Hypoxic Culture Induces Expression of Sialin, a Sialic Acid Transporter, and Cancer-Associated Gangliosides Containing Non–Human Sialic Acid on Human Cancer Cells

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Abstract

Tumor hypoxia figures heavily in malignant progression by altering the intracellular glucose metabolism and inducing angiogenic factor production, thus, selecting and expanding more aggressive cancer cell clones. Little is known, however, regarding hypoxia-induced antigenic changes in cancers. We investigated the expression of *N*-glycolyl sialic acid (NeuGc)-G_{M2}, a cancer-associated ganglioside containing non–human sialic acid, NeuGc, in human cancers. Cancer tissues prepared from patients with colon cancers frequently expressed NeuGc-G_{M2}, whereas it was virtually absent in nonmalignant colonic epithelia. Studies on cultured cancer cells indicated that the non–human sialic acid was incorporated from culture medium. Hypoxic culture markedly induced mRNA for a sialic acid transporter, sialin, and this accompanied enhanced incorporation of NeuGc as well as *N*-acetyl sialic acid. Transfection of cells with sialin gene conferred accelerated sialic acid transport and induced cell surface expression of NeuGc-G_{M2}. We propose that the preferential expression of NeuGc-G_{M2} in cancers is closely associated with tumor hypoxia. Hypoxic culture of tumor cells induces expression of the sialic acid transporter, and enhances the incorporation of non–human sialic acid from the external milieu. A consequence of this is the acquisition of cancer-associated cell surface gangliosides, typically G_{M2}, containing non–human sialic acid (NeuGc), which is not endogenously synthesized through CMP-*N*-acetyl sialic acid hydroxylase because humans lack the gene for the synthetic enzyme. As hypoxia is associated with diminished response to radiotherapy and chemotherapy, NeuGc-G_{M2} is a potential therapeutic target for hypoxic cancer cells. (Cancer Res 2006; 66(6): 2937-45)

Introduction

Hypoxia is a common characteristic of locally advanced tumors, and emerging evidence indicates that it has a profound effect on malignant progression and responsiveness to therapy (1–3). Tumor hypoxia causes clonal selection and expansion of cancer cells through somatic mutations, which can adapt and overcome the oxygen-deficient environment. Such advanced cancer cells have more aggressive properties such as higher invasive and metastatic activities, and they are known to enhance tumor angiogenesis by secreting vascular endothelial growth factor; they also undergo a metabolic shift to anaerobic glycolysis by inducing several intracellular glycolytic enzymes and glucose transporters (known as the Warburg effect). Because the hypoxia-resistant cancer cells are less susceptible to radiotherapy and chemotherapy, it is of particular importance to find a cell surface antigen selectively expressed on the hypoxia-adapted cancer cells, which could be used as a target for cancer therapy.

Only recently, hypoxia-induced cancer progression has been noted to affect the expression of cell surface antigens. We previously reported that hypoxic culture of tumor cells has a profound effect on the cell surface expression of cancer-associated carbohydrate determinants including sialyl Lewis A and sialyl Lewis X (4–6). In the present article, we show that hypoxic culture of tumor cells induces a unique cell surface ganglioside antigen, which would be useful for the immunotherapy of cancers.

It is well known that carbohydrate determinants undergo drastic changes during the course of malignant transformation (7, 8). Carbohydrate determinants which preferentially appear on tumors are used for the diagnosis and trial therapy of cancers. The ganglioside, G_{M2}, has long been known to be expressed on various cancers (9, 10). G_{M2}-KLH conjugates (refs. 11–14; reviewed in refs. 15, 16) and humanized anti-G_{M2} antibodies (17–20) are now under clinical trial. Preliminary results on G_{M2} vaccine therapy trial indicated that it would be beneficial, if the immune reaction directed to the determinant is elicited (11, 12, 21, 22).

G_{M2} ganglioside, as well as other monosialogangliosides in humans, contains one *N*-acetyl sialic acid (NeuAc) residue. On the other hand, non–human sialic acid, *N*-glycolyl sialic acid (NeuGc), is also known to preferentially appear on cancer cells (23–26). Carbohydrate determinants containing *N*-glycolyl sialic acid are synthesized by CMP-NeuAc hydroxylase (27, 28), which is active in cells and tissues of various animals except humans. In humans, the gene for the enzyme is nonfunctional because of a partial deletion in the sequence (29–31); hence, human cells and tissues lack *N*-glycolyl sialic acid. Many human cancers, however, are known to frequently contain carbohydrate determinants having NeuGc (32), which is known to be immunogenic in humans. It is designated as the Hanganutziu-Deicher (H-D) antigen, and is regarded as a good target for vaccination therapy of cancers (33, 34).

These findings suggest that G_{M2} containing NeuGc can be expected to have a higher cancer specificity than conventional G_{M2} which contains NeuAc. In this article, we investigated if

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NeuGc-containing G_{M2} is expressed in human cancer cells, and whether tumor hypoxia is involved in its expression.

Materials and Methods

Cells, antibodies, hypoxic culture, and flow cytometric analysis. LS174T cells were obtained from Tohoku University and Caco-2 and ZR-75-1 cells were from the American Type Culture Collection (Manassas, VA). These cells were maintained in DMEM supplemented with 10% FCS. In some experiments, chicken serum (JRH Bioscience, Lenexa, KS) or human serum AB (Gemini Bio-Products, Woodland, CA) was used instead of FCS. In some other experiments, cells were cultured in the chemically defined medium CHO-S-SFM-II (Life Technologies, Grand Island, NY). Free NeuAc was obtained from Nacalai Tesque (Kyoto, Japan), and NeuGc was prepared as described previously (35). For hypoxia experiments, the cells were cultured at 37°C with 5% CO_2/94% N_2/1% O_2 in a multi-gas incubator (Juji Field, Inc., Tokyo, Japan) as described previously (4).

MK1-16 (anti-NeuAc-G_{M2}, murine IgM) and MK2-34 (anti-NeuGc-G_{M2}, murine IgM) were originally generated in our laboratory (36) and distributed by Seikagaku Kogyo Inc., Tokyo, Japan. The antibody MK1-8, which is reactive to both NeuAc-G_{M2} and NeuGc-G_{M2} (murine IgM), was prepared as described previously (37). Affinity-purified rabbit anti-human sialin antibody was obtained from Alpha Diagnostic International (San Antonio, TX), and a monoclonal anti-human β-actin antibody clone AC-15 (mouse IgG_1) was from Sigma (St. Louis, MO). Anti-CD31 (PCAM-1) antibody (JC70A, mouse IgG_1) was from DAKO (Glostrup, Denmark).

Caco-2M cells were prepared from Caco-2 cells by magnetic cell sorting with MACS (Miltenyi Biotec GmbH, Gladbach, Germany) using anti-NeuAc-G_{M2} antibody MK1-16. Briefly, washed Caco-2 cells were suspended in 500 μL of PBE buffer, followed by the addition of MK1-16 antibody and incubation on ice for 15 minutes. After washing, cells were suspended in 800 μL PBE buffer, supplemented with 200 μL rat anti-mouse IgM microbeads (Miltenyi), and incubated on ice for 10 minutes. The magnetically labeled cells were separated on a MACS separation column (Miltenyi), which was placed in the magnetic field of a magnetic cell separator (Vario MACS, Miltenyi). Cells strongly expressing NeuAc-G_{M2} were thus enriched and designated as Caco-2M cells.

