Urokinase Secretion from Human Colon Carcinomas Induced by Endogenous Diglycerides

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ABSTRACT

Colon tumor cells are more responsive to certain growth modulators in their local environment in vivo than are normal colonocytes. Examples of this class of compounds are the fecal diglycerides (DGs) (E. Friedman et al., Cancer Res., 49: 544-548, 1989), which may act as endogenous tumor promoters. The concentration found in vivo, fecal DGs composed of oleic, myristic, and palmitic fatty acids induced mitogenesis of all classes of benign tumor cells and of half of the resected carcinomas tested in primary culture, but induced no detectable mitogenesis of normal colonocytes. Colon tumor cells also exhibit selective responses to these endogenous modulators as measured by another biological parameter, secretion of urokinase. Addition of the fecal DG dimyristin led to release of 17 times more urokinase from carcinomas than from normal colonocytes. Fecal DGs also induced a 13-fold increase in urokinase mRNA synthesis in colon carcinoma cells and induced secretion of active urokinase from each of five resected carcinomas. Colon carcinomas, at both the primary site and metastatic to the liver, secreted the M, 55,000 form of urokinase constitutively and secreted the same form upon treatment with fecal DGs. An increase in the steady-state level of urokinase secretion by saturated-chain DGs exhibited a strong dependency on the chain length of the fatty acid residues, those of 14 and 16 carbons having the greatest activity. Thus, fecal DGs composed of oleic, myristic, and palmitic acid residues induce two biological activities selectively in colon tumor cells, each of which would enhance tumor development. Selective mitogenesis would increase adenoma and carcinoma cell number relative to normal colonocyte number, and induction of the proteolytic enzyme urokinase would aid local invasion of the carcinoma within the bowel wall.

INTRODUCTION

DGs composed of oleic, palmitic, myristic, and stearic fatty acid side chains were identified in fecal extracts from each of eight normal volunteers (1). These fecal DGs at concentrations found in vivo are selective mitogens for both colon adenomas and carcinomas without detectable activity on normal cells (1). The mechanism of DG-induced mitogenesis in adenoma and carcinoma cells is activation of a M, 63,000 membrane phosphoprotein by enhanced tyrosine phosphorylation (2). This phosphoprotein was detected in carcinoma and adenoma cells, but not in normal cells by immunoblotting with antiphosphotyrosine antibody (2).

In earlier studies we had observed that the phorbol ester tumor promoter TPA induced proliferation of benign tumor cells, but not normal colonocytes (3, 4), a result parallel to the DG study (1). The TPA receptor within cells, protein kinase C, is activated by minute quantities of DGs released by membrane lipid hydrolysis following binding of certain growth factors (5).

Our biochemical analysis showed that colonic DGs are present at concentrations averaging 100 to 400 μmol (1) and from their fatty acid composition are likely to be derived from dietary fat. Thus, we speculate that DGs are present following fatty meals high enough concentrations within the colon to directly activate protein kinase C.

A second activity of TPA on resected colon casualties was the induction of urokinase secretion from clinically advanced benign tumors (those adenomas with dysplastic or villous components) and the induction of elevated levels of urokinase secretion from carcinomas (3, 6). Urokinase-type plasminogen activator is believed to play a central role in the invasive growth of cancer cells (7, 8), with multiple steps including activation from a prourokinase form, binding of the active form to specific membrane receptors, and interaction of the active form with urokinase-specific high-affinity inhibitors. One control point in this system is urokinase mRNA synthesis, which can be enhanced by TPA (9). We therefore decided to test whether those DGs observed within the colon could act as endogenous modulators of the urokinase system and induce elevated levels of urokinase from resected carcinomas.

MATERIALS AND METHODS

Cell Culture. Portions of carcinomas were received from Surgical Pathology, Memorial Sloan-Kettering Cancer Center; partially digested to epithelial organoids with hyaluronidase, neuraminidase, and collagenase; and cultured exactly as described (2) and used for only one experiment in this study, induction of urokinase mRNA (Fig. 2).

