Fecal Diglycerides as Selective Endogenous Mitogens for Premalignant and Malignant Human Colonic Epithelial Cells

Eileen Friedman, Per Isaksson, Joseph Rafter, Brigitte Marian, Sidney Winawer, and Harold Newmark

ABSTRACT

Diglycerides (DGs) have been found in fecal extracts at concentrations which induce mitogenesis of adenoma and some carcinoma cells but not normal cells in primary culture. DGs containing stearic, oleic, palmitic, and myristic acid side chains were found in fecal extracts from each of eight subjects. Synthetic 1,2-DGs, containing the fatty acids found in endogenous fecal DGs, induced mitogenesis in cultures of premalignant cells from each of 13 adenomas, covering all histological classes, and in cultures from two of four carcinomas. The potent adenoma mitogen, dimyristin, had no mitogenic activity on cultures of normal colonic epithelial cells from seven different subjects. These results suggest DGs may act as endogenous mitogens in the development of human colon cancer. The extent of adenoma mitogenesis was correlated with the chain length of the saturated R-groups: 16 > 14 > 12 > 10 > 8 >> 18. DGs with oleic acid residues, C18:1, were among the most active, while substitution of even one fatty acid residue with a stearic acid residue, C18:1, reduced or eliminated mitogenic activity. Dimyristin also induced enhanced levels of urokinase secretion from carcinoma cells, in parallel to the phorbol ester tumor promoter, 12-O-tetradecanoylphorbol-13-acetate. These results imply that DGs found in the colon induce a selective growth of benign colonic tumors and some carcinomas, and may enhance the invasive capacity of carcinomas, while leaving normal cells unaffected.

INTRODUCTION

Colon cancer develops through a series of preneoplastic cell stages. In the earliest identified stages the cells look normal histologically but display aberrantly high proliferation rates with delayed maturation. At later preneoplastic stages found in individuals symptomatic with familial polyposis, the cells lose all ability to mature to a normal endstage and exhibit even more aberrant proliferation. Preneoplastic cells are converted to benign neoplasms, adenomas, which themselves evolve through histological stages into dysplastic cells and finally into malignant cells. These cell stages have been observed clinically, described histologically, studied for physiological controls in organ and in tissue culture, and are now being analyzed molecularly. It is very likely that cells at premalignant cell stages differ genetically from normal cells. Several recent studies have uncovered genetic changes in adenomas (1, 2) and in cells from familial polyposis patients with a genetic predisposition to develop colon cancer (3, 4). Possibly normal colonic cells are converted into preneoplastic cells, then into more advanced premalignant cells by a series of genetic changes. The genetic changes which make colonic cells premalignant must also, directly or indirectly, confer susceptibility to tumor promoters such as TPA. TPA does not induce proliferation of normal cells (5). However, TPA stimulates proliferation of two classes of cells, preneoplastic cells from patients symptomatic with familial polyposis and premalignant tubular adenoma cells (5, 6). TPA also induces secretion of urokinase from more advanced adenomas (those with dysplastic and villous components), and increases the level of secretion of urokinase from carcinomas (6, 7).

Tumor promoters are distinctive long-chain fatty acids, oleic, palmitic, stearic, and myristic, which would act as selective mitogens for only preneoplastic, premalignant, and malignant cell growth and have no effect on normal cell proliferation. In addition, these DGs would enhance urokinase secretion, and hence invasive capability, of carcinomas.

MATERIALS AND METHODS

Specimen Procurement. Individual adenomas were removed by colonoscopy. A portion of the side of the head of the adenoma was removed and placed immediately in cold, antibiotic-containing wash medium (9) for transport to the laboratory. The stalk and tip of the adenoma were never selected because of their importance in diagnosis. The adenomas selected were always 1 cm or more in diameter, as sampling of smaller adenomas might compromise their diagnosis. Portions of carcinomas were received from Surgical Pathology. Colonic mucosal biopsies of normal subjects were taken from the rectosigmoid following preparatory with mild tap water enema and sigmoidoscopy, and immediately placed in cold wash medium, as above. All specimens were numbered to protect patient confidentiality. All procedures and assays were approved by the Institutional Review Board of Memorial Hospital.

