

## Deoxycholate induces DNA damage and apoptosis in human colon epithelial cells expressing either mutant or wild-type p53

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### **Abstract:**

Diets rich in fat result in higher concentrations of secondary bile acids or their salts in the colon, which may adversely affect cells of the colonic epithelium. Because secondary bile acids are thought to be genotoxic, exposing colon epithelial cells to secondary bile acids may induce DNA damage that might lead to apoptosis. The requirement for the p53 tumor suppressor gene in such events is unknown. In particular, the effects of secondary bile acids on colon epithelial cells having different p53 tumor suppressor gene status have not been examined. Therefore, HCT-116 and HCT-15 human colon adenocarcinoma cells, which express the wild-type and mutant p53 genes, respectively, were exposed to physiological concentrations of deoxycholate. The cells were then analyzed for evidence of DNA damage and apoptosis. After 2 h of incubation with 300  $\mu$ M deoxycholate, both cell lines had greater levels of single-strand breaks in DNA as assessed by the comet assay. After 6 h of exposure to deoxycholate, HCT-116 and HCT-15 cells showed morphological signs of apoptosis, i.e., membrane blebbing and the presence of apoptotic bodies. Chromatin condensation and fragmentation were also seen after staining DNA with 4',6-diamidino-2-phenylindole. Other apoptotic assays revealed greater binding of annexin V-fluorescein isothiocyanate, as well as greater post-enzymatic labeling with dUTP-fluorescein isothiocyanate, by both cell lines exposed to deoxycholate. These data suggest that deoxycholate caused DNA damage in colon epithelial cells that was sufficient to trigger apoptosis in a p53-independent manner.

### **Keywords:**

Apoptosis; Bile acids; Deoxycholate; DNA damage; p53

### **Article**

#### 1. INTRODUCTION

Colon cancer is one of the major health concerns in the US. Based on statistics from the American Cancer Society, there will be 93 800 new cases of colon cancer and 47 700 deaths from the disease this year [1]. These figures have prompted research regarding the etiology of colon cancer in order to prevent and treat the disease. Inherited mutations of critical tumor suppressor genes, such as the adenomatous polyposis coli (APC) gene, can cause colon cancer [2]. However, there may also be a non-familial or environmental basis for colon cancer. Along this line, gene mutation and colon tumorigenesis seem to be the long-term consequence of unhealthy dietary habits. For example, epidemiological studies indicate that the traditional Western diet, which is often low in plant products and high in red meat and fat, increases the risk of colon cancer [3]. Apparently, the Western diet supplies carcinogens and/or cancer-promoting substances that work directly or indirectly to cause damage to the DNA of colon epithelial cells, leading to gene mutation and tumorigenesis.

The role of dietary fat in colon tumorigenesis has been examined in particular. The risk of colon cancer is increased by diets high in fat [4], although this is not always the case [5]. Nevertheless, it is generally thought that high fat diets have cancer-promoting effects by way of bile acids or their salts [6]. High fat diets stimulate greater secretion of bile acids from the gall bladder into the small intestine to facilitate fat digestion and absorption [7]. Much of the bile acids are recycled, but a significant pool escapes recycling and reaches the colon, where resident bacteria convert this pool into secondary bile acids. One of the most predominant secondary bile acids is deoxycholic acid, which is a cancer-promoting substance as demonstrated in rats by its ability to increase proliferation of colonic crypt cells [8] and potentiate the stomach tumorigenic effect of the chemical carcinogen, N-methyl-N'-nitro-N-nitrosoguanidine [9]. The notion that deoxycholic acid and other secondary bile acids contribute to the development of colon cancer is supported by the finding that elevated levels of secondary bile acids are present in the colon residual fluid or fecal material of human subjects with colon cancer [10].

Several possible mechanisms have been examined to explain the role for bile acids in colon tumorigenesis. It does not appear that bile acids are direct mutagens or carcinogens, because no bile acid-DNA adducts have been detected either in the colon and liver of rats that were administered various bile acids by gavage, or in cultured cells exposed to bile acids [11]. Other studies have focused on the ability of secondary bile acids to act as tumor promoters by stimulating signal transduction and cancer-promoting genes. Activation of protein kinase C, which is a common event triggered by many tumor promoters, was reported in cultured colon cells that were exposed to deoxycholic acid [12]. Additionally, activation of the transcription factors, AP-1 [13] and NF- $\kappa$ B [14], as well as the NF- $\kappa$ B –responsive cyclooxygenase-2 (COX-2) gene [15], has been reported. Transcriptional activation of COX-2 may promote colon tumor cell growth and proliferation, since expression of COX-2 has been found in colon tumor tissue but not normal colon tissue [16].

In investigating another possible role of bile acids in colon tumorigenesis, it was established recently that bile acids can induce single-strand breaks in DNA of colon cells in a process that might involve oxygen free radicals [17]. Thus, it is conceivable that oxidative DNA damage could lead to gene mutation and tumorigenesis [18]. Normally, however, DNA damage increases the levels of p53 tumor suppressor protein, which transcriptionally activates the WAF1/CIP1/p21 gene whose protein product triggers cell cycle arrest to permit DNA repair [19]. However, if the DNA damage is irreparable, apoptosis is initiated. Therefore, the ability of colon epithelial cells to undergo apoptosis when exposed to bile acids that cause severe DNA damage might prevent them from being transformed into cancerous cells. On the other hand, it is thought [20] that continuous exposure of colon epithelial cells to bile acids and other genotoxic agents often associated with the Western diet can lead to selective survival of cells having unrepaired DNA. As such, the apoptosis-resistant cells could accumulate mutations to the APC tumor suppressor gene, thus allowing the initial development of a benign tumor or polyp. Eventually, these early tumor cells would develop mutations in a few other pivotal genes, including the p53 tumor suppressor gene, thus transforming the polyp into a malignant tumor [2].

The ability of different types of cells to undergo apoptosis after sustaining DNA damage can be either dependent or independent of the p53 gene or protein [21]. It is unknown whether p53 gene status is important in permitting colon epithelial cells to undergo apoptosis when exposed to secondary bile acids at concentrations that induce significant DNA damage. Accordingly, in this study, we determined whether deoxycholate was able to induce DNA damage sufficient to initiate apoptosis in HCT-116 and HCT-15 human colon adenocarcinoma cell lines expressing wild-type p53 [22] and mutant p53 [23] genes, respectively.

