Effects of Sodium Selenite on Deoxycholic Acid-induced Hyperproliferation of Human Colonic Mucosa in Short-Term Culture

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Abstract
It has been shown that in vitro incubation of human colonic biopsies with the secondary bile acid deoxycholic acid (DCA) leads to the hyperproliferation of colonic crypt cells with an expansion of the proliferative zone, which is regarded as a biomarker of increased cancer risk. Sodium selenite (SSE), on the other hand, has been implicated as a protective agent in experimental studies, but toxic effects were reported as well, depending on the dose of SSE. To elucidate the effects of SSE on human colonic mucosa, biopsies from endoscopically normal sigmoid colon tissue of 30 subjects were incubated with 5 μM DCA or a combination of 5 μM DCA and SSE in concentrations of 5, 10, 20, 50, 80, and 100 μM, respectively. Equimolar NaCl incubations served as a control. Proliferating cells were labeled by bromodeoxyuridine immunohistochemistry, and the labeling index (LI) was computed. In the experiments using 5, 10, and 20 μM SSE, the whole crypt LI was significantly lower after DCA + SSE incubation (0.136, 0.118, and 0.110, respectively) compared to that after incubation with DCA alone (0.172, 0.157, and 0.165, respectively; P < 0.01). The corresponding LIs during DCA + SSE incubation were comparable to the LIs obtained after NaCl incubation (average LI = 0.14). Contrary to this finding, severe cell damage was observed in the biopsies that were incubated with the higher SSE concentrations of 50 μM and above. The antiproliferative effects of SSE may indicate a possible protective effect in the prevention of human colon cancer development. However, the observed toxic effects of higher SSE concentrations strongly suggest the need for additional studies before general recommendations for the use of SSE in colon cancer prevention can be made.

Introduction
Cancer of the colon is one of the leading causes of cancer deaths in Western countries (1). Numerous epidemiological and experimental studies have indicated that certain dietary factors play an important etiological role. In particular, a high consumption of dietary fat and meat seems to be associated with increased colon cancer risk (2), whereas a high intake of fibrous fruits and vegetables is regarded as a major protective factor (3–5). Increased fecal excretion of secondary bile acids (DCA and lithocholic acid), which is generally found in populations consuming such a high-risk diet (6), has been implicated as a pathogenetic mechanism. A tumor-promoting effect has been demonstrated for both bile acids in experimental studies in which the rectal instillation of these sterols increased the tumor yield in carcinogen-treated rats (7). Furthermore, there is evidence that bile acids may promote colon cancer by free radical-generating mechanisms (8). Thus, the protective effect of fibrous fruits and vegetables has also recently been explained by their antioxidant vitamin and trace element content (9).

Studies reporting low plasma levels of selenium in patients with colonic polyps indicate a possible protective role of this trace element in colon cancer development (10). In a small-scale clinical trial, Cahill et al. (11) showed a significant reduction of colonic crypt cell proliferation in eight patients with colonic adenomas after 4 weeks of selenium supplementation. Hyperproliferation of colonic crypt cells with expansion of the proliferative zone to the upper 40% of the crypt is generally regarded as a sign of increased cancer risk (12). Thus, the antiproliferative effect of selenium has been interpreted as a possible method of colon cancer protection. In experimental studies, selenium showed convincing inhibitory effects on various kinds of tumors in rodents. In a literature review by Combs and Combs (13), 49 of 55 studies revealed a protective effect of selenium on cancer development. In most of these studies, inorganic SSE was added to food in pharmacological doses of 1–5 mg/kg food. However, severe toxic side effects have also been reported, depending on the nature (organic or inorganic) and dose of selenium used (14). We have shown previously that the short-term incubation of biopsies from the human ascending and descending colon with the secondary bile acid DCA resulted in hyperproliferation of colonic crypt cells with an expansion of the proliferative zone, which is regarded as a pre-neoplastic type of proliferation (15, 16). To elucidate the possible protective and/or harmful effects of selenium in human colon cancer development, we investigated the effects of different doses of SSE on DCA-induced hyperproliferation in biopsies from the human sigmoid colon.

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2The abbreviations used are: DCA, deoxycholic acid; SSE, sodium selenite; LI, labeling index; BrdUrd, bromodeoxyuridine; PGE2, prostaglandin E2.
Patients and Methods

During routine colonoscopy using an Olympus CF10L colonscope (Olympus Optical, Hamburg, Germany), biopsies were obtained from endoscopically normal-appearing mucosa of the sigmoid colon from 30 subjects (16 males and 14 females; age, 19–78 years) in whom inflammatory bowel disease, polyps, or tumors have been ruled out by complete colonoscopy. Written informed consent was obtained from every patient. Indications for endoscopy were visible or occult fecal blood loss (n = 11), abdominal discomfort (n = 14), and diarrhea of unknown origin (n = 5). The examination was performed after an overnight fast and bowel preparation with oral Golytely solution (59 g/liter polyethylene glycol 4000, 5.68 g/liter Na2SO4, 1.68 g/liter NaHCO3, 1.46 g/liter NaCl, and 0.75 g/liter KCl).

