Na+/monocarboxylate transport (SMCT) protein expression correlates with survival in colon cancer: Molecular characterization of SMCT

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We report an extensive characterization of the Na+/monocarboxylate transporter (SMCT), a plasma membrane protein that mediates active transport of monocarboxylates such as propionate and nicotinate, and we show that SMCT may play a role in colorectal cancer diagnosis. SMCT, the product of the SLC5A8 gene, is 70% similar to the Na+/I− symporter, the protein that mediates active I− uptake in the basolateral surface of thyrocytes and other cells. SMCT was reported in the apical surface of thyrocytes and formerly proposed also to transport I− and was called the apical I− transporter. However, it is now clear that SMCT does not transport I−. Here we demonstrate a high-affinity Na+/H+ symporter. We show that, whereas thyroidal Na+/H+ cotransporter system is regulated by thyroid-stimulating hormone (TSH), a major regulator of thyroid function. We determined the cellular and subcellular localization of this transporter in the thyroid, kidney, and colon. Strikingly, we show that higher levels of SMCT expression in Duke C colorectal cancer samples obtained from 113 patients correlated with significantly longer disease-free survival.

Results and Discussion

SMCT Expression in Thyroid Cells Is TSH-Independent. Given the homology between SMCT and NIS and based on the experimentally tested NIS secondary structure model (Fig. L4), we have proposed one for SMCT (Fig. 1B), which predicts that SMCT traverses the membrane 13 times, its N terminus faces the extracellular milieu, and its C terminus faces the cytoplasm. Interestingly, as many as 8 of the 12 cysteines found in NIS are conserved in SMCT (at positions 130, 173, 270, 297, 308, 344, 396, and 480), and most of the NIS residues identified as critical from the study of NIS mutations that cause congenital iodide transport defect are also conserved in SMCT (Val-57, Arg-122, Thr-352, Gly-393, and Gly-536) (12–14).

Because SMCT expression was initially demonstrated in the thyroid (5), we used our affinity-purified anti-SMCT Abs against the last 17 amino acid residues of mouse SMCT to probe membrane fractions from FRTL-5 cells, a line of highly functional rat thyroid cells (15). These Abs exhibited a very high affinity for SMCT (Kd = 10 nM; data not shown). Immunoreactivity was observed against a single, broad ~75-kDa polypeptide (Fig. 2A, left lane), whose electrophoretic migration was slower than that predicted by the secondary structure model (Fig. 1A). We confirmed this prediction by demonstrating that treatment of the membrane fractions with peptide N-glycosidase F, an enzyme that removes N-linked carbohydrates, caused SMCT to migrate as an ~60-kDa polypeptide (Fig. 2A, right lane).

The predicted cytosolic orientation of the SMCT C terminus (Fig. IB) was demonstrated by detecting immunofluorescence in permeabilized (Fig. 2B Center) but not in nonpermeabilized FRTL-5 cells (Fig. 2B Left). There was no immunoreactivity when only the secondary Ab was added (Fig. 2B Right). Further confirmation was obtained by flow cytometry (Fig. 2C): a fluorescence shift was observed only in permeabilized cells, where the Ab had access to the epitope (Fig. 2C Right).

Because NIS expression and targeting to the plasma membrane...
are regulated by TSH in thyroid cells (16), we examined whether TSH had similar effects on SMCT. TSH was withdrawn from the FRTL-5 cell culture medium for 7 days, and SMCT expression was assessed by immunoblotting the FRTL-5 membrane fractions. In marked contrast to NIS (Fig. 2D Top), SMCT was expressed in both the presence and absence of TSH (Fig. 2D Middle). Cell surface biotinylation revealed that SMCT plasma membrane targeting was also not regulated by TSH (Fig. 2D Bottom).

