Decreased Levels of 1,2-sn-Diacylglycerol in Human Colon Tumors

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Abstract

We have found that in 15 of 15 primary human colon tumors there was a significant decrease (by about 40%) in the levels of diacylglycerol when compared to paired adjacent normal mucosa samples. Assays on the same samples indicated that this decrease was seen both in tumors that did and did not display mutations in codon 12 of c-K-ras. These results, taken together with previous studies on protein kinase C, suggest that the protein kinase C signal transduction pathway is suppressed in human colon cancer.

Introduction

The enzyme PKC, a Ca²⁺- and phospholipid-dependent protein serine/threonine kinase, plays a central role in signal transduction, growth control, and phorbol ester-mediated tumor promotion (for review see Refs. 1–3). The function of PKC is linked to the action of several growth factors and other agonists via activation of a specific phospholipase C. This results in the hydrolysis of phosphatidyl inositol 4,5-bisphosphate, yielding inositol 1,4,5-triphosphate, which stimulates the release of Ca²⁺ from intracellular storage sites, and 1,2-sn-diacylglycerol (DAG), which binds to PKC in a 1:1 stoichiometry and activates the enzyme, particularly at low Ca²⁺ concentrations. This activation is specific for DAGs that have the 1,2-sn configuration (4). There is also evidence that the phorbol ester tumor promoters activate PKC by binding to the same regulatory region of PKC as DAGs, thus usurping their function (5). DAGs are also of interest since rodent fibroblasts and kidney cells transformed by activated ras oncogenes display increased cellular levels of DAG (6–10), presumably due to activation of a cellular phospholipase C and/or phospholipase D, but the precise mechanism is not known. Other types of evidence also indicate synergistic interactions between ras oncogenes and PKC in the transformation of rodent fibroblasts (11). It should be stressed that PKC belongs to a multigene family, encoded by at least 6 genes, but the specific functions of the individual isoforms in cell transformation are not known (3). Our laboratory has been interested in the possibility that DAGs and the PKC pathway may play an important role in human colon cancer. DAG can be detected in normal human fecal specimens (12, 13), which appears to be derived, at least in part, from the action of intestinal bacteria (12). Furthermore, Friedman et al. (13) found that DAG can enhance mitogenesis in colon adenoma and carcinoma cells in cell cultures. On the other hand, we have found that primary human colon tumors generally display decreased levels of PKC (14). The latter finding was recently confirmed and extended by Kopp et al. (15). In view of these findings and the fact that human colon tumors frequently display mutations in the 12th codon of the c-K-ras gene (16), the present study was undertaken to assay the absolute level of DAG in a series of primary human colon tumors and to assay the same tumor samples for mutations in c-K-ras.

Materials and Methods

Tissues. Surgically resected samples were collected from the operating rooms of Columbia-Presbyterian Medical Center. Tumor tissue and a portion of the corresponding normal mucosa (near the histologically normal margins of the resection) were immediately rinsed with cold phosphate-buffered solution. Areas of obvious necrosis were avoided. Within 30 min after resection, the tissue samples were frozen in liquid nitrogen and stored at −70°C until analysis. Surgical pathology reports were obtained on all of the specimens used in the present study.

Lipid Extraction. Samples were extracted by a modification of the method of Bligh and Dyer (17). Briefly, frozen tissue samples were homogenized in 1 m NaCl in a 10:1 dilution using a polytron homogenizer. To 100 µl of homogenate were added 100 µl of 1 m NaCl and 750 µl of chloroform:methanol (1:2. v/v) in a Eppendorf tube. The monophase was mixed vigorously. Then, 250 µl of chloroform and 250 µl of 1 m NaCl were added. The phases were separated after 1 min of centrifugation at 5000 x g. The aqueous phase was discarded and the chloroform phase was dried under nitrogen gas and stored at −70°C.

Detection of Mutations in the c-K-ras Oncogene. A rapid nonradioactive method, developed in our laboratory (19), was used for the detection of ras mutations, with minor modification. This method is based on PCR amplification of the human c-K-ras first exon sequence followed by specific restriction endonuclease digestion to diagnose primer-mediated deletion sites (19). High molecular weight DNA was amplified without the extraction of DNA from tissue homogenates. Ten µl of a tissue homogenate (10 mg wet weight of tissue per ml of saline) were added to 25 µl distilled water and heated at 100°C for 10 min. After cooling on ice, 5' and 3' end primers, deoxynucleotide triphosphates, Taq DNA polymerase and PCR reaction buffer were added and DNA was amplified using a Perkin-Elmer Cetus Thermal Cycler. Additional details were as described previously (19).
Results and Discussion

A total of 16 tumor and paired normal mucosal samples were assayed for DAG content and codon 12 c-K-ras mutations. The surgical pathology reports on these tissue specimens indicated that there were 15 colon tumors: 2 adenomas; 2 Dukes' Stage A tumors; 5 Stage B tumors; and 6 Stage C tumors. The location of these tumors within the colon ranged from the cecum to the rectum (Table 1). The additional sample represented a pancreatic carcinoma which had metastasized to the small intestine and which was resected to relieve intestinal obstruction (Table 1).