## 2. MATERIALS AND METHODS

### *2.1. Materials*

HCT-116 and HCT-15 human colon adenocarcinoma cell lines were purchased from the American Type Culture Collection (Rockville, MD). All reagents were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated.

## ***2.2. Cell culture and treatment***

HCT-116 cells were propagated in McCoy's 5A medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin (10 µg/ml), and 0.2% amphotericin B (250 µg/ml). HCT-15 cells were propagated in RPMI-1640 medium supplemented with 10% fetal bovine serum, 1% of penicillin/streptomycin (10 µg/ml), and 0.2% amphotericin B (250 µg/ml). Cells were kept at 37°C in a water-humidified atmosphere of 5% CO<sub>2</sub>/95% air. The media were changed every other day, and the cells were split every 5 days. Cell number and viability were determined by counting an aliquot of trypan blue-stained cells with a hemocytometer.

For the experiments, cells were seeded into either six-well polystyrene plates or 100 mm petri dishes and allowed to reach about 70% confluency. Then, the cells were exposed to 300 µM sodium deoxycholate (NaDOC), i.e., the sodium salt of deoxycholic acid, for 2–20 h depending on the experiment. They were harvested by using Non-enzymatic Cell Dissociation Solution (Sigma) and analyzed as detailed below.

## ***2.3. Assessment of cellular DNA damage***

After exposure to either 0 or 300 µM NaDOC for 2 h, cells were assessed for DNA damage using the comet assay [24], a procedure also described in our most recent work [25,26]. Briefly, cells were suspended in 0.5% agarose that had been dissolved in phosphate-buffered saline (PBS). Aliquots of the cell/agarose suspension were pipetted onto frosted glass slides, which had been pre-coated with the agarose solution. After allowing the cell/agarose suspension to congeal, the slides were then immersed in ice-cold lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 300 mM NaOH, 10% DMSO and 1% Triton X-100) for 1 h. Next, the slides were placed in a horizontal electrophoresis tank that was filled with alkaline buffer (300 mM NaOH, 1 mM EDTA). They were left undisturbed in the tank for 20–25 min, before electrophoresis was performed for 20 min at 20 V (300 mA).

After electrophoresis, the slides were washed with a neutralization buffer (0.4 M Tris-Cl, pH 7.5) for 5 min and then stained with 45 µl of ethidium bromide (20 µg/ml). The slides were viewed on an Olympus BX-60 fluorescence microscope equipped with a SPOT digital camera. For each of the six replicate experiments, 50 nucleoids were scored (at 400 × magnification) on a scale of 0 (no DNA damage) to 4 (severe DNA damage) in regard to the appearance of a comet tail-like shape, reflecting single-strand breaks in DNA. To calculate the total comet scores reported, the individual scores for each of the 50 nucleoids were added. Although subjective, this scoring method compares favorably with computer imaging analysis [27].

## ***2.4. Examination of the cells for morphological signs of apoptosis***

After exposure to either 0 or 300 µM NaDOC for 6 h, cells were examined for morphological signs of apoptosis (membrane blebbing and presence of apoptotic bodies) using an Olympus IX-70 inverted, light microscope equipped with another SPOT digital camera.

## ***2.5. Annexin V-FITC binding assay***

After exposure to either 0 or 300 µM NaDOC for 6 h, cells were washed twice with cold PBS. Binding of annexin V-FITC to the cell surface, which is an early marker of apoptosis, was determined with a kit from Pharmingen (San Diego, CA). The washed cells were very gently resuspended in a solution comprised of 100 µl of 1 x binding buffer, 5 µl of Annexin V-FITC, 5 µl of propidium iodide (50 µg/ml), and a small drop of Anti-fade solution (Bio-Rad, Hercules, CA) and allowed to incubate in the dark for 15 min at room temperature. After incubation, the cell suspension was applied onto a glass slide and covered with a glass cover slip. The edges of the cover slip were sealed with fingernail polish. Slides were viewed immediately on the Olympus BX-60 fluorescence microscope/SPOT digital camera. For each of the four replicate experiments, three randomly selected microscopic fields were examined at 400 x magnification. The presence of any annexin V-FITC-labeled cells (green fluorescence) were then counted in each of the fields. Finally, to calculate the annexin binding scores reported, all of the annexin V-FITC-labeled cells present in the three microscopic fields were added.

## ***2.6. Staining of cellular DNA with 4',6-diamidino-2-phenylindole (DAPI)***

After exposure to either 0 or 300  $\mu$ M NaDOC for 6 h, cells were washed three times and resuspended in PBS. Aliquots of the cell suspension were pipetted onto lysine-coated glass slides for centrifugation in a Stat Spin Cytofuge. To fix the cells, slides were immersed in 4% formaldehyde solution in PBS for 25 min and stored in 70% ethanol at  $-20^{\circ}\text{C}$  for at least 24 h. Before staining, the slides were washed three times with PBS. Then, they were immersed in 1  $\mu\text{g}/\text{ml}$  DAPI for 10 min. For some experiments, sulforhodamine 101 (10  $\mu\text{g}/\text{ml}$ ) was used as a counterstain. After washing in PBS, a drop of anti-fade solution was applied onto each slide. The slides were covered with glass cover slips, whose edges were then sealed with fingernail polish. They were examined (at 400X magnification) on the Olympus BX-60 fluorescence microscope/SPOT digital camera. For each experiment, performed in triplicate, 200 randomly selected cells were scored either for the absence or presence of DNA condensation and fragmentation, which are characteristic features of apoptosis. To calculate the DAPI staining scores reported, the number of cells showing the apoptotic features was expressed as a percentage of the total number of cells scored.