Primary Culture of Tumors and Normal Epithelial Cells. Adenomas, carcinomas, and normal tissue samples were partially digested to epithelial organoids and primary cultured in highly supplemented, serum-free Dulbecco's modified Eagle's medium modified as described for medium NCTC168 (10). The cultured cells were judged epithelial by the presence of cytokeratins and an epithelial cell surface epitope, and by electron-microscopy, junctional complexes, and brush borders (5, 10).

Growth fractions were obtained by continuous labeling with 5 μCi/ml [3H]thymidine at 20 Ci/mmole followed by autoradiography as described (6, 10). Earlier studies had shown that continuous labeling of cells with 5 μCi/ml [3H]thymidine arrested cycling cells in late S/G2, so that the fraction of labeled cells in a culture reached a plateau value in 2–3 days (10), giving the growth fraction. Unlike the majority of continuous cell lines which have close to a 100% growth fraction, carcinomas, adenomas, and normal colonocytes from resections are composed of a nonproliferating fraction and a growth fraction, which in tumors has been as low as 2% (10). Mass culture measurements of total DNA content or total cell number would probably not be sensitive...
enough to show differences in growth in short-term studies of tumors with a low growth fraction.

Diacylglycerol Micelle Preparation. The DGs were all Sn,1,2-forms purchased from Sardary Research Labs, New London, Ontario. Some DGs were supplied dissolved in hexane. If the DGs were crystalline at room temperature, they were then dissolved in dimethyl sulfoxide to give 10 mg/ml stocks (16-29 mM for the DGs tested in this study). Aliquots of DGs at the concentrations to be tested in vitro were suspended by a 10-s sonication at the highest setting of a Branson sonifier in the Dubcco's modified Eagle's medium used for primary culture also containing 10 μg/ml of the water-stripped surfactant polysorbate 80 (Hoffman La-Roche). The medium (10) contains 1 mg/ml fatty acid-free bovine serum albumin (Sigma), 0.1 mM each phosphoethanolamine and ethanolamine, 0.3 μM linoleic acid, and 10 mM deoxycholic acid, so the sonication is believed to form micelles. The DG preparations were sonicated immediately before addition to the cells.

Other Cell Culture Techniques. The fluorimetric protease assay utilizes cleavage of the fluorescein substrate glt-gly-arg-aminomethylcoumarin to release the coumarin substrate (6).

Fecal Analysis. Stool specimens were collected from eight healthy volunteers, homogenized for 2 min in stomacher bags, and 5 g taken for analysis. These were extracted with chloroform:methanol (2:1); water; acetic acid (1.0, 0.13, 0.9) (11). The final extract was concentrated under nitrogen gas and stored at -20°C until thin-layer chromatography, which was performed on plates with 0.25-mm silica gel-G (Merck). The developing system was toluene:acetone:dihydroethylether:ethylacetate:acetic acid (80:30:10:10:0.2). A series of aliquots of the concentrated extract were spotted on the plate together with the following lipid standards: synthetic 1,2-saturated-chain diglycerides of chain length 12 to 18, and 1,2-diolein, with Rf values of 0.67 to 0.72; palmitic acid, Rf = 0.57; oleic acid, Rf = 0.59; tripalmitin, Rf = 0.91; monopalmitin, Rf = 0.28; cholesterol oleate, Rf = 0.98; methyl stearate, Rf = 0.83; deoxycholic acid, Rf = 0.08; cholesterol, Rf = 0.59. The diglyceride zone, from Rf 0.65 to 0.75, was eluted three times with 3 ml each chloroform:methanol (2:1). These samples were then hydrolyzed, extracted, and methylated as previously reported (12). Gas chromatography was performed on a 180-cm glass column (0.3 cm i.d.) packed with 3% SP-2401 on 100/120 Supelcoport (Supelco Canada, Ltd., Oakville, Ontario), with helium as the carrier gas. The column temperature was programmed from 145°C to 265°C at a rate of 4°C per minute with a 10-min dwell period at the initial temperature. The instrument used was a Packard Model 438 gas chromatograph equipped with a flame-ionization detector and a Hewlett-Packard 3390A (Palo Alto, CA), integrating recorder. Gas chromatographic-mass spectrometric analysis was performed on a VG 70-250 integrated mass spectrometer data system (VG Analytical, Manchester, UK). Fatty acids originating from the hydrolyzed DGs were identified by comparing their mass spectra with those of known derivatized standards and quantitated with the aid of the internal standard, nonadecanoate.