An Endogenous Na⁺-Dependent Monocarboxylate Transport System, Likely to Be SMCT, Is Present in Thyroid Cells. We examined the monocarboxylate transport properties of FRTL-5 cells by using 

[14C]nicotinate as a substrate. We chose nicotinate because, unlike other monocarboxylates, it is not transported by monocarboxylate transporter 1 (MCT1), a different protein expressed in most cell types, including FRTL-5 cells (17, 18). In contrast to SMCT activity, MCT1-mediated monocarboxylate transport is not Na⁺-dependent, but its activity is large enough to interfere with measurements of SMCT-mediated transport. Although derivatives of α-cyanoenaminamidase such as α-cyano-4-hydroxycinnamate (CHC) are considered specific inhibitors of MCT1, these compounds also block the mitochondrial pyruvate transporter and the anion exchanger AE1 (18), limiting their usefulness. When SMCT was expressed in oocytes, modest inhibition of propionate-evoked currents in the presence of 1 mM CHC was observed (6). Further, in our experiments, CHC moderately inhibited the Na⁺-dependent components of monocarboxylate uptake (not shown). Thus, to prevent MCT1-mediated activity from being factored in, we used 50 μM [14C]nicotinate as a substrate in steady-state transport assays in FRTL-5 cells maintained in the presence or absence of TSH for 7 days. [14C]Nicotinate uptake was clearly Na⁺-dependent and displayed very similar characteristics in the presence and absence of TSH (Fig. 2E). Determination of kinetic parameters for [14C]nicotinate uptake was performed at concentrations from 4.5 to 600 μM. The data displayed typical Michaelis–Menten behavior with $K_m = 53 \pm 5.8$ μM and $V_{max} = 72 \pm 7.3$ pmol/μg of DNA per 2 min in the presence and absence of TSH (Fig. 2F). The Eadie–Hofstee transformation showed linearity of data, compatible with the presence of a single transport system (not shown). In conclusion, these findings demonstrate the existence of an endogenous high-affinity Na⁺-dependent monocarboxylate uptake system in thyroid cells not regulated by TSH, characteristics that make it likely for this system to be SMCT. Further studies are necessary to reveal the specific physiological role of SMCT in the thyroid.

Fig. 2. SMCT protein expression and function in FRTL-5 cells. (A) Immunoblot analysis of membrane fractions from FRTL-5 cells (~60 μg of protein) incubated either with or without peptide $N$-glycosidase F overnight at 37°C, electrophoresed, and immunoblotted with anti-mouse SMCT Ab. (B) Indirect immunofluorescence of FRTL-5 cells with anti-mouse SMCT Ab followed by fluorescein-conjugated anti-rabbit IgG. (Center) Nonpermeabilized conditions. (Left) Permeabilized with 0.1% Triton X-100. (Right) Without primary Ab. (C) FACS analysis of nonpermeabilized (Left) and permeabilized (Right) FRTL-5 cells with anti-SMCT Ab. (D) Membrane fractions (20 μg) from FRTL-5 cells grown in the presence or absence of TSH were electrophoresed and immunoblotted with either anti-rat NIS (Top) or anti-mouse SMCT Abs (Middle). (Bottom) Immunoblot analysis of biotinylated cell surface polypeptides with anti-mouse SMCT Ab. (E) [14C]Nicotinate steady-state uptake in FRTL-5 cells in the presence or absence of TSH and in the presence of Na⁺ (shaded bar) or choline (open bar). (F) Kinetic analysis of [14C]nicotinate uptake in FRTL-5 cells in the presence (continuous line) or absence (broken line) of Na⁺.

