Epigenetic Silencing of the Sulfate Transporter Gene DTDST Induces Sialyl Lewisx Expression and Accelerates Proliferation of Colon Cancer Cells

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Abstract

Colon cancer cells express the carbohydrate determinant sialyl Lewisx, while they exhibit markedly decreased expression of its sulfated derivative, sialyl 6-sulfo Lewisx. In contrast, normal colonic epithelial cells strongly express sialyl 6-sulfo Lewisx, but they virtually do not express sialyl Lewisx. Impaired sulfation was therefore suggested to occur during the course of malignant transformation of colonic epithelial cells and was assumed to be responsible for the increased sialyl Lewisx expression in cancers. To elucidate the molecular biological background of the impaired sulfation in cancers, we studied the expression levels of mRNA for 6-O-sulfotransferase isoenzymes, PAPS synthases and transporters, and a cell membrane sulfate transporter, DTDST, in cancer tissues. The most striking decrease in cancer cells compared with nonmalignant epithelial cells was noted in the transcription of the DTDST gene (P = 0.0000014; n = 20). Most cultured colon cancer cells had a diminished DTDST transcription, which was restored when cultured with histone deacetylase inhibitors. Suppression of DTDST transcription under the control of a tet-off inducible promoter resulted in increased sialyl Lewisx expression and reduced sialyl 6-sulfo Lewisx expression. Unexpectedly, the growth rate of the cancer cells was markedly enhanced when transcription of DTDST was suppressed. These results show that the decrease in the transcription of the sulfate transporter gene is the major cause of decreased expression of sialyl 6-sulfo Lewisx and increased expression of sialyl Lewisx in colon cancers. The results also suggest that the diminished DTDST expression is closely related to enhanced proliferation of cancer cells. Cancer Res; 70(10): 4064–73. ©2010 AACR.

Introduction

Malignant transformation is frequently associated with a drastic alteration in the surface expression of carbohydrate determinants (1–4). Some carbohydrate determinants that are preferentially expressed in cancers are clinically applied for diagnosis of cancers (5, 6). However, the underlying mechanism that leads to expression of altered carbohydrate determinants, as well as their pathophysiologic significance, is not always clear.

The sialyl Lewisx determinant is a typical cancer-associated glycan (1–3, 7–9), but the molecular mechanism responsible for preferential expression of the determinant in cancer has not been fully elucidated. Previously, we showed that differentiated normal colonic epithelial cells express the sulfated carbohydrate determinant sialyl 6-sulfo Lewisx, but they hardly express any of its nonsulfated form, sialyl Lewisx (10). In contrast, colon cancer cells strongly express sialyl Lewisx, while they exhibit markedly decreased sialyl 6-sulfo Lewisx expression compared with normal colonic epithelial cells. Impaired 6-sulfation was therefore suggested to occur during the course of malignant transformation of colonic epithelial cells and to be responsible for the preferential expression of sialyl Lewisx in cancers (8–10). The sialyl Lewisx thus appearing in cancer cells plays an important role in E-selectin-mediated cancer cell adhesion to vascular endothelial cells during the course of tumor angiogenesis (11) and distant metastasis (1, 7, 8). In this study, we tried to elucidate the molecular biological background of the impaired 6-sulfation in cancers. It has long been known that carbohydrate sulfation is generally suppressed in colon cancers compared with nonmalignant colonic epithelial cells (12–14) with a few exceptions (15), and this was expected to be related to the decreased 6-sulfation in cancers.

Materials and Methods

Clinical samples, real-time reverse transcription-PCR analyses, and immunohistochemical staining. Tumor specimens were obtained from 20 patients with primary colorectal cancer at surgical operation and processed as described previously (16, 17). The median age of patients was 59.8 years.
The carcinomas were staged according to Dukes classification (18). Malignant and nonmalignant tissues of each specimen were used for RNA extraction. Nonmalignant mucosa was scraped off using slide glasses, and tissue specimens of cancer were carefully excised to eliminate noncancerous tissue components. Specimens were powdered in liquid N2, and total cellular RNA was extracted with guanidine isothiocyanate and purified by cesium chloride gradient centrifugation. Total cellular RNA was reverse transcribed into cDNA using SuperScript II with Oligo(dT) primer per manufacturer’s instructions (Invitrogen). Real-time reverse transcription-PCR (RT-PCR) analyses were done using ABI PRISM 7000 (Applied Biosystems). The TaqMan probe IDs (Applied Biosystems) for five 6-sulfotransferases (GlcNAc6ST-1, HEC-GlcNAc6ST, I-GlcNAc6ST, GlcNAc6ST-4, and C-GlcNAc6ST; refs. 19–23), two PAPS synthases (PAPSS1 and PAPSS2; refs. 24, 25), two PAPS transporters (PAPST1 and PAPST2; refs. 26, 27), a sulfate transporter (DTDST; DTDST; ref. 28), and two hexosamine 6-sulfatases (29–31) are listed in Supplementary Table S1.

