Am J Physiol Gastrointest Liver Physiol 291: G288–G296, 2006.
First published February 23, 2006; doi:10.1152/ajpgi.00324.2005.

Sulfide-detoxifying enzymes in the human colon are decreased in cancer and upregulated in differentiation

S. Ramasamy,[1] S. Singh,[2] P. Taniere,[3] M. J. S. Langman,[1] and M. C. Eggo[1]

[1]*Division of Medical Sciences, Department of Medicine, University of Birmingham;* [2]*Department of Gastroenterology, Good Hope Hospital, Sutton Coldfield; and* [3]*Department of Cellular Pathology, University Hospital NHS Trust, Queen Elizabeth Hospital, Birmingham, United Kingdom*

Submitted 14 July 2005; accepted in final form 21 February 2006

Ramasamy, S., S. Singh, P. Taniere, M. J. S. Langman, and M. C. Eggo. Sulfide-detoxifying enzymes in the human colon are decreased in cancer and upregulated in differentiation. *Am J Physiol Gastrointest Liver Physiol* 291: G288–G296, 2006. First published February 23, 2006; doi:10.1152/ajpgi.00324.2005.—H_2S is highly toxic and selectively inhibits butyrate oxidation in colonocytes. Ineffective detoxification may result in mucosal insult, inflammation, and ultimately in colorectal cancer (CRC). Rhodanese can detoxify H_2S and is comprised of two isoenzymes: thiosulfate sulfurtransferase (TST) and mercaptopyruvate sulfurtransferase (MST). Using specific antisera to discriminate TST from MST, we found that only TST could detoxify H_2S. In sections of normal colon, both enzymes were located on the luminal mucosal surface, and they were expressed in the colonocytes but not in the mucin-secreting goblet cells. Expression of both enzymes was focally lost in ulcerative colitis and markedly reduced in advanced colon cancer, the disease progression correlating with the decreased expression of MST and TST. In HT-29 cells, a human colon cancer cell line, TST activity and expression were significantly increased by butyrate and by histone deacetylase inhibition, agents that promote HT-29 cell differentiation. Sulfide (0.1 mM) also increased TST activity, but higher sulfide concentrations (0.3–3 mM) were toxic. Preincubation in butyrate to increase TST expression, decreased sensitivity of the cells to sulfide toxicity. We conclude that decreased expression of TST (or MST) is a tumor marker for CRC. TST expression is increased in colonocyte differentiation. Dysregulation of TST expression and activity resulting in inability to effectively detoxify could be a factor in the cell loss and inflammation that accompany ulcerative colitis and ultimately then in CRC.

colorectal cancer; thiosulfate sulfurtransferase; butyrate; HT-29

IN THE HUMAN COLON, anaerobic bacteria produce hydrogen sulfide (H_2S) (25), which inhibits the oxidation of short-chain fatty acids (SCFA) (18, 19). SCFAs are the main energy source (>70%) for colonocytes (18) and also contribute 5–10% of the total energy supplies in humans. Inhibition of their oxidation in the colon will result in colonocyte starvation and death, resulting in inflammation. This, the "energy deficiency" hypothesis of Roediger (18), is postulated to lead to ulcerative colitis (UC). Many studies have shown that patients with UC have increased colorectal cancer (CRC) risk, with Prior et al. (16) estimating this as an 11-fold excess. On the basis of these studies, we suggest that the expression of enzymes important in sulfide detoxification is essential to preserve a healthy mucosa and that derangements in expression may accompany UC and CRC.

We recently showed that colonic mucosal rhodanese can detoxify hydrogen sulfide (15) and thus might protect against mucosal injury. Rhodanese is a mitochondrial enzyme, present in all living organisms from bacteria to humans, and is thought to play a central role in cyanide detoxification (12, 28, 29). Cloning data have shown the presence of two isoenzymes: thiosulfate sulfurtransferase (TST; E.C.2.8.1.1) and mercaptopyruvate sulfurtransferase (MST; E.C.2.8.1.2) of very similar size and biochemical properties (13, 14). Both MST and TST can transfer sulfur from either mercaptopyruvate or thiosulfate to mercaptoethanol or potassium cyanide (KCN), respectively, but they differ markedly in their K_m for these reactions (13, 14). In an elegant study using gene arrays to examine differences in gene expression in colonic mucosa from cancerous and normal tissues, Birkenkamp-Demtroder et al. (3) identified TST as a potential tumor marker (3, 4). Confirmatory data of TST protein or activity assays were not, however, presented, and data of MST regulation were not reported. To date, discrimination of MST and TST has not been attempted, and it is not known whether it is MST or TST that is important in the detoxification of H_2S. In this study, we have used specific antisera to determine the contribution of each isoenzyme to H_2S detoxification. We used these antisera to examine localization of MST and TST in normal colon and to compare the expression levels in paired normal and tumor tissue. Colonic samples from UC patients at various phases of disease severity were also examined.

We determined the regulation of expression of MST and TST in the colonic cancer cell line HT-29, which can use butyrate as an energy source (8). We examined the effects of sulfide and butyrate on the activity and expression of the two enzymes and the ability of butyrate to protect cells against sulfide toxicity. We then explored whether any effect of butyrate in protecting cells against sulfide damage is likely to be through protection as an energy source or as a modulator of histone acetylation.

MATERIALS AND METHODS

Cell culture and treatments. The human colon cancer cell line HT-29 was maintained in phenol red-free RPMI 1640 medium (Sigma, Poole, UK) supplemented with 10% FCS (First Link, Birmingham, UK) and antibiotics (100 U/ml penicillin G and 100 μg/ml streptomycin sulfate) and incubated in a humidified atmosphere of 5% CO_2-95% air at 37°C. For the experiment, cells were seeded in 25-cm^2 tissue culture flasks (double seal, Iwaki, Japan). When cells

Address for reprint requests and other correspondence: M. C. Eggo, The Medical School, Dept. of Medicine, Univ. of Birmingham, Birmingham B15 2TH, UK (e-mail: M.C.Eggo@Bham.ac.uk).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

were 70% confluent, they were incubated in 2 mM sodium butyrate for 24 h. The cell layer was washed with Hank's balanced salt solution (HBSS) before incubation in 5 ml medium containing varying doses of sodium hydrogen sulfide, ($NaHS \cdot H_2O$) (Sigma). To minimize the loss of volatile $NaHS \cdot H_2O$, the caps were sealed, and medium pH was maintained by the addition of 10 mM HEPES (pH 7.0).

Assays of cell viability. The ability of propidium iodide (PI) to enter permeabilized, dead cells but not living cells was used to determine cell viability. PI intercalates with double-stranded DNA, which amplifies fluorescence. After 24 h of preincubation with and without 2 mM butyrate, cells were treated with varying doses of NaHS (Alfa Aesar, UK) for 24 h. Floating cells were transferred into 15-ml tubes and centrifuged, and the pellet was resuspended in 0.2 ml RPMI containing 10 μg/ml PI (Sigma). The adherent cells were washed with HBSS and incubated in 1 ml of RPMI medium containing 10 μg PI for 10 min. Adherent cells were washed with HBSS and removed from the dish with 0.125% trypsin in Ca/Mg-free HBSS. PI fluorescence of the floating and the adherent cell fractions was measured (522 nm excitation, 612 nm emission) using a FLUOstar OPTIMA plate reader (BMG Labtech, Aylesbury, UK). Floating and adherent cells were combined and frozen at −80°C to disrupt nuclei, and PI (final concentration 10 μg/ml) was added for 30 min for an assay of total DNA. The percentage of sulfide-induced cell cytotoxicity was calculated by dividing the sum of PI fluorescence of the floating and adherent cells by the total cell PI fluorescence reading after freeze-thaw treatment.

