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Bile Salt Induction of Apoptosis in Goblet Cells of the Normal Human Colonic Mucosa: Relevance to Colon Cancer

Hanan Samaha[1], Carol Bernstein[2], Claire M. Payne[2,3], Harinder S. Garewal[4], Richard E. Sampliner[4,5] and Harris Bernstein[2]

[1]Department of Nutrition and Food Science, University of Arizona, Tucson, AZ USA 85721 [2]Department of Microbiology and Immunology, University of Arizona, Tucson AZ USA 85724 Fax: (602) 626-2100 [3]Arizona Research Laboratories, University of Arizona, Tucson, AZ USA 85721 [4]Veterans Affairs Hospital, Tucson AZ USA (85713) [5]Department of Internal Medicine, University of Arizona, Tucson AZ USA 85724

ABSTRACT

Substantial evidence indicates that bile salts have a role in the etiology of colon cancer. The mechanism of action of bile salts, however, is largely unknown. We show here that the bile salt sodium deoxycholate (NaDOC), at high physiologic concentrations, induces apoptosis (programmed cell death) in a select colonic epithelial subpopulation (immature and mature goblet cells). Induction of apoptosis by NaDOC was determined using light and electron microscopy.

The colonic goblet cells of four normal human subjects showed substantial induction of apoptosis (45% to 63% of goblet cells were apoptotic) upon treatment with 1 mM NaDOC for three hours. In contrast, the normal appearing colonic mucosa from six colon cancer patients showed relatively low induction of apoptosis (only 1% to 23% of goblet cells were apoptotic) after the same treatment. Among six patients with non-malignant polyps, four resembled the cancer patients in that the frequency of apoptotic cells in the normal portion of their colon was low after NaDOC treatment (2% to 21%); the other two had frequencies similar to that of normal individuals (61% and 63%).

These new findings suggest a novel hypothesis related to the role of bile salts in colon carcinogenesis. It is known that a high fat diet produces high levels of bile salts in the intestinal tract, and that bile salts cause DNA damage. Studies have also shown that cells with unrepaired DNA damage are subject to apoptosis. We hypothesize that when an individual ingests a high fat diet over several decades, bile salt-induced apoptosis occurs chronically and, as a result, a population of apoptosis-resistant cells can be selected. When such a population becomes established, cells with carcinogen caused DNA damage remain viable instead of entering the apoptosis pathway. Such cells may then replicate and undergo mutation at sites of damage. These mutations may then lead to colon cancer.

KEYWORDS

Apoptosis, goblet cells, bile salts, colon cancer.

INTRODUCTION

Epidemiological evidence has long indicated that diet plays an important role in colon cancer [1-4]. Cheah [5], in a review of the possible causative agents in colon cancer (e.g. fecal mutagens, ketosteroids, dietary fats and bile acids), concluded that fecal bile acids are the most strongly implicated. Bile acids are physiologically important steroidal surfactants that are secreted into the small intestine to emulsify fat. High bile salt concentrations are known to accompany a high fat, low fiber Western-type diet [6,7]. The mechanism, however, by which bile salts promote colon cancer is not known.

Kulkarni et al. [8] and Kandell and Bernstein [9] have shown that bile salts cause DNA damage in mammalian cells. Since bile salts act as promoters and are not primary carcinogens, the effects of bile salts on cells and their consequences may be fairly complex. Our novel finding that bile salts induce apoptosis, a physiologic form of cell death [10], in human colonic epithelial cells prompts this report.

It has been known for some time that bile salts are cytotoxic to cells. Previous studies evaluating the cytotoxicity of bile salts, however, generally employed high concentrations that caused cell lysis, and used indicators of cell lysis as measures of the effects of the bile salts [11-13]. Such studies primarily measured necrosis, a passive form of cell death which is trauma induced [14].

These studies did not evaluate the more regulated form of cell death, apoptosis, in response to bile salt treatment. In apoptosis, the cell takes an active role in its own death, a form of programmed cell suicide. Apoptosis occurs during embryogenesis [15], metamorphosis [16], tissue atrophy during hormone withdrawal [17-19], and after treatment with some DNA damaging agents [20]. The process of apoptosis may have evolved, in part, in multicellular organisms to promote the overall survival of the organism through the deletion of damaged cells, a form of cellular euthanasia [21]. Apoptosis may benefit humans by eliminating cells that might otherwise replicate their DNA past unrepaired damages leading to increased mutation, and, hence, increased probability of cancer [22].

Apoptotic cells were originally defined by characteristic margination of the chromatin, nuclear fragmentation and condensation of the nucleus and cytoplasm as seen in the electron microscope [10]. A biochemical characteristic subsequently reported to be associated with some cell types entering apoptosis has been cleavage of DNA at internucleosomal linker regions, resulting in a "ladder" of DNA fragments of 180-200 base pairs and multiples, thereof [23-30]. However, a "ladder" of DNA fragments is also associated with necrosis in some cell types [31,32] and in addition may be artifactually introduced during DNA isolation [33]. Other phenomena associated with apoptosis in some cell types such as the transcriptional activation of the long interspersed nuclear element and *in situ* DNA fragmentation have also been found to lack specificity for apoptotic cells alone [34]. Thus, the "gold standard" for determination of apoptosis has been observation of the characteristic morphological changes by electron microscopy [10,32,35-42]. Once apoptosis is established as occurring under a given set of circumstances using the "gold standard", other less time-consuming methods that correlate with the ultrastructural appearance of apoptosis can be used to quantitate the degree of apoptosis occurring with the cells in question.