RESULTS

High Diglyceride Concentrations Found within Colon. A prominent, broad diglyceride (DG) band was found when fecal extracts from each of eight volunteers, on unrestricted diets, were analyzed by thin-layer chromatography. The DGs were hydrolyzed to release their constitutive fatty acids, which were then identified by gas chromatography and mass spectrometry (“Methods and Materials”). Fecal DGs were composed of oleic acid (C\textsubscript{18:1}), stearic acid (C\textsubscript{18:0}), palmitic acid (C\textsubscript{16:0}), and myristic acid (C\textsubscript{14:0}) (Table 1). Mean values were 447, 407, 185, and <22 μM, respectively. A wide range of values for the three major fatty acids was observed, from 28 to 1324 μM. The DGs found within the colon must be mixed chain DGs, as diolein, dipalmitin, distearin, and dimyristin were not found in any of the samples after gas chromatography-mass spectrometric analysis. The major endogenous DG structures must contain mixtures of palmitic and stearic, palmitic and oleic, and oleic and stearic fatty acid residues, along with some minor species containing myristic acid residues.

Diglycerides Induce Mitogenesis of Premalignant Cells. Previous studies from this laboratory have shown that the phorbol ester tumor promoter TPA induced mitogenesis in preneoplastic cells from familial polyposis symptomatic patients and in premalignant tubular adenoma cells (5). Therefore, the putative endogenous tumor promoters, the fecal diglycerides, were tested for mitogenic activity on adenoma cells. If one assumes fecal DGs consist of mixed fatty acid chains, and uses the values from Table 1 as a guide, most fecal DGs must be present at 50-500 μM, with the upper concentrations usually less than 300 μM. In the first experiments, the effect of saturated fatty acid chain length on adenoma mitogenesis was tested. A series of DGs were added to parallel primary cultures of tubulovillous adenoma 1051 (A), and dimyristin (C\textsubscript{14:0}) and dilaurin (C\textsubscript{12:0}) to cultures of dysplastic tubular adenoma 1053 cells (B), and growth fractions determined (“Materials and Methods”). Hatched areas, control values (mean value ± standard error) across the entire figure so that any deviations from the control are readily apparent.

Table 1 Quantification of fatty acids from fecal diglycerides: fatty acids separated by gas chromatography (μg/g wet weight feces)

<table>
<thead>
<tr>
<th>Subject</th>
<th>C\textsubscript{16:0}</th>
<th>C\textsubscript{18:1}</th>
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<tr>
<td>5</td>
<td>101.5</td>
<td>372.6</td>
<td>372.6</td>
</tr>
<tr>
<td>6</td>
<td>45.9</td>
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<td>38.9</td>
</tr>
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<td>7</td>
<td>53.2</td>
<td>64.0</td>
<td>28.3</td>
</tr>
<tr>
<td>8</td>
<td>45.2</td>
<td>69.5</td>
<td>61.2</td>
</tr>
<tr>
<td>Mean</td>
<td>47.3</td>
<td>126.3</td>
<td>115.7 μg/g</td>
</tr>
<tr>
<td>Range</td>
<td>185-396 μM</td>
<td>447-3 μM</td>
<td>407-μM</td>
</tr>
</tbody>
</table>

* Oleic acid and stearic acid residues incompletely separated on gas chromatogram so total value halved.
* Assuming 1 wet weight g of feces = 1 ml; C\textsubscript{12:0} residues present in seven of eight samples at <5 μg/ml so the maximum concentration of myristic acid residues was <22 μM. A recovery of 80% was calculated using known quantities of sn-1,2-DG standards.