Preparation of cell homogenate. Cells were washed twice with HBSS to remove serum and placed on ice with 500 μl of cell lysate buffer containing 0.1% Triton X-100 in HBSS for 3 min. The lysed cells were scraped from the flask and centrifuged at 13,000 rpm for 15 min in a cold room; the supernatant was transferred to a fresh tube and used for immunoprecipitation, enzyme assay, and Western blotting.

Immunoprecipitation of MST and TST. HT-29 cell lysate (30 μl) was incubated overnight at 4°C with 2 μl of custom-made specific rabbit polyclonal antibodies, raised to epitopes at the COOH termini of TST and MST (Eurogentec, Seraing, Belgium). A Blast search was used to design the antigens for TST {[C]KEGHPVTSEPSRPE} and MST {[C]KEGHPVTSEPSRPE}. Cysteine [C] is added to the NH_2 terminus for an attachment via m-maleimidobenzoic acid *N*-hydroxyl-succinimide ester to keyhole limpet hemocyanin. The antigens are unique and found only in TST and MST, respectively. The TST and MST complexes were immunoprecipitated with protein A conjugated to agarose (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoprecipitates were separated by centrifugation for 30 s at 4°C. After the immunoprecipitates were washed three times with 0.1% Triton X-100 in HBSS, antigens were dissociated from the agarose-protein A beads by boiling in sample buffer as described in *Western blots*.

Immunohistochemistry. Immunostaining of 4-μm, paraffin-embedded, formaldehyde-fixed colonic sections was performed as described previously using the ABC technique (17). Fifteen samples (paired normal and disease) both for UC and for cancer were stained for MST and TST and counterstained with hematoxylin. The tumor tissues were staged according to the pTNM pathological classification (22). Comparisons were made between the proportion of positively staining cells in paired sections of normal and cancerous tissue. Ten fields were examined at high power, and results were expressed as the percentage of positively staining cells compared with the normal control.

Enzyme assays. Principles of the assays were based on the colorimetric method for the determination of thiocyanate formation (23, 24). TST activity was measured using sodium thiosulfate ($Na_2S_2O_3$) as substrate, as described by Aminlari and Gilanpour (1) based on the work of Sorbo (23, 24). The assay mixture contained 50 mM $Na_2S_2O_3$, 50 mM KCN, 40 mM sodium glycine buffer (pH 9.2), and 10–20 μl cell homogenate or 0.1–0.5 μl rhodanese (Sigma, R1756) in a final volume of 150 μl with Milli-Q water. The MST assay was based on a modification of the Vachek and Wood method (26) and Jarabak's method (9) using freshly prepared 3-mercaptopyruvate as the sulfur donor and cyanide as the sulfur acceptor. The assay mixture contained 225 mM 2-methyl-2-aminopropanediol (Bis buffer), 15 mM sodium 3-mercaptopyruvate, and 12.5 mM KCN and 10–20 μl cell homogenate in a final volume of 100 μl with Milli-Q water. The H_2S detoxification assay was based on the TST assay, using freshly prepared $NaHS \cdot H_2O$, at a final concentration of 50 mM as substrate in place of $Na_2S_2O_3$. $NaHS \cdot H_2O$ was dissolved in ethanol to remove putative contaminants of sodium thiosulfate and sodium sulfide (Na_2S), neither of which is soluble in ethanol. The small amount of ethanol-insoluble material was removed by centrifugation. The reactions were initiated at 37°C for 10 min by adding KCN. After the incubation, reactions were terminated by the addition of 50 μl of 13.9% formaldehyde followed by 0.025 M ferric nitrate (150 μl). The reaction product of ferric thiocyanate was detected at 460 nm in a plate reader. Each sample was assayed in triplicate, and a cell lysate buffer control was subtracted from the values.

Two commercial preparations of rhodanese were used: a highly purified preparation (Sigma R1756–130 U/mg) and an impure preparation (Sigma R1425–13 U/mg). These were assayed for TST and MST activity. The impure preparation was active in both enzyme assays, whereas the highly purified preparation only possessed TST activity.

Western blots. The cytoplasmic protein concentration of cell homogenates was determined using the Bio-Rad DC protein microassay kit (Bio-Rad Laboratories, Hempstead, UK) with BSA as standard. Proteins (100 μg) were heated at 100°C for 15 min in sample buffer containing 2% SDS, 10% 2-mercaptoethanol, 10% glycerol, and 62.5 mM Tris·HCl (pH 6.8), with bromophenol blue. Proteins were separated by electrophoresis on 15% SDS-polyacrylamide gels with a 7.5% stacking gel as described previously (17). Custom-made, affinity-purified, specific rabbit polyclonal antibodies to 15-amino acid sequences in TST and MST were used with enhanced Lumi Glo chemiluminescent substrate (KPL) for detection. Confirmation of equal loading was shown by stripping the membranes in 2% SDS, 1% 2-mercaptoethanol, and 62.5 mM Tris·HCl, pH 6.8, at 60°C for 30 min and reprobing with antisera to actin (The Binding Site, Birmingham, UK). Densitometry was performed and quantified using Total lab v2005 1D densitometry analysis software (Nonlinear Dynamics Limited). Corrections were made for differences in protein loading using the actin internal control, although these did not differ by >2%.

Statistical analysis. Statistical calculations were performed using Instat software package (Graphpad software). Duplicates or triplicate samples were used for all assays. Experiments were repeated at least twice, and data were analyzed using ANOVA and the Tukey-Kramer multiple-comparisons posttest.

RESULTS

Characterization of the antibodies to MST and TST. To determine the specificity of TST and MST antibodies, lysates from HT-29 cells were incubated with either MST or TST

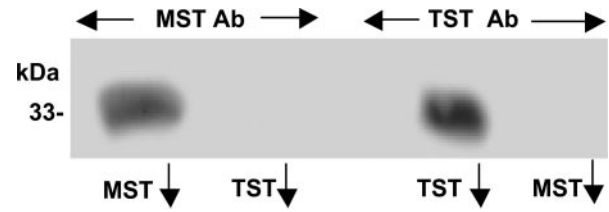

Fig. 1. Western immunoblotting of immunoprecipitates of mercaptopyruvate sulfurtransferase (MST) and thiosulfate sulfurtransferase (TST) from HT-29 cell lysates, probed with antisera to MST and TST. The 55-kDa IgG band was evident in the immunoprecipitates but is not shown on the blot for clarity. Arrows = immunoprecipitates.

antisera and immunoprecipitated with protein A-agarose. The washed immunoprecipitates were analyzed by Western immunoblotting. Blots were probed with either MST or TST antisera, and the data are shown in Fig. 1. MST antisera only recognized MST immunoprecipitates and not TST immunoprecipitates. Conversely, probing Western blots with TST antisera showed reactivity only in TST immunoprecipitates and no reactivity in MST immunoprecipitates, confirming that the antisera were specific.

