Inhibition of Human Colonic Epithelial Cell Proliferation in Vivo and in Vitro by Calcium

Michel Buset, Martin Lipkin, Sidney Winawer, Shanti Swaroop, and Eileen Friedman

ABSTRACT

Nine patients at high risk of developing colon cancer were placed on daily p.o. supplementation of 1500 mg of calcium for 4–8 weeks. The colonic epithelial cells in six of these patients showed a statistically significant decrease in their [3H]thymidine labeling indices in tissue culture so that they resembled those of patients at low risk of developing colon cancer. The three nonresponders had similar labeling indices before and after calcium supplementation. Biopsies from each of nine high-risk patients exhibited a decrease in proliferation when they were cultured in vitro with a high level of CaCl₂ (2.2 mM compared with the 0.1 mM optimum value for proliferation). Two adenomas and two carcinomas showed a different pattern of response than normal cells, exhibiting no inhibition of growth at 2.2 mM CaCl₂. These data indicate that the growth inhibition induced by high levels of extracellular calcium levels is lost at a stage in tumor development before cells become malignant.

INTRODUCTION

Enhanced epithelial cell proliferation in patients at increased risk of developing colon cancer reflects an expansion of the proliferative compartment in their colonic crypts. A more quiescent epithelium is found in patients at lower risk of developing colon cancer (1–3). In a previous study from one of us (4), 10 patients placed on dietary calcium supplementation showed an average decrease in colonic epithelial cell proliferation as assayed by [3H]Tdr labeling of colonic biopsies in an organ culture system. Therefore, reversion of this biomarker associated with increased risk suggested that dietary calcium was potentially an effective means of intervention.

To study this phenomenon more directly we decided to utilize a more readily manipulable system, tissue culture of the colonic epithelial cells. In organ culture a colonic biopsy is placed directly into culture medium without digestion, pulsed with [3H]Tdr for 1 h; then the tissue is formalin fixed, very carefully sectioned so that the entire test tube-like crypt from the base to the top (about 50 cells long) is contained within one section, and processed for autoradiography. In tissue culture, in contrast, the epithelial cells simply are cultured out from the partially digested colonic epithelial layer as patch cultures in the presence of [3H]Tdr, fixed, directly coated with emulsion, and scored under a microscope. In this tissue culture method, as in organ culture, the high-risk and low-risk subjects have been distinguished. Individuals at increased risk for the development of colon cancer generally have a higher overall labeling index than patients at lower risk. In an earlier study (5), labeling indices were determined for 11 subjects symptomatic with either adenomas or carcinomas, 15 subjects at increased risk defined by a positive family history of colon cancer but asymptomatic, and 5 subjects from families free of colon cancer for 2 or more generations. Their mean [3H]Tdr LI were, respectively, 12.2, 17.2, and 5.7%, although occasionally a high-risk patient would have a LI less than 10%. Therefore, this simple measurement reflects an abnormal excess proliferation which can be seen in patients at increased risk of developing colon cancer and is conspicuously absent in individuals at low risk.

MATERIALS AND METHODS

Tissue Culture Procedures for Biopsies from Patients on Calcium Intervention Study. Colonic mucosal biopsies were cultured on gelatin films in NCTC medium supplemented with 15% fetal calf serum and 2.2 mM CaCl₂ exactly as detailed (5), with the exception that the pentagastrin concentration utilized was 5 µg/ml. All the data in Tables 2–4 were obtained by this method. These partial digests were placed on gelatin films in 0.2 ml of medium and allowed to attach for 45 min at 37°C in a CO₂ incubator; then 1 ml of medium containing a lethal level of [3H]Tdr (5 µCi/ml) was added and the cells were cultured for 24 h. The very high level of [3H]Tdr ensured that each cell would pass through S phase only once during the labeling period and then be held in late S-G₂ as the badly damaged chromatin could not condense and undergo mitosis. During this time the colonic epithelial cells of the digest migrated from the attached explant to form a flat patch on the surface of the Petri dish. The monolayer cells were then fixed twice with 5 ml methanol/dish, air dried, coated with undiluted NTB-2 Kodak emulsion, exposed for 10 days, developed, and counterstained with hematoxylin. All cells in each epithelial patch colony were read by microscopy.