For flow cytometric analysis, cells were harvested at a semiconfluent stage and stained with the respective antibody using purified antibody (1.0 μg/mL) at 4°C for 30 minutes. The cells were then washed twice with PBS containing 2% FCS and stained with a 1:200 dilution of FITC-conjugated goat anti-mouse immunoglobulin (Silenus Laboratories, Hawthorn, Victoria, Australia) at 4°C for 30 minutes. Binding of the antibodies to the cells was evaluated by flow cytometry done with FACSCalibur (Becton Dickinson, Mountain View, CA).

HPLC analysis of NeuGc and NeuAc in cultured cells. Cells were harvested by cell scraper and washed twice with cold PBS buffer. The cell pellets were sonicated with a sonic dismembrator (Artek Systems Corp., Farmingdale, NY) in 400 μL PBS buffer on ice. The sonicate was centrifuged at 100,000 × *g* for 1 hour, and the supernatant was then removed to another tube for free-sialic acid analysis of cells. PBS (200 μL) was added to the pellet, which was sonicated and again centrifuged at 100,000 × *g*. The resulting pellet, called the "membrane-bound" fraction, was used for HPLC analysis. The bound sialic acid was hydrolyzed using a final 0.1 N trifluoroacetic acid at 80°C for 2 hours, and the residue was dried on a centrifugal evaporator (EYELA, Tokyo, Japan). The released sialic acids were labeled with 1,2-diamino-4,5-methylene dioxybenzene (DMB; Dojindo, Tokyo, Japan) at 50°C for 2 hours (38, 39). DMB-sialic acid derivatives from different fractions were then analyzed by HPLC using octadecyl silane column (Wakopak, Wako, Osaka, Japan), and eluted with 7% methanol and 9% acetonitrile in water at 1.0 mL/min flow. The eluate was monitored at an emission wavelength of 448 nm and excitation wavelength of 373 nm (38). For all HPLC chromatograms, sialic acids were quantified by comparison with known quantities of DMB-NeuAc/Gc derivatives used as standards, and the concentrations were calculated. A bicinchoninic acid protein assay kit was used for protein quantification and was done according to the manufacturer's instructions (Pierce, Rockford, IL).

Uptake and kinetic analysis of the incorporation of radiolabeled NeuAc and NeuGc. Caco-2 cells cultured under normoxic or hypoxic conditions for the indicated days were harvested and washed with cold PBS buffer twice. The cells were incubated in a six-well plate in 2 mL PBS buffer containing 5 μL of [^{3}H]NeuAc (1 mCi/mL, American Radiolabeled Chemicals, Inc., St. Louis, MO) and [^{14}C]NeuGc at room temperature for 30 minutes. For kinetic analysis experiments, the desired concentrations of nonradioactive NeuAc or NeuGc were also added to the cells. After washing twice with 2 mL PBS, cells were dried and radioactivity was counted in vials containing 5 mL scintillator (Wallac, OptiPhase "SuperMix," United Kingdom) using a liquid scintillation counter (Beckman, United Kingdom). For preparation of [1-^{14}C]NeuGc, [1-^{14}C]pyruvic acid sodium salt (Amersham Biosciences Co., Piscataway, NJ) was reacted with *N*-glycoyl mannosamine in the presence of sialic acid aldolase (Toyobo Co., Tokyo, Japan) in 50 mmol/L sodium phosphate (pH 7.4) at 37°C. After 18 hours, the reaction mixture was subjected to acetylation and purified to give peracetylated [1-^{14}C]NeuGc. The compound was then deprotected under basic conditions to give the final product, [1-^{14}C]NeuGc.

RT-PCR analysis and Western blotting. Cellular total RNA was extracted using Isogen (Wako). According to the instruction manual of SuperScript First-Strand Synthesis System for RT-PCR kit (Invitrogen, Carlsbad, CA), reverse transcription into cDNA was done using 1 μL oligo(dT)$_{12\text{-}18}$ as the initiation primer and preincubated with 1 μL of a 10 mmol/L deoxynucleotide triphosphate mix at 65°C for 5 minutes and placed on ice for at least 1 minute. The synthesis was achieved using SuperScript II RT at 42°C for 50 minutes and terminated at 70°C for 15 minutes in a final reaction volume of 21 μL. One microliter of the retrotranscription reaction was subjected to PCR amplification using specific primers. RT-PCR analysis was done using HotAtarTaq PCR kit (Qiagen, Valencia, CA). The primers, cycle numbers, and T_m for RT-PCR analysis used in this study were: 5′-GATTTGGGATCCTTGGCACTG-3′ and 5′-GTACCCACGGCACTGACTTC-3′ (28 cycles, 59°C) for sialin (SLC17A5), which give a 432-bp product; 5′-AGTAACCACCAAGTACGTGCTGTG-3′ and 5′-GTGTTTGAAGAAGAGCAGCCGGTG-3′ (40 cycles, 63°C) for β4GalNAc-T, which give a 538-bp product; 5′-GACCCTCTTGGAACTCTTGCC-3′ and 5′-CCAAACTGACTTCATCGCACA-3′ (30 cycles, 59°C) for ST3Gal-V, which give a 560-bp product; 5′-TTGGGAGAAGGACAACCTTC-3′ and 5′-CCAGGCAGCAACAGACAGTA-3′ (28 cycles, 55°C) for ST3Gal-VI, which give a 648-bp product; 5′-TCATCGTGGCTGAACTCTTCAG-3′ and 5′-TCACACTTGGGAATCAGCCCC-3′ (22 cycles, 60°C) for GLUT1, which give a 314-bp product; and 5′-AAGGTCATCCATGACAAC-3′ and 5′-CACCCTGTTGCTGTAGCCA-3′ (22 cycles, 57°C) for G3PDH, which give a 489-bp product. A reaction without cDNA or reverse transcriptase product was done as negative control to exclude the possibility of amplification of contaminating genomic DNA. Aliquots of each reaction were fractionated by electrophoresis through 2% agarose gels including ethidium bromide. After electrophoresis, the intensities of the bands were monitored by printgraph (Atto Bioinstrument, Tokyo, Japan).