Effect of MST and TST on the detoxification of NaHS·H_2O. In these experiments, NaHS·H_2O was used in place of Na_2S (15) because it is soluble in ethanol. We used its differential solubility in ethanol to separate NaHS·H_2O from Na_2S and from sodium thiosulfate, the normal substrate of TST, neither of which is ethanol soluble. Using a commercial preparation of rhodanese, which did not express MST activity, we examined the effect of increasing concentrations of NaHS·H_2O on product formed as shown in Fig. 2. Background controls were performed at each concentration of NaHS·H_2O and subtracted from sample. At concentrations >100 mM, linearity was lost. Controls using heat-treated enzyme (56°C, 30 min) or TST treated with 0.1% SDS showed loss of all activity against NaHS·H_2O as substrate and against $Na_2S_2O_3$ (data not shown). In controls substituting ovalbumin at the same concentration as TST, there was no product formation. The rate of product formed using NaHS·H_2O was considerably less than that formed from $Na_2S_2O_3$ when equivalent substrate concentrations (50 mM) were used (196 vs. 6,600 nmol·U^{-1}·min^{-1}).

To determine the individual contribution of MST and TST in the detoxification of H_2S, we immunoprecipitated TST and MST from an impure rhodanese preparation. This preparation contained both MST and TST activity when assayed. The TST antiserum was mixed overnight at 4°C with the enzyme preparation, and the antigen/antibody was removed with protein A-agarose. MST is less stable than TST, and incubations with MST antiserum were continued for 1 h at room temperature before the removal of the antigen/antibody complex with protein A-agarose. The supernatants were used in enzyme assays to determine their ability to detoxify NaHS·H_2O, mercaptopyruvate, and thiosulfate. Controls were enzyme incubated identically except that the antisera were omitted. Results are expressed relative to the activity in these controls for all assays.

Table 1 shows that immunoprecipitation and removal of TST from the preparation abolished >90% of the activity of the preparation against NaHS·H_2O and against $Na_2S_2O_3$ (TST assay) as substrates. MST activity was hardly affected and was >95% of the control. Conversely, when MST activity was immunoprecipitated from the reaction, there was no loss of enzyme activity against NaHS·H_2O or $Na_2S_2O_3$, whereas MST activity was lost. We conclude that TST is responsible for detoxification of H_2S.

Table 1. *Effect of immunoprecipitation of TST or MST from a rhodanese preparation on assays for NaHS, TST and MST*

%Control	NaHS Assay, %	TST Assay, %	MST Assay, %
TST immunoprecipitated (n = 3)	7.8±0.1	9.1±0.1	95.7±2.4
MST Immunoprecipitated (n = 1)	94.2±0.7	92.8±1.3	6.4±0.2

Values are means ± SD. Experiments performed in duplicate. NaHS assay uses 50 mM NaHS:H_2O as substrate, thiosulfate sulfurtransferase (TST) assay uses 50 mM $Na_2S_2O_3$ as substrate, and mercaptopyruvate sulfurtransferase (MST) assay uses 225 mM mercaptopyruvate as substrate.

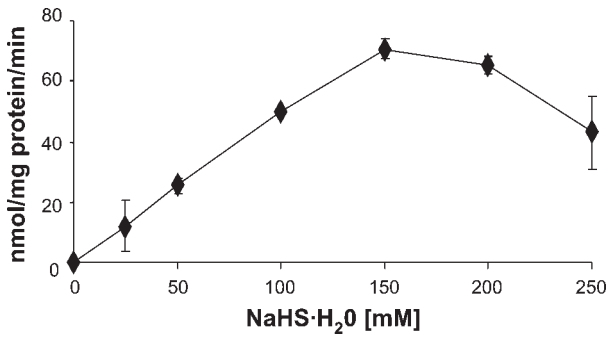

Fig. 2. Ability of a purified preparation of rhodanese expressing only TST activity to detoxify NaHS·H_2O. An ethanolic extract of NaHS·H_2O was freshly prepared, and increasing concentrations were incubated with enzyme.

Comparison of TST and MST in normal colon and tumor tissue and in UC. Figure 3, *A-D*, shows sections of normal colon sections for TST (Fig. 3, *A* and *C*) and MST (Fig. 3, *B* and *D*). Figure 3, *A* and *B*, are high-power magnifications showing a cross-sectional view of colonic crypt. In neither figure were goblet cells immunostained (marked with arrows), but the absorptive colonocytes showed strong cytoplasmic staining. Figure 3, *C* and *D*, which shows the crypts, demonstrates that the mucosal surface is heavily stained, but cells at the crypt base are unstained. The distribution of staining for both MST and TST (Fig. 3, *C* and *D*) was very similar.

Matched pairs (n = 15) of normal and colonic adenocarcinoma staged according to the pTNM pathological classification (tumor invasion, nodal involvement, metastases) were examined by immunohistochemistry. Representative sections of colon cancer are shown in Fig. 3, *E* and *F*. The percentage of cells staining positive for TST with tumor stage is shown in Table 2. In all tumors, staining was ≤50% of the paired control. The percentage of positively stained cells decreased as tumor stage increased. Similar results were found for MST. Figure 3, *G* and *H*, shows staining for TST and MST in a section where normal, dysplastic tissue, and cancer coexist. This section exemplifies the pattern of staining in normal > dysplasia > cancer and suggests that loss of MST and TST is an early event in the multistep process leading to colon carcinogenesis.

For UC (Fig. 3*I*), the mucosa, when present, stained for both MST and TST, whatever the severity of the active inflammation. In some instances, the number of positive cells (see Fig. 3, *K* and *L*) varied within the same section, suggesting regulation of enzyme expression.

Regulation of TST and MST activity and expression in vitro in HT-29 cells. The effects of butyrate (2 mM) on TST and MST activity and expression are shown in Fig. 4, *A* and *B*, respectively. Also shown on this figure are the effects of suberoylanilide hydroxamic acid (SAHA; 5 μM), a specific inhibitor of histone deacetylase enzymes (HDAC). Cells were incubated for 24 h with the agents, and activities were determined in the lysates as described in the MATERIALS AND METHODS. TST activity was significantly increased by butyrate (42%) and SAHA (34%) in HT-29 cells. There was no signif-

icant effect on MST activity. The effects of SAHA at 24 h were identical to those of butyrate.

