SLC5A8 Triggers Tumor Cell Apoptosis through Pyruvate-Dependent Inhibition of Histone Deacetylases

Muthusamy Thangaraju,1 Elangovan Gopal,1 Pamela M. Martin,1 Sudha Ananth,1 Sylvia B. Smith,2 Puttur D. Prasad,1 Esta Sterneck,1 and Vadivel Ganapathy1

Departments of 1Biochemistry and Molecular Biology and 2Cellular Biology and Anatomy, Medical College of Georgia, Augusta, Georgia and 3Laboratory of Protein Dynamics and Signaling, Center for Cancer Research, National Cancer Institute, Frederick, Maryland

Abstract

Tumor cells up-regulate glycolysis but convert pyruvate into lactate instead of oxidizing it. Here, we show that pyruvate, but not lactate, is an inhibitor of histone deacetylases (HDAC) and an inducer of apoptosis in tumor cells and that SLC5A8, a Na+/monocarboxylate cotransporter, is obligatory for this process. We found that SLC5A8 is expressed in nontransformed breast epithelial cell lines but silenced by DNA methylation in tumor cell lines. The down-regulation of the gene is also evident in primary breast tumors. When MCF7 breast tumor cells are transfected with SLC5A8 cDNA, the cells undergo pyruvate-dependent apoptosis. Butyrate and propionate also induce apoptosis in SLC5A8-expressing cells, whereas lactate does not. The differential ability of these monocarboxylates to cause apoptosis in SLC5A8-expressing MCF7 cells correlates with their ability to inhibit HDACs. Apoptosis induced by SLC5A8/pyruvate in MCF7 cells is associated with up-regulation of p53, Bax, tumor necrosis factor–related apoptosis-inducing ligand (TRAIL), TRAIL receptor (TRAILR) 1, and TRAILR2 and down-regulation of Bcl2 and survivin. Lactate dehydrogenase isozymes are differentially expressed in nontransformed cells and tumor cells such that the latter convert pyruvate into lactate. Silencing of SLC5A8 coupled with conversion of pyruvate into lactate in tumor cells correlates with increased HDAC activity in these cells compared with nontransformed cells. Our studies thus identify pyruvate as a HDAC inhibitor and indicate that the Na+-coupled pyruvate transport underlies the tumor-suppressive role of SLC5A8. We propose that tumor cells silence SLC5A8 and convert pyruvate into lactate as complementary mechanisms to avoid pyruvate-induced cell death. (Cancer Res 2006; 66(24): 11560–4)

Introduction

SLC5A8 is a Na+-coupled transporter for short-chain fatty acids (acetate, propionate, and butyrate), lactate, pyruvate, and nicotinate (1–5). Accordingly, SLC5A8 has been named sodium-coupled monocarboxylate transporter 1. SLC5A8 is the first plasma membrane transporter postulated to function as a tumor suppressor. Silencing of its expression by epigenetic mechanisms represents an early event in the progression of colorectal cancer, and reexpression of the gene in colon tumor cell lines induces apoptosis and prevents colony formation (6, 7). However, it is not known how the transport function of SLC5A8 is related to its putative tumor-suppressive role. Butyrate is an inhibitor of histone deacetylases (HDAC), and HDAC inhibitors show promise in the treatment of cancer (8, 9). Short-chain fatty acids, including butyrate, are produced in the colonic lumen by bacterial fermentation of dietary fiber (10, 11). Butyrate is an important energy substrate for normal colonocytes. It induces differentiation in normal colonocytes but causes apoptosis in colon cancer cells (10, 11). The tumor-selective sensitization of the cells to apoptosis by butyrate involves tumor cell-specific induction of death receptor pathway (12, 13). The SLC5A8-mediated entry of butyrate into colonic epithelial cells may explain its tumor-suppressive role in the colon. Recent studies have shown that the expression of SLC5A8 is under the control of the transcription factor CAAT/enhancer binding protein ß in certain tissues (14).

Butyrate is not found at high concentrations anywhere else in the body other than the colonic lumen; yet, the expression of SLC5A8 is silenced in cancer of noncolonic tissues (10, 11). Even in the colon cancer, the tumor epithelial cells lose their polarity and monolayer organization, indicating loss of contact of the tumor cells with luminal contents. Therefore, we asked whether there are other endogenous metabolites present in circulation, which might be substrates for SLC5A8 and function as HDAC inhibitors and tumor-specific apoptosis inducers. Here, we identify the ubiquitous metabolite pyruvate as a HDAC inhibitor and show that the Na+-coupled pyruvate transport underlies the tumor-suppressive role of SLC5A8.

