Downregulation of a human colonic sialyltransferase by a secondary bile acid and a phorbol ester

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Li, Ming, Ravi Vemulapalli, Asad Ullah, Leighton Izu, Michael E. Duffey, and Peter Lance. Downregulation of a human colonic sialyltransferase by a secondary bile acid and a phorbol ester. Am. J. Physiol. 274 (Gastrointest. Liver Physiol. 37): G599–G606, 1998.—Fecal constituents such as bile acids and increased sialylation of membrane glycoproteins by α-2,6-sialyltransferase (HST6N-1) may contribute to colorectal tumorigenesis. We hypothesized that bile acids and phorbol ester [12-O-tetradecanoylphorbol-13-acetate (TPA)] would upregulate HST6N-1 in colonic cells. However, deoxycholate (DOC) (300 µmol/l), a secondary bile acid, and TPA (20 ng/ml) decreased expression of an ~100-kDa glycoprotein bearing α-2,6-linked sialic acid in a colon cancer cell line (T84) in vitro. HST6N-1 mRNA levels were reduced ~80% by treatment (24 h) with DOC or TPA but not by cholate, a primary bile acid. Treatment (24 h) with DOC or TPA decreased activity of this enzyme to 30% and 13% of control, respectively. Thus DOC and TPA both downregulated, and did not upregulate, α-2,6-sialyltransferase expression in vitro, but by different transduction pathways. As colorectal tumors grow, their progressive removal from the fecal milieu that normally downregulates this enzyme may favor invasion and metastasis.

glycosyltransferase expression; gene expression regulation; colorectal neoplasia

MOST MEMBRANE PROTEINS and many secreted proteins bear oligosaccharides that are heterogeneous and exhibit tissue-specific patterns of expression. Sialic acids, in α-2,3- or α-2,6-linkage to a penultimate galactose residue, occupy terminal positions in many N-glycan oligosaccharides of colonic and other membrane glycoproteins. Sialylated N- and O-glycans have been implicated in the development and metastatic spread of colorectal carcinoma (3, 9, 22).

Highly specific glycosyltransferases catalyze the post-translational addition of the individual sugars that comprise N-glycan and other oligosaccharides; sialic acids are transferred to acceptor oligosaccharides by one of the sialyltransferases, members of the glycosyltransferase family of enzymes. It is widely accepted that tissue-specific or disease-associated oligosaccharide expression is primarily a function of variations in glycosyltransferase expression (20). We reported that levels of β-galactoside α-2,6-sialyltransferase (HST6N-1) mRNA were more than threefold greater in human adenocarcinomatous tissue than adjacent histologically normal colon (16). Whether increased colonic epithelial expression of this enzyme is related causally to neoplastic transformation and progression is unknown, as are the oligosaccharide products and mechanisms involved.

Knowledge of the agents and mechanisms that regulate glycosyltransferase expression is scarce. n-Butyrate, a product of colonic bacterial fermentation detectable in portal blood, causes differentiation-related changes in colonic and hepatic cells in vitro (21, 27). We reported that culture of human colonic (16) and hepatic (24) cells in the presence of n-butyrate for less than 24 h caused >80% reduction in HST6N-1 mRNA expression by posttranscriptional mechanisms.

Normal adults discharge into the small intestine ~30 g/day of conjugated (primary) bile salts that are mostly incorporated with cholesterol and lecithin into mixed micelles and large vesicles (7). Reabsorption of bile salt monomers in the terminal ileum is normally efficient, but unabsorbed dihydroxy (secondary) bile acids, such as taurodeoxycholate (TDC), stimulate colonic secretion of electrolytes and water, causing diarrhea. Deconjugation by colonic bacteria to deoxycholic acid (DOC) and other free secondary bile acids further increases secretory potency. The intracellular mediator for the action of bile salts on colonic epithelial cells was shown to be Ca2+ (6). Application of TDC to isolated T84 cells (a human colon cancer cell line) activated K+ and Cl− conductances that were obligatory for secretion via an inositol 1,4,5-trisphosphate (IP3)-mediated release of Ca2+ from intracellular stores (5).