For Western blotting, cells were harvested, washed twice with cold PBS, and cell pellets were added to the lysis buffer, containing 40 mmol/L HEPES (pH 7.4), 150 mmol/L NaCl, 0.5% sodium deoxycholate, 1% NP40, 0.1% SDS, and Complete Mini Protease Inhibitor Cocktail (Roche, Mannheim, Germany). The solution was incubated on ice for 30 minutes. After centrifugation, the supernatant was transferred to another tube, and sample buffer solution with 2-ME for SDS-PAGE (Nacalai Tesque) was added, and then incubated at 100°C for 10 minutes. Protein (10 μg) was loaded per lane, electrophoretically separated on SDS-polyacrylamide gel and transferred to Immobilon-P Transfer Membrane (Millipore Corporation, Bedford, MA). Transferred membrane was incubated with blocking buffer containing 3% bovine serum albumin in TBS-T buffer at 4°C for 4 hours, and was stained with anti-human sialin or anti-human β-actin antibody at room temperature for 1 hour. Horseradish peroxidase–conjugated anti-mouse or anti-rabbit IgG antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were used at a dilution of 1:5,000 in TBS-T buffer containing 1% BSA. After washing with TBS-T, protein bands were visualized with enhanced chemiluminescence Western blotting detection reagents (Amersham Biosciences, Buckinghamshire, United Kingdom) and exposed to high-performance

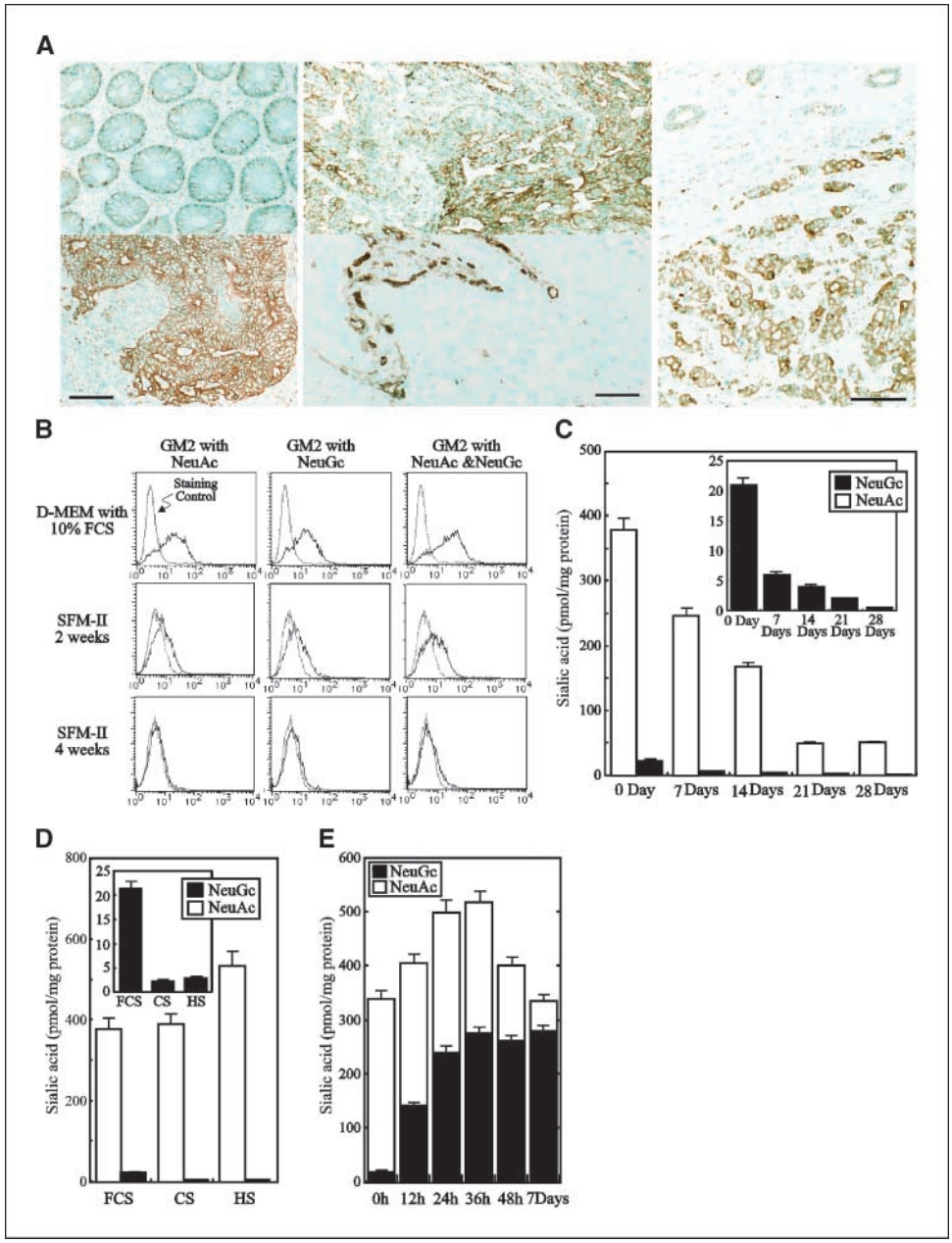

Figure 1. Surface expression of NeuGc-G_{M2} and presence of membrane-bound NeuGc in cultured human colon cancer cells, LS174T, and its dependence on culture medium. *A,* expression of NeuGc-G_{M2} in human cancer tissues. *Left,* immunohistochemical staining with the anti-NeuGc-G_{M2} antibody (MK2-34) of normal colonic epithelial cells (*top*) and cancer cells (*bottom*) in tissue sections prepared from a patient with colon cancer. *Middle,* consecutive sections of a colon cancer tissue stained with the MK2-34 antibody (*top*) and anti-CD31 antibody (*bottom*). *Right,* immunohistochemical staining of breast cancer tissue with the MK2-34 antibody. *Bar,* 100 μm. *B,* flow cytometry of LS174T cells cultured in DMEM supplemented with 10% FCS and in chemically defined medium CHO-S-SFM-II (*SFM-II*). NeuAc-G_{M2} was detected with monoclonal antibody MK1-16, NeuGc-G_{M2} with MK2-34. The antibody MK1-8, which reacts to both species of G_{M2}, was used as a control. *C,* time course of the decrease of membrane-bound NeuAc and NeuGc in the LS174T cells cultured in chemically defined SFM-II medium as ascertained by HPLC analysis. *D,* HPLC-analysis of membrane-bound NeuAc and NeuGc contents in the LS174T cells cultured in DMEM with 10% of FCS, chicken serum (*CS*), and human serum (*HS*). *E,* time course of the change in membrane-bound NeuAc and NeuGc contents in the LS174T cells cultured in DMEM + 10% FCS, supplemented with 4 mmol/L free NeuGc, as analyzed by HPLC. *D* and *E,* the NeuGc levels with expanded ordinates (*insets*). *C,* representative of two similar experiments; *D* and *E,* representative of three independent experiments.

chemiluminescence film of Hyperfilm enhanced chemiluminescence (Amersham Biosciences).

Immunohistochemical examination of human cancer tissues. Frozen sections of 10-μm thickness were prepared from surgical specimens obtained from 16 patients with colorectal cancer who underwent an operation at the Aichi Cancer Center Hospital. The avidin-biotin complex technique for the immunohistochemical examination was done as described in the instructions for the kits (Vectastain) provided by Vector, Inc. (Burlingame, CA) using MK2-34 antibody as the first antibody. For confocal microscopic analysis, polyclonal rabbit anti-human sialin and monoclonal anti-CD31 were used as primary antibodies in addition to MK2-34. Goat anti-rabbit IgG, anti-murine IgG_1, and anti-murine IgM antibodies labeled with Alexa Fluor 488 or 594 (Molecular Probes, Inc., Eugene, OR) were used as secondary antibodies. An inverted confocal laser scanning microscope (Bio-Rad, Radiance 2100) equipped with LaserSharp 2000 software was used for observation.