Tissue Culture Procedures for in Vitro Calcium Modulation Studies. Each biopsy was very finely minced so that no large pieces (>1 mm) remained, and placed into 3 ml of antibiotic-containing wash medium (6) plus 1 ml each of hyaluronidase, neuraminidase, and collagenase (5). Colonic bacteria often adhered to mucus secreted by the epithelial cells, so an addition was made of 0.1 ml of 20% Mucomyst (Mead-Johnson), a solution of acetylcysteine. Digestion occurred at room temperature on a blood rotator, so the digests were continuously gently shaken for 3–5 h to remove mucus, instead of the 1 h used in the simpler method described in the paragraph above. The digest was plated in serum-free NCTC 168 medium supplemented with transferrin, insulin, hydrocortisone, insulin, pentagastrin, sodium deoxycholate, epidermal growth factor, and selenous acid as described (5), but also contained 3 × 10⁻⁴ m isobutylmethyl xanthine, and 10⁻⁵ m each of ethanolamine and phosphoethanolamine. The digests consisted of groups of epithelial cells and partly digested colonic crypts. A substrate consisting of 10 µg of fibronectin (Bethesda Research Laboratories), 10 µg of collagen I (Vitrogel), and 30 µg of bovine serum albumin (globulin free; Sigma Chemical Co.) (7, 8) was prepared in serum and calcium-free unsupplemented NCTC 168, and stored in plastic tubes at −20°C; 1-ml aliquots were incubated for 2–6 h in 35-mm dishes in a CO₂ incubator. Then the solution was aspirated carefully so that the surface was not scratched, and the tissue digests were immediately applied. Occasionally dishes were prepared 1 day to 1 week before use and stored in the refrigerator. Before use each dried dish was wetted with 1 ml of serum and calcium-free unsupplemented NCTC 168, which was carefully aspirated before the digest was added. Attachment of the cells was allowed to proceed for 45 min in the CO₂ incubator. Then 1 ml of medium containing either 0.1 or 2.2 mM CaCl₂ (or a range of concentrations from 0.01 to 2.2 mM for Fig. 3) and 5 µCi [3H]Tdr/ml were added.
and the cells were incubated for 24 h in an atmosphere of 3% O₂ and 5% CO₂ (Heraeus incubator). Cultures were fixed and processed for autoradiography as described above.

### Patient Characteristics

The individuals on the dietary supplementation were 4 women and 5 men, of mean age 55 ± 4 (SE) years. Their characteristics are listed in Table 1, patients 1–9. The normal biopsies analyzed in vitro were obtained from 7 men and 2 women at increased risk for the development of colon cancer, with a mean age of 58 ± 3 years, as detailed in Table 1. The subjects in this study were on an unrestricted western-style diet and none were taking calcium supplementation prior to the study; none had familial polyposis or were in familial polyposis kindreds. All procedures and assays were approved by the Institutional Review Board of Memorial Hospital. All biopsies were number coded to protect patient confidentiality. Biopsies were taken between 10 a.m. and 12 noon, and were immediately placed into culture. Biopsies were always taken from the rectosigmoid following preparation with mild tap water enema and sigmoidoscopy.

### RESULTS

#### In Vitro Analysis of Colonic Epithelial Cells from Subjects on Dietary Supplementation with Calcium

Nine subjects classified as being at increased or average risk for the development of colon cancer ("Materials and Methods") were studied. The [³H]dThd LI of each colonic mucosal biopsy was measured ("Materials and Methods"). Subsequently each subject was placed on a daily supplementation of 1500 mg of calcium in the form of calcium carbonate for 6 weeks, and rebiopsied. Two subjects were biopsied after 4 weeks of dietary supplementation and one after 8 weeks because of the difficulty in scheduling these patients. The [³H]dThd LI was determined for each biopsy of calcium supplementation was unknown to the scorer.

