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Advances in Brief

Altered Intracellular Calcium Regulation in Human Colorectal Cancers and in "Normal" Adjacent Mucosa[1]

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Abstract

Intracellular calcium [$(Ca^{2+})_i$] was measured in human colonic epithelia derived from control mucosa from noncancer patients, from grossly "normal" mucosa taken 10–30 cm proximal to primary colorectal cancers, and from colorectal cancers. $(Ca^{2+})_i$ was measured first in cells bathed by calcium-free solution and again after adding 1 mM calcium. Compared to control mucosa, $(Ca^{2+})_i$ was 27% lower in cancer cells in the presence of 1 mM extracellular calcium, whereas $(Ca^{2+})_i$ was elevated more than 3-fold in the adjacent "normal" mucosa from cancer patients in both media. These results suggest altered $(Ca^{2+})_i$ levels in malignant cells and the presence of a "field defect" in $(Ca^{2+})_i$ regulation in "normal" colonic mucosa adjacent to colorectal cancers.

Introduction

$(Ca^{2+})_i$[3] is an important second messenger in numerous cellular processes including cell proliferation (1). The level of $(Ca^{2+})_i$ is regulated, in part, by the phosphatidylinositol signal transduction pathway that when stimulated causes the formation of inositol 1,4,5-triphosphate which subsequently leads to the cytoplasmic release of calcium from intraorganelle storage sites. The formation of inositol 1,4,5-triphosphate from phosphatidylinositol 4,5-biphosphate also generates DAG which, combined with the increase in $(Ca^{2+})_i$, results in increased PKC activity. In turn, PKC may decrease or increase cell calcium by activating processes that lower $(Ca^{2+})_i$ and/or by affecting the response of the signal transduction pathway at one or more sites (1). Because involvement of the phosphatidylinositol signal transduction pathway in growth control and differentiation is becoming increasingly evident (2–5), recent studies have measured PKC activity and DAG levels in human colonic mucosa. These studies found that both PKC activity and DAG levels are significantly decreased in malignant cells from primary human colon cancers compared to "normal" adjacent colonic tissue from cancer patients (6–9). No corresponding measurements of $(Ca^{2+})_i$, the other arm of this signal transduction pathway, have been reported.

In this study, we have determined the levels of free $(Ca^{2+})_i$ in cells from primary human colon cancers, in grossly "normal-appearing" mucosa located adjacent (proximal) to malignant tumors, and in normal mucosa from patients with benign disease. Our experimental protocol examined cells first in calcium-free solution and then in response to the addition of physiological levels of extracellular calcium, $(Ca^{2+})_o$. The purpose of this maneuver was to determine whether $(Ca^{2+})_i$ regulation was altered in either the malignant or adjacent "normal" colonocytes from patients with colorectal cancer. Because tissues in this latter group were obtained at a distance 10–30 cm proximal to the cancer, any alterations exhibited by these "normal" cells might be considered to be indicative of a "field defect" in $(Ca^{2+})_i$ regulation rather than being a result of their proximity to the cancer. We have also examined "normal" tissue from the colon of a 31-year-old male patient with familial adenomatous polyposis, a known premalignant condition, undergoing prophylactic proctocolectomy (pathology negative for malignancy).

Materials and Methods

Specimens. Left-sided human colon specimens were received immediately following resection from operating rooms at the University of California, San Diego; the Veterans Administration Medical Center; and Kaiser Permanente in San Diego. Tissue utilized in this study included left-sided colonic mucosa from 11 patients resected for traumatic injuries and/or resected or biopsied for benign disease (NORMAL), 9 colon and rectal adenocarcinomas (CANCER), and 10 samples of grossly normal mucosa that were located 10–30 cm proximal to an adenocarcinoma (ADJACENT). We also analyzed left-sided colonic mucosa from a 31-year-old male who underwent prophylactic proctocolectomy for familial adenomatous polyposis syndrome but had no pathological evidence of cancer.

Tissue Preparation. Mucosa was rapidly and atraumatically separated from the underlying submucosa and then minced with a scalpel. The tissue was placed into a solution of HBSS which was nominally free of calcium, magnesium, and phenol red, and to which was added 2 mg/ml collagenase (Boehringer Mannheim, Indianapolis, IN). The solution was oxygenated (95% O_2-5% CO_2) at pH 7.4 while being stirred gently at a constant 37°C for 30 min. The suspension was then centrifuged at a low speed for 60–90 s and the pellet was resuspended in HBSS plus 1 mM $CaCl_2$.