The results were calculated using the comparative Ct method. Relative transcripts were determined by the formula 2\(^{-\Delta\Delta Ct}\). The ΔCt value was determined by subtracting the average GAPDH Ct value from the average target Ct value. The results of real-time RT-PCR were ascertained also with conventional RT-PCR, and the conditions and primers for conventional RT-PCR are summarized in Supplementary Table S1.

Frozen sections of 10-μm thickness for immunohistochemical examination were prepared from surgical specimens for immunohistochemistry. Anti-DTDST antibody was obtained from Abnova. The avidin-biotin complex technique for immunohistochemistry. Anti-DTDST antibody was obtained from Abnova. The avidin-biotin complex technique was done as described in the kit instructions (Vectastain, Vector; ref. 10).

Cell culture and flow cytometric analyses. Cultured human colon cancer cell lines, C-1, Caco-2, Colo201, Colo205, Colo320, Co-R-1, HCT15, HCT116, HT29, LoVo, SW480, SW1083, T84, and WiDr, were from American Type Culture Collection (ATCC) and Japanese Collection of Research Bioresources. The cells were cultured in the presence of butyrate (4–8 mmol/L), trichostatin A (100 ng/mL), FK228 (2.5–10.0 ng/mL), 5-aza-2-deoxycytidine (5–30 μmol/L), or NaClO3 (12.5–25 μmol/L) for 3 days and subjected to RT-PCR analysis. The ΔCt value was determined by subtracting the average GAPDH Ct value from the average target Ct value. The results of real-time RT-PCR were ascertained also with conventional RT-PCR, and the conditions and primers for conventional RT-PCR are summarized in Supplementary Table S1.

Expression vectors for DTDST. Human DTDST cDNA was obtained from Quick-Clone human placenta cDNA (from Clontech). Transient transfections were done with the pIRE/DTDST vector and Lipofectamine 2000 according to the manufacturer’s protocols. Lentivirus-mediated shRNA knockdown of the DTDST gene was done in LS174T colon cancer cells using pLKO.1-based lentiviral DTDST shRNA vectors purchased from Sigma-Aldrich. Tetracycline-repressible expression (37) was carried out using RevTet-Off Gene Expression Systems (Clontech) according to the manufacturer’s protocols. DTDST cDNA was excised with EcoRI from pIREshygro, blunted, and cloned into the HpaI site of the phevTRE vector containing a hygromycin resistance gene to generate the plasmid phevTREhygro/DTDST. The phev/Tet-Off-neo vector and phevTREhygro/DTDST vector were transfected separately into RetroPack PT67 packaging cells, and the resulting virus-containing supernatants were used to serially infect HT-29 cells. G418 (500 μg/mL) was used for screening of HT-29 cells in the initial phev/Tet-Off virus infection. Infection of HT-29 cells with phevTRE virus and screening with G418 and hygromycin were done in the presence of 0.5 μg/mL doxycycline to keep DTDST expression suppressed. Clones were tested for DTDST mRNA expression on removal of doxycycline. Most clones showed a dramatic increase in DTDST mRNA levels.