Generation of cells transfected with *Cmah* and *sialin* cDNA. LS174T cells transfected with the gene for murine CMP-NeuAc hydroxylase (*Cmah*) were obtained by transfection of the cells with pcDNA3.1(+) NFLAG mHyd/*Cmah* (27) using LipofectAMINE 2000 reagent (Invitrogen), according to the manufacturer's protocol. Cells were allowed to grow for 2 days before being subjected to selection for the ability to grow in medium containing 750 μg/mL geneticin (G418-sulfate, Invitrogen). Caco-2 cells transfected with the *sialin* gene were obtained with pIRESneo3/*Sialin* on BTX Electro Cell Manipulator (Harvard Apparatus, South Natick, MA) under the conditions of 500 V and 300 μF in 400 μL PBS buffer. Human sialin cDNA in pCMV6-XL5 had been obtained from Origen Technologies, Inc. (Rockville, MD). Sialin cDNA was isolated from *Not*I and *Eco*RI sites and was ligated to the pIRESneo3 vector at *Not*I/*Eco*RI site by ligase. Cells expressing the sialin gene were selected by culture in the medium containing 400 μg/mL geneticin (G418-sulfate, Invitrogen), followed by a limiting dilution.

Results

Human cancer tissues and cultured cell lines express cancer-associated G_{M2} ganglioside containing non–human *N*-glycolyl sialic acid. When surgical specimens from patients

with colon cancers were tested for the expression of NeuGc-G_{M2}, 6 out of 16 samples (37.5%) were found to strongly express the determinant, and 8 out of 16 samples (50.0%) expressed it moderately or weakly. In some cancer tissues, NeuGc-G_{M2} showed patchy distribution and dependency on tumor vasculatures. An example of such cancer tissue is shown in Fig. 1*A* (*middle, top*), together with the distribution pattern of CD31 in the consecutive section (*middle bottom*), which indicates localization of blood vessels. Cancer cells distant from the blood vessels (*bottom right*) express NeuGc-G_{M2} more strongly than cancer cells in the vicinity of the blood vessels (*top left*). In some other cancer tissues, NeuGc-G_{M2} distributed homogeneously as is shown in Fig. 1*A* (*bottom left*). Nonmalignant colonic epithelial cells virtually failed to express the determinant (Fig. 1*A, top left*). Similar findings were also obtained with breast cancers, in which cancer cells strongly expressed NeuGc-G_{M2} whereas nonmalignant ductal epithelial cells did not (Fig. 1*A, right*). Among breast cancer tissue samples, 6 out of 12 (50.0%) were found to strongly express NeuGc-G_{M2}.

When cultured human cancer cell lines maintained *in vitro* were tested for expression of the NeuAc and NeuGc-containing carbohydrate determinants, several cell lines including LS174T, SW1083, IMR32 and YT cells were found to express both NeuAc-G_{M2} and NeuGc-G_{M2}, whereas several other cell lines including Caco-2 and ZR-75-1 cells express only NeuAc-G_{M2} but not NeuGc-G_{M2} (see below). Flow-cytometric analysis of a representative cell line, LS174T, is shown in Fig. 1*B*.

***N*-glycolyl sialic acid in G_{M2} ganglioside on cultured human cancer cells is acquired from the external culture medium.** Because human cells are known to lack the enzyme CMP-NeuAc hydroxylase required for the synthesis of NeuGc, the NeuGc present on cancer cells has been regarded to be acquired from the external milieu, because human cancer cells cultured *in vitro* with FCS or transplants in nude/SCID mice more frequently contain NeuGc (40–42). The expression of NeuGc-G_{M2} in LS174T cells was thought to be due to the incorporation of NeuGc from the DMEM culture medium supplemented with 10% FCS, which is known to contain free NeuGc (38).

When the LS174T cells were cultured in the chemically defined medium, CHO-S-SFM II lacking NeuGc, the expression of NeuGc-G_{M2} was time-dependently decreased as shown in Fig. 1*B*. A decreased expression of NeuGc-G_{M2} was evident after 2-week culture of the cells in serum-free CHO-S-SFM II medium, and its expression was virtually abrogated after 4-week culture. In line with this, the amount of membrane-bound NeuGc as analyzed by HPLC showed a significant decrease when cultured in the CHO-S-SFM II medium (Fig. 1*C, inset*), and fell below the detection limit after 28 days of culture. These findings were compatible with the idea that NeuGc was incorporated into the cells from the culture medium. This was further confirmed by the finding that culture of the cells in chicken or human serum, which is known to contain only NeuAc but essentially no NeuGc, also resulted in a prominent decrease of NeuGc content in the cells (Fig. 1*D, inset*). LS174T cells cultured in the CHO-S-SFM II medium supplemented with 4 mmol/L of free NeuGc acquired membrane-bound NeuGc time-dependently (Fig. 1*E*), which also confirmed the active incorporation of NeuGc from the culture medium. NeuGc comprised ~5% of the membrane-bound sialic acid in LS174T cells cultured in DMEM supplemented with 10% FCS. However, it accounted for 81.3% of the total membrane-bound sialic acid in LS174T cells cultured for 7 days in the presence of 4 mmol/L free NeuGc in the culture medium (Fig. 1*E*).

An unexpected finding was that the expression of NeuAc-G_{M2}, as well as NeuGc-G_{M2}, showed a substantial decrease along with the cell culture in the chemically defined medium (Fig. 1*B*). In HPLC analyses, the amount of membrane-bound NeuAc was also decreased time-dependently in the cells cultured in the CHO-S-SFM II medium (Fig. 1*C*). On the other hand, cells cultured in chicken or human serum, both of which are known to lack NeuGc but contain NeuAc (38), did not show any decrease in NeuAc