The effects on enzyme activity were reflected in the Western blot analyses showing enzyme expression (Fig. 4*B*). Densitometry measurements of Western blots of two separate experiments, corrected for actin expression, showed that TST expression was increased by 2.31 ± 0.18-fold (mean ± SD, $n = 2$) by butyrate and that MST expression was increased 1.15 ±

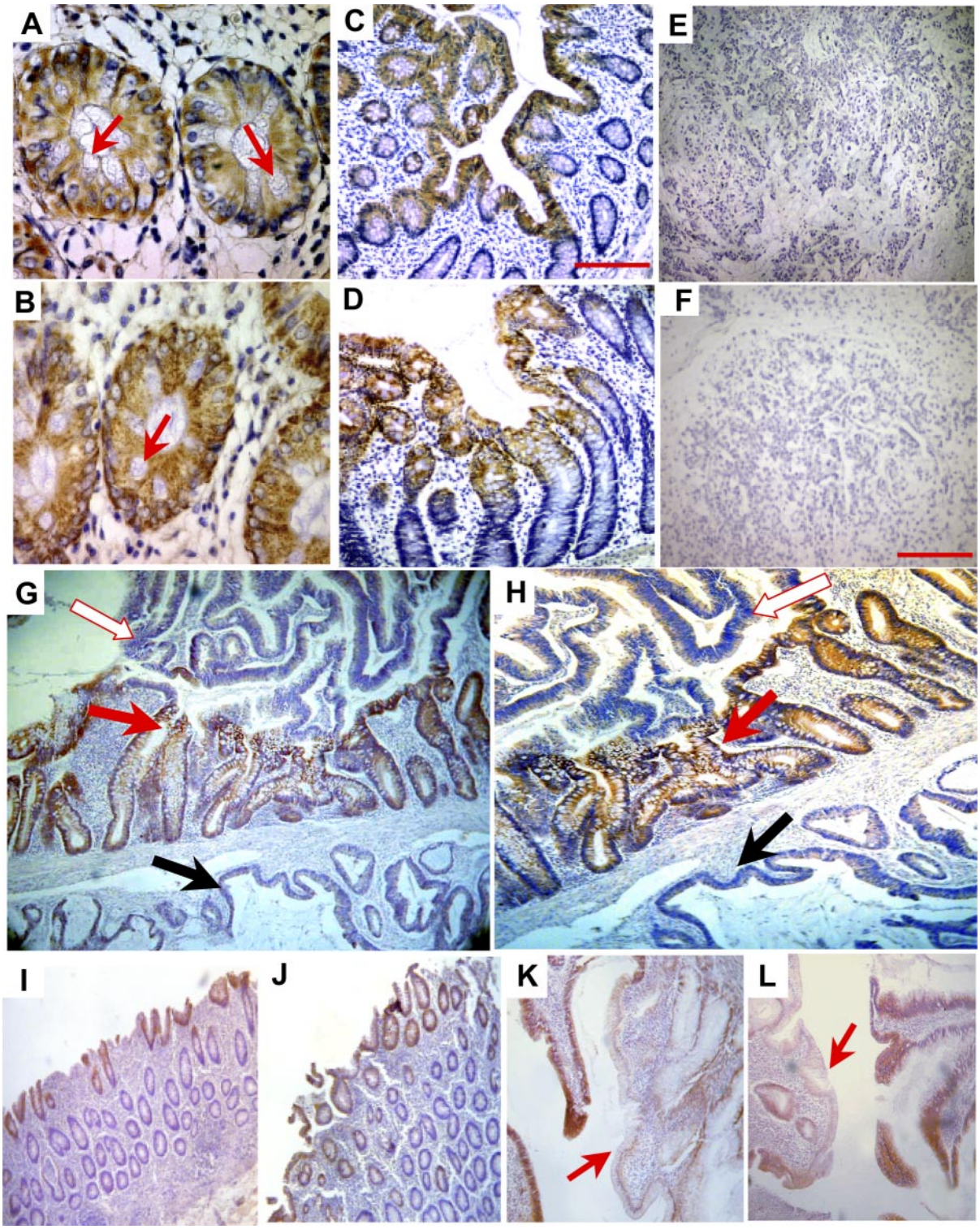

Fig. 3. TST and MST expression in normal colon, in colorectal cancer (CRC) and in ulcerative colitis (UC). *A* and B: normal tissue stained for TST and MST, respectively (×400). *C* and *D*: normal tissues stained for TST and MST, respectively (×100). *E* and *F*: CRC tissues stained for TST and MST (×100). *G* and *H*: staining for TST and MST, respectively, in section showing normal (red arrow), dysplastic (white arrow), and cancer (black arrow) tissue (×260). *I-L*: colon sections from a patient with UC stained for TST and MST. *K* and *L* (×100): the heterogeneous staining for TST and MST, respectively, found in some UC sections. Scale bar, 200 μm.

Table 2. *Percentage of cells staining positive for TST with tumor stage (T2–T4)*

% Positive	*N*	T2	T3	T4
100	15	0	0	0
21–50	0	3	0	0
11–20	0	1	1	0
1–10	0	1	3	3
0	0	0	0	3

Numbers refer to number of patients. *N,* normal sample; T, tumor sample.

0.01-fold. After SAHA treatment, there was a 1.67 ± 0.11-fold increase for TST expression and for MST; the fold increase was 1.19 ± 0.11.

To distinguish the effects of butyrate as an energy source for colonocytes from its effects as an inhibitor of HDAC, we determined the effect of acetate, a two-carbon SCFA, which, similar to butyrate, is metabolized by the cells but does not possess HDAC inhibitory activity (7). Cell were treated with various doses of acetate (0–30 mM) for 24 h. TST and MST activities were measured in cell lysates, but there were no significant effects (data not shown).

Effect of sulfide on TST and MST activity and expression in HT-29 cells. To investigate the effects of sulfide on TST and MST activities and enzyme expression, HT-29 cells were treated with and without $NaHS \cdot H_2O$ at various doses (0.1, 0.3, and 1 mM) for 24 h as shown in Fig. 5. Sulfide at low concentrations (0.1 mM) increased TST (28%) and MST (29%) activity significantly at 24 h, but higher concentrations of sulfide (1 mM) reduced enzyme activity significantly (Fig. 5*A*). Densitometry measurements of Western blots (shown in Fig. 5*B*) showed that 0.1 mM $NaHS \cdot H_2O$ increased TST expression by 1.51 ± 0.07-fold (mean ± SD, $n = 3$) and MST expression by 1.47 ± 0.18-fold (mean ± SD, $n = 3$). At 0.3 mM, TST expression was not significantly different from untreated controls (0.96 ± 0.22), and higher doses were inhibitory. Regulation of MST expression was similar.

Time course of toxicity of sulfide on TST and MST activity and expression in HT-29 cells. HT-29 cells were exposed to 2 mM $NaHS \cdot H_2O$ for varying times to determine the time course of its toxicity measured both as enzyme activity and as protein expression (Fig. 6). For both TST and MST, there was a significant decrease in activity by 16 h. The Western blots showed considerable degradation of MST within 4 h of addition of $NaHS \cdot H_2O$, although expression of the full-length form was maintained. For TST, Western blots showed considerable degradation at 16 h. For both enzymes, the activity reflected the expression of the full-length forms.