Materials and Methods

Cell culture. MCF7, T47D, and ZR75.1 cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA) and cultured in DMEM containing 10% bovine serum (FBS), 100 units/mL penicillin, 100 µg/mL streptomycin, 2 mmol/L glutamine, and 1 mmol/L pyruvate. For the pyruvate-free experiments, cells were cultured in the same medium but in the absence of pyruvate. Human breast epithelial cells HMEC (Clonetics) and MCF10A (ATCC) were grown in complete mammary epithelial growth medium (Cambrex, Walkersville, MD) containing 100 ng/mL cholera toxin and 2 mmol/L pyruvate. The nontransformed human breast epithelial cell line HBL100 (provided by Dr. Sukumar, Johns Hopkins University, Baltimore, MD) was grown in DMEM/F12 containing 10% FBS, 100 units/mL penicillin, 100 µg/mL streptomycin, 2 mmol/L glutamine, and 1 mmol/L pyruvate.

Reverse transcription-PCR. For the analysis of the expression of various genes, RNA prepared from the cell lines was used for semiquantitative reverse transcription-PCR (RT-PCR). RNA (2 µg) was reverse transcribed using the GeneAmp PCR System (Roche). Hypoxanthine phosphoribosyltransferase 1 mRNA was used as an internal control. Gene-specific PCR primers were designed based on the nucleotide sequences available in Genbank.
Protein analysis. For Western blot analysis, cell lysates were prepared by sonication in 10 mmol/L Tris-HCl buffer (pH 7.6) containing protease inhibitors (50 mmol/L NaF, 0.2 mmol/L vanadate, 1 mmol/L phenylmethylsulfonyl fluoride, 5 μg/mL aprotinin, 1 μg/mL pepstatin A, and 2 μg/mL leupeptin) and 1% Triton X-100. Protein (50 μg) was fractionated on SDS-PAGE gels and transferred to Protran nitrocellulose membrane (Schleicher & Schuell). Membranes were blocked with bovine serum albumin, exposed to primary antibody at 4°C overnight followed by treatment with appropriate secondary antibody, conjugated to horseradish peroxidase at room temperature for 1 hour, and developed by Enhanced Chemiluminescence SuperSignal Western System (Pierce). Primary antibodies were obtained as follows: histone H4 (Upstate); histone H4 (Lys16), p53, Bax, and Bcl2 (Santa Cruz Biotechnology, Santa Cruz, CA); survivin (R&D Systems); tumor necrosis factor–related apoptosis-inducing ligand (TRAIL), TRAIL receptor (TRAILR) 1, and TRAILR2 (Abcam); and actin and poly(ADP-ribose) polymerase (PARP; Sigma).

Flow cytometry. Cells were fixed in 50% ethanol, treated with 0.1% sodium citrate, 1 mg/mL RNase A, and 50 μg/mL propidium iodide, and subjected to fluorescence-activated cell sorting (FACS; FACCalibur, Becton Dickinson) analysis. For Annexin V analysis, cells were stained with FITC-labeled Annexin V and propidium iodide following the manufacturer's instructions (FACS Annexin V-FITC Apoptosis Detection kit, Miltenyi Biotec).

Measurement of HDAC activity in a cell-free system. The measurement of HDAC activity in a cell-free system was done using a commercially available kit (BioVision). Cell lysates were used for the measurement of HDAC activity. Lysate protein (50 μg) was incubated with or without monocarboxylates (0.01–1 mmol/L), and the reaction was initiated by the addition of HDAC substrate. The reaction was then terminated and the deacetylated product was measured.

Transfection and colony formation assay. MCF7 cells were transfected in 10-cm dishes with pcDNA3.1 or SLC5A8 expression construct along with pEGFP-N1 to check the transfection efficiency using Fugene 6 and Opti-MEM. The following day, cells were trypsinized and seeded into six-well plates (10,000 per well) or 24-well plates (1,000 per well) in DMEM.
without pyruvate. After 24 hours, cells were exposed to 750 μg/mL G418 and different concentrations of pyruvate, lactate, butyrate, propionate, and acetate for 2 weeks, changing the medium every 3 days. Cells were washed with PBS and fixed in 100% methanol for 30 minutes followed by staining with KaryoMax Giemsa stain for 1 hour. The wells were washed with water and dried overnight at room temperature. Finally, cells were lysed with 1% SDS in 0.2 N NaOH for 1 hour and the absorbance of the released dye was measured at 630 nm.