Fluorescence Staining and Measurement of Intracellular Calcium. FURA2/AM (Calbiochem, San Diego, CA) was added to the cell suspension to a final concentration of 5-μM, and the fluorescent probe was allowed to load into the cells at 37°C over 40 min. The cells were rinsed to remove the excess FURA2/AM, resuspended in HBSS (calcium free), and placed into a quartz cuvet positioned in a SPEX AR-CM DM3000 spectrofluorometer (SPEX Industries, Edison, NJ). The cuvet chamber was temperature regulated (37°C) and the suspension was stirred gently via a magnetic stirring motor in the base of the chamber. The cells were alternately excited at 340 and 380 nm, and emission was measured at 505 nm. Fluorescence intensities for each excitation wavelength were recorded first in calcium-free HBSS for 60 s and then for 140 s after increasing calcium in the bathing solution to 1 mM by addition of $CaCl_2$ stock solution. Subsequently, calibration of the FURA signal was accomplished on the same cell preparation by adding digitonin (Calbiochem) to the solution at a final concentration of 60 μM for 100 s to maximize the binding of Ca^{2+} to FURA, followed by the addition of 60 mM ethyleneglycol bis (β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (Sigma Chemical Co., St. Louis, MO) to remove all Ca^{2+} and determine the emission for unbound FURA. All data were corrected for cell autofluorescence which was determined by scanning the same cells prior to FURA loading. Calculation of $(Ca^{2+})_i$

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[3] The abbreviations used are: $(Ca^{2+})_i$, intracellular calcium concentration; DAG, diacylglycerol; $(Ca^{2+})_o$, extracellular calcium concentration; PKC, protein kinase C; HBSS, Hanks' balanced salt solution.

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Table 1 *(Ca^{2+})$_i$ concentrations (nmol/liter) determined in calcium-free and 1 mM (Ca^{2+})$_o$ solutions*

δ is the change in (Ca^{2+})$_i$ following the addition of 1 mM (Ca^{2+})$_o$. High and Low Adjacent groups subdivide the Adjacent group according to relative value.

Normal			Cancer			Adjacent			High adjacent			Low adjacent			Familial adenomatous polyposis		
0 mM	1 mM	δ	0 mM	1 mM	δ	0 mM	1 mM	δ	0 mM	1 mM	δ	0 mM	1 mM	δ	0 mM	1 mM	δ
133	207	74	158	257	99	416	617	201				416	617	201	888	1842	954
202	351	149	76	111	35	1083	1651	568	1083	1651	568						
165	271	106	104	165	61	430	774	344				430	774	344			
139	217	78	144	180	36	1367	1709	342	1367	1709	342						
181	319	138	124	173	49	395	609	214				395	609	214			
133	223	90	109	139	30	405	608	203				405	608	203			
125	250	125	155	220	65	1920	3689	1769	1920	3689	1769						
116	225	109	182	258	76	320	523	203				320	523	203			
100	172	72	122	173	51	329	523	194				329	523	194			
176	256	80				289	457	168				289	457	168			
192	320	128															
151 ± 10[a] (11)[b]	256 ± 17 (11)	104 ± 8 (11)	130 ± 11 (9)	186 ± 17 (9)	56 ± 7 (9)	695 ± 178 (10)	1116 ± 321 (10)	421 ± 155 (10)	1457 ± 246 (3)	2350 ± 670 (3)	893 ± 443 (3)	369 ± 21 (7)	587 ± 38 (7)	218 ± 22 (7)	(1)	(1)	(1)

[a] Mean ± SEM.
[b] Numbers in parentheses, *n*.

was made using the Grynkiewicz equation (10) and the ratio of bound (340 nm excitation) to unbound (380 nm excitation) emission intensities.

Results and Discussion

Table 1 and Fig. 1 summarize the results of these experiments. In calcium-free media, cells from primary human colon cancers (CANCER) had (Ca^{2+})$_i$ levels similar to those of normal colonocytes from noncancer patients (NORMAL). Upon addition of 1 mM extracellular calcium to the suspension, (Ca^{2+})$_i$ increased in each group, but both the increment and final level of (Ca^{2+})$_i$ were significantly lower in the tumor cells ($P < 0.01$ and < 0.001, respectively, by the unpaired Student *t* test).

In contrast, grossly "normal" (Adjacent) mucosal cells from our cancer patients consistently displayed markedly higher levels of (Ca^{2+})$_i$ in both calcium-free and 1 mM (Ca^{2+})$_o$ solutions relative to cells from patients with benign disease ($P < 0.005$ and < 0.02, respectively) and to the cancer cells ($P < 0.01$ and < 0.02). As a consequence, the rise in (Ca^{2+})$_i$ in response to the addition of 1 mM (Ca^{2+})$_o$ was 4- to 8-fold greater than in the Normal ($P < 0.05$) and Cancer ($P < 0.05$) groups, respectively (Table 1). Therefore, these Adjacent cells, which appear grossly normal and were taken 10–30 cm proximal to the tumor, display significant up-regulation of (Ca^{2+})$_i$ relative to both normal cancer-free colonic mucosa and to their malignant counterparts. Similar results of elevated (Ca^{2+})$_i$ were observed in the mucosa of the patient with familial adenomatous polyposis who had not yet developed any cancer. Taken together, these findings suggest that a field defect involving up-regulation of (Ca^{2+})$_i$ may be present prior to the development of the malignant phenotype. This conclusion is consistent with other reports of field defects in colonic mucosa from cancer patients wherein increased proliferation (11), altered ion transport (12), and microscopic changes in crypt structure (13) have been observed at sites distant to the tumor.