In addition to causing diarrhea, secondary bile acids are thought to be an etiological risk factor for colorectal cancer (25). Higher fecal concentrations of DOC were reported in patients with colorectal cancer and adenomatous polyps compared with control subjects (1, 26). Bile acids are tumor promoters in experimental animal models of colon cancer (19). Colonic epithelial cell hyperproliferation in response to cytotoxicity has been proposed as the mechanism responsible for the tumor-promoting effects of bile acids (15, 28).

We hypothesized that altered membrane sialylation through increased HST6N-1 expression could be one of
the cellular effects of colonic bile acids with tumor-promoting consequences. Unexpectedly, however, DOC and 12-O-tetradecanoylphorbol-13-acetate (TPA), a phorbol ester and another tumor promoter, caused selective downregulation rather than upregulation of HST6N-1 expression. Both agents altered HST6N-1 gene expression by direct transcriptional mechanisms. The transduction pathways for the bile acid and phorbol ester signals, respectively, were mediated by Ca\(^{2+}\)- and protein kinase C (PKC)-dependent mechanisms.

**MATERIALS AND METHODS**

**Reagents**

n-Butyrate, cholic acid, DOC, the Ca\(^{2+}\) ionophore A-23187, and TPA were obtained from Sigma Chemical (St. Louis, MO). Sambucus nigra agglutinin (S. nigra) was from E. Y. Laboratories (San Mateo, CA). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) standards were from Bio-Rad Laboratories (Hercules, CA). Enhanced chemiluminescence Western blotting detection reagents were from Amersham (Arlington Heights, IL). 1,2-bis(2-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid-acetoxymethyl ester (BAPTA-AM) was from Calbiochem (San Diego, CA). Fura-2-AM was purchased from Molecular Probes (Eugene OR). [\(^{32}\)P]dATP (3,000 Ci/mmol) and [\(^{2-}\)AM was purchased from Molecular Probes (Eugene, OR). Bovine serum albumin was from Amersham (Arlington Heights, IL). 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid-acetoxymethyl ester (BAPTA-AM) was from Calbiochem (San Diego, CA). Fura-2-AM was purchased from Molecular Probes (Eugene OR). [\(^{32}\)P]dATP (3,000 Ci/mmol) and [\(^{2-}\)AM was purchased from Molecular Probes (Eugene, OR).

**MATERIALS AND METHODS**

**Cell Viability**

Cell viability was determined by trypan blue exclusion. Cells were seeded at the same time from a single parent culture. Cultures were incubated without (control) or with DOC or TPA. Timing of the addition of TPA or DOC was staggered. Treated (12, 24, or 48 h) and control cultures were harvested at the same time, after careful washing to remove cells that had detached during incubation. Harvested cells were incubated with trypan blue and counted using a hemocytometer. From each culture, four fields of duplicate preparations were counted for the percentage of cells that excluded the dye.

**Isolation of RNA and Northern Analysis**

Isolation of total RNA, gel electrophoresis, Northern blotting, hybridization with radiolabeled cDNA probes, and quantitation of mRNA levels were performed as described previously (16, 24). Cells were cultivated to confluence, treated with test compounds, or maintained in medium unsupplemented with test compounds and harvested for isolation of total RNA. The following cDNA probes were used: human HST6N-1 cDNA was isolated previously by us (14), human \(\beta-1,4\)-galactosyltransferase (GaT1) cDNA was a gift from Dr. M. N. Fukuda [Masri et al. (17)], human N-acetylgalactosaminyltransferase (GnT I) cDNA was a gift from Dr. H. Schachter [Schachter et al. (23)], and rat \(\beta\)-galactoside-\(\alpha\)-2,3-sialyltransferase (ST3N) cDNA was a gift from Dr. J. C. Paulson [Wen et al. (29)]. The densities of DNA-RNA hybrids were determined by spectrophotometric scanning of autoradiographs, and results were normalized for intensity of staining with ethidium bromide.