### 2.7. TUNEL assay

After exposure to either 0 or 300  $\mu$ M NaDOC for 20 h, cells were fixed as above. To detect the DNA fragmentation associated with apoptosis, the TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) assay was performed using a kit with a set of instructions from Promega (Madison, WI). Following the PBS wash, cells were treated with permeabilizing solution (0.2% Triton X-100 in PBS) for 5 min on ice. The cells were washed again with PBS, and then 50  $\mu\text{l}$  of equilibration buffer was applied to the slides for 10 min of incubation at room temperature. A mixture of terminal deoxynucleotidyl transferase (TdT), nucleotide mix (containing FITC-12-dUTP), and equilibration buffer were pipetted onto the slides and coverslipped for incubation in a humidified chamber at  $37^{\circ}\text{C}$  for 60 min in the dark. The reaction was terminated by immersing the slides in SSC Buffer for 15 min. Following washes in PBS, slides were counterstained with 1  $\mu\text{g}/\text{ml}$  propidium iodide for 15 min. Stained slides were washed with deionized water, and a drop of anti-fade solution was applied onto each slide. After covering with a glass cover slip and sealing the edges with fingernail polish, the samples were examined by fluorescence microscopy as before. For each experiment, performed in triplicate, 100 randomly selected cells were scored for either the absence or presence of green fluorescence (TUNEL-labeling). To calculate the TUNEL scores reported, the number of cells showing the green fluorescence was expressed as a percentage of the total number of cells scored.

### 2.8. Statistical analysis of data

Data were analyzed for significant differences ( $P < 0.05$ ) by analysis of variance (ANOVA) and Fisher's Protected Least Significant Differences Test (version 9.0, SYS STAT, Chicago, IL).

## 3. RESULTS

To determine if NaDOC was able to induce single-strand breaks in DNA of HCT-15 and HCT-116 cells, the comet assay was performed with the results presented in Fig. 1. As shown by the representative photos, the untreated control samples for both cell lines had nucleoids that did not have 'comet tails'. That is, the DNA remained within the perimeter of the spherical masses giving them the appearance of 'comet heads', indicative of a minimal level of DNA single-strand breaks. Nonetheless, upon complete scoring of the nucleoids in multiple experiments, a small number of the nucleoids from control cells exhibited small comet tails (not shown by the photo), reflecting a baseline level of DNA single-strand breaks. Hence, the average comet scores for control HCT116 and HCT-15 cells were  $38 \pm 5$  and  $31 \pm 6$ , respectively.

In contrast, as shown by the other representative photos in Fig. 1, HCT-116 and HCT-15 cells that were exposed to 300  $\mu$ M NaDOC for 2 h had significant numbers of nucleoids with larger comet tails, indicative of higher levels of DNA single-strand breaks. The comet assay scores were  $105 \pm 16$  and  $88 \pm 6$  for HCT-116 and HCT-15 cells, respectively. Because the extent of DNA damage seen in many of the cells was substantial, it was suspected that this situation was irreparable overall and sufficient to initiate apoptosis with continued incubation of the cells with NaDOC. This possibility was investigated next.

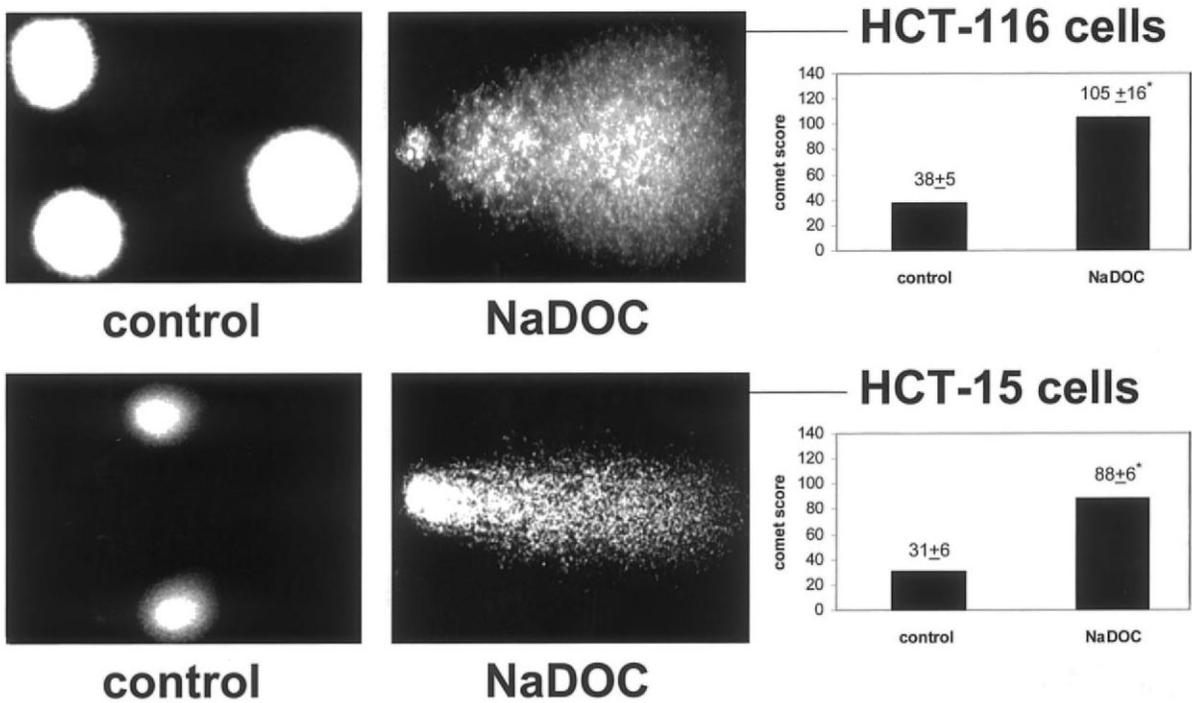


Fig. 1. Comet assay images (sample photos at 1000 ×) and comet scores of the HCT-116 and HCT-15 cells after exposure to either 0 or 300 μM sodium deoxycholate (NaDOC) for 2 h. The comet scores are the mean ± SD of six separate experiments. Scores with a superscript asterisk are significantly different than the respective control scores ( $P < 0.05$ ).