Within the Adjacent tissue group were three patients with significantly greater (Ca^{2+})$_i$ levels relative to the remainder of the group. Even if data from these three patients are separated into their own category, both this new group (High Adjacent) and the remaining group (Low Adjacent) exhibit significantly

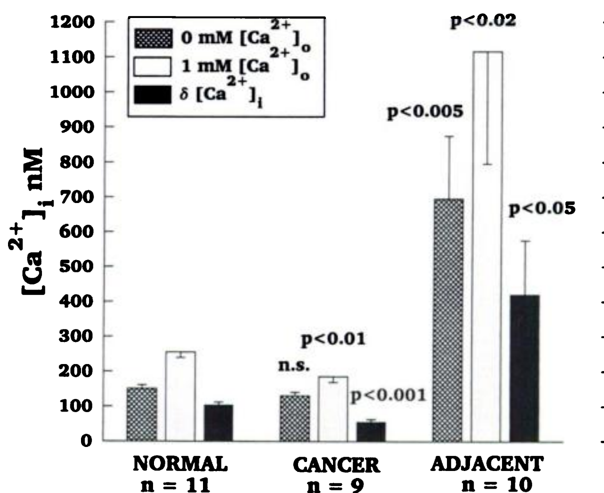

Fig. 1. Mean (Ca^{2+})$_i$ ± SEM (nM) for Normal, Cancer, and Adjacent tissues bathed by calcium-free and 1 mM (Ca^{2+})$_o$ physiological saline solutions. Delta (δ) designates the rise in (Ca^{2+})$_i$ following the addition of 1 mM (Ca^{2+})$_o$. p < 0.05 indicates the means are significantly different from those of the NORMAL group by unpaired t-test.

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elevated $(Ca^{2+})_i$ compared with both tumors and controls. Furthermore, the $(Ca^{2+})_i$ levels in the High Adjacent subset more closely resemble the high levels exhibited in the cells from the patient with familial adenomatous polyposis.

One of the High Adjacent patients was an 83-year-old female with a Dukes' A, moderately well-differentiated colon cancer. The second patient was a 74-year-old male with local recurrence 1 year following transanal excision and irradiation of a rectal cancer. The third patient was a 23-year-old female who presented with a Dukes' D, poorly differentiated adenocarcinoma of the colon. While the advanced age at presentation was the only atypical finding concerning the first patient, the recurrence of cancer in the second patient and the young age of the third patient, as well as the aggressive nature of her tumor, differentiate these latter two cases from the typical colon cancer patients that make up the majority of the Adjacent group. It is possible that these patients represent a subset population who develop colon cancers which are clinically or pathologically more aggressive, similar to patients with familial adenomatous polyposis. These patients may have a quantitatively different colonic field defect, with greater $(Ca^{2+})_i$ up-regulation, than do "typical" colon cancer patients.

Details of the interplay between $(Ca^{2+})_i$ and DAG and PKC levels remain largely unknown. Our results demonstrating decreased $(Ca^{2+})_i$ in malignant cells (Cancer) compared to adjacent mucosa (Adjacent) and to control mucosa (Normal), together with recent reports demonstrating decreased PKC activity and DAG levels of in primary human colon cancers *versus* adjacent tissue (6–9), support the hypothesis that the phosphatidylinositol pathway is down-regulated in human colon cancers. One plausible consequence of this down-regulation in tumor cells would be the loss of surges in $(Ca^{2+})_i$ to levels that have been associated with normal differentiation or apoptosis (cell death) in other cell types (2–5, 14, 15).

Our finding that Adjacent tissue has markedly elevated $(Ca^{2+})_i$ compared to Normal specimens seems contrary to what might be expected based on reports that both PKC activity and DAG levels are not significantly different between these groups (8, 9). However, it has been demonstrated previously that one expression of a field defect is the up-regulation of proliferation throughout the colon of cancer patients (11). Moreover, it has been shown recently that adjacent mucosa taken 15 cm from the cancer has an increased number of aberrant crypts which resemble the microscopic adenomatous polyps found in familial polyposis (13) consistent with an increased proliferative state of the mucosa. Thus, it is tempting to speculate that the markedly increased $(Ca^{2+})_i$ in our Adjacent group and the observation that $(Ca^{2+})_i$ is even more elevated in patients with aggressive cancers or a precancerous condition such as familial adenomatous polyposis reflects a role for $(Ca^{2+})_i$ as a signal for proliferation and, perhaps, as a factor in cancer development.

The relationships between genetic mutations, altered cell signaling, and regulation of $(Ca^{2+})_i$ are only just beginning to be examined. A clearer understanding of $(Ca^{2+})_i$ regulation and how that regulation is altered in both malignant tissues and tissues at increased risk of developing malignancy may improve our understanding of the biology of colorectal cancer. These findings are of particular interest because the observation of elevated $(Ca^{2+})_i$ in "normal" colonocytes some distance away from the primary colonic cancer suggests a field defect in $(Ca^{2+})_i$ homeostasis.

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