We have now used light and electron microscopy to establish the occurrence of apoptosis in immature and mature goblet cells of the human colonic mucosa. This

apoptosis occurred in response to exposure to high physiologic concentrations of the most common bile salt found in the human colon, sodium deoxycholate (NaDOC). We correlated the occurrence of apoptosis as seen in the electron microscope with the appearance of such cells in one micron, toluidine blue stained epoxy-embedded sections in the light microscope.

We then tested the levels of apoptosis that occur in goblet cells within biopsies from the normal mucosa of normal individuals, and from the normal appearing mucosa of low-risk polyp patients, cancer patients and high-risk polyp patients. The goblet cells from normal subjects and low risk polyp patients showed high levels of apoptosis when treated with NaDOC, while goblet cells from cancer patients or high risk polyp patients showed low levels of induced apoptosis. It is possible that this bioassay, which measures apoptosis using light microscopic criteria, may develop into a useful biomarker to identify patients at risk for colon cancer.

MATERIALS AND METHODS

A. Patient recruitment

Patients were recruited at the time of colonoscopy in the endoscopy laboratories at the Tucson Veterans Affairs Medical Center and the University Medical Center. Since all patients recruited into the study had a colonoscopy procedure performed, an assessment of polyp burden was known at the time the mucosal samples were taken for the "induced-apoptosis" bioassay.

B. Tissue procurement

Biopsy specimens were taken from the colonic mucosa approximately 20 cm from the anal verge during the colonoscopy procedure. For each subject, seven separate biopsies were obtained and treated as described below.

C. "Induced-apoptosis" bioassay

The seven biopsies from each patient were placed, respectively, in vials containing 0, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mM NaDOC, made up in sterile Eagle's Modified Tissue Culture Media (Sigma) containing 10% heat-inactivated fetal calf serum, non essential amino acids (Sigma), penicillin, streptomycin and HEPES buffer. The vials had been preincubated for 45 minutes in a CO^2-humidified incubator maintained at 37°C to allow for pH equilibration before the start of the incubation period. The biopsies were incubated for three hours at 37°C in a CO^2-humidified incubator. The vials were then removed from the incubator, the tissue culture media replaced with cold 3% glutaraldehyde fixative made up in 0.1 M phosphate buffer (pH 7.2), and the tissue fixed for 3 hours at 4°C. Subsequently, the fixed tissue was stored in 0.1 M phosphate buffer (containing 10% sucrose) at 4°C. The tissue was processed for epoxy embedment as described by Payne et al. [43,44]. Briefly, the tissue was post-osmicated, dehydrated in a graded series of alcohols and embedded in Spurr's epoxy resin. One micron semi-thin sections were then prepared using glass knives, stained with toluidine blue O and the number of "darkly-stained" (apoptotic) and "lightly-stained" (non-apoptotic) goblet cells were quantitated by light microscopy. At least 200 goblet cells obtained from 10 or more different crypts were scored from two separate blocks at each dose of NaDOC, and the percentage of apoptotic cells determined.

RESULTS

A. Determination of Assay Conditions and Methods of Measurement

NaDOC was used in the apoptosis bioassay because NaDOC is the most common

bile salt in human feces [45]. We found that concentrations greater than 1.0 mM (i.e. from 1.5 to 3.0 mM) produced lysis of epithelial cells in a high proportion of the cases from normal subjects, polyp patients and patients with a history of colon cancer. Therefore, data were analyzed only from the samples treated with 0, 0.5 and 1.0 mM NaDOC. We further found that goblet cells were the epithelial cells most sensitive to NaDOC-induced apoptosis. Thus, goblet cells were the cells that were scored in our assay.

Representative one micron epoxy sections of human colonic biopsies stained with toluidine blue O are shown in Figure 1. Apoptotic goblet cells (treated with NaDOC) have very densely stained cytoplasm (Figure 1B) compared with the cytoplasm of untreated goblet cells (Figure 1A). The criteria used to score goblet cells were condensation and/or margination of the chromatin or increased density of cytoplasm surrounding the mucin granules in profiles where the nucleus was out of the plane of section.

We have carefully evaluated this staining pattern at the ultrastructural level and determined that the dark staining cells seen in one micron epoxy sections by light microscopy correspond to classic apoptotic cells by electron microscopy. Figure 2A shows examples of goblet cells that were untreated and appear normal. Figures 2B and 2C show goblet cells that were treated with NaDOC and have characteristics of apoptosis, particularly chromatin condensation and margination and increased density of the cytoplasm.

B. Experimental Parameters of the Apoptosis Bioassay: Effect of Incubation in Tissue Culture Media on Cells

Figure 3 shows an electron micrograph of normal goblet cells from a tissue specimen that was incubated in the absence of bile salts. The cells contain large numbers of mucin granules and have normal nuclei with evenly dispersed chromatin and prominent nucleoli. This illustrates that the *in vitro* incubation adequately preserved the normal ultrastructure of the colonic epithelial cells. On the other hand, as can be seen in Figures 2B and 2C, treatment with 1.0 mM NaDOC caused some tissue degeneration after a three hour incubation at 37°C.