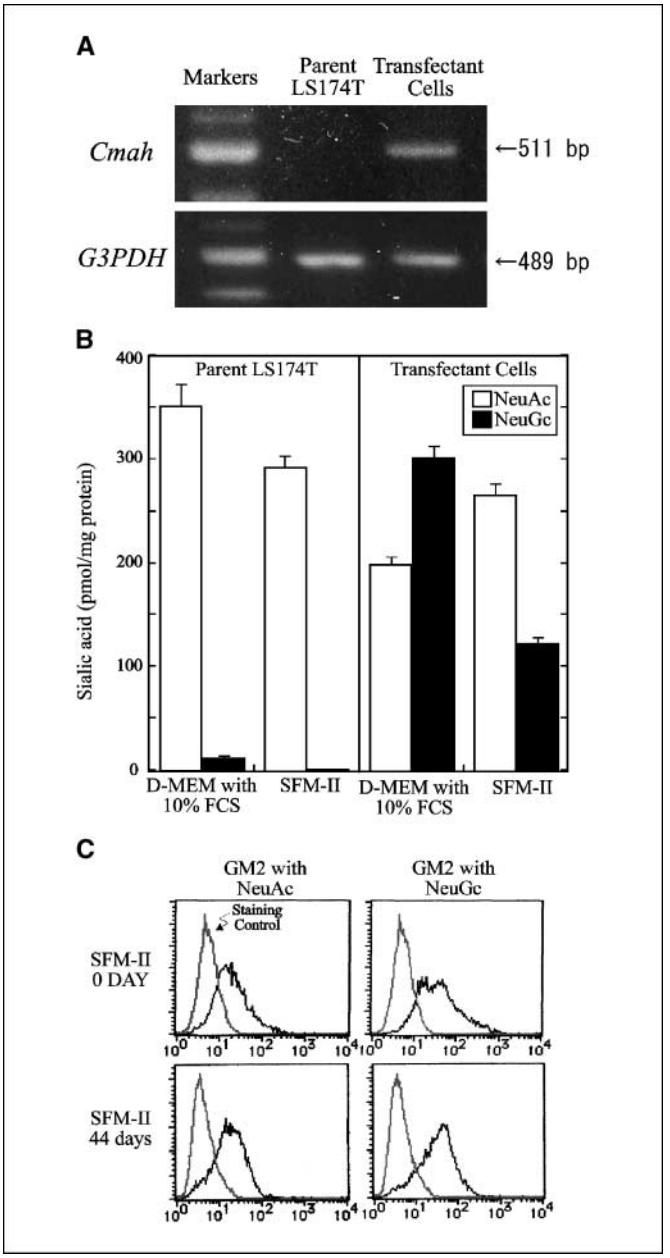

Figure 2. LS174T cells transfected with murine CMP-NeuAc hydroxylase cDNA. *A,* RT-PCR analysis of parental LS174T and transfectant cells for mRNA for murine CMP-NeuAc hydroxylase gene (*Cmah*). The primers used for *Cmah* were U: 5′-ATTTATGTTGGCGACAC-3′ and L: 5′-TAAGATTGTTGAGCTGA-3′, respectively. *B,* HPLC analysis of membrane-bound sialic acid content of parental LS174T and transfectant cells cultured in DMEM with 10% FCS or in the chemically defined medium, CHO-S-SFM-II (*SFM-II*). Representative of three similar experiments. *C,* flow cytometry of transfectant cells cultured in DMEM with 10% FCS or in chemically defined medium CHO-S-SFM-II (*SFM-II*) for 44 days. NeuAc-G_{M2} was detected with monoclonal antibody MK1-16 (murine IgM), NeuGc-G_{M2} with MK2-34.

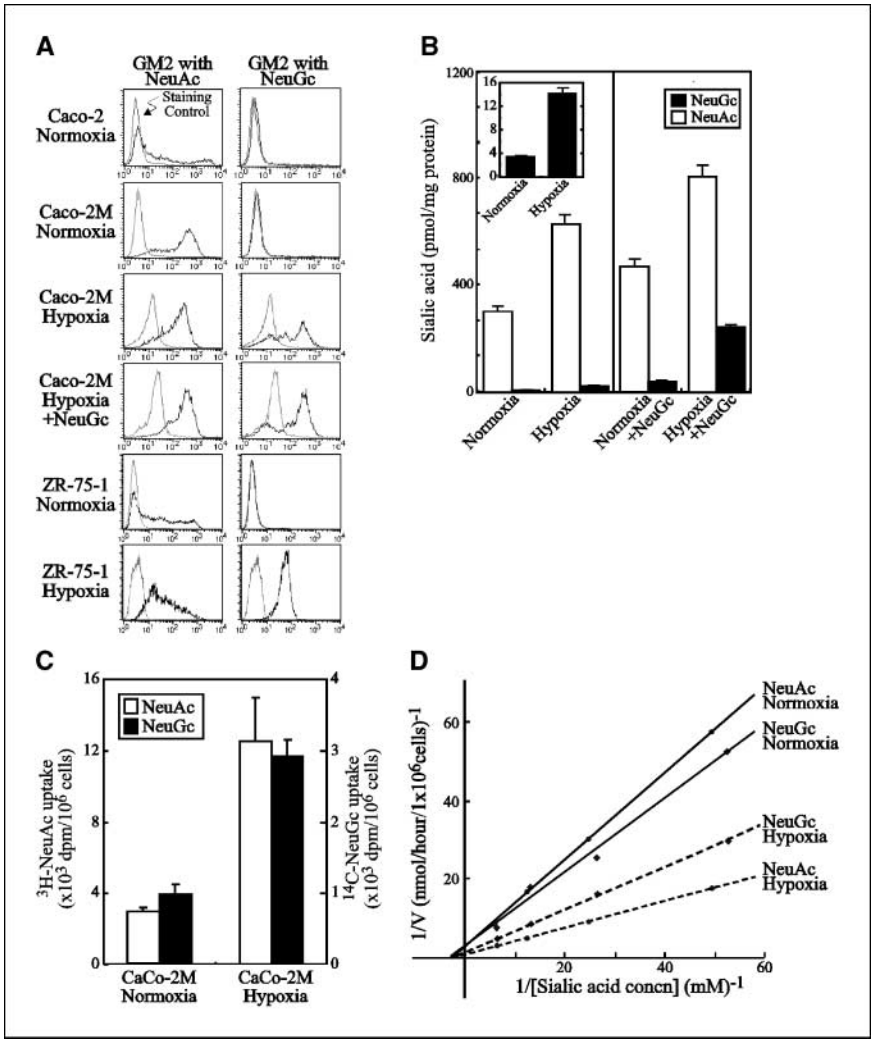

Figure 3. Effect of hypoxia on the expression of NeuGc-G_{M2} and content of membrane-bound NeuGc in cultured human cancer cells, Caco-2 and ZR-75-1. *A,* flow cytometric analysis of Caco-2 and ZR-75-1 cells. NeuAc-G_{M2} was detected with monoclonal antibody MK1-16, and NeuGc-G_{M2} with MK2-34. Parental Caco-2 cells lacked NeuGc-G_{M2} and were heterogeneic in terms of NeuAc-G_{M2} expression under normoxic condition. Cells expressing NeuAc-G_{M2} were enriched by MACS using MK1-16, and designated as Caco-2M cells, which were used for further experiments. ZR-75-1 cells were used without enrichment by MACS. Caco-2M cells were cultured under normoxic or hypoxic (1% O_2 for 7 days) conditions in DMEM containing 10% FCS, supplemented with/without 4 mmol/L free NeuGc. Hypoxic culture of ZR-75-1 cells was also done for 7 days under the same conditions. *B,* HPLC analysis of membrane-bound sialic acid content in Caco-2M cells, which were cultured under normoxic or hypoxic conditions as in (*A*). Representative of three similar experiments. *C,* Caco-2M cells were precultured under normoxic or hypoxic (1% O_2 for 4 days) conditions in DMEM containing 10% FCS, and incorporation of [^{3}H]NeuAc or [^{14}C]NeuGc was measured for 30 minutes at 4°C in a six-well plate. *Columns,* means of three independent experiments; *bars,* ±SD. *D,* kinetic analyses of cellular incorporation of [^{3}H]NeuAc and [^{14}C]NeuGc from external medium (Lineweaver-Burk plot).

content (Fig. 1*D*). These findings suggested that NeuAc was also actively incorporated from the culture medium as well as NeuGc. The decrease in NeuAc content in the cells cultured in the CHO-S-SFM II medium reached a plateau after 21 days of culture (Fig. 1*C, open columns*), which would reflect the activity of the endogenous enzymes for *de novo* NeuAc synthesis within the LS174T cells.

