspases but not activation of the proinflammatory caspase-1. A, whole-cell lysates of HT-29 cells treated with 500 μM DC and normalized for protein content were run on SDS-PAGE and blotted with the indicated anti-caspase antibodies. Upper bands, the uncleaved zymogen of the respective caspase. Upon induction of apoptosis with DC, the protein amount of the respective procaspase decreases while cleavage products, which are detected by the specific antibodies, occur. The activation of caspase-8 is shown solely by the decrease of the zymogen form over time. B, caspase-1 (ICE) is not cleaved as indicated by stable amounts of zymogen over time. No cleavage products occur. MAPK-1 does not decrease indicating that the total content of protein was stable during the process of apoptosis.
To assess the functional role of caspase cleavage in DC-mediated apoptosis, the broad spectrum tripeptide inhibitor of caspases z-VAD-fmk was added to the cells 1 h prior to DC. Although the inhibitor had no proapoptotic effect, z-VAD-fmk led to complete abrogation of apoptosis, determined by flow cytometry and DNA laddering in HT-29 cells incubated with DC (Fig. 4, A and C). The caspase-1 like caspase inhibitor YVAD-CHO did not inhibit apoptosis (Fig. 4A). Hence, the proapoptotic members of the caspase family of cysteine proteases are functionally involved in the apoptotic response of colorectal cancer cells to bile salts, whereas the proinflammatory member caspase-1 (ICE) is not.

UDC efficiently suppresses apoptosis caused by glycochenodeoxycholate and anti-Fas in hepatocytes (12). We compared the contraprotective activity of UDC in HT-29 and in the human hepatic cancer cell line Hep-G2. Preincubation with UDC (1 h at 500 μM) did not decrease apoptosis in HT-29 (Fig. 4, A and B). Concentrations up to 1 mM of UDC lacked any contra-apoptotic effects as well (data not shown), whereas DC-mediated apoptosis was efficiently suppressed by UDC in Hep-G2 cells. UDC itself had no proapoptotic or caspase-activating effect at the indicated time points (Fig. 4, A–C). These data suggest that UDC effects differ in hepatic and colon tumor cell lines.

To determine whether the apoptosis-inhibiting capacity of z-VAD-fmk correlates with caspase cleavage in DC-treated HT-29 cells, we analyzed caspase cleavage in Western blots. z-VAD-fmk almost completely inhibited caspase cleavage (Fig. 4D), supporting the functional importance of caspase activation in DC-mediated apoptosis.

After the detection of bile salt-induced cleavage of caspases in HT-29, we investigated the activation of caspase-1 and caspase-3 in the cytoplasm of bile salt-treated HT-29 cells by colorimetric assays with substrates indicating caspase-1 and caspase-3 like activity. The results presented in Fig. 5 point to activation of caspase-3, whereas caspase-1 is not activated. Again, the functional role of caspase-3 and the lack of caspase-1 in apoptotic signaling induced by DC in HT-29 cells was emphasized.

**DISCUSSION**

In our studies, we demonstrated that specific bile salts induce apoptotic cell death in colon cancer cell lines. The activation of the caspase cascade is functionally involved because inhibition of caspases reverses the proapoptotic effect of toxic bile salts. The CD95...
pathway does not seem to mediate bile salt-related death in colon cancer cells. This is in striking contrast to hepatocytes, which respond to bile salts via CD95. Also, the cytoprotective bile salt UDC did not inhibit apoptosis in colon cancer cells, whereas in hepatocytes and hepatic tumor cell lines, UDC is a powerful inhibitor of apoptosis induced by a variety of proapoptotic substances.

Epidemiological studies, results from in vitro and in vivo mutagenesis experiments, and data on bile salt-induced proliferation and signaling (16) imply a cocarcinogenic effect for certain bile salts in the development of colorectal cancer (1, 2). DC constitutes the major fraction of bile salts in the colon. Because of its hydrophobicity, the largest portion is found in the feces, but considerable amounts up to 700 μM DC was measured in fecal water (17–20). This portion, at least theoretically, can get into contact with colonic epithelial cells. We investigated the effect of different bile salts on various colon epithelial cancer cell lines. Induction of apoptosis in all examined cell lines by the unconjugated deoxy derivative of C, DC was observed, suggesting that dehydroxylation is critical for apoptosis induction. Conjugation with glycine residues resulted in a complete lack of activity. In this respect, it is important that bile salts can directly mediate mitochondrial permeability transition in isolated mitochondria from hepatocytes (8, 29). The zymogen form of caspase-2 has been shown to be released from mitochondria after opening of the permeability transition pore and to be subsequently activated during the apoptotic process (30). We were able to detect cleavage of caspase-2 in HT-29 cells treated with DC as well, indicating DC-dependent mitochondrial permeability transition in HT-29.

However, the observation that coadministration of UDC or its taurine and glycine conjugates with toxic bile salts resulted in abrogation of the cytotoxicity in hepatocytes and even in a sarcoma cell line via stabilization of the mitochondrial permeability transition pore (8, 29) was not reproducible in our colon cancer cell lines. Therefore, UDC differs in its capacity to protect hepatic cancer cells and colorectal cancer cells from apoptosis. Bile salt-mediated induction of the mitochondrial permeability transition in colorectal cancer cell lines and the lack of UDC-mediated inhibition of the mitochondrial permeability transition are under current investigation.

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5. Unpublished results.
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