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Tumor-Microenvironment Interactions: The Fucose-Generating FX Enzyme Controls Adhesive Properties of Colorectal Cancer Cells

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ABSTRACT

Extravasation of tumor cells is a pivotal step in metastasis formation. This step is initiated by an interaction of extravasating tumor cells with endothelial cells. Among the molecules mediating tumor-endothelium interactions are selectins and their fucosylated ligands. In a previous study, we demonstrated that the fucose-generating FX enzyme regulates the expression of selectin ligands by B and T lymphocytes and by head and neck squamous cell carcinoma cells. It was also shown that the FX enzyme regulated important interaction parameters between these cancer cells and endothelial cells. The present study was aimed to determine whether the FX enzyme controls adhesive interactions between colorectal cancer cells and endothelial cells. The results clearly indicate that this is indeed the case. Overexpressing the FX enzyme by the transfer of FX cDNA to low FX-expressing colorectal cancer cells resulted in an increased adhesive capacity of the transfectants to activated endothelial cells and to recombinant E-selectin. Down-regulating FX levels in colorectal cancer cells expressing high levels of endogenous FX by transfection with small-interfering RNA resulted in a down-regulated expression of the selectin ligand sialyl Lewis-a and a decrease in the adhesive capacity of the transfectants to activated endothelial cells and to recombinant E-selectin. These transfection experiments also indicated that manipulating the levels of the FX enzyme affected global cellular fucosylation and altered the interaction of colorectal cancer cells with some extracellular matrix components such as fibronectin. We also found that highly metastatic colorectal cancer variants express higher levels of FX and of sialyl Lewis-a than low metastatic variants originating in the same tumors. These results lead us to hypothesize that the FX enzyme controls the capacity of colorectal cancer to extravasate and form metastasis. If this hypothesis will be confirmed the FX enzyme could become a target molecule for metastasis prevention.

INTRODUCTION

Fucose is a component of many surface-localized and secreted molecules. It decorates the terminal portions of N-, O-, or lipid-linked glycans and modifies the core of some N-linked glycans (1). Terminal fucosylated glycans in humans constitute several blood group antigens and function as selectin ligands (2). Fucosylation of these ligands determines their ability to bind to the selectin family of cell adhesion molecules and therefore controls pivotal steps of selectin-dependent leukocyte and tumor cell adhesion and trafficking (1, 3, 4). In addition to its role in adhesive reactions, fucosylation influences Notch and Cripto signaling events (5–9).

The final steps of fucose biosynthesis are mediated by the GDP-D-mannose 4,6 dehydratase generating GDP-mannose-4-keto-6-D-deoxymannose. This sugar is converted to GDP-L-fucose by the FX enzyme, functioning both as an epimerase and a reductase (10, 11). The GDP-L fucose is then transported to the Golgi. Fucosylation of mammalian glycans is catalyzed by distinct fucosyltransferases, with catalytic activities characterized by specificity for specific glycoconjugate substrates and a requirement for GDP-fucose (12).

The generation of FX knockout mice enabled us to conclude that fucosylation events are essential for fertility, early growth, and development, as well as for intercellular adhesion (1). FX knockout resulted in a massive intrauterine mortality. Live-born FX-null mice exhibited a virtually complete deficiency of cellular fucosylation and a postnatal failure to thrive. FX (−/−) adults suffer from an extreme neutrophilia, myeloproliferation, and absence of leukocyte selectin ligand expression reminiscent of LAD-II/CDG-IIc (1).

All these studies demonstrate that fucosylation plays an important role in development and in adult physiology by influencing at least two different pathways: biosynthesis of selectin ligands and signaling through the Notch and Cripto pathways.

Previous studies from our laboratory demonstrated that the FX enzyme is involved in the biosynthesis of sLe-x in activated T or B cells (13). In head and neck squamous cell carcinomas, the FX enzyme plays a key role in the biosynthesis of selectin ligands such as sialyl Lewis-a (sLe-a) and in the interaction of these cancer cells with endothelial cells (14, 15). In lymphocytes, as well as in head and neck squamous cell carcinomas, the FX enzyme is regulated by outside-in signaling (13, 14).