C. Distribution of Apoptotic Cells and Type of Enterocyte Affected

Of the three distinct morphologic types of differentiated enterocytes within the human distal colonic mucosa (columnar, goblet and enterochromafin cells), only the goblet cells were found to undergo apoptosis in response to bile salts at high physiological levels. Bile salts were found to induce apoptosis in virtually all locations of goblet cells within the crypt, representing both immature and mature mucous-secreting cells. Although the goblet cell normally undergoes differentiation [46] followed by senescence and death [47] by apoptosis [40,48], this uninduced apoptosis is restricted to cells at the luminal surface [40,48]. Ijiri and Potten [49] have shown that intestinal cells can undergo apoptosis in response to DNA-damaging agents such as chemotherapeutic drugs and radiation. A specificity for a specific cell type, however, was not indicated using these apoptosis-inducing agents.

D. Evaluation of Induced Apoptosis in Patients with Different Risks for Colon Cancer

1) Normal patients:- The dose-response curves obtained with colonic biopsies from four normal subjects are shown in Figure 4A. The biopsies in this group were similar in showing a strong induction of apoptosis, reaching 45% to 63% apoptotic goblet cells at 1.0 mM NaDOC. This contrasts with the low frequency of spontaneously occuring apoptotic goblet cells (0 to 4%)

found in the zero dose controls.

2) Cancer patients:- Figure 4B shows the results obtained upon NaDOC treatment of biopsies from the *normal-appearing mucosa* of the colons of six patients with a history of colon cancer. The percentages of apoptotic cells within these biopsies, after treatment with 1.0 mM NaDOC, were substantially lower (1%-23%) than those observed in biopsies from normal subjects.

3) Patients with non-malignant polyps:- Figure 5 shows the results obtained from six unselected polyp patients. The dose-response curves from these cases segregated into two patterns (low induction or high induction of apoptosis). Goblet cells within biopsies from two polyp patients showed strong induction of apoptosis by 1.0 mM NaDOC (61%-63%) similar to the induction of apoptosis shown by goblet cells from normal individuals at 1.0 mM NaDOC (Figure 5A). Goblet cells from four polyp patients showed weak induction of apoptosis (2%-21%) similar to the induction of apoptosis shown by goblet cells from cancer patients at 1.0 mM NaDOC (Figure 5B). The two polyp patients whose goblet cells showed strong induction of apoptosis (and therefore low risk for colon cancer by the bioassay) were also determined to be clinically at low risk (both had a past history of small polyps with no recurrence at the time the current biopsies were taken). The four patients whose goblet cells showed low induction of apoptosis (and therefore high risk by the bioassay) were considered at increased risk clinically [one patient had an early onset of polyps at 48 years of age, a second had multiple polyps with one more than 3 cm. in size (villous adenoma type), a third had 2, 5 and 3 polyps removed at 3 month intervals (the 5 polyps were removed when the present biopsy was taken), and the fourth had 5 tubular adenomatous polyps removed at the time of biopsy; this same patient also had a prior history of a tubulovillous adenoma.

DISCUSSION

We have shown, for the first time, that NaDOC, a naturally occurring bile salt, induces a high level of apoptosis in the colonic goblet cells of normal subjects, while the normal-appearing portion of the colon of subjects with colon cancer are relatively resistant to NaDOC induction of apoptosis. The finding that bile salts readily induce apoptosis in goblet cells of normal individuals indicates that a normal protective response exists in individual damaged cells, which acts for the overall benefit of the organism. The relative failure of non-neoplastic epithelial cells from colon cancer and high risk polyp patients, to undergo apoptosis is an abnormal cellular response. A deficiency of a protective cell suicide pathway could, therefore, be detrimental to the survival of the individual.

Based on these findings we propose a model, outlined in Figure 6, which attempts to explain the increasing risk of developing colon cancer with age and exposure to agents such as bile acids. When an individual ingests a high fat and low fiber diet over several decades, the colonic lumen is exposed to chronically high concentrations of bile salts that have been produced to aid in digestion of fat. Bile salts have already been shown to cause DNA damage in mammalian cells [8,9]. Apoptosis serves to protect humans from cancer by eliminating cells with unrepaired DNA damage [20,22]. High levels of fat ingestion, causing high concentrations of NaDOC in the colon, leads to high frequencies of colonic epithelial cell death due to apoptosis. Over time, however, continuation of this process may select for survival of mutant cells, including mutant progenitor goblet cells, which have become relatively resistant to apoptosis induction. Prolonged intake of a high fat diet will result in the repopulation of the colonic mucosa by apoptosis-resistant cells. Any unrepaired DNA damage caused by carcinogens would

remain in the genome of apoptosis-resistant goblet cells. Replication of these DNA damaged cells could lead to further mutations with the resultant accumulation of genetic abnormalities, some of which may lead to neoplasia. The model is also consistent with the "better dead than wrong" hypothesis to explain the role of apoptosis in eliminating cells with DNA damage [50].

Our novel hypothesis is supported by recent evidence of Magnuson et al.[51] showing that when rats were chronically fed the bile acid cholic acid, the cells within their colonic crypts developed resistance to induction of apoptosis by the carcinogen azoxymethane. Both cells within normal appearing colonic crypts and cells within aberrant crypt foci (regarded as preneoplastic lesions of colon cancer) were resistant to induction of apoptosis.

Apoptosis is ordinarily triggered by much lower concentrations of inducing agents than necrosis [52]. Thus, apoptosis, which is induced by sub-micellar concentrations of NaDOC [53], is likely to have a more important role than necrosis in protecting against colon carcinogenesis. Our work indicates that 1.0 mM of NaDOC induces apoptosis within colonic goblet cells. Van Fassen et al. [45] showed that deoxycholic acid occurs at about 1.85 mM in the feces of individuals with a mixed Western diet. Thus, concentrations of bile acids within the physiologic range (i.e. levels that accompany a high fat diet) are sufficient to induce apoptosis in our *in vitro* assay system.