SMCT Is Not Inhibited by High Concentrations of Short-Chain Fatty Acids (SCFAs). We analyzed SMCT expressed in X. laevis oocytes during injection of SMCT cRNA by using the two-microelectrode voltage clamp technique (19, 20). When SMCT-expressing oocytes were placed in a NaCl buffer and voltage-clamped at −50 mV, the holding current was recorded when 1 mM propionate was added to the bath (Fig. 3A). In agreement with previous results (6, 7, 9), the addition of propionate caused an inward positive current of ∼5–70 nA, indicating a net transfer of positive charge into the oocyte, attributable to SMCT activity (Fig. 3A Right). Thus, SMCT-mediated propionate transport is electrogenic, i.e., it involves the translocation of at least two Na⁺ ions per propionate anion. When propionate was added in the absence of external Na⁺ in the medium (choline was used to replace Na⁺), no inward current was evoked, indicating that SMCT-mediated transport is completely Na⁺-dependent (Fig. 3A Center); in control water-injected oocytes, propionate at concentrations up to 7.5 mM did not induce an inward current (Fig. 3A Left). Therefore, the observed currents were generated by SMCT activity. We then investigated the transport of several monocarboxylates (1 mM) besides propionate: nicotinate, L- and D-lactate, pyruvate, butyrate,
Functional properties of SMCT in *X. laevis* oocytes. The membrane potential was clamped at −50 mV. (A) Propionate (1 mM) did not evoke a current in control oocytes (Left), whereas in SMCT-expressing oocytes, 1 mM propionate evoked an inward current that was 100% Na⁺-dependent (Center and Right). (B) Currents evoked by various substrates (1 mM) were normalized to the current elicited by 1 mM propionate in the same oocyte (n = 3). (C) Kinetic analysis of propionate transport (n = 3). (D) The pH dependence of propionate-evoked inward currents was examined in the range of 5–9. At each pH, the current evoked by 1 mM propionate was normalized with respect to that elicited at pH 7.4. (E) Application of ibuprofen alone (up to 2 mM) did not alter the holding current. When applied in the presence of propionate, ibuprofen inhibited the 1 mM propionate-evoked current in a dose-dependent manner. (F) Ibuprofen inhibited the propionate-evoked current with a *K*ᵢ of 73 ± 9 μM (n = 3). (G) Kinetic analysis of nicotinate transport.

We also examined SMCT activity as a function of extracellular pH (ranging from 5 to 9). At each pH, the propionate-evoked current was normalized to that elicited at pH 7.4 in the same oocyte. At pH values lower than 6.5, transport activity decreased with decreasing pH; however, peak SMCT activity was virtually identical in the pH range 6.5–9.0 (Fig. 3D), unlike the reported narrow pH dependence of mouse SMCT (9). The nonsteroidal antiinflammatory drug ibuprofen inhibited the propionate-evoked current with a *K*ᵢ value of 73 ± 9 μM (Fig. 3E and F). Further, kinetic analysis of human SMCT (hSMCT)-mediated nicotinate transport revealed a *K*ᵢ value of 390 ± 36 μM (Fig. 3G), in agreement with Gopal et al. (8) (*K*ᵢ = 296 ± 88 μM) for murine SMCT, whereas in rat FRTL-5 cells the *K*ᵢ for nicotinate was ∼5- to 6-fold higher (Fig. 2F), probably because of species differences. Interestingly, in FRTL-5 cells 500 μM ibuprofen completely inhibited the Na⁺-dependent component of [14C]nicotinate uptake as well (not shown).

**SMCT Is Localized on the Apical Surface of Thyroid, Colon, and Kidney Epithelial Cells.** Because SMCT exon 1 was reported to be hypermethylated in colon cancer (11), and SMCT transports SCFAs, which are the major energy source of colonocytes (21), we investigated the cellular and subcellular localization of SMCT in human and rat colon tissues. When extracts from human colon samples were probed, an ∼75-kDa polypeptide was detected (Fig. 4A, left lane), whose electrophoretic migration was slower than that predicted by the hSMCT molecular mass (∼67 kDa). During treatment with peptide *N*-glycosidase F, the polypeptide migrated at an apparent molecular mass of ∼65 kDa (Fig. 4A, right lane), indicating that SMCT is glycosylated in human colon. Two bands were detected by immunoblot analysis of rat colon tissue: a broad ∼85-kDa one and a narrow ∼54-kDa one, corresponding to the maturely and immunochemically glycosylated polypeptides, respectively (Fig. 4B Upper). The immunochemically glycosylated precursor becomes detectable when fractions are enriched for integral membrane proteins by alkaline extraction (22). Immunohistochemistry analysis revealed that SMCT expression was restricted to the apical surface of the colonocytes and was absent from the surrounding goblet cells and underlying tissue layers (Fig. 4B Lower). This finding is consistent with the notion that SMCT mediates the translocation of SCFAs from the colonic lumen to the epithelial cells. SMCT migrated as an ∼75–80-kDa band in immunoblot analyses of rat kidney and thyroid (Fig. 4C and D). SMCT was also expressed apically in the brush border of renal proximal tubules (Fig. 4C Lower) and to a lesser extent, but still apically, in the distal parts of...
the nephron. In the thyroid, as reported in ref. 5, SMCT was expressed apically as well (Fig. 4D Lower).