Transfection of CMP-NeuAc hydroxylase gene induces constitutive expression of NeuGc-containing G_{M2}. In order to know the effect of endogenous *de novo* synthesis of sialic acid on the expression of carbohydrate determinant, we introduced the murine cytidine monophospho-*N*-acetylneuraminic acid hydroxylase (*Cmah*) gene to LS174T cells. The transfectant cells significantly expressed *Cmah* mRNA (Fig. 2*A*), and contained a much larger amount of NeuGc than the parental LS174T cells, either under normal culture conditions with DMEM + 10% FCS, or in the serum-free CHO-S-SFM II medium (Fig. 2*B*). Expression of NeuGc-G_{M2} on the transfectant cells was maintained even after 44 days of culture in the serum-free CHO-S-SFM II medium supplemented with 0.84 mmol/L free NeuAc.

The *Cmah*-transfected cells contained 290 pmol/mg protein of NeuGc when cultured in DMEM, against 120 pmol/mg protein when cultured in the serum-free CHO-S-SFM II medium (Fig. 2*B*). On the other hand, the parental LS174T cells accumulated 150 to 280 pmol/mg protein of NeuGc when cultured in the medium containing exogenous NeuGc supplement (Fig. 1*E*). These results indicate that the amount of exogenously incorporated sialic acid is comparable to that supplied by the *de novo* synthesis. Another finding was that the mean fluorescence intensity of expression of G_{M2} containing NeuGc on the transfected cells (Fig. 2*C*) was not much different from that in parental cells cultured in the usual medium (DMEM + 10% FCS), which contained ~20 pmol/mg protein of NeuGc (Fig. 1*B*). This would suggest that only a small amount of NeuGc-containing sialoconjugate suffices for immunologic detection of the determinant in flow cytometry.

Hypoxia induces NeuGc-containing G_{M2} on human cancer cells. Another line of cultured human colon cancer cells, Caco-2, expressed NeuAc-G_{M2}, but no NeuGc-G_{M2} (Fig. 3*A, first row*). Because expression of NeuAc-G_{M2} on Caco-2 cells was heterogenous, NeuAc-G_{M2}-high expresser cells were enriched from parental Caco-2 cells by magnetic cell sorting with MACS (Miltenyi) using anti-NeuAc-G_{M2} antibody. The sorted cells were designated Caco-2M cells, and were used throughout the experiments described in this report. Caco-2M cells strongly expressed NeuAc-G_{M2}, but still lacked virtually any appreciable expression of NeuGc-G_{M2} (Fig. 3*A, second row*), although Caco-2M cells were maintained in the same medium (DMEM + 10% FCS) as LS174T.

We found that hypoxic culture of Caco-2M cells resulted in the significant expression of NeuGc-G_{M2} on Caco-2M cells (Fig. 3*A, third row*). Inclusion of free NeuGc in the culture medium further enhanced hypoxia-induced induction of NeuGc-G_{M2} expression on Caco-2M cells (Fig. 3*A, fourth row*). Hypoxic culture also induced significant expression of NeuAc-G_{M2} on a human breast cancer cell line, ZR-75-1, which, under usual normoxic culture conditions, expressed NeuAc-G_{M2} but not NeuGc-G_{M2} (Fig. 3*A, fifth* and *sixth rows*).

HPLC analysis of membrane sialic acid showed that hypoxic culture of Caco-2M cells for 7 days resulted in a 4.6-fold increase in the NeuGc content of the cells (Fig. 3*B*). Hypoxic culture in the presence of free NeuGc in the medium resulted in a more prominent increase of NeuGc, which reached as much as 233 pmol/mg protein. These findings led us to the working hypothesis that incorporation of sialic acid from the culture medium to the cancer cells is facilitated by hypoxia.

Experiments using radioactive sialic acid indicated a significant enhancement in cellular sialic acid uptake by hypoxia for both NeuAc and NeuGc (Fig. 3*C*). The apparent K_m for cellular sialic acid uptake was within the range of 0.34 to 0.50 mmol/L (Fig. 3*D*), which is comparable to the reported K_m for sialin, a sialic acid transporter (0.2-0.7 mmol/L; refs. 43, 44).

Hypoxia induces transcription of a sialic acid transporter (sialin) in cancer cells. Because the best-known transporter of sialic acid across the cell membrane is sialin (SLC17A5), and the observed K_m for sialic acid incorporation coincided with that of sialin; next, we studied the effect of hypoxia on the expression of genes for sialin and related transporters. The results indicated that transcription of the sialin gene is strongly induced by hypoxia (Fig. 4*A*). LS174T cells, which showed significant incorporation of sialic acid under usual normoxic culture conditions, had a high level of constitutive sialin mRNA expression, which was comparable to that in hypoxia-induced Caco-2M cells (Fig. 4*A*).

Hypoxia did not induce gene transcription of a GalNAc transferase, β4GalNAc-T, and sialyltransferases ST3Gal-V and ST3Gal-VI (Fig. 4*A*), which are involved in synthesis of the carbohydrate framework of the G_{M2} ganglioside. The transcription of GLUT1, the sugar transporter previously known to be induced by hypoxia, was significantly increased in hypoxia-treated Caco-2M cells and served as a positive control. Similar results were obtained when a cultured breast cancer cell line, ZR-75-1, was used instead of CaCo-2M cells (Fig. 4*A*).

Results of Western blotting using a commercial anti-sialin antibody showed a significant induction of the sialin protein in CaCo-2M cells as well as in ZR-75-1 cells cultured under hypoxic conditions (Fig. 4*B*).

Transfection of sialic acid transporter sialin gene induces expression of non–human sialic acid on cancer cells. We next tried transfection of the sialin gene to Caco-2M cells. A weak expresser clone and two strong expresser clones were obtained by the limiting dilution method (Fig. 5*A*). Results of Western blotting with anti-sialin antibody indicated significant production of the sialin protein in transfectant clones (Fig. 5*B*). HPLC analyses revealed a significant amount of NeuGc in the strong expresser clones (Fig. 5*C*). The incorporation of NeuGc sialic acid correlated with the degree of expression of the sialin gene among the transfectant clones (Fig. 5*C, inset*). Whereas a weak expresser transfectant clone (Sialin-1, Fig. 5*D*) failed to express NeuGc-G_{M2}, two strong expresser clones (Sialin-2 and Sialin-3, Fig. 5*D*) significantly expressed the determinant, indicating that increased transcription of sialin gene led to the surface expression of the NeuGc-containing G_{M2} ganglioside.