Effect of butyrate preincubation on $NaHS \cdot H_2O$ effects on TST and MST activity and expression in HT-29 cells. To determine whether by inducing TST expression with butyrate in HT-29 cells, cells could be protected from sulfide toxicity, cells were preincubated with butyrate (2 mM) for 24 h to allow increased enzyme expression, before the addition of various doses (0.1, 0.3, 1, and and 3 mM) of $NaHS \cdot H_2O$. TST and

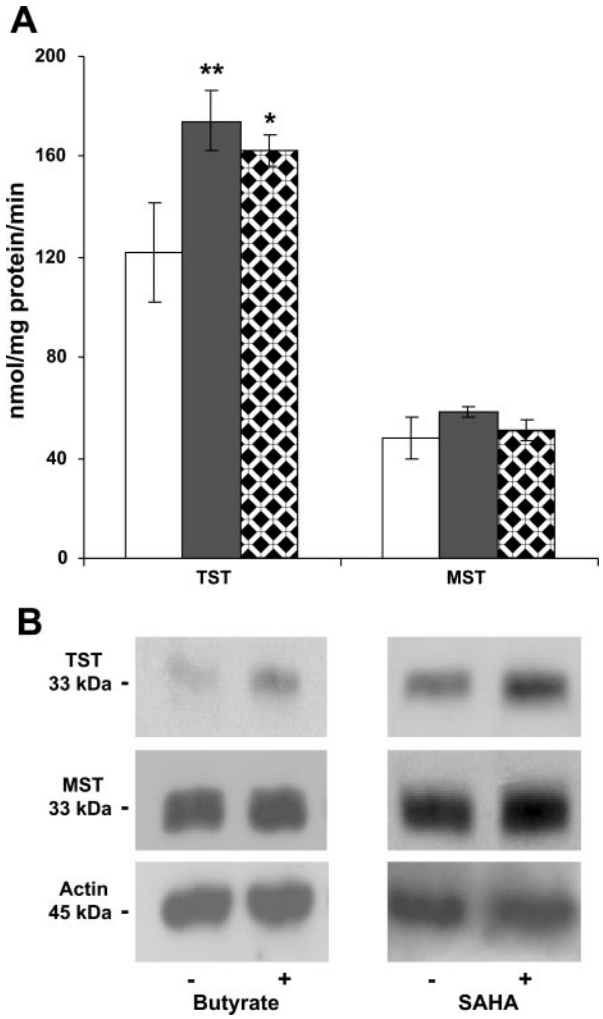

Fig. 4. Effect of butyrate and suberoylanilide hydroxamic acid (SAHA) on TST and MST activity and expression in HT-29 cells. *A*: TST and MST activities were measured after 24 h treatment with and without butyrate (2 mM) and SAHA (5 μM). *B*: Western immunoblotting showing TST and MST expression in HT-29 cell lines treated with and without butyrate or SAHA. Actin was used as an internal control of protein loading. Open bars for control and solid bars for butyrate (2 mM) and dotted bars for SAHA (5 μM). Statistical analyses were carried out using ANOVA and Tukey-Kramer multiple-comparisons posttest ($n = 6$).

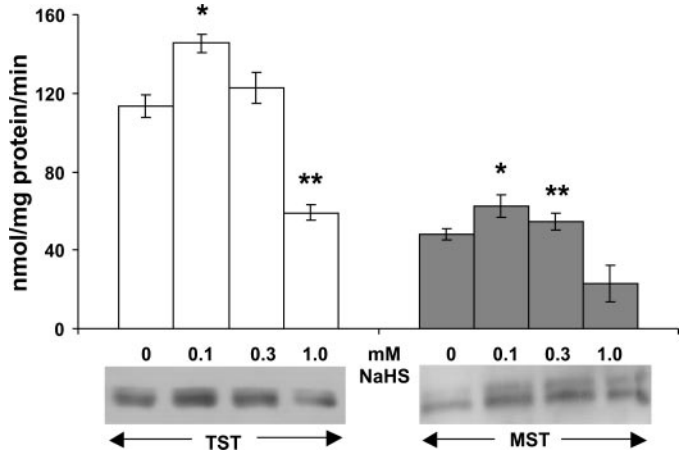

Fig. 5. Effect of sulfide on TST and MST activity and expression in HT-29 cells. TST and MST activities were measured after 24 h treatment with various doses of $NaHS \cdot H_2O$. Western immunoblotting (beneath) shows TST and MST expression in HT-29 cell lysates following treatment with $NaHS \cdot H_2O$. Open bars for TST and solid bars for MST. Statistical analyses were carried out using ANOVA and Tukey-Kramer multiple-comparisons posttest ($n = 3$).

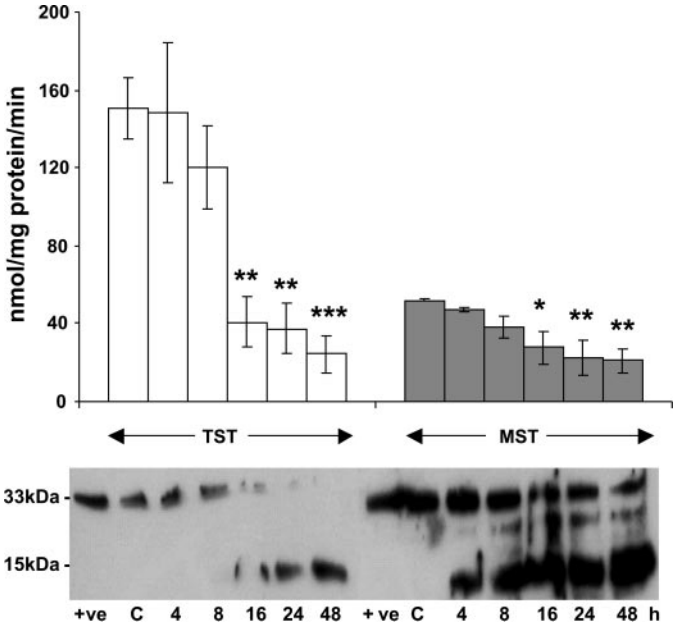

Fig. 6. Time course of toxicity of sulfide on TST and MST activity and expression in HT-29 cells. TST and MST activities were measured after treatment with sulfide (2 mM) at various time points. Western immunoblotting (beneath) shows the time course of effect of sulfide (2 mM) on TST and MST degradation in HT-29 cells. Open bars for TST and solid bars for MST. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ were considered significant.

MST activities were measured as shown in Fig. 7, *A* and *B*, respectively. TST and MST expression is shown in Fig. 7*C*.

When incubated with 0.3 mM $NaHS \cdot H_2O$, HT-29 cells not preincubated with 2 mM butyrate showed a marked and significant decrease in TST activity as shown in Fig. 7*A*. In contrast, when preincubated in butyrate, there was no inhibitory effect of sulfide on TST activity at this concentration of $NaHS \cdot H_2O$. The dose-response curve had been shifted to the right by butyrate preincubation. Higher concentrations of $NaHS \cdot H_2O$ ($\geq$1 mM) were inhibitory, although in the butyrate-preincubated cells, some rescue was evident.

For MST, butyrate preincubation did not significantly affect the toxicity of $NaHS \cdot H_2O$ as shown in Fig. 7*B*. Although the cells were slightly less susceptible to the inhibition by $NaHS \cdot H_2O$ when preincubated with butyrate, these effects were not significant.

Western blotting (Fig. 7*C*) showed that when cells were not preincubated in butyrate, there was dose-dependent degradation of full-length TST and MST. When preincubated in butyrate, despite the loss of enzyme activity (shown in Fig. 7, *A* and *B*), the integrity of both TST and MST structure was maintained.