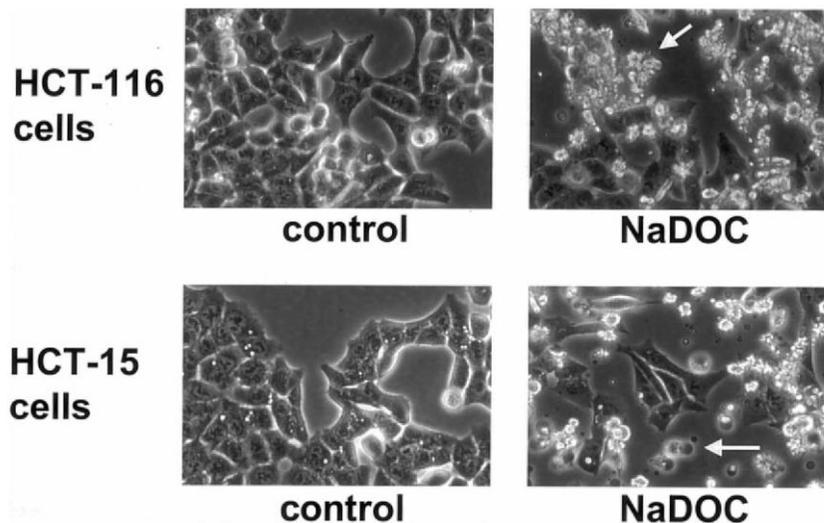


Fig. 2. Morphological changes to both HCT-116 and HCT-15 cells after exposure to 300 μM NaDOC for 6 h. Representative photos are shown. The white arrows point to examples of membrane blebbing and apoptotic bodies.

To determine whether exposing HCT-116 and HCT-15 cells to 300 μM NaDOC for 6 h resulted in apoptosis, the cells were first examined for morphological signs of apoptosis by light microscopy (Fig. 2). Consistent with the occurrence of apoptosis, membrane blebbing and apoptotic bodies were present.

To unequivocally determine that exposing HCT-116 and HCT-15 cells to NaDOC leads to apoptosis, three other apoptotic assays were performed. The annexin V-FITC binding assay was done first. It detects the translocation of phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane during the early stages of apoptosis. The annexin V-FITC binds to the translocated PS, producing green fluorescence at the cell surface as shown by the representative photos in Fig. 3. Control HCT-116 and HCT-15 cells had low annexin binding scores of  $8 \pm 3$  and  $7 \pm 3$ , respectively. In contrast, the HCT-116 and HCT-15 cells had high annexin binding scores of  $46 \pm 10$  and  $49 \pm 15$ , respectively, after exposing the cells to 300 μM NaDOC for 6 h.

Further evidence for NaDOC-induced apoptosis in HCT-116 and HCT-15 cells was obtained after staining their DNA with DAPI for fluorescence microscopy (Fig. 4). Chromatin condensation and fragmentation were present in  $46 \pm 5\%$  of HCT-116 cells and  $43 \pm 17\%$  of HCT-15 cells, whereas virtually none of the control cells had these features.

HCT-116 and HCT-15 cells were also examined for the presence of DNA fragmentation after exposure of the cells to  $300 \mu\text{M}$  NaDOC for 20 h. More specifically, the TUNEL assay was done (Fig. 5) to detect free 3'-OH ends of the fragmented DNA, which arise due to the action of endonucleases that are activated during the late stages of apoptosis. Upon scoring,  $26 \pm 6\%$  of HCT-116 cells and  $57 \pm 2\%$  of HCT-15 cells were TUNEL-positive, whereas virtually none were detected in the respective control samples.

Although all of these effects on the cells were achieved with  $300 \mu\text{M}$  NaDOC, lower concentrations ( $50$  and  $100 \mu\text{M}$ ) also induced noticeable DNA damage and signs of apoptosis in both HCT-116 and HCT-15 cells (data not shown).

#### 4. DISCUSSION

The gastrointestinal tract is exposed to numerous dietary constituents and also ingested xenobiotics. Some of these substances produce undesirable secondary effects as they are processed. For example, eating high amounts of fat increases the secretion of bile acids to help fat digestion and absorption [7]. As a consequence, the concentration of secondary bile acids is increased in the colon. An estimate of the colonic concentration can be inferred from the amount of secondary bile acids excreted from the body. Total bile acids in fecal water were as high as  $1 \text{ mM}$ , and deoxycholate approached concentrations as high as  $0.8 \text{ mM}$  in human subjects given added fat in the diet [28]. Therefore, it is reasonable that the  $0.3 \text{ mM}$  NaDOC used in the present study falls within the physiological range. However, it is unknown whether such a concentration of deoxycholate in vivo results in any DNA damage and apoptosis in colon epithelial cells. It must be considered that under physiological conditions, other types of matter entering the colon (e.g., unabsorbed calcium, phytochemicals, and indigestible dietary fiber) could inhibit the adverse effects of deoxycholate. As such, these dietary constituents would influence whether a certain concentration of deoxycholate will induce DNA damage and apoptosis but also development of colon cancer.

Exposing both HCT-116 and HCT-15 cells to NaDOC resulted in DNA damage, i.e. single-strand breaks as detected by the comet assay performed under alkaline conditions. Our results are consistent with recent work by others who showed that secondary bile acids induced DNA damage in HT-29 [17] and Caco-2 [29] human colon adenocarcinoma cells. The extent of DNA damage sustained by the HCT-116 and HCT-15 cells were similar, as indicated by the comet scores. It is not entirely clear how NaDOC induces the DNA damage, but there is support for the notion that free radicals are involved. Apparently, secondary bile acids stimulate the production of reactive oxygen species (ROS) and also reactive nitrogen species (RNS). In considering