Substantial evidence indicates that most colonic carcinomas originate in adenomas [54,55], which may project into the bowel lumen in the form of a polyp. Adenomas are classified according to their microscopic architecture as tubular, tubulovillous or villous [56]. Adenomas result from a disturbance of growth and differentiation, and may show abnormal cytological features that are expressed as varying degrees of dysplasia, the severest being carcinoma *in situ*. A high adenoma burden is considered an important risk factor for developing adenocarcinoma. Important morphological features that determine the malignant potential of adenomas are their size, histological type and grade of dysplasia [56].

Since our results indicate that the *normal-appearing mucosa* of high risk polyp patients (risk determined by increased polyp size, poor histological grade and severe degree of dysplasia) are deficient in the natural defense process of apoptosis, we anticipate that an *in vitro* induced-apoptosis bioassay could be developed for use as an intermediate biomarker to help identify patients at high risk for colon cancer. The wide variation in NaDOC-induction of apoptosis among patients with non-malignant polyps, and the fact that low apoptotic response correlates with other risk factors, suggests that this bioassay may prove useful as a prognostic marker of the susceptibility of such patients to colon cancer. Polyp patients whose goblet cells show low bile salt-induced apoptosis (similar to that of colon cancer patients), could undertake a change in life-style (including a switch to a low-fat, high fiber diet and frequent monitoring), which may be preventive against colon cancer.

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RESUMEN

Evidencias substanciales indican que

las sales biliares juegan papel en la etiología del cáncer del colon. Sin embargo se desconoce profundamente el mecanismo de acción de las sales biliares. Nosotros demostramos que la sal biliar deoxicolato de sodio (NaDOC), a altas concentraciones fisiológicas induce la apoptosis (muerte celular programada) en una selecta subpoblación del epitelio del colon (células caliciformes maduras e inmaduras). La inducción de la apoptosis fue determinada por medio de microscopía de luz y electrónica.

Las células caliciformes del colon, de cuatro sujetos humanos normales, demostraron inducción substancial de la apoptosis (45 a 63% de las células fueron apoptóticas), en el tratamiento con 1mM, NaDOC, por tres horas. En contraste, la mucosa de apariencia normal, de seis pacientes con cáncer de colon, demostró relativamente baja inducción de la apoptosis (solamente 1 al 13% de las células caliciformes fueron apoptóticas), después del mismo tratamiento. Entre seis pacientes con pólipos no malignos, cuatro se asemejaron a los pacientes de cáncer, con una baja frecuencia de células apoptóticas (2 al 21%), en la porción normal del colon después del tratamiento con NaDOC. Los otros dos pacientes presentaron frecuencias similares a los individuos normales (61 y 63%).

Estos nuevos hallazgos sugieren una nueva hipótesis en relación al papel de las sales biliares en la carcinogénesis del colon. Es conocido que una dieta rica en grasas produce altos niveles de sales biliares, las cuales causan daño al ADN. Algunos estudios también han sugerido que las células con daños no reparados en el ADN, están sujetas a la apoptosis. Nosotros lanzamos como hipótesis que: cuando un individuo ingiere una dieta rica en grasas por varias décadas, la inducción de apoptosis mediada por sales biliares ocurre crónicamente, y como resultado, puede ser seleccionada una población de células resistentes a la apoptosis. Cuando tal población se establece, las células que presentan un daño al ADN, con capacidad carcinogénica, permanecen viables en lugar de entrar en la vía de la apoptosis. Tales células pueden replicarse y sufrir mutación en los sitios del daño, estas mutaciones pueden conducir al cáncer de colon.

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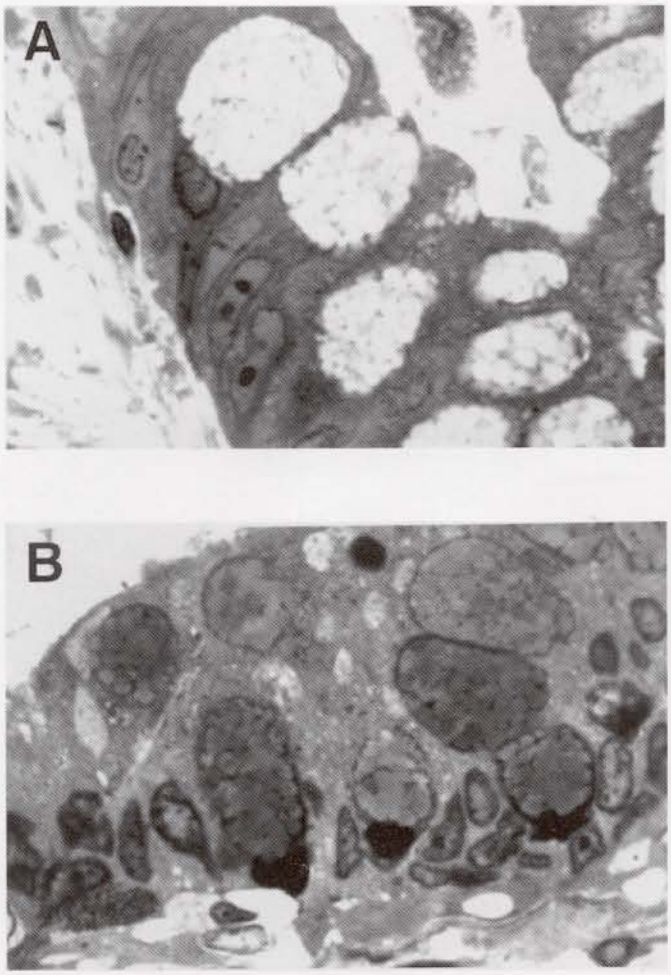

FIGURE 1:- Light micrographs of biopsies of the normal mucosa of a non-colon cancer patient with adenomatous polyps (low risk group). **[A]** Tissue incubated for 3 hours at 37oC in the absence of NaDOC. **[B]** Tissue incubated for 3 hours at 37°C in the presence of NaDOC. Note the dark staining of the nucleus and cytoplasm of the goblet cells, which are identified by the abundant mucin granules. (One micron epoxy sections, Toluidine blue O stain; X 2,600).