Six of the subjects showed a statistically significant decrease in overall LI following dietary calcium supplementation (Tables 2 and 3; Fig. 1), while 3 showed no change. Analysis of the data (Table 3) showed that the colonic cells formed flat epithelial patches equally well before and after dietary intervention. The colony sizes for the 6 responding subjects and the 3 nonresponding subjects were identical within statistical analysis (t test; see legend for Table 3). The parameter which did vary was the number of labeled cells per colony, which was significantly decreased after calcium intervention in the responder group (118 ± 42 versus 36 ± 16 cells; P < 0.05). This translated significantly decreased after calcium intervention in the responder was the number of labeled cells per colony, which was significantly decreased after calcium intervention in the responder group (118 ± 42 versus 36 ± 16 cells; P < 0.05). This translated into a significant decrease, P < 0.003, in [³H]dThd LI of 16.1 ± 2.5% to 5.2 ± 1.3%. The nonresponders had a similar number of labeled cells per colony before and after calcium (50 ± 8 versus 61 ± 4 cells), and no decrease in LI as a result (7.4 ± 0.9% to 6.9 ± 1.3%; Table 3).

#### Tissue Culture Analysis of Colonic Epithelial Cell Proliferation

The mean [³H]dThd LI was determined by the central dot where the ends of each box mark the limits of the SE. Values for LI before and after dietary intervention are shown (Fig. 1). After 8 weeks because of the difficulty in scheduling these patients. The [³H]dThd LI was determined for each biopsy before and after calcium intervention. The nonresponders showed a statistically significant decrease in [³H]dThd LI after calcium compared to the nonresponders either before or after calcium. The nonresponding group had a low fraction of proliferating cells before the intervention trial and thus a rather quiescent epithelium, which was not typical of this high risk group. The total cells and total colonies analyzed before and after dietary supplementation were similar, showing that analyses were comparable.

#### Tissue Culture Analysis of Colonic Epithelial Cell Proliferation Yielded Characteristic Results per Patient

The data of Tables 2 and 3 are dependent on the relative stability of the LI as a marker. Patient biopsies have been analyzed by this tissue culture method for the last 2–3 years in this laboratory, so we examined our records for patients who had been analyzed twice, but were not on the intervention study. Each of three patients had LIs measured on two occasions. These were identical within statistical variation (t test). Therefore the tissue culture of...
CALCIUM MODULATED EPITHELIAL CELL GROWTH

Table 3 Effect of an in vivo calcium supplementation on [3H]thymidine labeling indices in vitro: analysis of data by population

<table>
<thead>
<tr>
<th>Patient</th>
<th>Date of first biopsy,</th>
<th>Mean % of [3H]thymd LI ± SE</th>
<th>Cultured at 0.1 mM CaCl2</th>
<th>Cultured at 2.2 mM CaCl2</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>3/83</td>
<td>6.9 ± 2.6</td>
<td>5.1 ± 0.8 (1909)</td>
<td>3.5 ± 0.8 (1399)</td>
</tr>
<tr>
<td>B</td>
<td>6/83</td>
<td>3.7 ± 0.8</td>
<td>9.3 ± 1.1 (1626)</td>
<td>3.4 ± 0.7 (1623)</td>
</tr>
<tr>
<td>C</td>
<td>3/85</td>
<td>5.2 ± 1.1</td>
<td>16.7 ± 3.4 (752)</td>
<td>8.8 ± 1.0 (2074)</td>
</tr>
<tr>
<td></td>
<td>9/84</td>
<td>16.3 ± 4.8</td>
<td>16.2 ± 1.5 (3177)</td>
<td>7.9 ± 1.3 (3950)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15.3 ± 2.6</td>
<td>35.1 ± 2.0 (3273)</td>
<td>24.7 ± 1.6 (2595)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>15.6 ± 1.0 (3136)</td>
<td>7.8 ± 1.0 (2423)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>16.3 ± 1.1 (3680)</td>
<td>13.6 ± 0.9 (1789)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>14.2 ± 1.2 (2688)</td>
<td>16.7 ± 1.8 (954)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5.8 ± 0.8* (1970)</td>
<td>1.1 ± 0.4 (2159)</td>
</tr>
</tbody>
</table>

*NS, nonsignificant P values, P > 0.01, were obtained for all LI comparisons in Table 2, group B; nonsignificant P values were obtained for all pooled data analysis in which mean and SE values are given unless a P value is stated.