DGlycercide Micelle Preparation. The DGs were all sn-1,2 forms purchased from Serdary Research Laboratories, New London, Ontario, and suspended by sonication in DMEM containing 10 μg/ml of water-stripped Polysorbate 80 (Hoffman La-Roche) immediately before addition to the H1-1 cells.

Urokinase-type Plasminogen Activator Assay. Cells were primary cultured in DMEM containing DGs for 72 h. The serum-free medium was then removed, debris was pelleted, and the supernatant was stored at −20°C until assay. The assay was performed using the synthetic substrate Spectrozyme-PL (H-n-norleucyl-hexahydro-tyrosyl-lysine-p-nitro anilide diacetate; American Diagnostics, Inc., New York, NY) for plasmin in the presence of plasminogen according to the manufacturer's
procedure. Briefly, a final reaction volume of 1 ml contained 0.1 µmol of substrate, 50 mM Tris-HCl-buffered saline at pH 7.9, 0.01% Triton X-100, 10 µg of plasminogen, and test samples ranging from 50 to 200 µl. The reaction mixture was incubated for 30 min to 2 h at 37°C, during which the reaction was linear (data not shown). Plasminogen was purified from outdated human plasma, as described (13). The reaction was stopped by addition of 50 µl of 50% acetic acid. Duplicate samples were assayed in the presence of 10 µg of goat anti-urokinase IgG to ensure that all proteolytic activity measured was due to urokinase-like activators. The specificity of this preparation of anti-urokinase antiserum has been documented (14). The yellow color developed was measured at 405 nm in a Varian DMS-90 spectrophotometer and evaluated in comparison with a urokinase standard curve run at the same time (data not shown). Units of urokinase were normalized to cell number using the tetrazolium assay (15). Briefly, cultures were washed once with PBS and then incubated in serum-free, glutamine-free DMEM containing 1 mg/ml of dimethylthiazol diphenyltetrazolium bromide in a CO2 incubator for 4 h. The dimethylthiazol diphenyltetrazolium bromide medium was aspirated, the cells were extracted in a small volume of dimethyl sulfoxide (0.2 to 1.0 ml), and color development was then assayed at a test wavelength of 570 nm and a reference wavelength of 630 nm, using a plate reader.

**Western Blot Analysis.** Culture medium (0.5 ml) for each assay was dialyzed against 0.1 M ammonium bicarbonate, lyophilized, and then redissolved in 0.1 ml of Tris-HCl-buffered saline at pH 7.9. Twenty µl of the concentrated material were electrophoresed on 4 to 30% gradient gels (Pharmacia LKB, Piscataway, NJ) in 0.025 M Tris, 0.0192 M glycine, 0.002% sodium dodecyl sulfate at pH 8.3. Urokinase (Winkinase from Washigton Laboratories, or urokinase from Calbiochem) was simultaneously electrophoresed as a standard. After transferring onto nitrocellulose, the blots were blocked with 0.3% gelatin in Tris-HCl-buffered saline and incubated with monospecific goat anti-urokinase (Accurate Biochemicals) followed by incubation with the substrate o-phenylene diamine and H2O2.

**Northern Blot Hybridization.** Total RNA was extracted by a modification of the method of Chomzynski and Sacchi (16) and blotted onto a nitrocellulose membrane using a Millipore slot blotter. Hybridization was performed under high-stringency conditions overnight at 65°C in 7% formamide: 1 M NaCl: 1% sodium dodecyl sulfate: 10% dextran sulfate. The urokinase clone pHUK-8 is a human cDNA clone (17), a 1.5-ko base fragment of the original clone pHUK-1 encoding amino acids 103 to 270, which was excised from the vector by restriction enzymes BamHI and BglII and 32P labeled by random priming. The probes were washed out by submerging the blots in boiling water for 5 min and then reprobed with a 32P-specific cDNA probe for GAPDH labeled by random priming, for an internal control for the amount of mRNA loaded per slot. Each total RNA preparation was analyzed for integrity of the ribosomal bands on a 1.0% agarose gel by ethidium bromide staining as a first analysis of integrity of the RNA preparation before any preparation was used for blotting.