Chromatin immunoprecipitation assays. Chromatin immunoprecipitation assays were done using ChIP-IT Express (Active Motif) according to the manufacturer’s instructions. The antibodies for acetylated-H3K9 (H3K9ac), trimethylated-H3K9 (H3K9me3), dimethylated-H3K9 (H3K9me2), trimethylated-H4K20 (H4K20me3), trimethylated-H3K4 (H3K4me3), and trimethylated-H3K27 (H3K27me3) were purchased from Abcam. Anti–dimethylated H3K27 (H3K27me2) was from Cell Signaling Technology. Anti–RNA polymerase II (Active Motif) and control anti-IgG were from Active Motif. The immunoprecipitation mixtures containing the sheared chromatin, protein-G magnetic beads, and each antibody were incubated for 4 hours at 4°C. The DNA-histone complexes with or without immunoprecipitation were incubated for 15 minutes at 94°C for reverse cross-linking and then treated with protease K. The DNA fragments were purified with QIAquick PCR Purification kit (Qiagen). PCR for the DTDST promoter region −258 to −363 was done with the primers 5′-GGGGCTCTAATTCGTTTCAAAAG-3′ and 5′-GAGTTTTGAGTGCAGGTGTTGCG-3′, using GeneAmp Fast master mix at 38 cycles of amplification.

Results

Real-time RT-PCR analysis of 6-sulfotransferase gene expression in human colon cancer tissues and nonmalignant colonic epithelial cells. Figure 1A shows a typical example of distribution patterns of sialyl Lewisα, a cancer-associated glycan, and its sulfated derivative, sialyl 6-sulfo Lewisα, in cancer tissue prepared from a patient with colon cancer. Nonmalignant colonic epithelial cells expressed the carbohydrate determinant sialyl 6-sulfo Lewisα, but virtually
no sialyl Lewis\(^x\). In contrast, colon cancer cells strongly express sialyl Lewis\(^x\), while they exhibit markedly decreased sialyl 6-sulfo Lewis\(^x\) expression compared with normal colonic epithelial cells. From the comparison of the structures of the two glycans, impaired sulfation at the C6 position of the N-acetylglucosamine moiety was assumed to have occurred during the course of malignant transformation of colonic epithelial cells, and this was thought to be responsible for the increased expression of sialyl Lewis\(^x\) in cancers.

To elucidate the molecular biological background of the impaired 6-sulfation in cancers, we first studied the expression levels in cancer tissues of mRNA for 6-O-sulfotransferase (6ST) isoenzymes. Results of RT-PCR analyses of five 6ST transcripts in colorectal cancer tissues and nonmalignant colonic epithelial cells are shown in Fig. 1B. Preliminary results on nonmalignant epithelial cells indicated that the major 6ST species in the human colon was I-GlcNAc6ST, followed by GlcNAc6ST-1. The amount of mRNA for the other three isoenzymes was almost negligible in nonmalignant epithelium. A marked decrease of I-GlcNAc6ST mRNA was detected in cancer cells compared with nonmalignant epithelial cells prepared from the same patient \((P = 0.000007; n = 20)\), whereas GlcNAc6ST-1 mRNA showed no statistically significant difference (Fig. 1B).

These results suggested a possibility that the suppressed transcription of the I-GlcNAc6ST gene, the major 6ST in the intestine, may account for the decreased expression of sialyl 6-sulfo Lewis\(^x\) in colon cancer cells. However, the significant levels of other 6ST isoenzymes in cancer cells, such as GlcNAc6ST-1 and HEC-GlcNAc6ST, capable of synthesizing sialyl 6-sulfo Lewis\(^x\), would detract from the contribution of I-GlcNAc6ST in explaining the decreased sialyl 6-sulfo Lewis\(^x\) expression in cancer cells. In addition, it was previously reported that I-GlcNAc6ST was not able to generate the sialyl 6-sulfo Lewis\(^x\) epitope in transfected cells (38).

We also obtained experimental results suggesting the very low efficiency, if any, of this enzyme in glycan 6-sulfation, whereas GlcNAc6ST-1 and HEC-GlcNAc6ST could efficiently synthesize sialyl 6-sulfo Lewis\(^x\) in the same cells (see Supplementary Fig. S1). These findings suggested that the decreased sialyl 6-sulfo Lewis\(^x\) expression in cancer cells may not be ascribed solely to the suppressed transcription of the gene for I-GlcNAc6ST. Accordingly, we studied the expression of genes other than 6STs that are involved in carbohydrate sulfation in human colon cancer tissues.