![Fig. 1. Effect of chain length of the saturated R-groups of synthetic 1,2-DGs on adenoma proliferation. DGs composed of 2-R groups C\textsubscript{16:0} (○), C\textsubscript{16:0} (●), C\textsubscript{16:0} (□), C\textsubscript{16:0} (△), and C\textsubscript{16:0} (■) were applied to parallel primary cultures of tubulovillous adenoma 1051 (A), and dimyristin (C\textsubscript{14:0}) and dilaurin (C\textsubscript{12:0}) to cultures of dysplastic tubular adenoma 1053 cells (B), and growth fractions determined (“Materials and Methods”). Hatched areas, control values (mean value ± standard error) across the entire figure so that any deviations from the control are readily apparent.](image-url)
concentrations of dipalmitin and dimyristin above 300 μM inhibited adenoma growth, but these high concentrations were not observed in vivo (Table 1).

Inhibitory Effect of Stearic Acid in a Diglyceride. The effects of dipalmitin and distearin on adenoma mitogenesis were compared on adenoma 1556 (Fig. 2). Dipalmitin at physiological concentrations of 100 and 150 μM stimulated mitogenesis by 41% while distearin at 50, 100, and 100 μM induced no mitogenesis. Therefore, the gradient of activity for DGs with saturated side chains was 16 > 14 > 12 > 10 > 8 > 18.

Stearic acid C₁₈:₀, oleic acid, C₁₈:₁, and linoleic acid, C₁₈:₂, are the same chain length but differ by the presence of no, one, or two double bonds, respectively. The DG which contained oleic acid residues at both positions 1 and 2 (diolein) was compared with DGs which contained a mixture of saturated and unsaturated long-chain fatty acids. Diolein induced mitogenesis of adenoma 1068 with an optimum at 100 μM (Fig. 3). Replacing the 1-position oleic acid with stearic acid to give the C₁₈:₀, C₁₈:₁ mixed DG eliminated the mitogenic stimulation. This loss of mitogenic stimulation continued if the oleic acid residue on position 2 was replaced with the more unsaturated fatty acid, lineolic, to give the DG structure C₁₆:₀, C₁₈:₂. Thus the presence of even one stearic acid residue on a DG was enough to eliminate its mitogenic effect. The inhibitory stearic acid residue was then replaced by a palmitic residue to give a DG of structure C₁₆:₀, C₁₈:₁. This compound was as mitogenic as diolein (Fig. 3). Thus decreasing the saturated fatty acid chain length just two carbons, from stearic to palmitic, restored mitogenic activity. Clearly, the DGs exhibit a strong structure-function relationship, possibly related to their membrane solubility.

In a third adenoma, 1550, distearin induced some mitogenic activity at 50 μM (29% increase over control values, P < 0.03) but exhibited no activity at 100 μM. This adenoma stimulated to proliferate by distearin was a tubular adenoma, while 1556 and 1068 were villotubular, adenomas more evolved toward carcinoma, by histological criteria. Possibly the activity of distearin is limited to the more primitive tubular adenoma class. Thus, two DGs expected to be found within the colon, those containing stearic acid residues: C₁₈:₀, C₁₆:₀ and C₁₈:₀, C₁₈:₁, would be expected to have little or no mitogenic activity, while the DGs C₁₆:₀, C₁₈:₁, C₁₆:₀, C₁₅:₀ and C₁₈:₁, C₁₄:₀ would be expected to be potent adenoma mitogens.