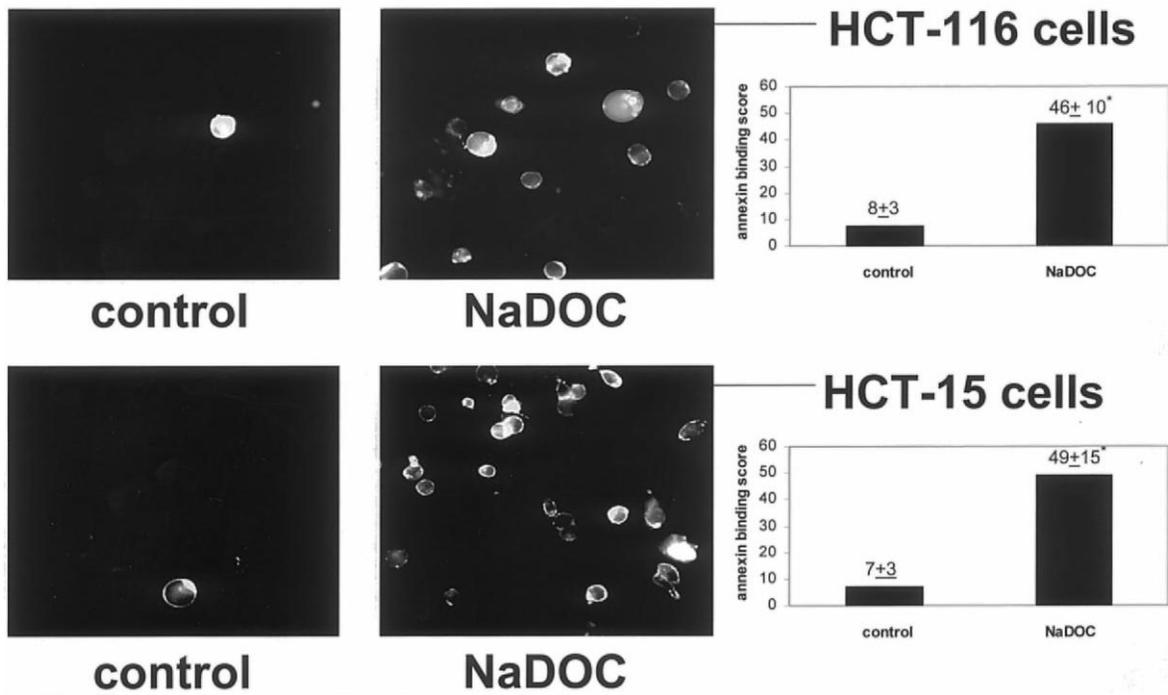


Fig. 3. Increased binding of annexin V-FITC to the outer membrane surface of HCT-116 and HCT-15 cells after exposure to 300  $\mu$ M NaDOC for 6 h, as observed by fluorescence microscopy (400 $\times$ ). Sample photos are shown. The annexin binding scores are the mean  $\pm$  SD of four separate experiments. Scores with a superscript asterisk are significantly different than the respective control scores ( $P < 0.05$ ).

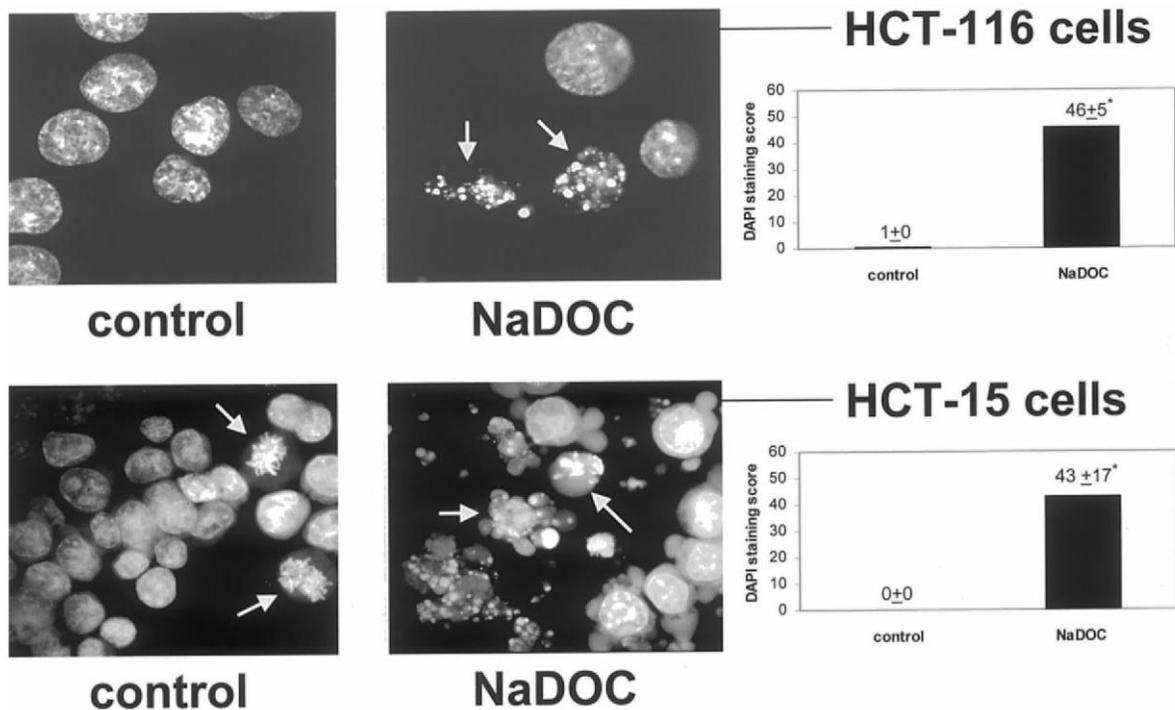


Fig. 4. Chromatin condensation and fragmentation in HCT-116 and HCT-15 cells after exposure to 300  $\mu$ M NaDOC for 6 h and examination by fluorescence microscopy (1000 $\times$ ). HCT-116 cells were stained with DAPI, and HCT-15 cells were stained with DAPI/ sulforhodamine 101. Sample photos are shown. In the photo of control HCT-15 cells, the white arrows point to cells undergoing mitosis with the chromatids highly visible. In the photos of NaDOC-treated HCT-116 and HCT-15 cells, the arrows point to examples of the chromatin condensation and fragmentation. Membrane blebbing is also visible in NaDOC-treated HCT-15 cells because of the sulforhodamine staining of protein. The DAPI staining scores are the mean  $\pm$  SD of three separate experiments. Scores with a superscript asterisk are significantly different than the respective control scores ( $P < 0.05$ ).