**RT-PCR analysis of genes involved in carbohydrate 6-sulfation in human colon cancer tissues and nonmalignant colonic epithelial cells.** We turned our attention to two PAPS synthases (PAPSS1 and PAPSS2) producing the donor substrate for sulfotransferases, two PAPS transporters (PAPST1 and PAPST2) in the Golgi membrane, a sulfate transporter (DTDST) localized at the cell membrane, and two 6-sulfohexosamine sulfatases (Fig. 1C). Transcripts for PAPSS1 showed a moderate increase in cancer \((P \leq 0.05; n = 20)\), whereas sulfated glycan expression decreased. Transcripts for PAPST1 showed no significant change. Transcripts for PAPST1 showed no significant change. PAPSS2
PAPST2 showed a moderate decrease ($P \leq 0.0001$ and $P \leq 0.0005$; respectively; $n = 20$). These changes, although not very remarkable, are in line with the decreased expression of sulfated glycans in cancers. Decreased expression of PAPSS2 had reportedly also occurred in cultured human prostate cancer cells (25, 39). The two sulfatases showed only a modest increase in cancers (statistically not significant).

The most prominent decrease, which is compatible with the loss of sulfated glycans in cancers, was observed with the transcript of DTDST ($P = 0.0000014$; $n = 20$; Fig. 1C). Its decreased transcription was apparent even in the relatively early-stage cancer tissues taken from patients with Dukes stages A and B and was ubiquitously observed in the cancers occurring in the right or left hemicolon (Fig. 1C). The decreased expression of DTDST was ascertained also at the protein level. Immunohistochemical staining of frozen tissue sections prepared from colon cancer patients with anti-DTDST antibody indicated that cancer cells express only low levels of DTDST protein, compared with adjacent nonmalignant colonic epithelial cells. This was consistently observed with all eight tested cases, and representative photos are shown in Fig. 1D.

**Effect of perturbation of sulfate incorporation on the expression of sialyl Lewis$^x$ and sialyl Lewis$^x$ glycans.**

As the transcription of sulfate transporter DTDST showed the most prominent decrease in cancers compared with

![Figure 1. Continued.](image_url)

C, results of real-time RT-PCR analyses of mRNA levels of genes involved in cellular sulfate metabolism, including two PAPS synthases (PAPSS1 and PAPSS2), a PAPS transporter [PAPST1 (rel4)], DTDST (SLC26A2), and two hexosamine-6-sulfatases (G6ase and GALNS; $n = 20$). Bottom, effects of Dukes stages and primary tumor sites on DTDST mRNA expression. Ca, cancer tissue; N, nonmalignant mucosa prepared from same patient. A paired t test was done to ascertain statistical significance between the level in cancer tissue and that in nonmalignant mucosa in B and C. N.S., not significant. See Supplementary Table S1 for probes.

D, examples of expression of DTDST protein in cancer tissue and adjacent nonmalignant mucosa. Ca, cancer tissue; N, nonmalignant mucosa. Bar, 200 $\mu$m.
nonmalignant epithelial cells, we next tested if perturbation of cellular sulfate incorporation indeed affects the cell surface expression of sialyl 6-sulfo Lewis^x and sialyl Lewis^x glycans. In our preliminary experiments, cultured colon cancer cells Colo201 were found to contain a considerable amount of HEC-GlcNAc6ST mRNA, but no detectable mRNA for I-GlcNAc6ST or GlcNAc6ST-1 (see Supplementary Fig. S2), and to moderately express sialyl 6-sulfo Lewis^x as well as sialyl Lewis^x under the usual culture conditions. When the Colo201 cells were cultured in the presence of NaClO_3, an inhibitor of eukaryotic cell sulfate incorporation (40–42), a significant attenuation of sialyl 6-sulfo Lewis^x expression was observed, which was accompanied by an increase of the nonsulfated form of the glycan, sialyl Lewis^x (Fig. 2A).

More clear results were obtained when ECV304 cells co-transfected with the genes for GlcNAc6ST-1 and fucosyltransferase VII (34) were used in similar experiments (Fig. 2B). These cells strongly expressed sialyl 6-sulfo Lewis^x and virtually no sialyl Lewis^x under usual culture conditions. Addition of NaClO_3 to the culture medium almost completely abrogated the sialyl 6-sulfo Lewis^x expression and induced prominent expression of sialyl Lewis^x (Fig. 2B). There was a clear reciprocal relationship between expression of sialyl 6-sulfo Lewis^x and sialyl Lewis^x, depending on the concentrations of NaClO_3 (Fig. 2C). This was not observed with the cells transfected with the gene for fucosyltransferase only, which expressed sialyl Lewis^x under normal conditions, and addition of NaClO_3 produced no change in its expression (Fig. 2B).