Survey of Adenomas. The efficacy of the DG structurally most similar to TPA, dimyristin, and the DG with the most common unsaturated side chain, diolein, were compared on three adenomas at 50 and 100 μM. The optimal concentration of each DG varied between adenomas, but the increases in tumor growth fraction induced by dimyristin and diolein, respectively, were 83% and 46% on adenoma 1197, 66% and 51% on adenoma 1210, and 27% and 54% on adenoma 1068. Thus dimyristin was slightly more active (mean increase 59% compared to mean increase of 50%), and selected for further screening. A summary of the mitogenic effect of the optimal concentration of dimyristin (50 or 100 μM) on 10 adenomas is shown in Fig. 4. Dimyristin increased adenoma growth fractions a mean of 90%. Therefore, this DG induced almost a doubling of the fraction of the population able to divide. Furthermore, dimyristin induced mitogenesis in each of the adenomas studied regardless of histological class demonstrating that it is a potent adenoma mitogen.
Dimyristin Does Not Induce Mitogenesis of Normal Colonic Epithelial Cells. Earlier studies from this laboratory had shown no stimulation of proliferation by TPA of normal epithelial cells from 18 adult subjects (5). Therefore, it was reasonable to suspect that DGs also would not induce mitogenesis of normal cells. Since dimyristin was the most potent of the DGs assayed on adenoma cells, this DG was tested on normal cells from seven subjects using a series of DG concentrations from 25 to 500 μM (Fig. 5). In no case was mitogenesis observed.

Effects of Diglycerides on Carcinoma Cell Growth. Mitogenesis experiments were performed on four primary-cultured carcinomas, using a range of dimyristin concentrations from 50 to 500 μM. Two of the carcinomas exhibited a mean 46% increase in growth fraction at the optimum concentration of 50 μM dimyristin. Two others exhibited an unexpected decrease to about 42% of control levels (only 50 μM data shown in Fig. 6 for brevity). There was no obvious histological difference between the two groups, as both groups consisted of two moderately differentiated carcinomas, one Dukes’ stage A and the other, Dukes’ stage C. Of course the histological criteria for Dukes’ classification are quite broad and the moderately differentiated group may contain many subgroups.

Comparison of Diglycerides with TPA. In our previous studies (6, 7), TPA was observed to induce mitogenesis of tubular adenoma cells, while at the same concentrations inducing growth inhibition of more advanced premalignant cells from adenomas containing moderate to severely dysplastic cells or villous cells. This inhibition, which was also seen to a lesser degree with carcinomas, was due to loss of some proliferating cells from the monolayer after the cells elongated then rounded up and formed loosely adherent clusters. None of these morphological effects were seen with any class of adenoma cell treated with any DG. The monolayers remained intact with the cells closely apposed, while the DGs induced mitogenesis instead of cell loss. Five of the adenomas stimulated to divide by dimyristin had also been treated with 10 ng/ml TPA. In adenomas which were mainly tubular in histology, TPA again induced mitogenesis as in our earlier studies (Fig. 7). In three other, more pathologically advanced adenomas, TPA induced loss of proliferating cells from the monolayer and a decrease in tumor growth fraction while parallel cultures continued to thrive and were growth-stimulated by dimyristin (Fig. 7, adenomas 1236, 1197, 1134). In three carcinomas, TPA induced some cell loss with concomitant growth inhibition (Fig. 6), while parallel cultures from two tumors were stimulated to divide by dimyristin. Thus TPA and DGs had different mitogenic activities on dysplastic and villous adenoma cells and one class of carcinoma cells. TPA induced urokinase secretion from each of 12 primary-cultured carcinomas (6, 7). Both TPA and dimyristin were observed to induce enhanced levels of urokinase secretion from colon carcinoma cells (Fig. 8).