**SMCT Is Properly Targeted to the Apical Surface in Polarized Madin–Darby Canine Kidney (MDCK) Cells.** MDCK cells have been extensively used to investigate polarized membrane protein targeting (23). We generated MDCK cells stably expressing functional hSMCT. SMCT expression was analyzed by FACS, followed by immunoblot analysis of membrane fractions from FACS-positive clones. An MDCK clone moderately expressing hSMCT was selected for further analysis; immunoreactivity against an 84-kDa polypeptide from this clone is shown in Fig. 5A. We assessed the functionality of SMCT in this system by conducting steady-state and kinetic analyses of [14C]nicotinate uptake. The MDCK hSMCT clone transported ~9 times more [14C]nicotinate than nontransfected MDCK cells (Fig. 5B) with a Km of 28 ± 83 μM. Because SMCT was initially reported to be an I– transporter, we examined whether SMCT-expressing MDCK cells translocate I–. Steady-state I– uptake experiments at saturating (20 μM, not shown) and supersaturating (80 μM) I– concentrations were conducted in MDCK cells stably expressing NIS (24) or SMCT (Fig. 5C). As expected, NIS-expressing MDCK cells displayed perchlorate-sensitive I– uptake. In contrast, SMCT-expressing MDCK exhibited no I– transport, further confirming previous reports and our own observations that SMCT does not mediate I– transport (Fig. 5C).

We analyzed SMCT polarized targeting by immunofluorescence directly on filter membranes. Monoclonal antibodies against gp135 and the Na+/K+-ATPase were used to monitor apical and basolateral markers, respectively (25). The en face (xy) view revealed clear apical localization of SMCT and gp135 (Fig. 5 D1 and D2). Cross sections in the xz direction confirmed this finding (Fig. 5 D4 and D5). The apical distribution of SMCT was confirmed by colocalization with gp135 (Fig. 5 D3 and D6).

**Higher Expression of SMCT in Duke C Human Colorectal Cancer Correlates with Longer Disease-Free Survival.** Because no high-affinity anti-SMCT Abs were available, other investigators had examined SMCT expression only at the transcriptional level (8, 9, 11). Using our Abs, we analyzed SMCT protein expression in Duke C (locally advanced lymph node-positive) human colon cancer samples (tumoral and paired peritumoral normal mucosa) by immunoblotting. Remarkably, SMCT was absent or markedly down-regulated in tumor tissue (Fig. 6A and B, asterisks) compared with adjacent normal mucosa in 14 of 15 pairs examined. Equal loading for each tissue pair was determined with monoclonal anti-β-tubulin (Fig. 6A) or anti-β-actin Abs (Fig. 6B). These samples, obtained from patients treated in New York City, represented a heterogeneous histopathological population (ranging from poorly to moderately differentiated adenocarcinomas). There was no correlation between the differentiation status of the tumor and the down-regulation of SMCT protein levels.

To extend our findings, we assessed SMCT expression by immunohistochemistry in tumor samples from colorectal cancer patients. To control for possible confounding factors affecting patient survival, all 113 analyzed tumors were stage Duke C, and cases with microsatellite instability were excluded. SMCT expression was evaluated in a tissue microarray-based immunohistochemical assay in triplicate and scored blindly on a scale from 0 (no staining) to 4 (strong staining) (Fig. 6C). The average score of triplicates was used in the following analyses. Tumor samples with staining intensities of 1.7 or less (75 of 113) were considered low-SMCT-expressing, whereas those with staining intensities higher than 1.7 (38 of 113) were considered high-SMCT-expressing: 66.4% of the tumors expressed low levels of SMCT. There was a significant correlation between staining levels and patient age (Spearman r = 0.27, P = 0.0018), i.e., patients with low-SMCT-expressing tumors tended to be younger. There were no correlations between SMCT expression and common genetic abnormalities linked to colorectal cancer (p53 and kras mutations or loss of heterozygosity in 18q) or other clinicopathological features [gender, grade, or tumor location (right or left colon or rectum)].