**Sialyltransferase Assay**

Specific \(\beta\)-galactoside-\(\alpha\)-2,3-sialyltransferase enzyme activity was assayed as described (8, 24), with modifications. T84 cells, either untreated (control) or treated with DOC, cholate, or TPA, were washed four times in ice-cold PBS, scraped from culture dishes in 0.4 ml of sodium cacodylate (50 mmol/l) buffer at pH 6.5 (150 mmol/l NaCl, 1% Triton X-100, and 20% glycerol), and homogenized. The homogenate was centrifuged at 10,000 g for 30 min at 4°C. The reaction mixture for each assay contained 0.21 nmol CMP-[\(^{14}\)C]NeuAc, 19.4 nmol CMP-[\(^{14}\)C]NeuAc, 1 mmol/l 2,3-dehydro-2-deoxy-NeuAc, 5 mmol/l MnCl\(_2\), 50 mmol/l sodium cacodylate, 150 mmol/l NaCl, and 195 μg of asialo-\(\alpha\)-3-acid glycoprotein as acceptor, in a final volume of 60 μl. Reactions, performed in duplicate, were initiated by the addition of 20 μl of supernatant from centrifuged cell homogenate and incubated at 37°C for 1 h. The radioactive reaction product was isolated by chromatography on Sephadex G-50 and quantitated by liquid scintillation spectrometry. The protein content of cellular homogenates was quantitated by the method of Bradford (2). Specific sialyltransferase enzyme activities of treated and control cultures were compared using Student’s t-test.

**Nuclear Transcriptional Assay**

Nuclei were isolated, and the run-on protocol was carried out as described previously (13, 24). Equal amounts of nascent radioisotopically labeled RNA transcripts (5 \(\times\) 10\(^7\) cpm/3 ml) were hybridized for 3 days to 2 μg of nitrocellulose-bound cDNA.

**Intracellular Ca\(^{2+}\) Measurements**

Cells on glass coverslips were loaded at room temperature with fura 2-AM (4 μmol/l) for 20 min in Ca\(^{2+}\)-free solution,
followed by incubation for 1 h in the usual Ca²⁺-containing medium for T84 cells. The coverslips were then placed in a Plexiglas chamber and mounted on the stage of an inverted microscope (Nikon Diaphot) equipped for epifluorescence using a ×40 oil-immersion lens, as described previously (5). Fura 2 fluorescence images at 340-nm excitation wavelengths were captured with a silicon intensified target video camera and analyzed using imaging software (Image 1/FL, Universal Imaging). Average whole cell ratio values were determined. For chelation of intracellular free Ca²⁺, cells were incubated for 20 min in Ca²⁺-free solution supplemented with BAPTA-AM (20 µmol/l), followed by incubation in regular medium without BAPTA-AM for 1 h.

RESULTS

Terminal α-2,6-Linked Sialic Acids and Sialyltransferase Expression

Lectin affinity of T84 cell lysates. Lysates of cells cultured in the absence or presence of DOC or TPA were electrophoresed and electroblotted to membranes that were incubated with S. nigra, a lectin with specific affinity for α-2,6-linked sialic acid (Fig. 1). Three bands were detected, corresponding to sialylated glycoproteins with approximate sizes of 100, 80, and 75 kDa. With the length of exposure required for demonstration of the ~100-kDa band in Fig. 1, top, the more intense ~80- and 75-kDa bands resemble a single broad band, but they are readily distinguishable in Fig. 1, bottom.

Intensity of the ~80- and 75-kDa bands was unaffected by incubation with DOC. From comparison of treated and control lanes, decreased expression of the ~100-kDa glycoprotein was first evident 4 h after exposure to 300 µmol/l DOC. Decreased expression of this glycoprotein, relative to control, was most pronounced at 24 and 48 h in the DOC experiment. Diminished expression of the ~100-kDa band, compared with control, was first seen at 12 h in TPA-treated cells and was still apparent at 24 h.

An overall decline in levels of sialylated glycoproteins was evident at the extremes of both sets of experiments. This can be seen from reduced intensity of the ~100-kDa bands in the control lanes of the DOC (top) and TPA (bottom) experiments, respectively, by 72 and 48 h compared with control ~100-kDa bands at earlier time points. Exhaustion of the medium, which was not changed for control or treated cultures, causing a generalized reduction of cellular synthetic capacity in the longer experiments, is the likely explanation. Because DOC or TPA treatment caused reductions of product level in <24 h, and considerably earlier than the more generalized decline in levels of sialylated glycoproteins, cell cultures were treated for ~24 h in subsequent studies of the mechanisms of action of these compounds.