Numerous reports indicated a correlation between a high expression of selectin ligands by epithelial cancer cells, notably colorectal cancer, and a high rate of metastasis and poor prognosis (16–18). The fucose-generating FX enzyme may thus be a pivotal element in cancer-associated perturbations of differentiation, survival, and proliferation, as well as in cancer cell extravasation. It is therefore important to find out if the FX enzyme is involved in controlling selectin ligand expression by colorectal cancer cells and in their interaction with endothelial selectin. The present study provides proof that this is indeed the case.

MATERIALS AND METHODS

Cell Lines. The human colorectal cancer cell lines: 474, 0485, 1086, 1203, 044, and 427 were established at the John Wayne Cancer Institute (Santa Monica, CA). The 474 and 1203 cell lines were derived from primary colorectal cancer tumors. The 1086 cell line was derived from a colorectal cancer liver metastasis. The 0485 cell line was derived from a lymph node metastasis. The cells were maintained in RPMI 1640 supplemented with 20% FCS, 2 mmol/L L-glutamine, 100 units/mL penicillin, 0.1 mg/mL streptomycin, and 12.5 units/mL nystatin. All of the medium components were obtained from Biological Industries (Beit-Haemek, Israel). KM12C, KM12L4, and KM12SM were kindly provided by Dr. Isaiah J. Fidler (Department of Cell Biology, M. D. Anderson Cancer Center, Houston, TX). The KM12C cell line was derived from a Duke's B2 colorectal cancer primary tumor. The KM12L4 and KM12SM variants originated in liver metastases that developed in BALB/c nude mice inoculated with KM12C cells to spleen or cecum, respectively (19). KM12C, KM12L4, and KM12SM cells were maintained in Eagle's MEM supplemented with 10% FCS, 2 mmol/L L-glutamine, 100 units/mL penicillin,

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0.1 mg/mL streptomycin, and 12.5 units/mL nystatin, 5 mmol/L sodium pyruvate, nonessential amino acids, and 2-fold vitamin solution (19). The colorectal cancer SW480 and SW620 cells were obtained from American Type Culture Collection (Manassas, VA). The SW480 cell line was derived from a primary colorectal cancer, whereas the SW620 line was derived from a lymph node metastasis of the same patient. SW480 cells were maintained in RPMI 1640 supplemented with 10% FCS. FX-cDNA SW480-transfected cells were maintained in the same medium supplemented with 800 μg/mL G418 (Calbiochem, San Diego, CA). SW620 cells were maintained in Leibovitz L-15 medium supplemented with 15% FCS, 2 mmol/L L-glutamine, 100 units/mL penicillin, 0.1 mg/mL streptomycin, 12.5 units/mL nystatin, 10 mmol/L HEPES buffer, and 0.075% sodium bicarbonate. The colorectal cancer HT-29 cell line was kindly provided by Dr. Livia Theodor (Gastroenterology Department, The Chaim Sheba Medical Center, Tel Hashomer, Ramat-Gan, Israel). The HT-29 cells were maintained in McCoy's modified medium (Invitrogen-Life Technologies, Inc., Paisley, Scotland, United Kingdom) supplemented with 10% FCS, 2 mmol/L L-glutamine, 100 units/mL penicillin, 0.1 mg/mL streptomycin, and 12.5 units/mL nystatin. All of the cell lines were routinely cultured in humidified air with 5% CO_2 at 37°C.