In Vitro Correlation to the in Vivo Study. One theory behind the study is that much of the ingested calcium will remain within the digestive tract and raise the concentration of calcium ions in the fecal water. It has been reported that 75-80% of ingested calcium is excreted through the colon (9). These calcium ions then would act directly upon the colonic epithelial cells which line the gut lumen in a highly indented single cell layer. To test this possibility the CaCl2 concentration in culture medium was varied and the effect on cell proliferation was measured. Biopsies from three of the patients in the dietary study (patients 6–8) were taken before p.o. calcium supplementation began. These biopsies and six others from patients at increased risk for colon cancer (see Table 1 for patient characteristics) were cultured in medium containing either a low CaCl2 level (0.1 mM) or a level characteristic of serum (2.2 mM), and the [3H]thymd LI was determined. Each of the biopsies from the nine patients showed a significant inhibition of growth (t test) by the higher calcium concentration (Tables 5 and 6; Fig. 2). After a 24-h exposure to the different calcium levels, the inhibition by 2.2 mM was even more striking (patient 15). These data imply that all the colonic epithelial cells from all high-risk patients should respond to higher levels of extracellular CaCl2 by a decrease in proliferation.

Figure 2. High extracellular calcium levels in vitro inhibit normal colonic epithelial cell growth, but are not inhibitory to adenoma and carcinoma cells. The boxes enclose the mean [3H]thymd LI (central dots) and mark the limits of the SE. The values obtained when cells were cultured at 0.1 mM CaCl2 or values at 2.2 mM. The normal biopsy from patient 15 was analyzed on day 1 and after 24 h of growth in different calcium levels (day 2). Adenoma 17 and carcinoma 19 were cultured for 8 days in the two calcium levels before [3H]thymd labeling. *, mean values statistically different (t test) P < 0.01; **, mean values differ with P < 0.01; ***., mean values differ with P < 0.005.

These nine biopsies which showed in vitro inhibition by calcium included two from the patients who were classified as nonresponders, patients 7 and 8 (Tables 2 and 3). The dose-response curve (Fig. 3) for one of these patients (patient 7)
CALCIUM MODULATED EPITHELIAL CELL GROWTH

Table 6 Elevated CaCl2 concentration in culture medium suppresses normal, but not neoplastic colonic epithelial cell proliferation: data analyzed by population

<table>
<thead>
<tr>
<th></th>
<th>Cultured at 0.1 mm CaCl2</th>
<th>Cultured at 2.2 mm CaCl2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal colonic epithelial cells cultured 1 day from 9 patients</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total no. of cells</td>
<td>22,563</td>
<td>17,883</td>
</tr>
<tr>
<td>Mean ± SE/patient</td>
<td>2,507 ± 314</td>
<td>1,987 ± 308</td>
</tr>
<tr>
<td>Total colonies</td>
<td>1,53</td>
<td>123</td>
</tr>
<tr>
<td>Mean colony size ± SE</td>
<td>144 ± 21</td>
<td>149 ± 21</td>
</tr>
<tr>
<td>Total labeled cells</td>
<td>3,768</td>
<td>1,913</td>
</tr>
<tr>
<td>Mean labeled cells/colony ± SE</td>
<td>24.9 ± 5.6</td>
<td>16.1 ± 3.7 P &lt; 0.1</td>
</tr>
<tr>
<td>[3H]dThd LI ± SE</td>
<td>16.7 ± 2.8%</td>
<td>10.7 ± 2.3% P &lt; 0.1</td>
</tr>
</tbody>
</table>

Tumors from 4 patients

| Total no. of cells | 9,964 | 13,416 |
| Mean ± SE/patient  | 2,412 ± 717 | 3,354 ± 1,261 |
| Total colonies     | 44     | 62     |
| Mean colony size ± SE | 226 ± 51   | 216 ± 45 NS*   |
| Total labeled cells | 1,264   | 2,859  |
| Mean labeled cells/colony ± SE | 35.4 ± 16.3 | 102 ± 84 NS    |
| Mean [3H]dThd LI ± SE | 12.2 ± 0.9% | 17.9 ± 3.2% NS |

* NS, not significantly different at P > 0.1.

The in vitro study demonstrated that a level of calcium equivalent to that found in serum inhibited the proliferation of colonic epithelial cells from each of nine subjects. This in vitro finding predicted that individuals at increased risk with abnormally elevated colonic cell proliferation should respond to dietary calcium intervention by decreased cell replication, provided that available intracolonic calcium levels can be elevated sufficiently. In fact, six of nine subjects who ingested supplementary dietary calcium in the dietary intervention study did show a significant decrease in colonic cell growth.