**Breast cancer profiling array.** The membrane (Clontech, Palo Alto, CA) was hybridized with a 32P-labeled human SLC5A8-specific cDNA probe under high stringency conditions, and the signals were quantified using the PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

**Results**

To identify endogenous metabolites that might be substrates for SLC5A8 and function as HDAC inhibitors and tumor-specific apoptosis inducers, we used human breast epithelial cell lines as a model. We first compared the expression of SLC5A8 between nontransformed (HMEC, HBL100, and MCF10A) and transformed (MCF7, T47D, and ZR75.1) breast epithelial cell lines to assess the relationship between SLC5A8 expression and tumor transformation status. We found that the transporter was expressed in nontransformed cell lines and that the expression was markedly reduced in transformed cell lines (Fig. 1A and B). This was evident at the level of mRNA (Fig. 1A), protein as observed by immunofluorescence analysis (Supplementary Fig. S1), and transport function (Fig. 1B). The silencing of SLC5A8 in transformed cell lines was due to DNA methylation as evident from the induction of expression of this gene on treatment with 5’-azacytidine, a demethylating agent (Fig. 1C). Comparison of SLC5A8 expression in breast tumors and normal breast tissue from the same patient revealed that this gene was down-regulated in 27 of 30 tumors (Fig. 1D). Overall, there was a 90% decrease in SLC5A8 mRNA levels in tumor tissues compared with corresponding normal tissues (P < 0.001, paired Student’s t test). There was a wide variation in the expression levels of this gene in normal tissue, potentially related to the physiologic status of the individual (e.g., hormone levels, pregnancy, and lactation).

Next, we evaluated the potential tumor-suppressive function of SLC5A8 in MCF7 cells, which do not express the transporter. We found that exogenous expression of SLC5A8 in these cells led to massive apoptosis (data not shown). This was puzzling because until now only the substrate butyrate was known to function as a HDAC inhibitor and cause apoptosis in tumor cells; but this compound was not present in the cell culture medium. However, MCF7 cells were cultured in the presence of pyruvate, another known substrate for SLC5A8. Therefore, we hypothesized that pyruvate may function as a HDAC inhibitor and an inducer of apoptosis in tumor cells. To test this hypothesis, we compared the effects of exogenous expression of SLC5A8 on apoptosis in MCF7 cells in the presence and absence of pyruvate in the culture medium. We found that the SLC5A8-mediated apoptosis was obligatorily dependent on the presence of pyruvate, as evident from the SLC5A8/pyruvate-dependent increase in cells with sub-G0/G1 DNA content, in Annexin V–positive cells (Fig. 2A), and in cleavage of the caspase substrate PARP (Fig. 2B). To determine if pyruvate is indeed a HDAC inhibitor, we analyzed the level of acetylation at Lys16 in histone H4 in MCF7 cells in the presence and absence of pyruvate in SLC5A8-expressing MCF7 cells (Fig. 2C). The acetylation status of histone H4 was low in these cells irrespective of the presence or absence of pyruvate when transfected with vector alone. However, exogenous expression of SLC5A8 enhanced the acetylation status in these cells but only when pyruvate was present.

Tumor cells up-regulate glycolysis and convert the glycolytic product pyruvate into lactate (15, 16). We compared the potencies of pyruvate and lactate to inhibit HDACs in a cell-free system (Fig. 3A). Whereas pyruvate inhibited HDACs (IC50 ≈ 80 μmol/L), lactate did not. Among the other monocarboxylates tested, butyrate was the most potent (IC50 ≈ 60 μmol/L), whereas acetate was the least potent. Similar results were obtained in intact SLC5A8-expressing MCF7 cells as evident from the changes in the acetylation status of histone H4 (Fig. 3B). We then compared the effects of these monocarboxylates on cell growth in MCF7 cells by a colony formation assay. In the absence of SLC5A8 expression, none of these monocarboxylates had any effect on cell growth.
However, pyruvate, butyrate, and propionate inhibited the growth of SLC5A8-expressing MCF7 cells, whereas acetate and lactate had no effect (Fig. 3C and D). The potencies of these monocarboxylates to inhibit HDACs paralleled their potencies to inhibit colony formation in SLC5A8-expressing MCF7 cells. The IC50 values for butyrate, pyruvate, and propionate in the colony formation assay were 37 ± 5, 72 ± 12, and 205 ± 40 μmol/L, respectively. Even at concentrations that were 10-fold higher than the corresponding IC50 values, these monocarboxylates showed minimal effect on vector-transfected cells under identical conditions (Supplementary Fig. S2).