To avoid selecting a threshold arbitrarily, we arranged the 113 tumors in order of increasing SMCT expression and divided them into two groups: low- and high-SMCT expression. Disease-free survival in both groups and log-rank P values were calculated for every possible grouping resulting from increasing the number of patients allocated to the low-SMCT-expressing group from 1 to 113, starting with the patient with the lowest SMCT tumor level. Patients with low-SMCT-expressing tumors showed shorter disease-free and overall survival in 108 of 113 groupings. These differences were statistically significant (log-rank test P < 0.05) in 11 of these groupings, suggesting that low expression of SMCT is a marker of poor prognosis in Duke C colorectal cancer (Fig. 6D). On a multivariate analysis, high SMCT protein levels were a strong predictor of longer disease-free survival (P = 0.027). Further, to make sure that longer disease-free survival was not the result of postsurgical chemotherapy with 5-fluorouracil, we conducted survival analyses with 81 patients from this group who were only treated surgically. Higher SMCT protein levels remained a strong predictor for disease-free survival in these patients (Fig. 6E). This finding is significant, given that it is currently impossible to predict accurately the probability of recurrence in Duke C colorectal cancer after surgery with or without chemotherapy (26–29). Considering that SMCT expression would be easy to ascertain in colon cancer samples obtained in routine colonoscopic screenings, these findings suggest that SMCT expression may be a significant marker for lower disease recurrence in Duke C colorectal cancer.

In conclusion, we have extensively characterized the SMCT
protein at multiple levels and shown that it may play a valuable role, not previously examined, in colon cancer management.

**Methods**

**Cloning.** The hSMCT cDNA was cloned from the Human Thyroid Gland Marathon-Ready cDNA (BD Biosciences Clontech). The primers used to amplify SMCT cDNA were 5’-GTGTCTCATCTGCTCAGGTTGCC and 5’-GCCGTGATTTAGCCTTTCAGCAT. The PCR parameters were 35 cycles of 95°C for 30 s, 53°C for 30 s, and 68°C for 2.5 min, followed by 68°C for 10 min, and 4°C to cool. The amplified cDNA was digested with BamHI and EcoRI and ligated into the pcDNA3 vector (Invitrogen). Sequencing primers were T7 promoter primer 5’-TACGGTCTCAAC, and 5’-CTCAGGTGTCC and 5’-CGCAGATTGACCGCAGTG, 5’-GCCGTGATTTAGCCTTTCAGCAT.

**Generation of Anti-SMCT Abs.** High-affinity site-directed polyclonal Abs against the C-terminal sequences of the rodent (amino acids 596–611, VELNFTHDSHKINGTRL) and human (amino acids 591–610, AFNHIELNSDQSGKSNGTRL) SMCT proteins were generated. Purification was performed as described in ref. 30.

**Cell Culture.** FRTL-5 rat thyroid cells were cultured as described in ref. 16. MDCK II cells were maintained in DMEM (Invitrogen) supplemented with 10% FBS (Gemini BioProducts, West Sacramento, CA), 1% glutamine, and 1% penicillin/streptomycin (Invitrogen) at 37°C in a 95% air/5% CO₂ atmosphere. For polarized culture, stably transfected MDCK cells or nontransfected MDCK cells were plated on 12-mm (1-cm² area) Transwell polyester filter units (0.4-µm pore size; Costar) at a density of 150,000 cells per filter unit and cultured for 5 days to allow development of polarity.