**RESULTS**

**Comparison of Urokinase Induction in Resected Carcinomas and in Normal Colonocytes.** Parallel primary cultures prepared from two resected carcinomas and from resected sections of normal large bowel from two patients were cultured in the presence of the fecal DG, dimyristin, at 0 to 100 µmol (Fig. 1). The mean constitutive level of urokinase secretion was 8-fold higher from the carcinomas than from the normal colonocytes (226 milliunits per 10⁶ cells) compared with 29 milliunits per 10⁶ cells. The urokinase assays in this study were found to be linear with respect to both the concentration of enzyme and time of incubation (data not shown) and displayed a complete inhibition of their lytic activity by goat anti-urokinase antiserum ("Materials and Methods," data not shown).

Addition of 25 to 50 µM dimyristin in detergent micelles increased the level of urokinase secretion by a mean of 370% from the two carcinomas. A peak of activity occurred at 25 to 50 µM dimyristin, with activity decreasing markedly, almost to constitutive levels, at 100 µmol. In contrast, 25 to 100 µM dimyristin induced no detectable increase in urokinase secretion from one preparation of normal colonocytes (Fig. 1, N#2), while doubling the low level of secretion from normal colonocytes taken from a second patient (Fig. 1, N#1). In colonocytes from the latter patient, the 2-fold increase with dimyristin occurred at all concentrations tested. This lack of a sharp dose-response curve in Normal Preparation 1, in sharp contrast to the peaks seen with the two carcinomas, suggests that the DG-Tween 80 micelles may have induced leakage of the urokinase from goblet cells in Normal Preparation 1, rather than active secretion. Supporting this interpretation is the observation that treatment of formalin-fixed sections of normal colon with a related detergent, 0.1% Triton X-100, allowed greater detection of goblet cell urokinase (18). Regardless of the explanation for the effect of DG micelles on normal colonocytes, in the presence of 25 to 50 µM dimyristin the carcinoma cells secreted a mean of 827 milliunits per 10⁶ cells, while the normal cells released far less urokinase, a mean of 49 milliunits per 10⁶ cells. Thus, when cultured in the presence of a fecal DG, carcinoma cells secreted 17 times more urokinase than did normal colonocytes.

**Fig. 1.** Dose-response curve measuring the effect of dimyristin on urokinase secretion from two surgically resected colon carcinomas (CA#1, CA#2) and two specimens of normal colon from resections (N#1, N#2).

**Diglyceride Induction of Elevated Levels of Urokinase mRNA Synthesis.** To answer the question of whether fecal DGs induced synthesis and then secretion of urokinase, or whether they simply induced release of intracellular stores of urokinase by membrane leakage, the level of urokinase mRNA was compared in DG-treated and vehicle-only treated colon carcinoma cells. The DG-sensitive human colon carcinoma cell line HI-1 (2) was utilized for this experiment as insufficient numbers of cells can be obtained from primary cultures of either colon carcinomas or normal colonocytes for mRNA analysis. Parallel T-150 flasks of subconfluent HI-1 cells were treated with 10 or 50 µM diolein in detergent micelles ("Materials and Methods") for 2
and 4 h, while control cells were exposed to the same medium containing only the vehicle for 4 h before RNA extraction (“Materials and Methods”). When normalized to levels of expression of the constitutive gene GAPDH (“Materials and Methods”), urokinase mRNA levels were observed to increase 5-fold after a 2-h treatment with 10 μm diolein (not shown) and 13-fold after 4 h of treatment (Fig. 2, Lanes 1 and 3). Diolein at the higher concentration of 50 μmol induced no detectable increase in urokinase mRNA level over control levels, when normalized to GAPDH mRNA levels (Fig. 2, Lanes 1 and 2). Thus, the fecal DG diolein at physiological concentrations induced an increase in steady-state levels of urokinase mRNA synthesis in colon carcinoma cells. Diolein at 1 and 10 μmol, but not at 50 μmol, induced detectable levels of secreted urokinase from HI-1 colon carcinoma cells (data not shown), results parallel to the mRNA induction. Thus, it is very likely that the increase in levels of the biologically active urokinase found in conditioned media of carcinoma cells treated with DG was due to increased levels of synthesis of urokinase, not simply release from intracellular stores.