Effects of butyrate preincubation on cytotoxicity in $NaHS \cdot H_2O$-treated HT-29 cells. The morphology of cells preincubated with or without 2 mM butyrate and treated with or without 1.0 mM $NaHS \cdot H_2O$ for 24 h is shown in Fig. 8. Treatment with $NaHS \cdot H_2O$ alone for 24 h was clearly cytotoxic, and the cells were floating (Fig. 8*A* compared with Fig. 8*B*). There is obvious cell shrinkage due to their detachment, the nuclei are dark, and the surrounding cytoplasm is translucent. With butyrate alone (Fig. 8*C*), there were changes in morphology, but cell shrinkage was not present, and the cells remained adherent. In those cells preincubated in butyrate and treated with $NaHS \cdot H_2O$, again, the cells remained adherent (Fig. 8*D*) and did not demonstrate the cytotoxicity seen in the cells not preincubated in butyrate (Fig. 8*B*). Some translucent cells are seen, but fewer than in $NaHS \cdot H_2O$ alone.

Cytotoxicity was also assayed by the binding of PI to cellular DNA, which is indicative of cell membrane permeabilization and cell death (30). Compared with control cultures, butyrate increased the amount of cellular DNA available to bind PI, i.e, cell death, by 15.8%. This was not unexpected, because butyrate is known to increase apoptosis in HT-29 cells (21). In cells incubated in NaHS (Alfa Aesar, UK) for 24 h, there was a dose-dependent increase in PI-bound DNA that reached 57.6% at 0.3 mM NaHS. This agreed with our observations using the microscope, which showed that cells were readily detached from the cell culture dish at this NaHS concentration. Preincubation in 2 mM butyrate for 24 h before 24 h treatment with 0.03 mM or 0.1 mM NaHS completely prevented the NaHS-stimulated rise of cell death of 13.4% and 46.5%, respectively. At 0.3 mM NaHS, butyrate reduced the 57.6% increase in cell death to 17.3%, compared with appropriate controls. At higher doses of NaHS, the protective effects of butyrate, although maintained, were less marked.

DISCUSSION

We have prepared antisera specific for MST and TST that allows us to discriminate these isoenzymes of rhodanese. We found that TST by itself, but not MST, was able to use $NaHS \cdot H_2O$ as a substrate. There was no evidence of cooperativity between the enzymes in effecting the detoxification of $NaHS \cdot H_2O$. Our data do not preclude the existence of other colonic enzyme(s) capable of detoxifying sulfide, but we have identified one candidate enzyme. Immunohistochemical staining of sections of normal colonic epithelium showed that TST and MST were expressed predominantly in the cytoplasm of

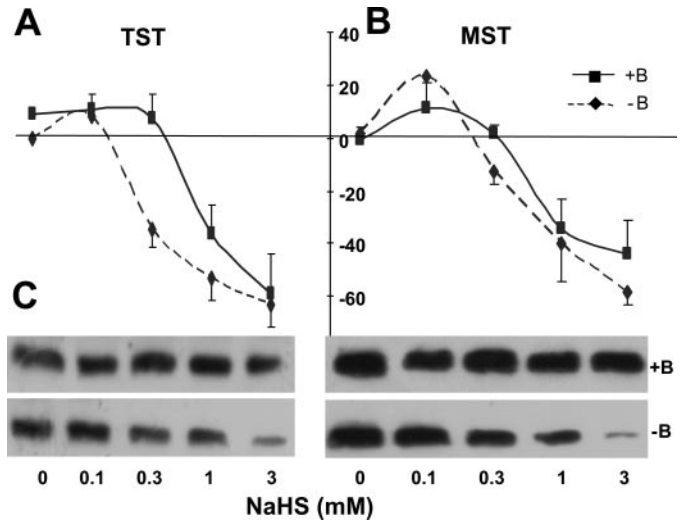

Fig. 7. Effect of butyrate preincubation on $NaHS \cdot H_2O$ effects on TST and MST activity and expression on HT-29 cells. *A*: TST activity in cell lysates from cells treated with or without 24 h preincubation with 2 mM butyrate and incubation with various doses of $NaHS \cdot H_2O$. *B*: MST activity in cell lysates from cells treated with or without 24 h preincubation with 2 mM butyrate and incubation with various doses of $NaHS \cdot H_2O$. *C*: Western immunoblotting showing TST and MST expression in HT-29 cell lysates following the treatment described in *A* and *B*. Statistical analyses were carried out using ANOVA and Tukey-Kramer multiple-comparisons posttest ($n = 2$).

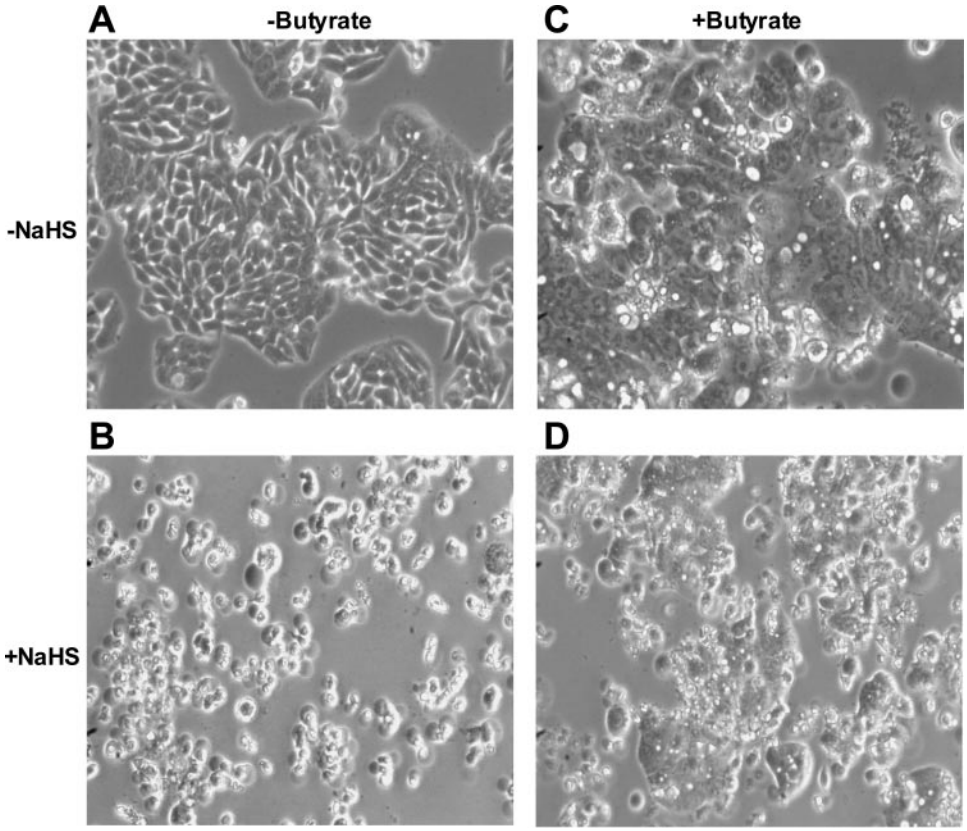

Fig. 8. Morphological effects of 24 h $NaHS \cdot H_2O$ treatment with and without 24 h butyrate (2 mM) pretreatment on HT-29 cells. *A*: control. *B*: 1 mM $NaHS \cdot H_2O$. *C*: 2 mM butyrate-pretreated control. *D*: 2 mM butyrate pretreatment with 1 mM $NaHS \cdot H_2O$.

the surface epithelium and the proximal part of the crypts where differentiation is highest. The bases of the crypts, where the pluripotent stem cells reside, were unstained by both antibodies. We hypothesize that the differentiated cells at the crypt surface are exposed to luminal H_2S and require means to detoxify this.