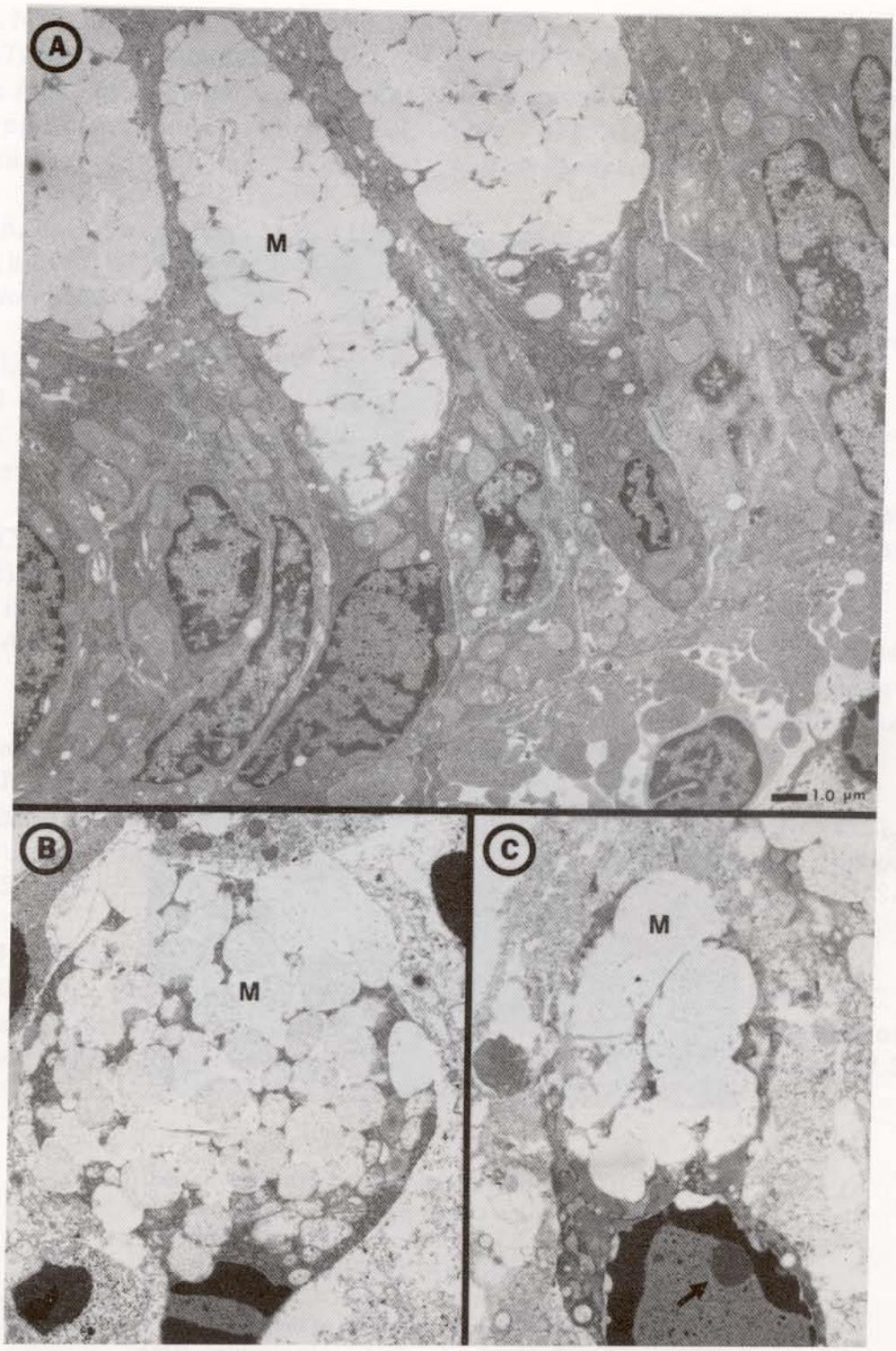

FIGURE 2:- Composite electron micrographs of the normal colonic mucosa of normal, neoplasia-free patients undergoing colonoscopy. The biopsies were incubated in vitro for 3 hours at 37°C in a CO_2-incubator in the presence or absence of NaDOC. (M=mucin granules; Uranyl acetate, Lead Citrate). **[A]** Incubation without NaDOC. Note the excellent tissue preservation and lack of apoptosis. **[B]** Incubation in the presence of 1 mM NaDOC. The goblet cell shown has characteristic features of apoptosis, including condensation and margination of chromatin and increase in nuclear and cytoplasmic electron density. [C] Incubation in the presence of 1 mM NaDOC. This goblet cell has characteristic features of apoptosis, including condensation and margination of chromatin, loss of nucleolar structure (arrows) and increase in nuclear and cytoplasmic electron density.