ROS, lipid peroxidation products were increased in rat hepatocytes after being exposed to bile acids [30], but this was inhibited by various antioxidants [31]. The bile acid-induced DNA damage in HT-29 cells mentioned above was inhibited when cells were pre-treated with the antioxidant, vitamin E, before exposure to the bile

acids [17]. The hydroxyl radical is the major ROS that damages DNA [32]. Whether the hydroxyl radical is responsible for the DNA-damaging effect of NaDOC remains to be established, however. For this reason, it would be desirable to measure the levels of 8-hydroxy-2'-deoxyguanosine (8-oxodG) as a marker of oxidative DNA damage [33] in NaDOC-treated HCT-116 and HCT-15 cells. In considering RNS, nitrotyrosine residues were detected immunocytochemically in the plasma membrane of HT-29 cells after exposure to NaDOC [34], suggesting that NaDOC stimulated the production of nitric oxide and/or peroxynitrite. Both of these RNS can damage DNA [35].

A normal response of cells to DNA damage is to undergo cell cycle arrest so that DNA repair can be implemented [19]. If the repair is unsuccessful, cells commit apoptosis to safeguard the genome. Therefore, our other data suggest that NaDOC induced irreparable DNA damage in both HCT-116 and HCT-15 cells, since the DNA damage caused by NaDOC was clearly followed by apoptosis, as determined by a panel of standard methods (microscopic examination for morphological changes, DAPI staining, and also annexin V-FITC and TUNEL assays). This finding expands previous research, where it was shown that NaDOC induced apoptosis in HT-29 and Caco-2 colon cells in one study [36], and also apoptosis not only in HT-29 but also FHC colonic cell lines in another study [37]. Nevertheless, we cannot rule out the possibility that the DNA damage caused by NaDOC was a coincidental event and that NaDOC may have induced apoptosis by some other means.

Because p53 tumor suppressor protein influences some of the critical events leading to apoptosis [38], a key question in our study was whether the presence of the normal or wild-type p53 gene is essential for NaDOC-mediated apoptosis. Accordingly, this was the reason for selecting HCT116 (wild-type p53) and HCT-15 (mutant p53) for our study because of their different p53 gene status [23]. The results of the morphological examination, DAPI staining, and also annexin VFITC binding and TUNEL assays show that both cell lines committed apoptosis when exposed to NaDOC. Therefore, these data suggest that NaDOC induced apoptosis in the colon epithelial cells in a p53-independent manner. It has been reported that other DNA-damaging agents, such as adriamycin, can also induce apoptosis in a p53-independent manner at least in human lymphocytes [39]. Other lines of evidence support the suggestion that NaDOC induces apoptosis in a p53-independent manner. First, NaDOC also induced apoptosis in rat fibroblasts that expressed p53 activity and in homologous rat fibroblasts that did not express p53 activity [40]. Secondly, like the HCT-15 colon cells used in the present study, both HT-29 [41] and Caco-2 [42] colon cells express mutant p53, and both undergo apoptosis when exposed to NaDOC [36]. Thirdly, in a similar study to ours, NaDOC induced apoptosis in not only HT-29 colon cells, but also in a human

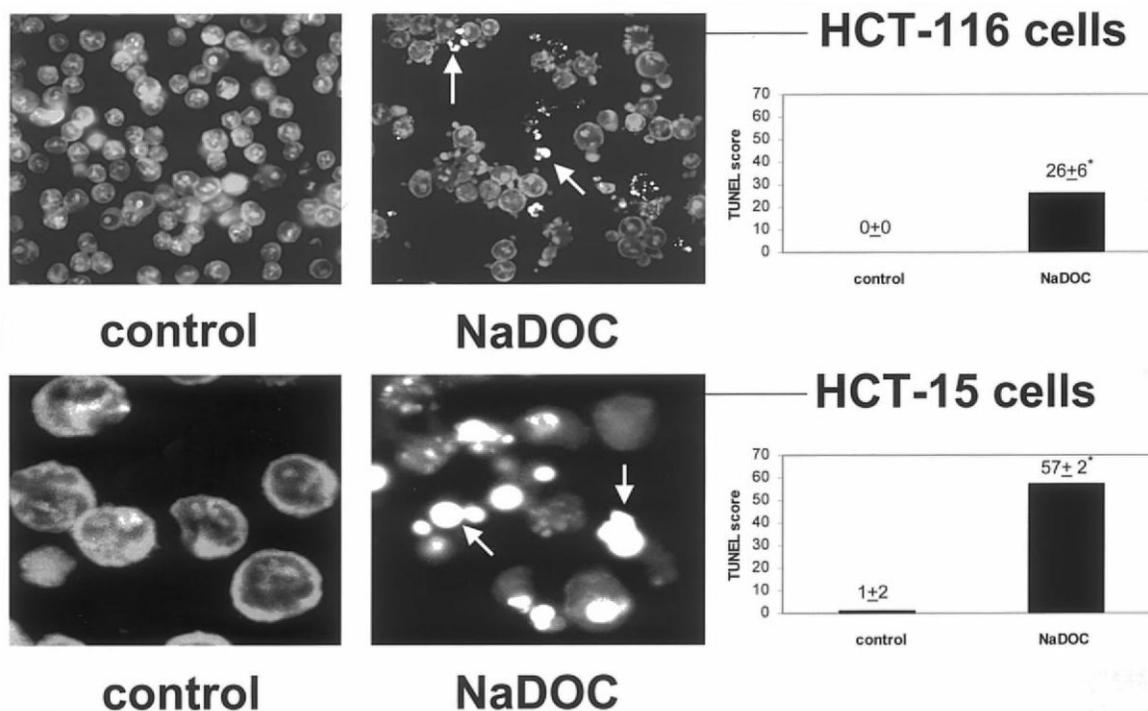


Fig. 5. TUNEL assay images (sample photos) and scores for HCT-116 and HCT-15 cells after exposure to 0 or 300  $\mu$ M NaDOC for 20 h and examination by fluorescence microscopy (400  $\times$  for HCT-116 and 1000  $\times$  for HCT-15). Sample photos are shown. White arrows point to examples of the green fluorescence resulting from the terminal deoxynucleotidyl transferase-mediated labeling of the 3'-OH ends of the fragmented DNA with dUTP-FITC. The TUNEL assay scores are the mean  $\pm$  SD of three separate experiments. Scores with a superscript asterisk are significantly different than the respective control scores ( $P < 0.05$ ).

fetal colonic mucosa cell line (FHC) expressing wild-type p53 [37].