DISCUSSION

Levels of extracellular calcium equivalent to those found in blood, 2.2 mm, inhibited the proliferation of colonic epithelial cells from each of nine subjects. This in vitro finding predicted that individuals at increased risk with abnormally elevated colonic cell proliferation should respond to dietary calcium intervention by decreased cell replication, provided that available intracolonic calcium levels can be elevated sufficiently. In fact, six of nine subjects who ingested supplementary dietary calcium in the dietary intervention study did show a significant decrease in colonic cell growth.

Why were there three “nonresponders”? Two of the biopsies which were growth inhibited by CaCl2 in vitro were taken from patients 7 and 8 who did not subsequently respond in the in vivo trial. Possibly the available extracellular calcium levels attained in the colons of these patients was not equivalent to the inhibitory level of 2.2 mm in the test culture medium. A second possible explanation is that each of the nonresponding patients (patients 7, 8, and 9) had a relatively quiescent epithelium (mean LI of 7.4 ± 0.8%) characteristic of normal cells even before the dietary trial. Possibly, dietary calcium could not further slow the proliferation of such slowly proliferating cells. However, patient 6 also had relatively quiescent epithelial cells whose proliferation was suppressed by dietary calcium supplementation, making this explanation less likely.

The in vitro study demonstrated that a level of calcium equivalent to that found in serum inhibited the proliferation of colonic epithelial cells. Elevated calcium levels are known to inhibit the proliferation of a number of epithelial cell types, including keratinocytes, mammary epithelial cells, esophageal epithelium, bronchial epithelium, and urothelium (7, 10–15). In some cell systems in which markers for differentiation are available, elevated calcium levels have been demonstrated to induce a terminal differentiation of the epithelial cells, with a concomitant growth limitation (10, 12, 13, 15–17). Human colonic epithelial cells are thus exhibiting a common epithelial cell trait. In the colon this modulation was brought about by a dietary modification (Ref. 4 and this study). This modulation may be due to an elevation of free ionized calcium in the fecal water. Blood levels of calcium are very tightly controlled, but the colon carries all of the solid effluent of the body, and unabsorbed calcium from the diet is carried in the digestive tract. The absorption efficiency of calcium from the diet is approximately 30%, so on a recommended daily allowance intake of calcium of 800 mg, about 650 mg enter into the total fecal contents. The fecal liquid phase is, in a sense, analogous to our culture medium. Increasing the calcium level in either induces growth inhibition, possibly by inducing cellular differentiation.

There are important differences of course between the two systems. In culture medium we have a very low level of the bile acid, and in the colon we have a very high level of this compound.
acid deoxycholic acid, $8 \times 10^{-9}$ M, which is present at 0.01 to 0.1 mM levels in the aqueous phase of the colonic lumenal contents. Culture medium does not contain free fatty acids, also found at high concentration in the gut lumen. Elevated levels of calcium administered by p.o. gavage in experimental animals have been shown to inhibit the toxicity of intrarectally instilled free bile acids and fatty acids (18, 19). Possibly in the nonresponsive patients the levels of these toxic agents were elevated and sequestered some of the available calcium. A second important difference between the studies is that the mucus layer which protects epithelial cells from injury in the body is stripped from the cultured cells to remove adherent bacteria. Thus the cultured cell system is a simplified system with cells spread out flat, and denuded of mucus. Cells are no longer organized in test tube-like crypt columns, and are unchallenged with the injurious agents, bile acids and fatty acids.

Colonic epithelial cells progress through several distinct biological preneoplastic and premalignant stages before they become malignant (20, 21). Our findings suggest that these cells lose ability to respond to the differentiation signal of high extracellular calcium by the time they evolve to an advanced benign tumor state, the villous adenoma. A thorough study of different histological classes of colonic adenomas has not yet been made, so the exact stage at which a response to extracellular calcium levels is lost has not been determined. However, the loss of a normal growth response to calcium was maintained in two malignant colonic tumors. Mouse epidermal cells treated with carcinogens develop into variant lines which also have lost the calcium switch signal to differentiate (22). These studies taken together indicate that the normal growth inhibition response to the calcium signal is lost before the evolution of malignant cells.

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REFERENCES


4. J. Rafter, personal communication.


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