Sialyltransferase (HST6N-1) mRNA levels and activity in cells treated with DOC and TPA. Expression of the sialyltransferase responsible for transfer of sialic acid to colonic glycoproteins in terminal α-2,6-linkage was studied by Northern analysis (Fig. 2). HST6N-1 mRNA level was reduced ~80% by exposure to 300 µmol/l DOC for 24 h, and inhibition was first seen after 2 h. TPA caused threshold and maximal (~85%) inhibition of HST6N-1 mRNA levels, respectively, at concentrations of 5 and 20 ng/ml. The inhibitory effect of TPA was first seen after exposure for 4 h.

Downregulation of β-galactoside α-2,6-sialyltransferase activity on exposure of T84 cells to DOC or TPA followed reductions in HST6N-1 mRNA level (Table 1). Relative activity after exposure for 24 h to DOC and TPA, respectively, was 30% and 13% of control. Specific sialyltransferase activity in T84 cells incubated with cholate was undiminished at 12 h and was reduced to 57% of control after 24 h.

Terminal N-glycan sialic acids are linked to galactose residues, which are linked in turn to N-acetylgalcosamines. N-Acetylglucosamine and galactose sugars, respectively, are transferred sequentially to oligosaccharide chains by the actions of N-acetylgalactosaminyltransferase (GnT I) and galactosyltransferase (GalT) enzymes. To investigate the specificity of HST6N-1 downregulation by DOC and TPA, HST6N-1, GalT, and GnT I mRNA levels were assessed in the same Northern blot (Fig. 2A). DOC, at a threshold concentration of
200 µmol/l, and TPA, at a threshold of 5 ng/ml, caused modest increases in GalT mRNA level. Slight decreases in GnT I mRNA level were discernible in T84 cells incubated with DOC or TPA.

### Specificity of Bile Acid Effects

Comparison of primary and secondary bile acids. DOC, a secondary bile acid, has greater secretory potency and, it is thought, tumor-promoting activity than primary bile acids such as cholate (19). Therefore, the effects of DOC and cholate on HST6N-1 mRNA expression by T84 cells were compared. DOC caused reductions as before, but HST6N-1 mRNA levels were unaltered by incubation of cells for up to 24 h in cholate concentrations of up to 300 µmol/l (Fig. 3).

DOC treatment of nonneoplastic colonic cells. The applicability to nonneoplastic colonic epithelium of bile acid effects reported in colon cancer cell lines in vitro is uncertain. The NCM460 cell line was established from normal human colonic epithelial cells (18). As in cancer cell cultures, NCM460 cell HST6N-1 mRNA expression was downregulated by incubation with DOC but not with cholate (Fig. 4).

Cell viability and morphology. T84 cell viability, after treatment with DOC or TPA according to a similar protocol to that used for the lectin affinity experiments depicted in Fig. 1, was determined by trypan blue exclusion (Table 2). Cell viability was 94% after all treatment periods up to 48 h with either compound. Cell morphology was not affected by cholate, but DOC caused pronounced changes (Fig. 5). Morphological changes caused by DOC were reversible (data not shown); DOC-treated T84 cells continued to grow after return to DOC-free medium and within 24 h were almost indistinguishable in appearance from cultures that had never been exposed to DOC. As further confirmation of reversibility, HST6N-1 mRNA levels returned almost to pretreatment levels 24 h after DOC-treated cells were shifted to DOC-free medium;
by 48 h, pretreatment HST6N-1 mRNA levels had been exceeded (data not shown).

DOC and TPA Regulation of HST6N-1 Gene Expression

Nuclear transcriptional assays. Possible mechanisms for DOC- and TPA-mediated reduction of HST6N-1 mRNA expression include decreased transcription, reduced processing of nuclear HST6N-1 mRNA precursors and increased degradation of mature transcripts. The influence of these agents on the rate of HST6N-1 transcription was assessed by the nuclear run-on reaction (Fig. 6). The effects of DOC and TPA on transcription of other glycosyltransferases (GalT, GnT I, and ST3N) were examined to evaluate further the specificity of potential regulatory effects. Linkages synthesized through the actions of GalT and GnT I were described above. β-Galactoside α-2,3-sialyltransferase (ST3N) is an alternative sialyltransferase to HST6N-1 that transfers sialic acid to galactose acceptors in an α-2,3- rather than an α-2,6-linkage.