Fecal Diglyceride Diolein Induction of Secretion of Urokinase from Primary-cultured Carcinoma Cells. Diolein, a DG composed of fatty acid residues 18 carbons in length with one double bond, has activity on resected colon carcinomas as well as established cell lines. Diolein markedly stimulated the secretion of urokinase from a third primary cultured colon carcinoma, No. 1442 (Fig. 3), with an optimum at 30 μmol and less effect at 40 to 90 μmol.

Effect of Diglyceride Chain Length on Induction of Urokinase Secretion. Parallel primary cultures of a fourth surgically resected carcinoma, No. 1475, were assayed with 25 μmol each of a series of synthetic sn-1,2-diglycerides (DGs). Each was composed of two identical saturated fatty acid R-groups of chain lengths 8 to 18. A dose of 25 μmol was chosen because it was an effective dosage in the experiments of Fig. 1. There was a marked effect of chain length with increasing induction of urokinase until a maximum was reached with 14 carbons (Fig. 4). The DG with 16 carbons in its side chains, dipalmitin, had less activity, while the DG with fatty acid side chains 18 carbons in length (distearin) was inactive. Thus, two of the three saturated chain DGs found in the human colon, those with fatty acid residues 14 and 16 carbons long, were potent inducers of urokinase secretion, while the DG containing fatty acids 18 carbons in length was inactive.

Effect of Stearic Acid Residues on Mixed DGs. Distearin was inactive (Fig. 4) in urokinase induction on No. 1475 resected carcinoma. However, most fecal DGs are mixed, that is, composed of different side chains (1). Therefore, the effect of one stearic acid residue on DG activity was examined. In a fifth surgically resected colon carcinoma, No. 1491, mixed DGs consisting of one palmitic and one oleic residue induced urokinase secretion (Table 1). The DG with the palmitic acid at position 1 (C_{16:0}, C_{18:1}) was more active, inducing 837 milliunits of urokinase per 10^5 cells, than the oleic-palmitic DG (C_{18:1}, C_{16:0}) with the palmitic residue at position 2, which induced secretion of 665 milliunits of urokinase per 10^5 cells. If a stearic acid residue were substituted for a palmitic at position 1, the activity of the DG dropped dramatically, inducing only 222 instead of 837 milliunits of urokinase per 10^5 cells. If a stearic acid residue was maintained at position 1 and the oleic residue in position 2 was substituted with more unsaturated fatty acids, either linoleic (C_{18:2}) or...
arachidonic (C20:4), the level of activity was restored back to that observed constitutively.

These results implied that DG activity might be related to the degree of solubility of the DG within the membrane lipids, with the flexibility of the unsaturated fatty acid chain acting to counter the inflexibility of the 18-carbon saturated chain. Two or four double bonds allowed greater flexibility of the unsaturated fatty acid side chain than one double bond, reducing the inhibition due to the stearic acid residue. However, all three DGs containing one stearic acid residue failed to induce elevated urokinase levels of secretion in this carcinoma, No. 1491, confirming the inhibitory effect of this fatty acid which was first observed with distearin in carcinoma No. 1475 in Fig. 4. The much smaller amounts of higher molecular weight forms can be visualized, regardless of their state of activity.

Relative Potencies of Fecal DGs in Urokinase Induction. The relative potencies of three biologically active fecal DGs were assayed on two surgically resected colon carcinomas in primary culture (dimyristin data also seen in Fig. 1). In carcinoma 1447, the unsaturated DG diolein was the most potent on a molar basis, with a sharp optimum at 25 μmol. Dimyristin and dipalmitin were less active, with the higher concentration of 50 μmol needed for optimum activity (Fig. 5). In carcinoma 1445, diolein was also the most potent DG on a molar basis with a peak of activity at 15 μmol (made by extrapolating the two measured values at 10 and 25 μmol to a peak to conform to the peaks found with the other DGs). The saturated chain DGs, dimyristin and dipalmitin, exhibited maximal activity at 25 μmol (Fig. 6). Thus, in two carcinomas, diolein exhibited optimal activity at half the molar concentration needed for optimal activity of the saturated chain DGs.