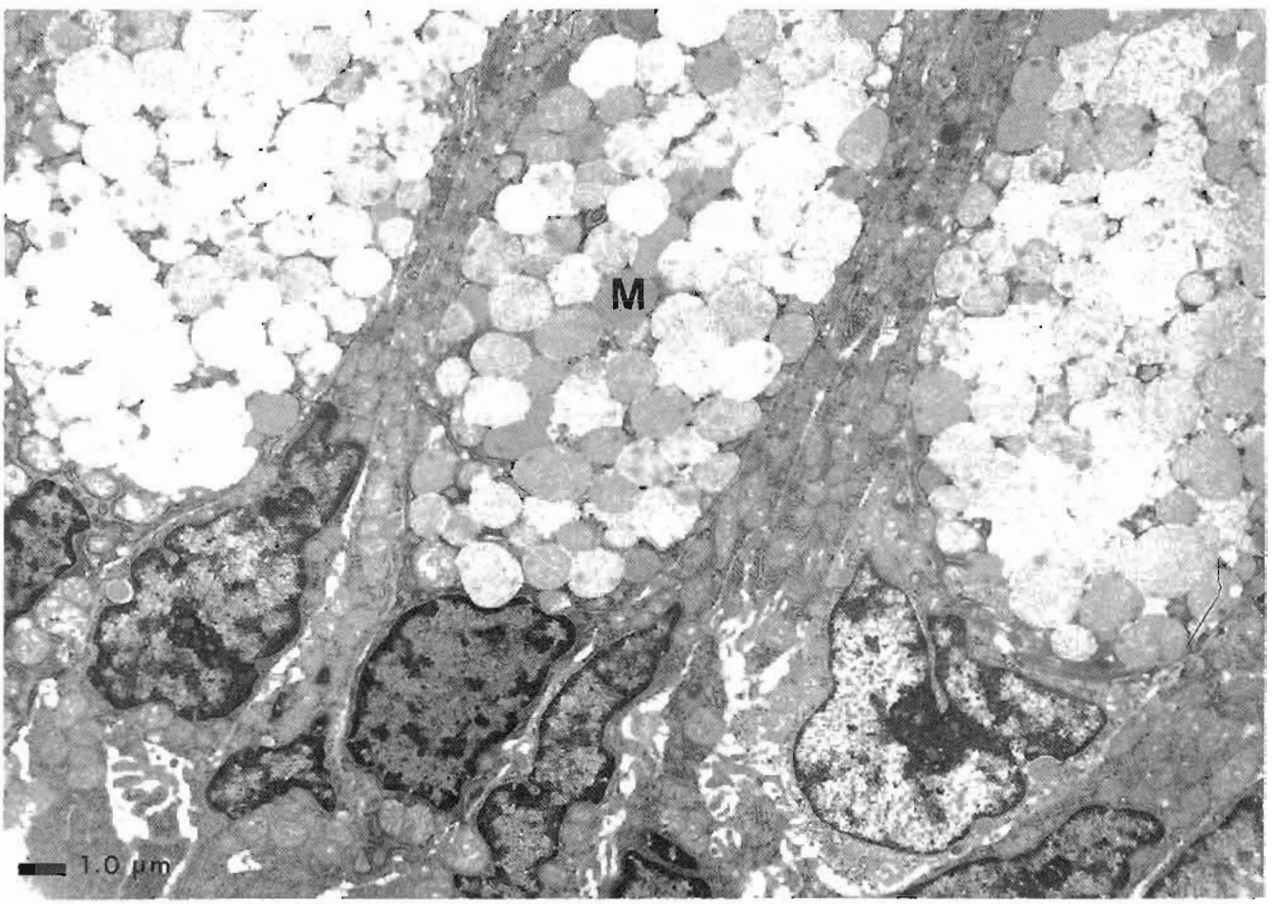

FIGURE 3:- Electron micrograph of the normal colonic mucosa of a polyp patient incubated in the absence of NaDOC for 3 hrs. at 37°C in a CO_2-incubator. Note the excellent tissue preservation and the absence of apoptotic cells. (M=mucin granules; Uranyl Acetate, Lead Citrate).