Finally, the signaling mechanism by which NaDOC induces apoptosis in HCT-116 and HCT-15 cells is unknown. However, previous studies suggest an involvement of the Fas receptor-mediated apoptotic pathway. Exposing rat hepatocytes to bile acids resulted in ligand-independent oligomerization of plasma membrane-bound Fas, recruitment of FADD, activation of caspase 8 and also a cascade of downstream caspases, and finally apoptosis [43]. Thus, since the Fas signaling pathway is functional in human colon cells [44], it is conceivable that NaDOC induced apoptosis in HCT-116 and HCT-15 cells in this manner. Studies are planned to consider this possibility.

## REFERENCES

- [1] American Cancer Society, The colon and rectum cancer resource center: colon and rectum cancer statistics, 2000.
- [2] K.W. Kinzler, B. Vogelstein, Lessons from hereditary colorectal cancer, *Cell* 87 (1996) 159–170.
- [3] M. Lipkin, B. Reddy, H. Newmark, S.A. Lamprecht, Dietary factors in human colorectal cancer, *Annu. Rev. Nutr.* 19 (1999) 545–586.
- [4] B.S. Reddy, Nutritional factors and colon cancer, *Crit. Rev. Food Sci. Nutr.* 35 (1995) 175–190.
- [5] E. Giovannucci, B. Goldin, The role of fat, fatty acids, and total energy intake in the etiology of human colon cancer, *Am. J. Clin. Nutr.* S 66 (1997) 1564S–1571.
- [6] R.W. Owen, Faecal steroids and colorectal carcinogenesis, *Scand. J. Gastroenterol.* 222(Suppl.) (1997) 76–82.
- [7] A.F. Hofmann, The continuing importance of bile acids in liver and intestinal disease, *Arch. Intern. Med.* 159 (1999) 2647–2658.
- [8] T. Hori, K. Matsumoto, Y. Sakaitani, M. Sato, M. Morotomi, Effect of dietary deoxycholic acid and cholesterol on fecal steroid concentration and its impact on the colonic crypt cell proliferation in azoxymethane-treated rats, *Cancer Lett.* 124 (1998) 79–84.

- [9] O. Kobori, T. Shimizu, M. Maeda, Y. Atomi, J. Watanabe, M. Shoji, Y. Morioka, Enhancing effect of bile and bile acid on stomach tumorigenesis induced by N'-methyl-N'-nitro-N-nitrosoguanidine in Wistar rats, *J. Natl. Cancer Inst.* 73 (1984) 853–861.
- [10] C.H. Imray, S. Radley, A. Davis, G. Barker, C.W. Hendrickse, I.A. Donovan, A.M. Lawson, P.R. Baker, J.P. Neoptolemos, Faecal unconjugated bile acids in patients with colorectal cancer or polyps, *Gut* 33 (1992) 1239–1245.
- [11] K. Hamada, A. Umemoto, A. Kajikawa, M.J. Seraj, Y. Monden, In vitro formation of DNA adducts with bile acids, *Carcinogenesis* 15 (1994) 1911–1915.
- [12] X.P. Huang, X.T. Fan, J.F. Desjeux, M. Castagna, Bile acids, non-phorbol-ester-type tumor promoters, stimulate the phosphorylation of protein kinase C substrates in human platelets and colon cell line HT29, *Int. J. Cancer* 52 (1992) 444–450.
- [13] F. Hirano, H. Tanada, Y. Makino, K. Okamoto, M. Hiramoto, H. Handa, I. Makino, Induction of the transcription factor AP-1 in cultured human colon adenocarcinoma cells following exposure to bile acids, *Carcinogenesis* 17 (1996) 427–433.
- [14] C.M. Payne, C. Crowley, D. Washo-Stultz, M. Briehl, H. Bernstein, C. Bernstein, S. Beard, H. Holubec, J. Warneke, The stress-response proteins poly(ADP-ribose)polymerase and NF- $\kappa$ B protect against bile salt-induced apoptosis, *Cell Death Different.* 5 (1998) 623–636.
- [15] F. Zhang, K. Subbaramaiah, N. Altorki, A.J. Dannenberg, Dihydroxy bile acids activate the transcription of cyclooxygenase-2, *J. Biol. Chem.* 273 (1998) 2424–2428.
- [16] H. Sano, Y. Kawahito, R.L. Wilder, A. Hashiramoto, S. Mukai, K. Asai, S. Kimura, H. Kato, M. Kondo, T. Hla, Expression of cyclooxygenase-1 and -2 in human colorectal cancer, *Cancer Res.* 55 (1995) 3785–3789.
- [17] L.A. Booth, I.T. Gilmore, R.F. Bilton, Secondary bile acid induced DNA damage in HT29 cells: are free radicals involved, *Free Rad. Res.* 26 (1997) 135–144.
- [18] B. Halliwell, Free radicals and antioxidants: a personal view, *Nutr. Rev.* 52 (1994) 253–265.
- [19] R.M. Elledge, W.-H. Lee, Life and death by p53, *BioEssays* 17 (1995) 923–930.
- [20] C.M. Payne, H. Bernstein, C. Bernstein, H. Garewal, Role of apoptosis in biology and pathology: resistance to apoptosis in colon carcinogenesis, *Ultrastruct. Pathol.* 19 (1995) 221–248.
- [21] C.C. Harris, Structure and function of the p53 tumor suppressor gene: clues for rational cancer therapeutic strategies, *J. Natl. Cancer Inst.* 88 (1996) 1442–1455.
- [22] Y. Take, M. Kumano, H. Teraoka, S. Nishimura, A. Okuyama, DNA-dependent protein kinase inhibitor (OK-1035) suppresses p21 expression in HCT116 cells containing wild-type p53 induced by adriamycin, *Biochem. Biophys. Res. Commun.* 221 (1996) 207–212.
- [23] R. Chinery, J.A. Brockman, M.O. Peeler, Y. Shyr, R.D. Beauchamp, R.J. Coffey, Antioxidants enhance the cytotoxicity of chemotherapeutic agents in colorectal cancer: a p53-independent induction of p21WAF1/CIP1 via C/EBP $\beta$ , *Nature Med.* 3 (1997) 1233–1241.
- [24] N.P. Singh, M.T. McCoy, R.R. Tice, E.L. Schneider, A simple technique for quantitation of low levels of DNA damage in individual cells, *Exp. Cell. Res.* 175 (1988) 184–191.
- [25] Y.-J. Pan, G. Loo, Effect of copper deficiency on oxidative DNA damage in Jurkat T-lymphocytes, *Free Radic. Biol. Med.* 28 (2000) 824–830.
- [26] M.K. Johnson, G. Loo, Effects of epigallocatechin gallate and quercetin on oxidative damage to cellular DNA, *Mutat. Res.* 459 (2000) 211–218.
- [27] P.J. McCarthy, S.F. Sweetman, P.G. McKenna, V.J. McKelvey-Martin, Evaluation of manual and image analysis quantitation of DNA damage in the alkaline comet assay, *Mutagenesis* 12 (1997) 209–214.
- [28] J. Stadler, H.S. Stern, K.A. Sing-Yeung, V. McGuire, R. Furrer, N. Marcon, W.R. Bruce, Effect of high fat consumption on cell proliferation activity of colorectal mucosa and on soluble faecal bile acids, *Gut* 29 (1988) 1326–1331.
- [29] M. Venturi, R.J. Hambly, B. Glinghammer, J.J. Rafter, I.R. Rowland, Genotoxic activity in human faecal water and the role of bile acids: a case study using the alkaline comet assay, *Carcinogenesis* 18 (1997) 2353–2359.
- [30] R.J. Sokol, B.M. Winklhofer-Roob, M.W. Devereaux, J.M. McKim Jr, Generation of hydroperoxides in isolated rat hepatocytes and hepatic mitochondria exposed to hydrophobic bile acids, *Gastroenterol.* 109 (1995) 1249–1256.