**Effect of the histone deacetylase inhibitor butyrate on DTDST transcript.** Most cultured human colon cancer cells were found to have decreased levels of DTDST transcript (Fig. 3A). The only exception was Caco-2 cells, which showed a moderate level of DTDST mRNA, but still less than the level observed in nonmalignant epithelial cells. Significant increases in DTDST mRNA were observed when these cancer cells were cultured in the presence of histone deacetylase (HDAC) inhibitors, and representative results are shown in Fig. 3B. The DNA methylation inhibitor 5-aza-2-deoxycytidine was not a potent inducer of DTDST mRNA. These results suggested that transcription of the DTDST gene was suppressed by some epigenetic silencing mechanism involving histone modification, but that DNA methylation was not closely involved.

![Figure 2](image-url) Effect of inhibition of cellular sulfate incorporation by sodium chlorate on expression of sialyl 6-sulfated glycans. A, cultured human colon cancer cell line Colo201 was cultured for 7 d with 12.5 mmol/L sodium chlorate and analyzed by flow cytometry for cell surface expression of sialyl 6-sulfo Lewis^x defined by G72 antibody and nonsulfated sialyl Lewis^x defined by CSLEX-1 antibody. B, cell surface expression of sialyl 6-sulfo Lewis^x and nonsulfated sialyl Lewis^x in ECV304 cells cotransfected with genes for GlcNAc6ST and fucosyltransferase VII (clone LS12; top) or in cells transfected with the gene for fucosyltransferase VII alone (clone 7ECV; bottom) was analyzed after culture for 7 d with 25 mmol/L sodium chlorate. C, inverse correlation between expression of sialyl 6-sulfo Lewis^x and nonsulfated sialyl Lewis^x in LS12 cells depending on sodium chlorate concentration in terms of mean fluorescence intensity ascertained by flow cytometric analyses.

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Silencing of the DTDST gene in cultured colon cancer cells correlated with promoter histone modification in chromatin immunoprecipitation assays (Fig. 3C). Only CaCo-2 cells, which had a moderate level of DTDST transcription, showed significant histone H3 lysine 9 acetylation and H3 lysine 4 methylation, which are key chromatin marks associated with active gene expression, in chromatin immunoprecipitation assays. A thick band observed with anti–pol II antibody was compatible with significant DTDST transcription in Caco-2 cells (Fig. 3C). On the other hand, histone H3 lysine 9 deacetylation and loss of H3 lysine 4 methylation were observed in cultured colon cancer cells with diminished DTDST transcription, such as SW480, SW1083, and HT29. Instead, increased methylation of H4 lysine 20, H3 lysine 27, and H3 lysine 9 was noted in some cultured colon cancer cells with diminished DTDST transcription (Fig. 3C). These results suggested that aberrant epigenetic chromatin events may be the underlying mechanism for the reduced DTDST transcription in cancer cells.

Effects of inducible expression of DTDST on glycan expression and cancer cell proliferation. To determine the direct effect of DTDST on sulfated glycan expression, we performed DTDST gene transfection experiments. Several experiments involving transient transfection of the DTDST gene into HT29 cells could induce only a weak expression of sialyl 6-sulfo Lewis^x^ (Fig. 4A), and trials for isolating stable transfectant clones were not successful; therefore, we attempted to use a tet-off inducible construct. The transfectant cells thus obtained expressed only a low level of DTDST transcripts under culture conditions with doxycycline (DOX+), but showed strongly induced expression of a large amount of DTDST transcripts on doxycycline removal (DOX−; Fig. 4B). When transcription of DTDST was induced, a significant expression of sialyl 6-sulfo Lewis^x^ was observed, together with an anonymous pan-6-sulfated antigen defined by the KN412 antibody. Interruption of DTDST expression by the addition of doxycycline induced a marked increase in sialyl Lewis^x^ expression, accompanied by the disappearance of sialyl 6-sulfo Lewis^x^ as well as the pan-6-sulfated antigen (Fig. 4C). The presence or absence of doxycycline did not affect the transcription of other genes involved in the sulfated glycan synthesis, such as 6-sulfotransferases, PAPS synthases, and PAPS transporters (Supplementary Fig. S3). DTDST knockdown experiments with lentiviral shRNA vectors also indicated a significant reduction in sialyl 6-sulfo Lewis^x^, further supporting the role of DTDST in the induction of sulfate glycan expression (Supplementary Fig. S4).