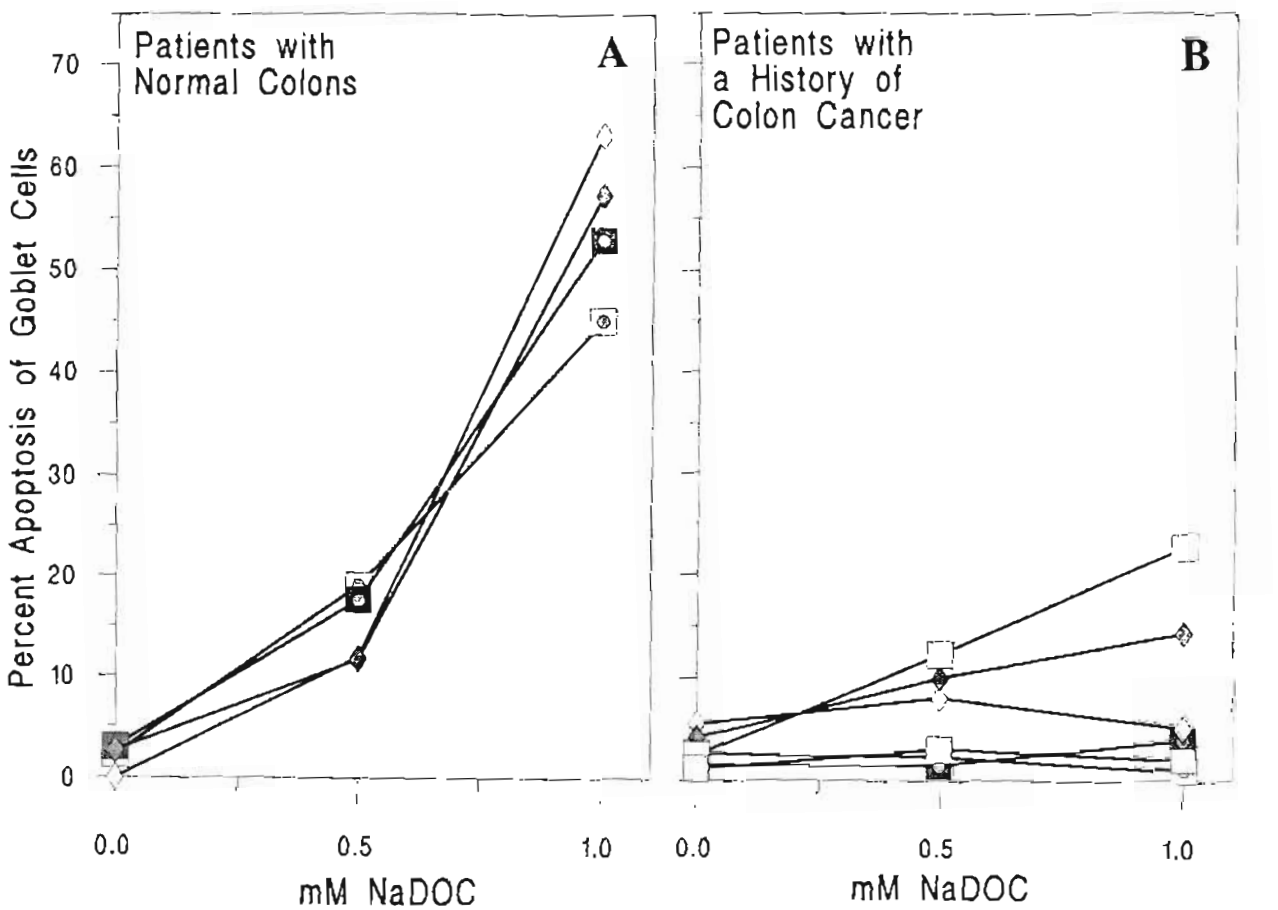

FIGURE 4:- Dose-response curves (percent apoptosis vs. NaDOC concentration) for goblet cells of the normal colonic mucosa derived from normal subjects [**A**] and from colon cancer patients [**B**]. Colonic goblet cells of normal subjects show a high induction of apoptosis at 1.0 mM NaDOC, whereas colonic goblet cells of cancer patients are relatively resistant to apoptosis at 1.0 mM NaDOC.