Immediately after biopsy, the specimens were immersed in BME medium (Eagle’s medium with Earle's salts, FCS, and antibiotic-antimycotic solution; Life Technologies, Inc., Paisly, United Kingdom) and transferred to the laboratory where incubation experiments were carried out within 15 min. For each incubation experiment, eight biopsies from every patient were incubated with either 5 μM DCA (n = 3 biopsies), a combination of 5 μM DCA + SSE (n = 3 biopsies), or sodium chloride (n = 2 biopsies), which served as a control solution, to determine the grade of DCA-induced hyperproliferation. Two different doses of SSE (5 and 10 μM) were tested in this experimental setup. Using this setup of intraindividual comparisons, the chance of confounding by variables that may affect cell proliferation, such as the age of the subjects (17, 18), was reduced. To determine the maximum tolerated dose of SSE for the biopsies in this in vitro experiment, we further incubated the biopsies of 10 patients with different doses of SSE. To facilitate intraindividual comparisons in this setup, a total of eight biopsies from each patient were divided into subgroups of two specimens, which were incubated with a combination of 5 μM DCA and SSE in concentrations of 20, 50, 80, and 100 μM, respectively (n = 2 biopsies each). This range of selenite concentrations was selected according to the putative luminal concentrations that could be reached in physiological situations, considering a malabsorption rate of 60% (19). Incubations were carried out for 4 h at 37°C in a Modular Incubating Chamber (Billups-Rothenberg Inc., del Mar, CA) using culture dishes with a total volume of 3 ml of BME medium. A total incubation time of 4 h has been shown to be effective in stimulating DNA synthesis (20) and colonic cell proliferation in short-term culture experiments (21). During the whole incubation period, the dishes were continuously gassed with carbogen gas (95% oxygen and 5% carbon dioxide) at 1 liter/min and gently rotated at 10 cycles/min on a rotary shaker (Heidolph, Kehlheim, Germany). The test solutions were made up fresh from reagents of the highest purity available (Sigma, St. Louis, MO).

Crypt cell proliferation was determined by BrdUrd immunohistochemistry as described previously (15, 16). Briefly, 200 μM BrdUrd (Sigma) and 20 μM fluorodeoxyuridine (Sigma) were added to the media after 2 h of incubation with the test solutions for another 2 h. The specimens were then fixed in PRIMAFIX (Camon, Wiesbaden, Germany), embedded in PARAPLAST (Monoject Scientific, Athy, Ireland), and section cut into 2-μm slices using a Leitz microtome (Leitz, Wetzlar, Germany). HCl was added to denature the DNA; afterward, the first antibody [mouse anti-BrdUrd in a 1:100 dilution (7580; Becton Dickinson, San Jose, CA)] was applied. Biotinylated antimouse immunoglobulin (1:100 dilution; Amersham RPN 1051), BrdUrd-labeled cells were visualized using diaminobenzidine solution (Serva, Heidelberg, Germany). Finally, the tissue was counterstained with nuclear fast red, and the histological slides were viewed under a Laborlux S microscope (Leitz) at ×625 magnification.

Statistical Analysis. Crypt cell proliferation was assessed in 20 longitudinaly sectioned crypts in the biopsies of every incubation setup. The number of BrdUrd-labeled cells and the total number of cells were counted according to the criteria described by Lipkin et al. (22). An average LI (the number of labeled cells divided by the total number of cells) was calculated for the whole crypt (LI1), as well as for five different longitudinal crypt compartments (LI1, crypt base) through LI4 (crypt surface) to obtain information about the distribution of labeled cells within the crypt. The LI for the upper 40% of the crypt (LI1,s) was evaluated separately as the upper crypt LI. Furthermore, the φh value was calculated as the ratio of labeled cells in compartments 4 + 5 divided by the number of labeled cells in the entire crypt. This value is regarded as the best discriminator for increased cancer risk (22, 23). The data were tested for normal distribution and equal variances by the Bartlett test. Because these requirements were not fulfilled, the nonparametric Friedman’s block test was used in the experiments with 5 and 10 μM SSE for comparisons with DCA and NaCl. The data from the DCA + SSE incubations revealed by the dose-finding experiments were compared with the DCA values from the preceding incubation experiments by the Mann-Whitney U test. Spearman’s rank test was used to evaluate a possible dose-response relationship of the selenite effects on whole crypt LI. The Winstat statistical software package (Greulich Software, Stuttgart, Germany) was used for analysis. Data are given as the mean ± SE, and differences with P < 0.05 were considered to be significant.