When the colon tumors were assayed for DAG content we found that all of them displayed lower levels of DAG than the corresponding adjacent normal colonic mucosa samples (Fig. 1). The absolute level of DAG in the 15 colon tumors had a mean value of 1.12 ± 0.44 nmol/mg protein, compared to 1.95 ± 0.77 for the mean value of the 15 normal mucosa samples. The average DAG content for the tumor tissues was 58.7 ± 13.5% that of the paired normal mucosa samples. This difference was significant ($P < 0.001$, paired t test).

When the patient samples were stratified according to Dukes' staging, there was a steady decline in the absolute DAG content of the tumor tissues from Stage A (average, 1.53 nmol/mg protein), to Stage B (average, 1.15 nmol/mg protein) to Stage C (average, 0.79 nmol/mg protein) (Fig. 1). However, the percentage of DAG in tumors versus normal mucosa samples did not fall concomitantly, because a parallel decline was also seen in the DAG content of the adjacent normal mucosa samples: Stage A, average, 2.75 nmol/mg protein; Stage B, average, 2.14 nmol/mg protein; Stage C, average, 1.38 nmol/mg protein. Obviously, a larger number of samples is required to determine the significance of the latter correlations. If the data were stratified according to the location of the tumor within the colon, no clear pattern was seen in terms of the DAG content of the tumors or normal mucosa samples. Neither the sex nor the age of the patient appeared to influence the DAG levels.

During the course of these studies, Sauter et al. (20) also found decreased levels of DAG in a series of human colon adenomas and carcinomas. This decrease was comparable to that seen in the present study. Thus, this finding appears to be a hallmark of human colon tumors, although the previous authors did not assay their tumor samples for c-K-ras mutations (see below). Our data and those of Sauter et al. (20) do not reveal whether the decreased levels of DAG found in human colon tumors are due to decreased synthesis or increased conversion of DAG to other metabolites. In addition, since the tumor and normal mucosa samples represent mixtures of cell types, further studies are required to determine the cellular basis for the decreased levels found in the tumor samples.

To our knowledge, DAG levels have not been systematically analyzed in other primary tumors. A striking finding was that an additional sample analyzed in the present study, a resected metastatic lesion to the small intestine of a pancreatic adenocarcinoma, revealed about a 3-fold increase in DAG content when compared to the adjacent normal small intestinal mucosa. The level of DAG in this tumor (2.2 nmol/mg protein) was also higher than the mean value (1.12 nmol ± 0.44 nmol/mg protein) of the 15 colon tumors. Since all of the tumor samples were analyzed as coded samples, the pancreatic tumor sample served, in a sense, as a “positive control.” In future studies it will be of interest to determine whether, in contrast to colon tumors, a series of pancreatic tumors display high levels of DAG.

All of the above tumors and adjacent normal mucosa tissues were analyzed for mutations in the 12th codon of the c-K-ras protooncogene, since such mutations are seen in 30–40% of human colon tumors (16). Using a rapid and simple PCR method (see “Materials and Methods” and Ref. 19) we found that 4 of the 15 colon tumors (27%) displayed codon 12 mutations, which were distributed as follows: 1 Dukes’ A; 1 Dukes’ B, and 2 Dukes’ C (Fig. 1). These tumors displayed decreases in DAG levels that were comparable to those seen in the remaining 11 colon tumors that did not carry this mutation. These findings stand in striking contrast to previous studies demonstrating that rodent fibroblast and kidney cell cultures transformed with an activated ras oncogene display increased levels of DAG (6–10). This discrepancy suggests that an activated ras oncogene can exert different effects on phospholipid turnover in different cell types. Furthermore, it seems likely that the decreased levels of DAG seen in primary colon tumors are unrelated to activation of the c-K-ras oncogene since they were seen in all of the 15 colon tumors, whether or not they displayed the codon 12 c-K-ras mutation (Fig. 1).

Previous studies with rodent fibroblasts provided evidence that activation of PKC enhances cell growth and can act synergistically with an activated ras oncogene in the process of cell transformation (11). The present data, taken together with...
previous studies, suggest that this may not be the case in human colon tumors. Thus, colon tumors contain not only decreased levels of DAG (Fig. 1), the normal endogenous activator of PKC, but also decreased levels of PKC enzyme activity (14, 15). Furthermore, in separate studies our laboratory has shown that, whereas overexpression of the \( \beta \) isoform of PKC enhances the growth of rodent fibroblasts, it suppresses the growth of a human colon cancer cell line (21). Taken together, these findings suggest that the role of PKC and an activated ras oncogene in growth control is highly dependent on the context of the specific cell type. It will be of interest, therefore, to determine whether in certain types of human tumors an activated ras oncogene might function synergistically with PKC to enhance malignancy. Obviously, such alternate roles of components of signal transduction might be of relevance to the pathogenesis of specific malignancies and the design of tumor-specific therapeutic agents.

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References

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