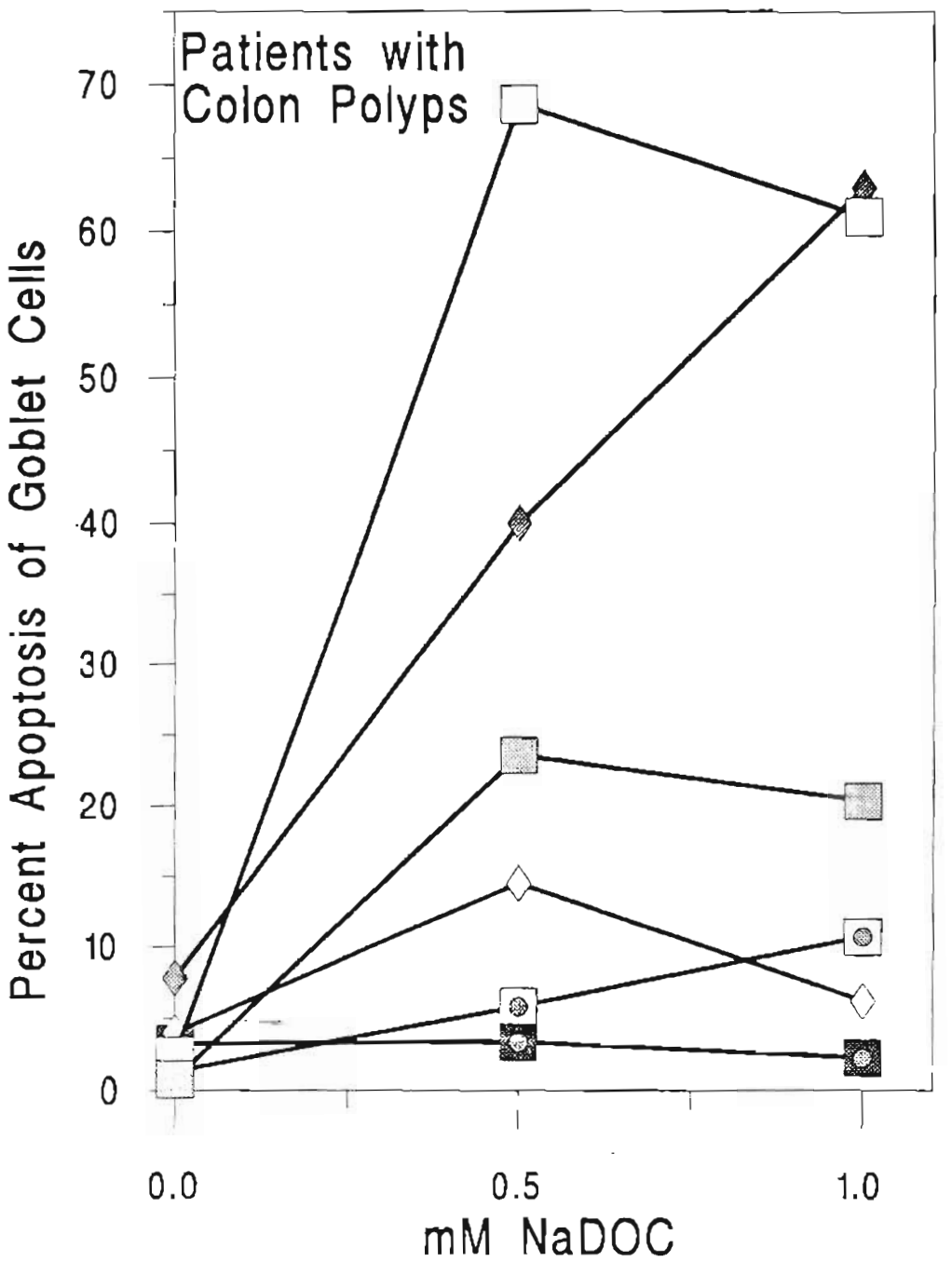

FIGURE 5:- Dose-response curves (percent apoptosis vs. NaDOC concentration) for goblet cells of the normal mucosa derived from low-risk (top two curves) patients with polyps and high-risk (lower four curves) patients with polyps. The two low-risk patients show a relatively high induction of apoptosis at both 0.5 mM NaDOC and 1.0 mM NaDOC, and the four high risk patients show a relatively low induction of apoptosis at these concentrations.

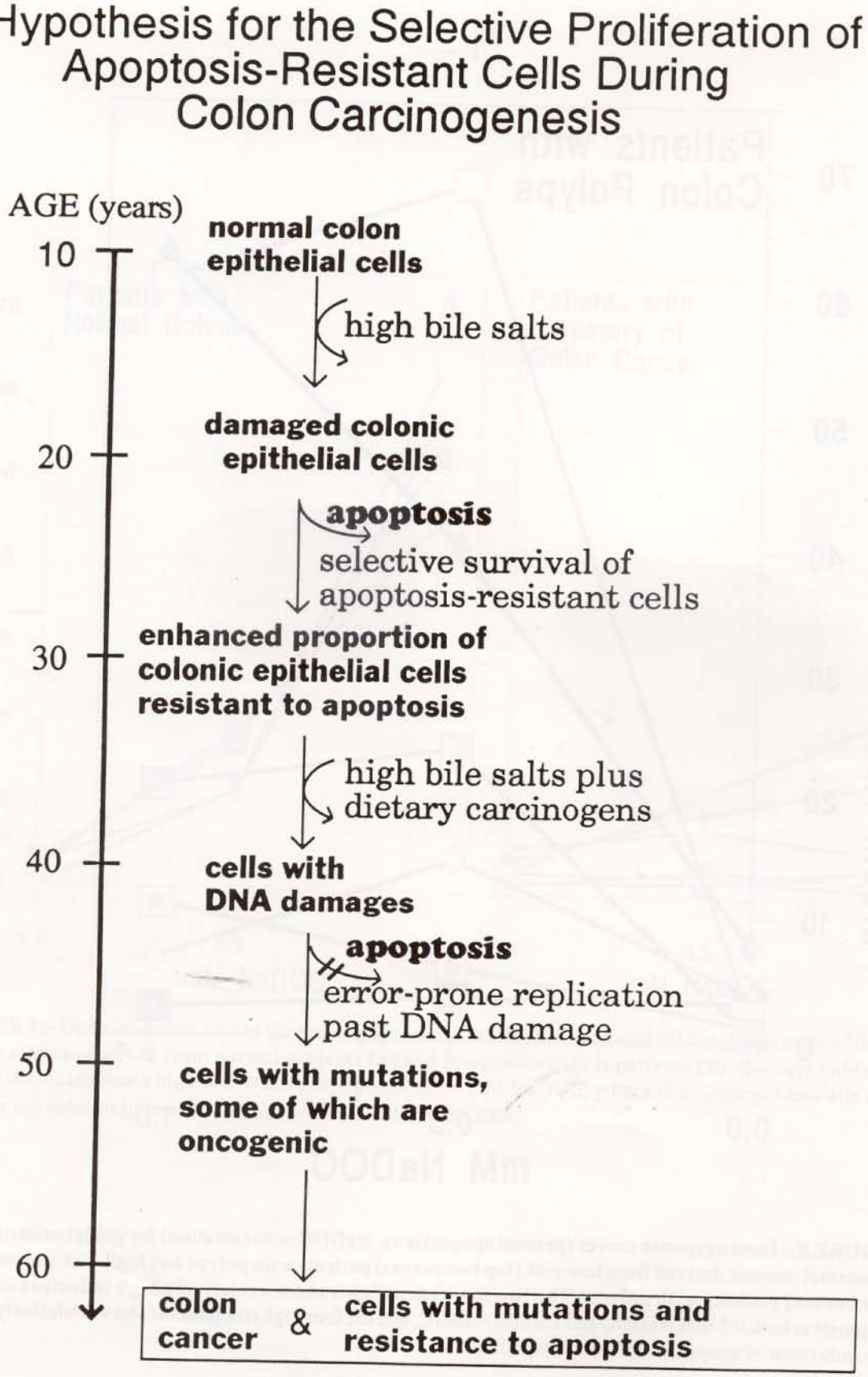

FIGURE 6:- Schematic model for generation of apoptosis-resistant cells in the etiology of colon cancer.