To explore this hypothesis, we used a human colon cancer cell line and examined MST and TST expression when the cells were induced to differentiate with butyrate. The activity and expression of TST was significantly increased by butyrate. Enzyme expression was increased more substantially than enzyme activity following butyrate treatment, which may be related to other effects of butyrate. Butyrate has been shown to induce markers of cell differentiation, e.g., alkaline phosphatase activity, brush-border hydrolases, (20, 27) as well as apoptosis. The balance between increasing differentiation while promoting apoptosis may be delicate and offers an explanation for the disparity in the activity/expression profile. MST activity and expression were not regulated markedly by butyrate under any conditions. These data show a separation in the regulation of the expression of the two isoenzymes. Although human TST and human MST show 32% similarity in their amino acid sequence and they are located adjacent to each other on the same chromosome (22q13.1), they are derived from different genes. Their subcellular localization differs (8), MST being cytoplasmic and TST mitochondrial, which is consistent with separation of function. Similarly, their tissue distribution also differs.

The effect of butyrate on colonocytes is complex. Butyrate can be catabolized by colonocytes, which provide them with energy, but butyrate can also function as an inhibitor of histone deacetylases. To control for the effects of butyrate as a fuel supply, we substituted acetate, which, similar to butyrate, is an SCFA catabolized by colonocytes but, unlike butyrate, does not inhibit histone deacetylases (7, 20). Acetate had no stimulatory effect on TST expression, indicating that the predominant mode of action of butyrate was as an inhibitor of histone deacetylases. To confirm this, we used SAHA, a more specific histone deacetylase inhibitor, and showed that SAHA was able to mimic the effects of butyrate. As for butyrate, enzyme expression was increased more substantially than enzyme activity, although the disparity was less marked than for butyrate. SAHA was less effective than butyrate in both assays. Again, the effects on MST activity and expression were minimal.

Low concentrations of sulfide (0.1 mM) increased TST and also MST activity and expression in HT-29 cells. Higher concentrations were toxic within 4 h, and rapid degradation of MST to a species of 15 kDa was seen. TST was also degraded in colonocytes treated with $NaHS \cdot H_2O$, but it was more stable, and marked degradation was not seen until 16 h postaddition. Cytotoxicity data using PI as a marker of cell permeability and damage confirmed these findings, dose-dependent significant increases in cell death being seen with sulfide. In 1.0 mM sulfide, most cells were nonadherent and PI assays showed almost 100% cell death. Under the conditions described in this paper, sulfide is clearly cytotoxic; however, when we performed similar experiments on cells cultured on 96-well plates sealed with alumaseal (Sigma), sulfide was not cytotoxic using either this assay and/or by observation. We conclude that the effects observed are critically dependent on experimental protocol. Leschelle et al. (10) did not find profound cytotoxic effects in their experiments in HT-29 cells, but rather an

adaptive response to preserve cell viability, although they found acute inhibitory effects on substrate oxidation and cytochrome-*c* oxidase. They reported that sulfide was unstable in cell culture medium, and we also found this; although under our conditions, even less sulfide remained, with only 15 ± 0.1% of 1 mM sulfide remaining after 1 h. We confirmed that sulfide levels in media showed a much more rapid decline in polystyrene tissue cultureware than in polypropylene, which had previously been reported by Furne et al. (6). Deplancke and Gaskins (5), using rat intestinal cells (IEC-8), found that treatment with $NaHS \cdot H_2O$ between 1 and <5 mM for 4 h increased the fraction of cells in G_2M, suggesting no toxicity, although high concentrations (5 mM) were toxic (5). Others have found that sulfide is cytotoxic (2, 5, 11). Although there seems to be agreement that sulfide inhibits metabolic functions, the conflicting data on cytotoxicity may be reconciled through experimental variations in attainable sulfide concentrations, alternative energy sources available, reactive oxygen species produced, prevailing antioxidant levels, prevailing oxygen tensions, and other factors as yet unrecognized.

Because butyrate enhanced TST activity, we examined the effects of a 24 h pretreatment with butyrate on the ability of HT-29 cells to detoxify sulfide. Butyrate pretreatment shifted the dose-response curve to the right, and cells were able to withstand exposure to 0.3 mM $NaHS \cdot H_2O$ for 24 h, whereas in its absence, enzyme activity was decreased by 50%. These experiments demonstrate that by inducing colonocyte differentiation, greater resistance to the toxic effects of sulfide is realized. The increased viability of the cells was also demonstrated by the lack of degradation of TST and MST in the butyrate-pretreated cells. Without butyrate pretreatment, we found that the cells rapidly degraded TST, but this degradation was prevented in the pretreated cells. The decreased cytotoxicity of sulfide in the butyrate-pretreated cells was particularly striking when cell morphology of HT-29 cells was examined. These effects were also evident in the PI assays measuring cytotoxicity, where butyrate completely prevented NaHS-induced cytotoxicity at 0.03 and 0.1 mM and significantly prevented the effects of higher doses.

These data showed that TST expression is regulated in colonocyte differentiation, which can be promoted by butyrate in vitro (and possibly also in vivo), and that this can protect the colonocytes from sulfide-induced cytotoxicity. We therefore examined whether dysregulation of expression occurred in UC and in CRC. In the UC patients where the mucosa was present, TST was expressed. In some sections, however, we observed that the mucosa was stained heterogeneously and some areas had much lighter staining. It is impossible to deduce whether this reduction is the initiating event in the onset of UC or merely correlates with the disease process; however, in the normal mucosa, all the crypt surfaces showed the same strong positivity. For all of the colon cancers examined, both TST and MST expression were markedly reduced and the decrease in expression was correlated to the depth of infiltration. These data support the findings of Birkenkamp-Demtroder et al. (3, 4) that TST is a tumor marker for colon cancer. They showed marked reductions in TST mRNA expression for cancer from Dukes' stages A-D compared with normal. Whether MST was also expressed on their gene array is not clear, but our data suggest that similar to TST, MST expression is lost in malignancy.

In the human colon, failure of colonocytes to differentiate appropriately may increase the exposure of colonocytes and other cells in the crypt to the toxic effects of luminal H_2S. This could result in cell death and the inflammation seen in UC. Persistent insult may result in the genetic changes seen in CRC. Elevations in H_2S, due to perturbations in sulfate-reducing bacteria, and/or inadequacies in its detoxification, due to reductions in TST expression, may lead to or aggravate inflammatory bowel disease that may ultimately progress to CRC.

ACKNOWLEDGMENTS

We thank Dr. E. Toescu and M. Chitolie for helpful advice and for allowing the use of a plate reader.