DISCUSSION

Diglycerides (DGs) found within the colon contain oleic acid, palmitic acid, stearic acid, and myristic acid residues. Synthetic DGs of these structures, at the concentrations found in vivo, when applied in vitro induce proliferation of premalignant and some malignant cells but not normal cells. Thus DGs may act as selective mitogens, enhancing adenoma growth and growth of one subgroup of carcinosmas, while inducing no response in normal cells. In addition, DGs induce enhanced levels of urokinase secretion from carcinoma cells, which is very likely to increase the invasive capability of the carcinoma cells. There is evidence suggesting that tumor promoters have acted on colon tumors in vivo. Two groups (13, 14) have found that colonic adenomas and carcinomas have elevated levels of a TPA-inducible enzyme, ornithine decarboxylase. Thus the tumors in vivo have properties consistent with tumor promotion ongoing in vivo, and when the colonic tumors are removed from the body and placed into primary culture, they retain their responsiveness both to TPA and to the postulated tumor promoters with properties like TPA, the fecal diglycerides.

One possible source of colonic DGs is the incomplete breakdown of dietary triglycerides. 2–10 g of lipids excreted per day are derived from foods incompletely digested by lipases within the small bowel (15). The three major fecal DG fatty acids are oleic, stearic, and palmitic (Table 1). These are also the major fatty acids from fecal triglycerides, which are composed of 29.7% palmitic acid, 24.1% stearic acid, and 22.5% oleic acid (15). Myristic acid, found in trace amounts in fecal DGs, is also found in fecal triglycerides at a low level (4.8%). These data

Fig. 5. Bar graph of growth fractions showing lack of mitogenic activity of dimyristin from 25 to 500 μM on normal cells from seven different subjects. O, control; □, 25 μM; ▪, 50 μM; ◊, 100 μM; ▣, 200 μM; □, 500 μM. Mean and standard error bars are shown.

Fig. 6. Bar graph showing tumor-growth fractions (mean and standard errors) of four carcinomas grown in the presence of 50 μM dimyristin (•) or 10 ng/ml TPA (□) compared to controls (C). MD-A, moderately differentiated, Dukes’ A stage carcinoma; MD-C, moderately differentiated, Dukes’ C-stage carcinoma.

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provide a mechanistic explanation for the epidemiological evidence linking a high fat diet with colon cancer development (16, 17). We hypothesize that the periodic exposure of premalignant and malignant colonic cells to DGs generated within various regions of the gut from partial breakdown of fecal triglycerides following a high fat meal, would provide a strong selective pressure for premalignant and malignant cell growth. In addition, repeated exposures of malignant cells to DGs could induce repeated bursts of urokinase secretion, aiding carcinoma invasion into the gut wall. Indeed, the area of the colon in which the fecal mass is most concentrated and stationary is the rectosigmoid, the area of the colon most prone to the development of carcinomas. Thus adenoma cells and some carcinoma cells would continue to amass within the colon, their growth stimulated by periodic DG exposures which would not affect the normal cells.

A second, minor source of fecal DGs, besides the diet, may be the breakdown products of cell membranes from the normal process of exfoliation of terminally differentiated epithelial cells at the tops of colonic crypts. These exfoliated cells are believed to be the source of the 2 g of lipids excreted per day by individuals on fat free diets (15). A third source of colonic DGs could be bacterial lipids from the breakdown of the lipid membranes of the many bacterial species within the gut.

Other investigators have shown that a short chain DG (Di-C16:0) induces ornithine decarboxylase activity and mitogenesis, but does not induce papilloma formation when applied to mouse skin pretreated with a carcinogen (18). In this system the short chain DG acted as a stage II tumor promoter, only inducing papilloma formation after a short treatment with TPA (19). We have shown that DGs induce mitogenesis of cells already at the adenoma or carcinoma stages and not examined their effects on colonic cells at preneoplastic stages.

REFERENCES

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