Results

A total of 171,634 cells was counted in all of the incubation experiments. As summarized in Tables 1 and 2, the total number of crypt cells and crypt height did not differ in the various incubation experiments, but the number of labeled cells was significantly higher after incubation with 5 μM DCA as compared to the values obtained after Na2SeO3 and NaCl incubation, respectively.

The calculated LIs obtained in the experiments using 5 μM Na2SeO3 are shown in Table 1. Total crypt LI (LI1,0) in the biopsies incubated with 5 μM DCA was 27% higher compared to the control run with NaCl (P < 0.01) and 26% higher compared to the combined incubation with 5 μM DCA + 5 μM Na2SeO3 (P < 0.05). No significant difference was noted between the incubations with NaCl and the coincubation of DCA + Na2SeO3. The LIs in the different crypt compartments showed an equal pattern, with lower values after coinoculation with DCA + 5 μM Na2SeO3 and incubation with NaCl compared to DCA incubation. These differences, however, were only significant for the basal crypt compartments (LI1, and LI3).

Similar results were obtained in the experiment using 10 μM Na2SeO3 (Table 1). The LIs after coinoculation with DCA + 10 μM Na2SeO3 were comparable to the values obtained after NaCl incubation but were always lower in comparison to the LIs obtained after DCA incubation. Differences were significant for whole crypt LI and the LIs for basal crypt compartments 1–3, whereas only a nonsignificant trend was found in upper crypt LI (LI1,s; P = 0.12).

Coincubation of the biopsies with 5 μM DCA + increasing
concentrations of Na₂SeO₃ in doses of 20, 50, 80, and 100 μM affected crypt cell morphology in a dose-dependent manner. Whereas crypt architecture was completely normal in the biopsies that were incubated with 20 μM Na₂SeO₃, cell damage of increasing severity was noted in the specimens incubated with Na₂SeO₃ concentrations of 50 μM and above. The observed toxic effects ranged from damage of the superficial epithelial layer with distortion of the nuclei to a complete destruction of crypt morphology. Due to these alterations of the crypt structure, a quantitative assessment of crypt cell proliferation according to the criteria of Lipkin et al. (22) was impossible.

The proliferation data from the biopsies incubated with 5 μM DCA + 20 μM Na₂SeO₃ were subjected to individual comparisons with the data obtained after incubation with DCA only from the preceding incubation experiments with 5 and 10 μM Na₂SeO₃. The observed close range of intra- and interindividual variations of the LIIs (variation coefficient, 16%) justified this procedure. As summarized in Table 2, the two groups did not differ in the total number of crypt cells or crypt height, but the number of labeled cells was significantly lower in biopsies that were coincubated with DCA + 20 μM Na₂SeO₃ (P < 0.05). Concomitantly, the LI for the whole crypt (LIT) was 33% lower after a combined incubation with DCA and Na₂SeO₃ than it was after an incubation with DCA only (P < 0.01). This was mainly due to the decreased LIIs for the basal crypt compartments 1–3, whereas only a trend to lower LIIs for the upper crypt compartments (LII4–5) was noted after coincubation with DCA + 20 μM Na₂SeO₃ (P = 0.12).

Fig. 1 describes the correlation of the Na₂SeO₃ concentrations tested and their effects on whole crypt LI. As indicated, there was a clear dose-response relationship between the antiproliferative effect and increasing doses of Na₂SeO₃.

**Discussion**

A high fat/low fiber diet has been implicated as a major risk factor in colon cancer development by the increasing fecal excretion of secondary bile acids DCA and lithocholic acid, which are known promoters for colon cancer in carcinogen-treated rats (7). In previous studies, we and others have shown that the incubation of biopsies from the human ascending and descending colon with DCA induces hyperproliferation of the colonic epithelium, which is regarded as a biomarker of increased susceptibility for neoplastic transformation (15, 16, 21). Also, in the present study, whole crypt LI was 27% higher after DCA incubation compared to that after incubation with the NaCl control (P < 0.01). These data are in line with the results of several experimental studies on carcinogen-treated rats and mice that showed that rectal instillation of DCA resulted in an increase in epithelial cell proliferation, colonic nuclear damage, and colon tumor incidence in these animals (7, 24, 25).