Distribution of sialin and NeuGc-G_{M2} in the hypoxic area of human cancer tissues. Next, we examined whether sialin and the NeuGc-G_{M2} ganglioside are localized in hypoxic areas of actual human tumor tissues. Anti-CD31 antibody, which stains endothelial cells, was used to ascertain the tumor vasculature. Results of

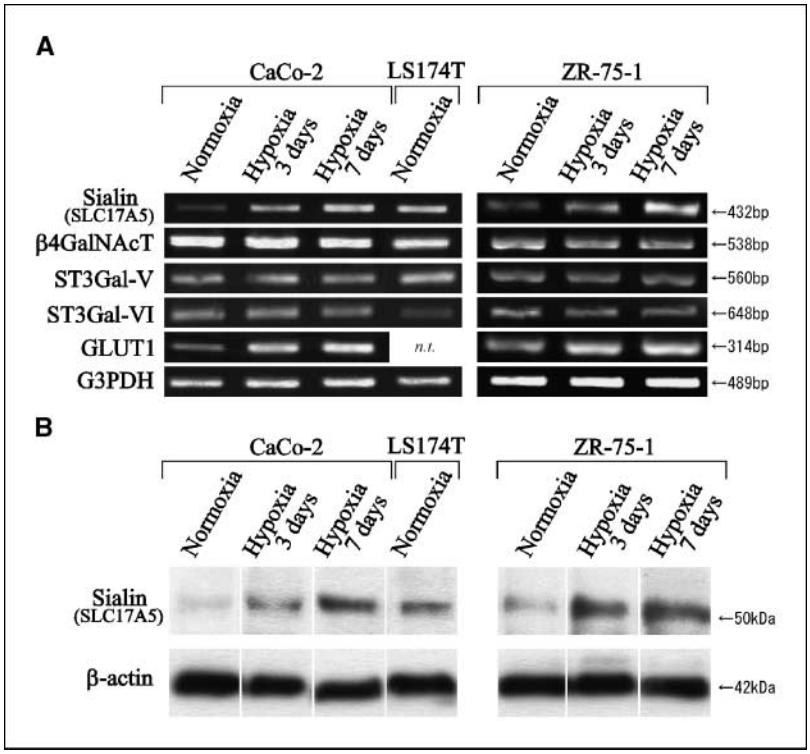

Figure 4. Hypoxia-induced expression of mRNA and protein for a sialic acid transporter, sialin (SLC17A5) in CaCo-2M, and ZR-75-1 cells. Results of RT-PCR are shown in (*A*). mRNA for glycosyltransferase involved in synthesis of the carbohydrate framework of the G_{M2} ganglioside, β4GalNAc-T, ST3Gal-V, or ST3Gal-VI were also evaluated. *GLUT1,* glucose transporter-1; *n.t.,* not tested. *B,* results of Western blotting using anti-sialin antibody. Staining with anti-β-actin antibody serves as a control.

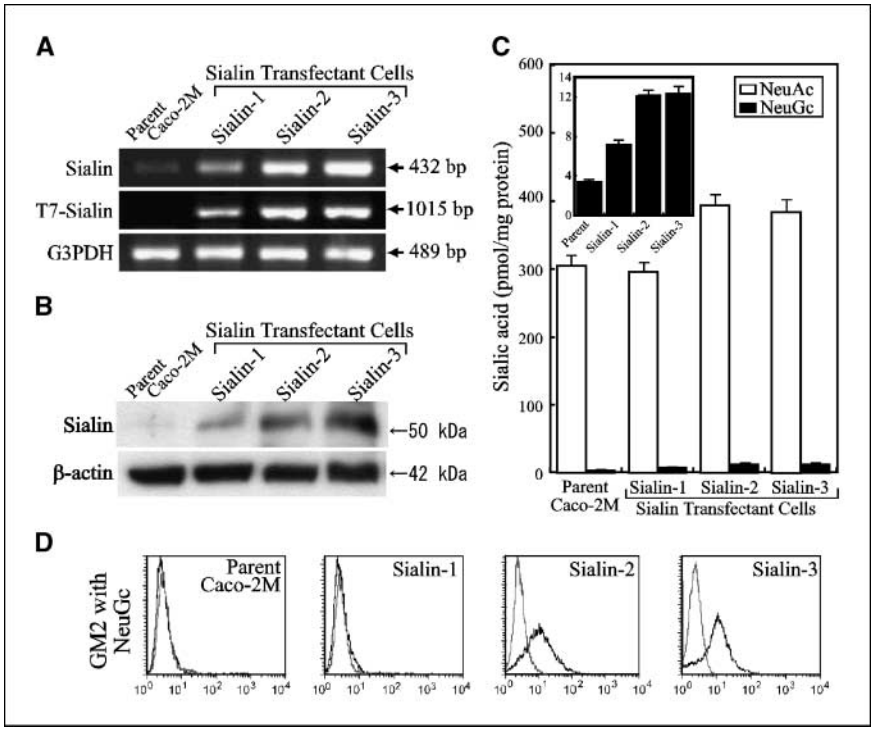

Figure 5. Induction of expression of NeuGc-G_{M2} in the cells transfected with sialin gene. The transfectant clone Sialin-1 is a low expresser and Sialin-2/-3 are high expressors. Results from parental Caco-2M cells are shown as control. *A,* RT-PCR analyses of the sialin-transfected clones using primers for the coding region of sialin (*Sialin*) and those for transfected vector (5′-primer taken from vector sequence and 3′-primer from sialin sequence; *T7-sialin*), and the primers for G3PDH. *B*, results of Western blotting using anti-sialin antibody. *C,* HPLC-analysis of membrane-bound sialic acid content in the parental Caco-2M cells and sialin-transfectant clones. *Inset,* NeuGc levels with expanded ordinate axis. Representative of three similar experiments. *D,* flow cytometry of the parental Caco-2M cells and sialin-transfectant clones. NeuGc-G_{M2} was detected with monoclonal antibody MK2-34 (murine IgM).

confocal microscopic analyses of human colon cancer tissue sections using anti-NeuGc-G_{M2} and anti-CD31 antibodies indicated that cancer cells distant from blood vessels preferentially express NeuGc-G_{M2}, compared with those in the vicinity of tumor vessels (Fig. 6*A*). Similarly, sialin was also localized preferentially in the cancer cells distant from the tumor vasculature (Fig. 6*B*). These findings were compatible with the proposal that tumor hypoxia induces expression of NeuGc-G_{M2} and sialin, because it is postulated that a distance of 70 to 100 μm from a blood vessel causes severe hypoxia from the Thomlinson and Gray's equation on the relationship between oxygen pressure and distance from a blood vessel, (45). Double staining of sialin and NeuGc-G_{M2}, indicated that the distribution of both molecules overlaps in the areas distant from tumor vasculature (Fig. 6*C*). In cancer cells, sialin was expressed on the granular structures in the cytoplasm, whereas membranous and patchy expression of NeuGc-G_{M2} was observed at the surface of cancer cells.