**Table 1** Effect of stearic acid residue in mixed diglycerides on urokinase secretion

<table>
<thead>
<tr>
<th>DG added</th>
<th>DG structure</th>
<th>Urokinase secreted/1000 cells</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tween-80 only</td>
<td>None</td>
<td>434 ± 27*</td>
<td>&lt;0.0025</td>
</tr>
<tr>
<td>Oleic-palmitic</td>
<td>C18:1C16:0</td>
<td>665 ± 22</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Palmitic-oleic</td>
<td>C16:0C18:1</td>
<td>837 ± 45</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Stearic-oleic</td>
<td>C18:0C18:1</td>
<td>222 ± 48</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>Stearic-linoleic</td>
<td>C18:0C20:4</td>
<td>559 ± 96</td>
<td>NS*</td>
</tr>
<tr>
<td>Stearic-arachidonic</td>
<td>C18:0C22:4</td>
<td>370 ± 48</td>
<td>NS*</td>
</tr>
</tbody>
</table>

* Mean ± SE.
* NS, not significantly different by the t test, P > 0.5.

**Fig. 5.** Dose-response curves measuring the relative potencies of fecal DGs on surgically resected colon carcinoma, No. 1447.

**Fig. 6.** Dose-response curves measuring the relative potencies of fecal DGs on surgically resected colon carcinoma, No. 1445.

Immunoblotting Demonstration of Identical M, 55,000 Urokinase Protein Secreted from Carcinomas. Immunoblotting was performed to verify the presence of urokinase-like proteins in culture medium. Surgically resected colon carcinomas 1442 and 1447 and liver metastasis 1475 all secreted a M, 55,000 urokinase molecule reactive with anti-urokinase antiserum (Fig. 7, Lanes B, E, and F, respectively). The M, 55,000 species was also observed when the resected colon carcinomas were treated with either diolein (Lanes A and D) or dimyristin (Lanes C and G). Coomassie blue staining of parallel gels (data not shown) demonstrated that the bovine serum albumin, present at 1 mg/ml in the serum-free medium used to culture the colonic cells, caused a distortion in the electrophoresis, seen as a bare area of gel directly above the urokinase band. This distortion caused the secreted urokinase to migrate more rapidly than purified urokinase run in a parallel lane (not shown). However, mixing experiments demonstrated comigration of urokinase secreted from these colon carcinomas and purified urokinase (Calbiochem or Winkinase from Winthrop Laboratories, data not shown).

This Western blot (Fig. 7) was performed to determine the major species of urokinase secreted constitutively and that secreted when cells were challenged with fecal DGs, so no attempt was made to analyze culture media from equivalent numbers of cells. Urokinase-type plasminogen activators of high molecular weights were previously found by us in conditioned media from both colorectal and gastric tumor explants (19), so it was possible that fecal DGs induced secretion of one of these high-molecular-weight activators. The advantage of using the Western blot is that all urokinase-related molecular forms can be visualized, regardless of their state of activity. Thus, both M, 55,000 and 33,000 active urokinase, M, 55,000 prourokinase, and complexes of urokinase with inhibitors will be detected. The blot in Fig. 7 clearly indicates that the major form of urokinase released, both in tumors at the primary site in the colon and liver metastases, is the M, 55,000 component. The much smaller amounts of higher molecular weight forms (M, 70,000 to 220,000) probably represent complexes of urokinase with plasminogen activator inhibitor type 1 and possibly other inhibitors.
carcinoma 1442 untreated: C. colon carcinoma 1447 treated with 50 µM dimyristin cells. No attempt at quantification to biological activity was made, as the anti-

by primary cultured colon carcinomas and métastases, whether the cells are concentrated in different subjects from 28 to 1324 nmol (1). We hypothesize that this abundance of fecal DGs is sufficient to induce urokinase secretion from colon tumor cells in vivo, as concentrations of 10 to 100 µmol are effective in vitro. The fecal DG dimyristin induced 17 times more urokinase secretion than do normal colonocytes. Thus, transformation has changed the colonocyte from a cell barely responsive to fecal DGs to a cell with a heightened response to this class of colonic biological response modulators.