- [31] R.J. Sokol, M. Devereaux, R. Khandwala, K. O'Brien, Evidence for involvement of oxygen free radicals in bile acid toxicity to isolated rat hepatocytes, *Hepatology* 17 (1993) 869–881.
- [32] A.P. Breen, J.A. Murphy, Reactions of oxyl radicals with DNA, *Free Radic. Biol. Med.* 18 (1995) 1033–1077.
- [33] T.M. Nicotera, S. Bardin, Electrochemical detection of 8-hydroxy-2-deoxyguanosine levels in cellular DNA, *Methods Mol. Biol.* 108 (1998) 181–190.
- [34] D. Washo-Stultz, N. Hoglen, H. Bernstein, C. Bernstein, C.M. Payne, Role of nitric oxide and peroxynitrite in bile salt-induced apoptosis: relevance to colon carcinogenesis, *Nutr. Cancer* 35 (1999) 180–188.
- [35] S. Burney, J.L. Caufield, J.C. Niles, J.S. Wishnok, S.R. Tannenbaum, The chemistry of DNA damage from nitric oxide and peroxynitrite, *Mutation Res.* 424 (1999) 37–49.
- [36] M.C. Marchetti, G. Migliorati, R. Moraca, C. Riccardi, I. Nicoletti, R. Fabiani, V. Mastrandrea, G. Morozzi, Possible mechanisms involved in apoptosis of colon tumor cell lines induced by deoxycholic acid, short-chain fatty acids, and their mixtures, *Nutr. Cancer* 28 (1997) 74–80.
- [37] A.J. Haza, B. Glinghammer, A. Grandien, J. Rafter, Effect of colonic luminal components on induction of apoptosis in human colonic cell lines, *Nutr. Cancer* 36 (2000) 79–89.
- [38] M.R. Bennett, Mechanisms of p53-induced apoptosis, *Biochem. Pharmacol.* 58 (1999) 1089–1095.
- [39] R.B. Gartenhaus, P. Wang, P. Hoffman, Induction of the WAF1/CIP1 protein and apoptosis in human T-cell leukemia virus type I-transformed lymphocytes after treatment with adriamycin by using a p53-independent pathway, *Proc. Natl. Acad. Sci. USA* 93 (1996) 265–268.
- [40] Z.-Y. Zheng, H. Bernstein, C. Bernstein, C.M. Payne, J.D. Martinez, E.W. Gerner, Bile acid activation of the gadd 153 promoter and of p53-independent apoptosis: relevance to colon cancer, *Cell Death Different.* 3 (1996) 407–414.
- [41] W.G. Zhang, X.W. Li, L.P. Ma, S.W. Wang, H.Y. Yang, Z.Y. Zhang, Wild-type p53 protein potentiates phototoxicity of 2-BA-2-DMBA in HT29 cells expressing endogenous mutant p53, *Cancer Lett.* 138 (1999) 189–195.
- [42] S. Djelloul, M.E. Forgue-Lafitte, B. Hermelin, M. Ma-reel, E. Bruyneel, A. Baldi, A. Giordano, E. Chastre, C. Gespach, Enterocyte differentiation is compatible with SV40 large T expression and loss of p53 function in human colonic Caco-2 cells. Status of the pRb1 and pRb2 tumor suppressor gene products, *FEBS Lett.* 406 (1997) 234–242.
- [43] W.A. Faubion, M.E. Guicciardi, H. Miyoshi, S.F. Bronk, P.J. Roberts, P.A. Svingen, S.H. Kaufmann, G.J. Gores, Toxic bile acids induce rodent hepatocyte apoptosis via direct activation of Fas. *J. Clin. Invest.* 103 (1999) 137–145.
- [44] J.A. Houghton, F.G. Harwood, A.A. Gibson, D.M. Tillman, The fas signaling pathway is functional in colon carcinoma cells and induces apoptosis, *Clin. Cancer Res.* 3 (1997) 2205–2209.