During the experiments using the tet-off inducible construct, a significant growth suppression of the transfectant cells was observed by expressing DTDST through removal of sialyl Lewis^x^ (Fig. 4A), and trials for isolating stable transfectant clones were not successful; therefore, we attempted to use a tet-off inducible construct. The transfectant cells thus obtained expressed only a low level of DTDST transcripts under culture conditions with doxycycline (DOX+), but showed strongly induced expression of a large amount of DTDST transcripts on doxycycline removal (DOX−; Fig. 4B). When transcription of DTDST was induced, a significant expression of sialyl 6-sulfo Lewis^x^ was observed, together with an anonymous pan-6-sulfated antigen defined by the KN412 antibody. Interruption of DTDST expression by the addition of doxycycline induced a marked increase in sialyl Lewis^x^ expression, accompanied by the disappearance of sialyl 6-sulfo Lewis^x^ as well as the pan-6-sulfated antigen (Fig. 4C). The presence or absence of doxycycline did not affect the transcription of other genes involved in the sulfated glycan synthesis, such as 6-sulfotransferases, PAPS synthases, and PAPS transporters (Supplementary Fig. S3). DTDST knockdown experiments with lentiviral shRNA vectors also indicated a significant reduction in sialyl 6-sulfo Lewis^x^, further supporting the role of DTDST in the induction of sulfate glycan expression (Supplementary Fig. S4).
of doxycycline. Fig. 4D (top) shows the growth curves and bromodeoxyuridine (BrdUrd) incorporation assays of the transfectant cells with the tet-off promoter–driven DTDST expression vector. Significant growth suppression is evident when transcription of DTDST was induced by removal of doxycycline (DOX−). This was a reversible effect, and interruption of DTDST transcription by addition of doxycycline led to recovery of the growth of cancer cells (DOX+). This was a paradoxical phenomenon because control cells without DTDST expression vector showed moderate growth suppression in the presence of doxycycline (DOX+) rather than in its absence (DOX−), most probably because of the mild cytotoxicity of doxycycline.

**Discussion**

Previously, we showed that the expression of 6-sulfated carbohydrate determinants such as sialyl 6-sulfo Lewisx is significantly reduced in colon cancer cells compared with nonmalignant colonic epithelial cells, and we proposed the reduction in 6-sulfation associated with carcinogenesis to be the major mechanism leading to preferential expression of the nonsulfated form of sialyl Lewisx in cancer cells (10). The results of the present study indicated that the decreased transcription of the DTDST gene figures heavily in the reduction of 6-sulfated glycan expression in cancer cells. Transcription of the DTDST gene was most prominently decreased in colon cancer cells compared with nonmalignant colonic epithelial cells among the genes involved in the cellular sulfate metabolism. Results using an inhibitor of sulfate incorporation and the tet-off inducible DTDST expression vector clearly indicated that the reduction in DTDST transcription level leads to loss of sialyl 6-sulfo Lewisx and induction of cancer-associated expression of sialyl Lewisx. A generalized decrease of glycan sulfation, especially that of mucin glycoproteins, has long been known to associate with...
malignant transformation of colonic epithelial cells (12–14). Decreased sulfation of the sialyl Lewis^x^ determinant, which is carried mainly by mucin glycoproteins, is in line with this notion. Sulfate deprivation does not uniformly affect the sulfated glycan expression. Some sulfated glycolipids such as sulfatides are reported to rather increase in the advanced stage of cancers (15). It is well known that sulfate deprivation using NaClO_3 strongly affects the expression of chondroitin sulfates, but it exerts much less effect on heparan sulfates, and all candidate enzymes involved in sulfation of sialyl 6-sulfo Lewis^x^ belong to the same gene family as chondroitin 6-O-sulfotransferases. The K_m value of the synthetic enzymes for PAPS, as well as other multiple factors, must be involved in the effect of sulfate deprivation, which is uneven depending on the molecular species.