Discussion

The results presented in this study showed that hypoxic culture of tumor cells induces expression of G_{M2} ganglioside containing the non–human sialic acid, NeuGc, on human cancer cells. This is mediated at least partly by the hypoxia-induced transcription of sialin, a sialic acid transporter, on cancer cells. Sialin facilitated the incorporation of sialic acid from culture medium *in vitro*, and is expected to facilitate cellular incorporation of sialic acids of dietary origin in cancer tissues *in vivo*. This leads to the accumulation of non–human sialic acid in the cancer-associated ganglioside, G_{M2}. Selection of hypoxia-resistant cancer cells by hypoxic environment in locally advanced tumor nests is known to result in the clonal expansion of cancer cells with higher invasive and metastatic activities. Our results suggest that NeuGc-containing G_{M2} could be a good marker for such exacerbated cancer cells.

The amount of non–human sialic acid incorporated by cancer cells was usually very small, comprising only a small percentage of the total sialic acid in the cancer cells under normal culture conditions. The chemical amount of NeuGc in these cells was generally far below the amount synthesized endogenously after the introduction of CMP-NeuAc hydroxylase gene. Eventually, however, it reached as much as 80% of the total cellular membrane-bound sialic acid. The amount of NeuGc in cancer cells seemed to depend heavily on its availability in the external medium, if the cells significantly expressed sialin. Interestingly, only a small amount of NeuGc was enough to confer antigenicity on cancer cells and enable immunologic detection using a specific antibody. Once induced, its expression persisted for a long time after the deprivation of exogenous NeuGc supply in the chemically defined culture medium. A long-term culture of 4 weeks or more, however, finally eliminated NeuGc-G_{M2} expression, indicating that it is not endogenously synthesized by a CMP-NeuAc hydroxylase–like enzyme in cancer cells.

The cancer-associated G_{M2} ganglioside had been earlier regarded to be preferentially expressed in tumors of neuroectodermal origin, such as melanoma, neuroblastoma, glioma, and small cell lung carcinoma. Later, it was found to be widely associated with common tumors including colon, stomach, liver, breast, ovary, and germ cell cancers (25, 36, 46–49). Among the cancer-associated gangliosides, G_{M2} is known to have a particularly high ability to elicit humoral immune response in humans, when conjugated with KLH and introduced with appropriate adjuvants (15, 16). G_{M2} has been applied in clinical trials for vaccine therapy of cancers (11–14). The main component of the hitherto applied G_{M2} vaccines is conventional NeuAc-containing G_{M2}. Clinical application of humanized monoclonal anti-G_{M2} antibodies is also in progress

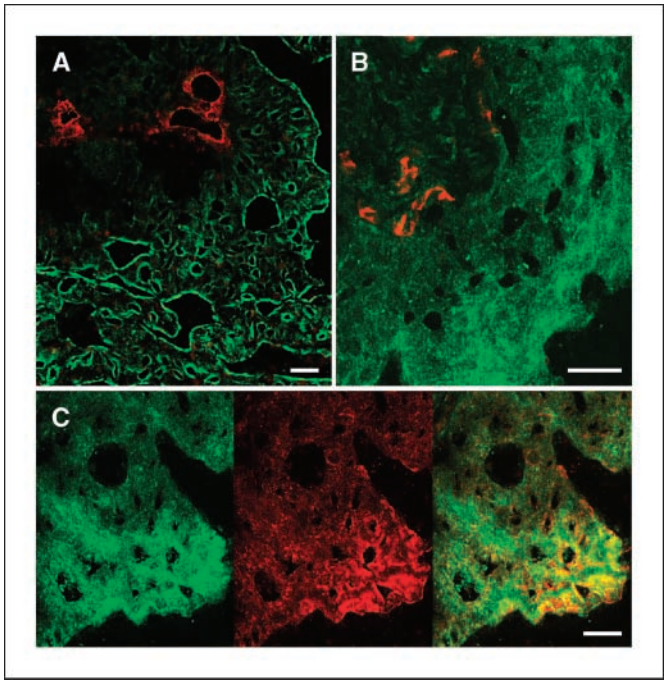

Figure 6. Distribution of NeuGc-G_{M2} and sialin in hypoxic areas in human colon cancer tissues. Results of confocal microscopic analyses of human colon cancer tissue sections. *A,* distribution of NeuGc-G_{M2} (*green*) and CD31 (*red*), indicating cancer cells (*bottom right*), which are distant from CD31-positive blood vessels preferentially expressing NeuGc-G_{M2}. *B,* distribution of sialin (*green*) and CD31 (*red*), indicating cancer cells (*bottom right*), which are distant from CD31-positive blood vessels, preferentially express sialin. *C,* double staining of sialin (*green*) and NeuGc-G_{M2} (*red*), indicating that these two molecules colocalize in the areas distant from tumor vasculature (*bottom*). *Bars,* 20 μm.

(17–20). These antibodies were also originally raised against conventional NeuAc-containing G_{M2}.

Although G_{M2} is preferentially expressed in cancers, the mechanism that leads to its cancer-associated expression is not fully elucidated. Some oncogenes are known to induce transcription of the gene for a GalNAc transferase, which is closely related to G_{M2} synthesis (50). Our analysis of human cancer cells indicated that the expression of NeuAc-G_{M2} showed no appreciable change when cultured under hypoxic conditions. Hypoxia affected mainly the sialic acid species of the G_{M2} ganglioside, and induced strong and selective expression of NeuGc-G_{M2} on cancer cells. As both species of G_{M2} ganglioside, NeuAC-G_{M2} and NeuGc-G_{M2}, are synthesized through the action of the common sialyltransferase (ST3Gal-V) and GalNAc transferase (β4GalNAcT), these findings imply that glycosyltransferases involved in the synthesis of the core structure of G_{M2} are not significantly affected by hypoxic culture of tumor cells. Our previous DNA-microarray analyses on the hypoxia-induced gene expression in colon cancer cells also failed to show any difference in the expression of the genes for ST3Gal-V and β4GalNAcT (4). This also suggests that cancer-associated expression of G_{M2} core structure per se is independent of hypoxic culture of tumor cells.

The selective induction of NeuGc-G_{M2} by hypoxia supports the idea that the specific effect of hypoxia is to expedite sialic acid transport from the external medium, leading to the induction of NeuGc-G_{M2} expression on the cells. G_{M2} having non–human sialic acid (NeuGc) is expected to have a higher cancer specificity and stronger antigenicity than the usual NeuAc-containing G_{M2} because it has the non–human sialic acid residue known to be immunogenic for humans. Its immunogenicity in humans would make it an ideal candidate for a better antigen for cancer immunotherapy. Hypoxia-adapted cancer cells are known to show a poor response to conventional radiotherapeutic and chemotherapeutic interventions. NeuGc-containing G_{M2} is therefore expected to be a better target for the therapy of hypoxia-adapted highly aggressive cancers.

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