Densitometric analysis of the autoradiograph showed that HST6N-1 transcription was decreased to 30% of control after exposure of T84 cells to DOC or TPA for 24 h, but ST3N transcription was unaffected. GalT transcription was increased to 230% and 150% of control, respectively, in cells exposed to DOC for 4 and 24 h, but was unaltered by TPA. GnT I transcript levels after 24-h exposure to DOC and TPA, respectively, were 72% and 80% of control. Both DOC and TPA increased transcription of the actin gene.

Signal-Transduction Pathways of DOC and TPA Effects

DOC-mediated increase of intracellular Ca2+. Activation of K+ and Cl− conductances in T84 by TDC (750 µmol/l), a secondary bile acid, was reported in T84 cells (5). The mechanism of this action was via IP3-mediated release of intracellular Ca2+. Before investigating whether HST6N-1 downregulation by DOC was Ca2+ mediated, we confirmed that the secondary bile acid DOC caused a rapid increase in the ratio of F340 to F380, indicative of increased [Ca2+]i. This response occurred

Table 2. Trypan blue exclusion by T84 cells after treatment with DOC or TPA

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<td>Control</td>
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<td>% Viable</td>
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Results are given as average % of viable cells ± SE. Cells were seeded at the same time from the same parent culture and incubated without (Control) or with DOC (300 µmol/l) or TPA (20 ng/ml). Timing of the addition of DOC or TPA was staggered, and all cultures were harvested 48 h after seeding. Harvested cells were incubated with trypan blue and counted using a hemacytometer. Four fields each of duplicate preparations from each culture were counted for the number of cells that excluded dye.
<10 min after exposure to DOC. A transient decrease and increase in the fluorescence ratio followed the initial rise. This behavior is similar to the rise in \([\text{Ca}^{2+}] \text{_{i}}\) and \([\text{Ca}^{2+}] \text{_{oscillations}}\) seen on exposure of T84 cells to TDC at the higher concentration of 750 µmol/l (5).

\(\text{Ca}^{2+}\)-mediated regulation of HST6N-1 expression. Exposure to DOC in the absence of extracellular \(\text{Ca}^{2+}\) prevented downregulation of HST6N-1 mRNA expression in T84 cells that had been preincubated with BAPTA-AM to chelate intracellular \(\text{Ca}^{2+}\) (Fig. 8). However, when intracellular \(\text{Ca}^{2+}\) was not chelated, lack of extracellular \(\text{Ca}^{2+}\) did not prevent downregulation of HST6N-1 expression, indicating that this action of DOC occurred as a consequence of the release of intracellular \(\text{Ca}^{2+}\). Downregulation of HST6N-1 by TPA was not \(\text{Ca}^{2+}\)-dependent.

To further substantiate the role of increased \([\text{Ca}^{2+}] \text{_{i}}\), in regulating HST6N-1 expression, T84 cells were treated with a \(\text{Ca}^{2+}\)-ionophore, A-23187, to increase \([\text{Ca}^{2+}] \text{_{i}}\) (Fig. 9). HST6N-1 mRNA expression was reduced by \(>80\%\) with exposure to A-23187 (5 µmol/l) for 4 h.

PKC-mediated regulation of HST6N-1 expression. Many of the tumor-promoting actions of phorbol esters are mediated by PKC (4, 12), and bile salts have been shown to activate PKC under certain conditions in vitro (11). Therefore, we investigated whether GF-109203X, a selective inhibitor of PKC (10), blocked downregulation of HST6N-1 by DOC or TPA in T84 cells (Fig. 10). GF-109203X (1 µmol/l) prevented inhibition of HST6N-1 mRNA expression by TPA but not DOC. The effect of GF-109203X on the inhibitory actions of \(n\)-butyrate was examined to define further the specificity of PKC-mediated regulation of HST6N-1 expression; \(n\)-butyrate-mediated downregulation of HST6N-1 was unaffected by inhibition of PKC.