**Colon Tumor Extract and Membrane Fraction Preparation.** Human colorectal tumors with paired adjacent normal tissue were collected after surgical resection from patients who consented according to institutional guidelines; the samples were immediately snap-frozen in liquid nitrogen. Proteins were extracted by brief homogenization on ice in lysis buffer (50 mM Tris-HCl, pH 7.5/150 mM NaCl/1% Nonidet P-40/0.5% sodium deoxycholate/1 mM EDTA/protease inhibitor mixture) followed by incubation for 30 min at 4°C with gentle agitation. After incubation, samples were sonicated for 10 s and centrifuged at 14,000 × g for 10 min. Membrane fractions were prepared as described in ref. 22.

**Deglycosylation Assays.** Membrane protein fractions or tissue extracts were deglycosylated with peptide N-glycosidase F (PROzyme, San Leandro, CA) and subjected to immunoblot analysis as described in ref. 31.

**Immunoblot Analysis and Cell-Surface Biotinylation.** These procedures were performed as described in ref. 22 with 13 nM affinity-purified anti-mouse SMCT Ab, 7 nM affinity-purified anti-hSMCT Ab, and 4 nM affinity-purified anti-rat NIS Ab for 1 h and monoclonal anti-β-actin 1:10,000 (Sigma) or anti-β-tubulin 1:3,000 Ab (Sigma).

**Immunofluorescence and Immunohistochemical Analyses.** FRTL-5 cells were seeded onto polylysine-coated coverslips. Cells were incubated with 70 nM primary Ab against mouse SMCT in PBS containing 1 mM CaCl₂ and 0.1 mM MgCl₂ (PBS/CM) with 0.2% BSA and 0.1% Triton X-100 for 1 h and processed as described in ref. 14. For immunohistochemistry, 5-µm colon tissue sections were deparaffinized and rehydrated and processed as described, with affinity-purified anti-mouse-SMCT Ab at 7 nM final concentration (32).

**Immunofluorescence Microscopy of Polarized MDCK Cells.** MDCK cell monolayers were fixed in 2% freshly prepared paraformaldehyde in PBS for 20 min at room temperature and quenched with 50 mM NH₄Cl in PBS/CM. For permeabilization, methanol at −20°C or 1% saponin was used. Cells were blocked in PBS/CM containing 10% goat serum (Invitrogen). The primary Ab used was a mAb.
against the ectodomain of gp135 (1:50 dilution; kindly provided by E. Rodriguez-Boulan). Secondary Abs used were Alexa 488-tagged anti-rabbit IgG and Alexa 568-tagged anti-mouse IgG. Images were obtained on a Radiance 2000 laser scanning confocal microscope (Bio-Rad, Hercules, CA) in a Z-series (top to bottom) were collected and analyzed with IMAGEJ software (National Institutes of Health) and PHOTOSHOP 5.0 (Adobe, San Jose, CA). Images presented here show individual confocal x-section and the entire x-section.

Transport Assays. [14C]Nicotinate was obtained from Moravek Biochemicals (Brea, CA). Cells grown in 12-well plates were incubated with buffered Hanks’ balanced salt solution containing 50 μM [14C]nicotinate and processed as described in ref. 1. Results are the average of at least three separate experiments performed in triplicate. For kinetic analysis, cells were incubated for 2 min with 4.5–600 μM [14C]nicotinate. Initial-rate data were analyzed by a nonlinear regression by using the following equation for [14C]nicotinate-dependent [14C]nicotinate uptake:

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v = V_{\text{max}}[\text{[14C]nicotinate}]/(K_m + [\text{[14C]nicotinate}]) + 0.07[\text{[14C]nicotinate}]/0.41.
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[1] The terms 0.07[14C]nicotinate + 0.41 correspond to background adjusted by the lowest level of data obtained with FRTL-5 cells in the presence of choline. Data were fitted by nonlinear least squares with the Marquard–Levenberg algorithm (33). Data were analyzed with the hazard ratio and the log-rank test (26). The Cox proportional-hazards model was used to assess the simultaneous contribution of the following covariates: sex, age, grade, tumor location (right/left colon), and SMCT protein tumor levels. A P value of <0.05 was considered to indicate statistical significance.

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