Although the possibility that reduced transcription of the 1-GlcNAc6ST also partly contributes to the loss of sialyl 6-sulfo Lewis^x^ expression cannot be completely ruled out, the significant levels of other 6ST isoenzymes such as GlcNAc6ST-1 and HEC-GlcNAc6ST in cancer cells, which were shown to be sufficient for sialyl 6-sulfo Lewis^x^ synthesis, would detract from the contribution of 1-GlcNAc6ST. For instance, although Colo201 cells lacked 1-GlcNAc6ST, the level of HEC-GlcNAc6ST was sufficient to synthesize sialyl 6-sulfo Lewis^x^ (Fig. 2). HT29 cells, which also lacked 1-GlcNAc6ST, expressed enough HEC-GlcNAc6ST transcripts to confer sialyl 6-sulfo Lewis^x^ expression when DTDST was supplied by the tet-off inducible vector (Fig. 4). The possibility that reduced transcription of the PAPS2 and PAPST2 genes may also contribute to the loss of sialyl 6-sulfo Lewis^x^ expression in cancer cells cannot be entirely set aside. However, the observed change in the mRNA levels was not prominent, and would be compensated by the increase of PAPSSI transcription in the case of PAPS2.

We previously proposed that the increased expression of the sialyl Lewis^x^ determinant, another cancer-associated glycan, is due to the reduction of disialyl Lewis^x^ expression. The disialyl Lewis^x^ determinant was preferentially expressed in nonmalignant epithelial cells, and the epigenetic silencing of the gene for the enzyme responsible for transfer of the second sialic acid residue on malignant transformation conferred the induction of cancer-associated sialyl Lewis^x^ expression (9, 43). The situation is similar also for sialyl Lewis^x^ in that the reduction of DTDST transcription is also mostly due to epigenetic silencing of the gene. These findings collectively imply that normal epithelial cells generally express highly complex glycans, and partial impairment of their synthetic pathway by epigenetic silencing of synthetic genes on malignant transformation induces the expression of cancer-associated glycans, which usually have less complex structures (incomplete synthesis; ref. 9). This well explains why earlier trials conducted by other researchers and ourselves (4, 16, 17) only partially succeeded in identifying the genes responsible for the cancer-associated expression of sialyl Lewis^x^ based on a positive correlation of sialyl Lewis^x^ expression with the transcription level of some particular glycosyltransferase gene.

Epigenetic silencing of the DTDST gene led to diminished sialyl 6-sulfo Lewis^x^ expression and to induction of sialyl Lewis^x^ expression. The physiologic function of the sialyl Lewis^x^ glycan was shown to serve as a ligand for selectins (44, 45). Interaction of sialyl Lewis^x^ on cancer cells with endothelial selectins was earlier shown to facilitate tumor angiogenesis (11) and hematogenous metastasis (2, 8, 9). Although this may provide an advantage to cancer cells, sialyl 6-sulfo Lewis^x^ is also capable of serving as a ligand for all three members of the selectin family (46). Surprisingly, the present study revealed that silencing the DTDST gene facilitates cell proliferation. Thus, the reduction of DTDST transcription confers a definite growth advantage on cancer cells.

Whether or not the sialyl 6-sulfo Lewis^x^ or the sialyl Lewis^x^ determinant per se is directly involved in regulation of cell proliferation is not fully clear at this moment. Although some earlier studies suggested an association between sialyl Lewis^x^ expression and cell proliferation (47, 48), the reduction of DTDST transcription will cause a generalized decrease in the sulfation reaction of not only sialyl Lewis^x^ but also various other molecules such as proteoglycans. Some of such sulfated molecules may well play a key role in regulating the proliferative activity of cancer cells. For instance, loss of 6-O-sulfation at GlcNAc of heparan sulfate, which is selectively affected by sodium chlorate treatments (41), is known to facilitate Wnt signaling (49, 50). In such a case, the loss of sialyl 6-sulfo Lewis^x^ and the acquisition of sialyl Lewis^x^ would be phenomena coincidental with DTDST silencing and serve as markers for highly proliferative cancer cells.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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