DISCUSSION

Wide variations between different tissue and cell types in levels of expression of the genes that encode N-glycan sialyltransferases have been thoroughly documented. However, relatively little is known of the responsiveness of these and other glycosyltransferase genes to extracellular signals or the extent to which levels of expression vary over time within the same tissue or cell type. In this study, a secondary bile acid...
We demonstrated that release of intracellular Ca2+ previously for a conjugated secondary bile acid, TDC, which causes a rise in [Ca2+]i (Fig. 1). Structural analyses of the oligosaccharides of this glycoprotein are in progress. From nuclear transcriptional assays (Fig. 6), it was evident that the effects of DOC and TPA on HST6N-1 expression were, at least in part, due to reduced primary transcription of this gene.

Several findings indicate that DOC- and TPA-mediated inhibition of HST6N-1 expression was relatively selective. Concurrent with HST6N-1 downregulation, both DOC and TPA caused modest upregulation of GalT mRNA expression (Fig. 2A). DOC increased GalT gene transcription, and both DOC and TPA increased transcription of the actin gene (Fig. 6).

The effects of DOC and TPA on T84 cells were not lethal and were reversible. Trypan blue exclusion was unimpaired by both agents. Morphological changes and downregulation of HST6N-1 mRNA expression were fully reversible within 24 to 48 h of shifting cells to DOC-free medium.

Different intracellular pathways mediated downregulation of HST6N-1 expression by DOC and TPA. Independent of the effect of DOC, corroborating evidence that an increase in [Ca2+]i can lead to downregulation of HST6N-1 expression came from experiments with A-23187; within 4 h of exposure to this Ca2+ ionophore, which causes a rise in [Ca2+]i, by allowing bath Ca2+ to enter cells, HST6N-1 mRNA levels fell by >80%. DOC, a deconjugated secondary bile acid, caused accumulation of cytosolic Ca2+ (Fig. 7), as had been shown previously for a conjugated secondary bile acid, TDC (5). We demonstrated that release of intracellular Ca2+ was obligatory for the downregulatory effect of DOC on HST6N-1 mRNA expression (Fig. 8). However, downregulation of HST6N-1 expression could occur in the absence of extracellular (bath) Ca2+; provided sufficient intracellular Ca2+ was available.

The effect of TPA on HST6N-1 expression was mediated, as expected, by PKC (Fig. 10) but activation of PKC by bile acids, as reported by Huang et al. (11) in an in vitro, cell-free model, did not appear to contribute to the effect of DOC on HST6N-1. Near-total inhibition of HST6N-1 mRNA expression in T84 cells by TPA or DOC confounded our initial hypothesis that such agents, thought to enhance neoplastic transformation, would induce this enzyme. As in the colon cancer cells, both agents also caused profound downregulation of HST6N-1 in a human colon epithelial cell line, NCM460, that was derived from a normal colon (Fig. 4).

We reported previously that n-butyrate downregulates HST6N-1 expression in T84 cells (16); thus DOC is the second fecal constituent that we have shown can alter sialyltransferase expression in short-term cultures of colonic cells in vitro. Our results in vitro raise the possibility that fecal constituents and other agents could regulate sialyltransferase expression in vivo but the physiological and pathophysiological significance of these potential effects is unknown.

Downregulation of HST6N-1 expression by DOC in malignant (T84) as well as nonneoplastic (NCM460) cells suggests that malignant transformation in colonic cells is not necessarily associated with aberrant or lost regulation of N-glycan sialyltransferase expression. We propose the following hypothesis. β-Galactoside α-2,6-sialyltransferase expression of normal and, initially, neoplastic colonic epithelial cells is downregulated by secondary bile acids, short-chain fatty acids, and, potentially, a variety of other fecal constituents. With malignant transformation and invasion, cells of a neoplastic clone are progressively removed from the fecal milieu. This leads to HST6N-1 disinhibition and increased α-2,6-sialylation of specific membrane proteins that could confer a selective advantage in subsequent steps of the metastatic cascade.

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