Interconversion between pyruvate and lactate is mediated by lactate dehydrogenase (LDH). LDH1 is a homotetramer of LDH-H and its kinetic features are suitable for the conversion of lactate into pyruvate; on the other hand, LDH5 is a homotetramer of LDH-M and its kinetic features are suitable for the conversion of pyruvate into lactate. Because pyruvate and lactate show differential effects on apoptosis in MCF7 cells following exogenous expression of SLC5A8, we investigated the expression of the two subunits (LDH-H and LDH-M) in nontransformed and transformed breast epithelial cell lines. As shown before in cancer of various tissues (17–20), we found that nontransformed cell lines express LDH-H more abundantly than tumor cell lines and that LDH-M is expressed at much higher levels in tumor cell lines than in nontransformed cell lines (Supplementary Fig. S3). Thus, tumor cells have the enzymatic machinery for effective conversion of pyruvate into lactate. This mechanism may reduce intracellular levels of pyruvate and consequently prevent HDAC inhibition and cell death. Tumor cell lines must then have higher HDAC activity compared with nontransformed cell lines. To determine whether this is true, we measured HDAC activity in these cell lines. We found that HDAC activity, monitored with a cell-free assay system, was 4- to 10-fold higher in tumor cell lines than in nontransformed cell lines (Fig. 4A). This was confirmed by HDAC activity in intact cells where the acetylation status of histone H4 was taken as the readout of HDAC activity (Fig. 4B).

To understand the mechanism of pyruvate-induced apoptosis, we monitored the expression of various proapoptotic and antiapoptotic genes (Fig. 4C). When SLC5A8-expressing MCF7 cells were cultured in the presence of pyruvate, there was up-regulation of many proapoptotic factors (p53, Bax, Bak, TRAIL, TRAILR1, and TRAILR2) and down-regulation of antiapoptotic factors (Bcl2, Bcl-W, and survivin). None of 18 different genes related to apoptosis was affected in the absence of pyruvate or in vector-transfected cells irrespective of the presence or absence of pyruvate. The changes in mRNA levels for p53, Bax, survivin, Bcl2, TRAIL, TRAILR1, and TRAILR2 correlated with changes in corresponding protein levels (Fig. 4D). These data show that the SLC5A8-mediated changes in the expression of apoptosis-related genes are obligatorily dependent on the presence of pyruvate in the extracellular medium, suggesting that the transporter-mediated concentrative entry of pyruvate is essential for the tumor-suppressive function of the transporter.

**Discussion**

These studies show for the first time that pyruvate is a HDAC inhibitor and a tumor cell-specific inducer of apoptosis. These data have profound implications in tumor progression and cancer treatment. Tumor cells up-regulate glycolysis and accumulate lactate in the culture medium (15, 16). The enhanced conversion of pyruvate into lactate in these cells is the result of induction of LDH5, which maintains the rate of glycolysis by keeping the NAD+/NADH ratio high. Our present studies provide additional mechanistic insight into the relationship between the enhanced conversion of pyruvate into lactate and tumor growth. We suggest that LDH5 is up-regulated in tumor cells as a means to prevent the accumulation of pyruvate despite the up-regulation of glycolysis and thus to evade the pyruvate-induced cell death. The tumor cell-specific induction of apoptosis by pyruvate is elicited by the ability of this metabolite to inhibit HDACs. The inhibition of HDACs in tumor cells leads to up-regulation of the proapoptotic factors p53, Bax, Bak, TRAIL, TRAILR1 and TRAILR2 and, at the same time, down-regulation of the antiapoptotic factors Bcl2, Bcl-W, and survivin. The induction of the death receptor pathway by pyruvate in tumor cells is similar to what has been described for the classic HDAC inhibitors (12, 13), corroborating our data that pyruvate induces this pathway via inhibition of HDACs. The concentration of pyruvate in circulation under normal physiologic conditions is ~100 μmol/L, a value comparable with the IC50 value for extracellular pyruvate to inhibit cell growth in SLC5A8-expressing tumor cells. These data are relevant to the down-regulation of the transporter in cancer. Silencing of SLC5A8 expression in cancer would prevent the entry...
of circulating pyruvate into tumor cells and thus avoid pyruvate-induced cell death. Therefore, pharmacologic means to increase the intracellular concentration of pyruvate in tumor cells via induction of SLC5A8 and/or inhibition of LDH5 may have potential in the treatment of cancer.

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References

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