Interestingly, coincubation of the biopsies with Na₂SeO₃ completely abolished this DCA-induced hyperproliferation, resulting in LIIs of 0.11–0.13 that were almost equal to the LIIs obtained after incubation with the NaCl control. However, the inhibition of crypt cell proliferation was only statistically significant in the basal crypt compartments. According to Risio et al. (23), hyperproliferation and the upward movement of the proliferating zone within the crypt do not begin at one time. Thus, it could be that the period of 4 h of incubation with DCA in the present study was too short to induce this second step of
premalignant proliferation, i.e., the expansion of the proliferative zone. Longer incubation periods with DCA and SSE may be necessary to show an inhibitory effect on the upper crypt compartments. The antiproliferative effect of Na₂SeO₃ on whole crypt LI followed a dose-response pattern when concentrations between 5 and 20 μM were used. However, doses of ≥50 μM Na₂SeO₃ were toxic to the colonic epithelium, revealing cell damage of increasing severity along with increasing doses of Na₂SeO₃. Depending on the various cell types, antiproliferative effects of selenium have been observed at concentrations ranging from slightly above the minimum dietary requirements to surprisingly high levels. Macromolecular synthesis in HeLa S₃ cells is already inhibited at intracellular selenium levels of above 0.5 ppm, and DNA synthesis is reduced by 60% at selenium concentrations of 2 ppm, but recovery from intoxication is still possible after exposures to 130 ppm selenium in this cell type. Other cell types, however, are more sensitive (26). In vitro studies on primary cultures of rat tracheal epithelial cells revealed an inhibition of cell growth by Na₂SeO₃ at concentrations of 20 μM, whereas concentrations between 6 × 10⁻³ and 6 × 10⁻² M even stimulated cell proliferation (27). The toxic effects of higher SSE concentrations have been postulated as a reason for the antiproliferative effect by the authors. In our study, toxic effects were obviously present at Na₂SeO₃ concentrations of 50 μM and above. Considering that 44–70% of orally ingested SSE is absorbed during passage through the short bowel (19), these toxic concentrations could principally be reached in the colonic lumen after supplementation with high doses of selenium. In contrast, dose-dependent inhibition of crypt cell proliferation without any signs of cell damage occurred at Na₂SeO₃ concentrations of 5–20 μM. Thus, the limited therapeutic range of selenium, which is well known from epidemiological and clinical observations, was also noted in our in vitro model.

It has been shown that selenium has a delaying effect on all mitotic phases of the cell cycle and also on the premitotic resting phase (G₂), thus creating conditions that are favorable for DNA repair (26). A modulating effect of selenium on several growth-regulatory proteins has been implicated as a mode of action. Because oxidative stress is one mechanism by which secondary bile acids exert a promoting effect on colon carcinogenesis (8), the activation of glutathione peroxidase by Na₂SeO₃ may be another factor for the protective effects of selenium (26). Besides, glutathione peroxidase has been shown to be involved in the regulation of fatty acid hydroperoxides during the production of prostaglandins (19). Several lines of evidence suggest that prostaglandins, especially PGE₂, play a role in colon carcinogenesis. It has been shown that colonic tumors contain higher PGE₂ concentrations than the surrounding healthy colonic epithelium (28). PGE₂ analogues have a proliferative effect on the colonic mucosa (29), and bile salts have been reported to enhance PGE₂ release from the rat colon (30). Several studies have shown that treatment with nonsteroidal anti-inflammatory drugs reduces colon tumor formation in animals and humans, an effect that has been explained by the inhibition of PGE₂ synthesis (31). Thus, possible effects on prostaglandin synthesis might also play a role in the antiproliferative and anticarcinogenic action of selenium.

Although the present study is descriptive in nature, the antiproliferative effects of SSE observed in our in vitro model may indicate a possible protective effect of selenium in the development of human colon cancer. In this respect, however, it must be considered that the pathogenetic model of colon cancer development involving hyperproliferation as an important step in the transformation of normal cells to neoplastic cells still needs further confirmation from clinical trials. An intervention trial by Greenberg et al. (32) did not result in a reduction of colonic polyp recurrence in patients with adenomatous polyps after 4 years of supplementation with vitamin C, vitamin E, and β-carotene, whereas Paganelli et al. (33) showed that rectal cell proliferation in polyt patients significantly decreased after the supplementation of their usual diets with vitamins A, C, and E. Thus, especially considering the observed toxic effects of higher selenite concentrations in the present in vitro study, additional studies involving experimental and clinical trials in subjects at high risk for colon cancer are essential before general recommendations for the use of selenium in colon cancer prevention can be made.

References


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