GRANTS

This work was supported by the Good Hope Hospital National Health Service Trust.

REFERENCES

1. **Aminlari M and Gilanpour H.** Comparative studies on the distribution of rhodanese in different tissues of domestic animals. *Comp Biochem Physiol B* 99: 673–677, 1991.
2. **Attene-Ramos MS, Wagner ED, Plewa MJ, and Gaskins HR.** Evidence that hydrogen sulfide is a genotoxic agent. *Mol Cancer Res* 4: 9–14, 2006.
3. **Birkenkamp-Demtroder K, Christensen LL, Olesen SH, Frederiksen CM, Laiho P, Aaltonen LA, Laurberg S, Sorensen FB, Hagemann R, and Orntoft TF.** Gene Expression in Colorectal Cancer. *Cancer Res* 62: 4352–4363, 2002.
4. **Birkenkamp-Demtroder K, Olesen SH, Sorensen FB, Laurberg S, Laiho P, Aaltonen LA, and Orntoft TF.** Differential gene expression in colon cancer of the caecum versus the sigmoid and rectosigmoid. *Gut* 54: 374–384, 2005.
5. **Deplancke B and Gaskins HR.** Hydrogen sulfide induces serum-independent cell cycle entry in nontransformed rat intestinal epithelial cells. *FASEB J* 17: 1310–1312, 2003.
6. **Furne J, Springfield J, Koenig T, DeMaster E, and Levitt MD.** Oxidation of hydrogen sulfide and methanethiol to thiosulfate by rat tissues: a specialized function of the colonic mucosa. *Biochem Pharmacol* 62: 255–259, 2001.
7. **Hinnebusch BF, Meng S, Wu JT, Archer SY, and Hodin RA.** The effects of short-chain fatty acids on human colon cancer cell phenotype are associated with histone hyperacetylation. *J Nutr* 132: 1012–1017, 2002.
8. **Hulin SJ, Singh S, Chapman MA, Allan A, Langman MJ, and Eggo MC.** Sulphide-induced energy deficiency in colonic cells is prevented by glucose but not by butyrate. *Aliment Pharmacol Ther* 16: 325–331, 2002.
9. **Jarabak R.** 3-Mercaptopyruvate sulfurtransferase. *Methods Enzymol* 77: 291–297, 1981.
10. **Leschelle X, Goubern M, Andriamihaja M, Blottiere HM, Couplan E, Gonzalez-Barroso MD, Petit C, Pagniez A, Chaumontet C, Mignotte B, Bouillaud F, and Blachier F.** Adaptative metabolic response of human colonic epithelial cells to the adverse effects of the luminal compound sulfide. *Biochim Biophys Acta* 1725: 201–212, 2005.
11. **Levitt MD, Furne J, Springfield J, Suarez F, and DeMaster E.** Detoxification of hydrogen sulfide and methanethiol in the cecal mucosa. *J Clin Invest* 104: 1107–1114, 1999.
12. **Nagahara N, Ito T, and Minami M.** Mercaptopyruvate sulfurtransferase as a defense against cyanide toxication: molecular properties and mode of detoxification. *Histol Histopathol* 14: 1277–1286, 1999.
13. **Nagahara N and Nishino T.** Role of amino acid residues in the active site of rat liver mercaptopyruvate sulfurtransferase. CDNA cloning, overexpression, and site-directed mutagenesis. *J Biol Chem* 271: 27395–27401, 1996.
14. **Nagahara N, Okazaki T, and Nishino T.** Cytosolic mercaptopyruvate sulfurtransferase is evolutionarily related to mitochondrial rhodanese. Striking similarity in active site amino acid sequence and the increase in the mercaptopyruvate sulfurtransferase activity of rhodanese by site-directed mutagenesis. *J Biol Chem* 270: 16230–16235, 1995.
15. **Picton R, Eggo MC, Merrill GA, Langman MJ, and Singh S.** Mucosal protection against sulphide: importance of the enzyme rhodanese. *Gut* 50: 201–205, 2002.

16. **Prior P, Gyde S, Macartney J, Thompson H, Waterhouse J, and Allan R.** Cancer morbidity in ulcerative colitis. *Gut* 23: 490–497, 1982.
17. **Ramsden JD, Cocks HC, Shams M, Nijjar S, Watkinson JC, Sheppard MC, Ahmed A, and Eggo MC.** Tie-2 is expressed on thyroid follicular cells, is increased in goiter, and is regulated by thyrotropin through cyclic adenosine 3′,5′-monophosphate. *J Clin Endocrinol Metab* 86: 2709–2716, 2001.
18. **Roediger WE.** The colonic epithelium in ulcerative colitis: an energy-deficiency disease? *Lancet* 2: 712–715, 1980.
19. **Roediger WE, Duncan A, Kapaniris O, and Millard S.** Reducing sulfur compounds of the colon impair colonocyte nutrition: implications for ulcerative colitis. *Gastroenterology* 104: 802–809, 1993.
20. **Siavoshian S, Segain JP, Kornprobst M, Bonnet C, Cherbut C, Galmiche JP, and Blottiere HM.** Butyrate and trichostatin A effects on the proliferation/differentiation of human intestinal epithelial cells: induction of cyclin D3 and p21 expression. *Gut* 46: 507–514, 2000.
21. **Singh B, Halestrap AP, and Paraskeva C.** Butyrate can act as a stimulator of growth or inducer of apoptosis in human colonic epithelial cell lines depending on the presence of alternative energy sources. *Carcinogenesis* 18: 1265–1270, 1997.
22. **Sobin LHWC.** *TNM Classification of Malignant Tumours*. New York: Wiley, 2002.
23. **Sorbo B.** Crystalline rhodanese. I. Purification and physicochemical examination. *Acta Chem Scand* 7: 1129–1136, 1953.
24. **Sorbo B.** Enzymic transfer of sulfur from mercaptopyruvate to sulfite or sulfinates. *Biochim Biophys Acta* 24: 324–329, 1957.
25. **Suarez F, Furne J, Springfield J, and Levitt M.** Insights into human colonic physiology obtained from the study of flatus composition. *Am J Physiol Gastrointest Liver Physiol* 272: G1028–G1033, 1997.
26. **Vachek H and Wood JL.** Purification and properties of mercaptopyruvate sulfur transferase of *Escherichia coli*. *Biochim Biophys Acta* 258: 133–146, 1972.
27. **Velcich A, Palumbo L, Jarry A, Laboisse C, Racevskis J, and Augenlicht L.** Patterns of expression of lineage-specific markers during the in vitro-induced differentiation of HT29 colon carcinoma cells. *Cell Growth Differ* 6: 749–757, 1995.
28. **Westley J.** Rhodanese. *Adv Enzymol Relat Areas Mol Biol* 39: 327–368, 1973.
29. **Westley J, Adler H, Westley L, and Nishida C.** The sulfurtransferases. *Fundam Appl Toxicol* 3: 377–382, 1983.
30. **Wrobel K, Claudio E, Segade F, Ramos S, and Lazo PS.** Measurement of cytotoxicity by propidium iodide staining of target cell DNA. Application to the quantification of murine TNF-α. *J Immunol Methods* 189: 243–249, 1996.