DISCUSSION

Fecal diglycerides are composed of mixed chains of stearic, oleic, palmitic, and myristic acid, and they range widely in concentration in different subjects from 28 to 1324 µmol (1). The DG concentrations which actually reach colonic cells are unknown. In this study, 1,2-DGs with the same fatty acid chains as those found in the colon induced urokinase secretion optimally at the relatively low concentrations of 15 to 50 µmol. DG concentrations over 100 µmol inhibit urokinase secretion, but are not toxic to the cells, which remain as an intact monolayer. However, DG levels higher than 100 µmol may never reach the cells in vivo. Our method of emulsifying DGs by sonication of DGs together with pharmaceutical-grade, water-stripped Tween-80 detergent and the fatty acid-free bovine serum albumin carrier may create far more efficient micelles for DG transport than those the body uses. This possibility must be considered when making any comparison of the in vivo DG concentrations with those concentrations active in vitro. In the small intestine, triglycerides and diglycerides form oily droplets surrounded by lysophospholipids, fatty acid soaps, and monoglycerides (20). Fatty acids, 2-monoglycerides, and 1-lyso-

lecithin are dispersed into bile salt micelles which are taken up by enterocytes which resynthesize triglycerides (20). We are the first group, to our knowledge, to demonstrate the presence of DGs within the colon of normal, healthy subjects (1), so there are as yet no data on DG transport. In the colon the long chain DGs probably remain as oil droplets, surrounded by amphoteric lipids. The bulk of the lipid is present as fatty acids from triglyceride digestion in the small bowel. Short-chain organic acids, like acetic and butyric, are present at high concentrations, 15 to 50 mmol. The fatty acids and organic acids are present free, or as sodium, potassium, calcium, or magnesium soaps, with varying solubilities. Secondary bile acids such as deoxy-

cholic acid, with concentrations ranging around 1 to 3 mmol, and some of the soaps with amphoteric properties could serve as emulsifying agents for colonic DGs. A series of diffusional barriers including mucin and an unstirred water layer must be traversed for DGs to enter colonocytes. Normal stem cells lay at the bottom of colonic crypts, protected under a mucus plug. Adenomas and carcinomas often have a smaller mucus coat than do normal cells, and many times the tumors project into the gut lumen. Both properties would make colon tumors better targets than normal colonocyte stem cells for the entry of DGs from the fecal stream.

A structure/function relationship was observed in urokinase induction by DGs, as had been observed in the mitogenesis study (1). The presence of one or two stearic acid residues in a DG was inhibitory in each of four urokinase induction assays in this study, and stearic acid residues had been inhibitory in mitogenesis assays with colon tumor cells (1). Dimyristin and dipalmitin were the most mitogenically active DGs, with shorter chain DGs less active (1), similar to the chain length depend-

cency observed for urokinase induction in this study. The unsat-

urated DG diolein was among the most potent DGs in stimu-

lating colon tumor cell proliferation (1) and was the most active DG in inducing urokinase on a molar basis in this study. Thus, fecal DGs had generally similar activities in studies of adenoma cell growth and of urokinase induction from carcinoma cells.

Urokinase secretion by carcinomas has been implicated by many studies to play a role in local tumor invasion and possibly in metastasis (7, 8). Urokinase mRNA synthesis is stimulated by TPA (9), and probably by fecal DGs, which increase constitutive levels of secretion. We postulate that colon tumor cells are periodically exposed to high concentrations of DGs from partial breakdown of triglycerides following a high-fat meal. These DGs would have three roles. (a) They would induce proliferation of adenoma cells, increasing the number of pre-

malignant cells which serve as targets for mutagens and, thus, increasing the likelihood of further mutations leading to malignancy (21–23). (b) They would increase the proliferation of some carcinoma cell types, increasing the number of malignant cells and thus, making it more likely that metastatic variants arise. (c) Fecal DGs would induce periodic waves of urokinase secretion from colon tumors, aiding invasion into the gut wall. Support for this hypothesis comes from clinical studies which have shown that adenomas arise with roughly equal frequency in all areas of the colon (24). However, adenomas develop into carcinomas most often in the rectosigmoid, the area of the colon in which the fecal mass is most stationary and concen-

trated, and therefore the area exposed for the longest time to fecal DGs.

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