Human Umbilical Vein Endothelial Cells (HUVECs). HUVECs were either purchased from Dr. Neomy Lanir (Department of Hematology and Bone Marrow Transplantation, Rambam Medical Center, Haifa, Israel) or prepared in our laboratory from umbilical cords as follows: endothelial cells were harvested by 0.25 mg/mL collagenase type II (Sigma, Holon, Israel). Cells were grown to confluence in tissue culture flasks precoated with fibronectin (20 μg/mL; Biological Industries, Beit- Haemek, Israel). Cells were established as primary cultures in M199 medium supplemented with 20% FCS, 50 μg/mL endothelial cell growth factor (Biomedical Technologies, Inc., Stoughton, MA), heparin (5 units/mL; Laboratoire Choay, Paris, France), and antibiotics. Cells from the third passage were taken for experiments.

Antibodies. The following antibodies were used in flow cytometry assays: anti-sialyl Lewis-a (sLe-a), anti-Lewis-a (Le-a), and anti-Lewis b (Le-b) were purchased from Seikagaku America (Falmouth, MA). Anti-sialyl Lewis-x (sLe-x) was purchased from American Type Culture Collection. Anti-Lewis-x (Le-x) and anti-CD24 were purchased from PharMingen (San Diego, CA). Anti-Lewis-y (Le-y) and anti-PSGL-1 were purchased from Serotec (Oxford, United Kingdom). Antihuman VIM2 antibodies were a kind gift from Dr. Walter Knapp (Institute of Immunology, University of Vienna, Vienna, Austria). Anti-CD62E was purchased from Southern Biotechnology Associates (Birmingham, AL). FITC-conjugated goat antimouse IgM and IgG were purchased from Jackson Immunosearch Laboratory, Inc. (West Grove, PA). The following antibodies were used in Western blotting assays: mouse monoclonal antihuman FX antibody (3G10/12) prepared in our laboratory, rabbit polyclonal antihuman FX antibody 701 (13) also prepared in our laboratory, anti-glyceraldehyde-3-phosphate dehydrogenase antibody (Chemicon International, Inc., Temecula, CA), and horseradish peroxidase-conjugated secondary goat antibody against mouse IgG (Jackson Immunosearch Laboratory, Inc.).

Plasmids. The plasmid pSUPER was kindly provided by Dr. Reuven Agami (Division of Tumor Biology, The Netherlands Cancer Institute, Plesmanlaan, Amsterdam, the Netherlands). The plasmid pRc/CMV was purchased from Invitrogen BV (Groningen, the Netherlands). The plasmid pEGFP-C was purchased from Clontech Laboratories, Inc. (Palo Alto, CA).

Construction of FX Small Interfering RNA (siRNA). FX mRNA suppression was achieved by using the pSUPER vector. Two gene-specific oligonucleotides were designed as follows: 5′-GATCCCC**AGACGCCGATCTCACGGAT**TTCAAGAGA**ATCCGTGAGATCGGCGTC**TTTTTTGGAAA-3′ and 5′-AGCTTTTCCAAAAA**AGACGCCGATCTCACGGAT**TCTCTTGAA**ATCCGTGAGATCGGCGTCT**GGG-3′. Regular characters represent regions required for the generation of the siRNA as previously described (20), and bold characters represent FX-specific complementary sequences. Both oligonucleotides were denatured at 95°C for 4 minutes, annealed at 70°C for 10 minutes, and cooled down slowly. Oligonucleotides were then phosphorylated by the use of T4 polynucleotide kinase at 37°C for 30 minutes. This oligonucleotide mixture was ligated into the pSUPER vector predigested with *Bgl*II and *Hin*dIII and pretreated with calf intestinal phosphatase. A mutated FX siRNA that served as control was generated by introducing a point mutation (G to C) at position 24 in the first FX-specific complementary sequence oligonucleotide shown above. This oligonucleotide was cloned to the pSUPER vector along with the second FX-specific complementary oligonucleotide shown above.

Flow Cytometry. Cells (5×10^5) were incubated for 45 minutes at 4°C with primary antibodies directed against the tested selectin ligand. After a wash with cell sorter medium (RPMI 1640 supplemented with 5% FCS and 0.01% sodium azide), the cells were incubated for 45 minutes at 4°C with FITC-conjugated goat antimouse IgG or IgM. After an additional wash, antigen expression on 5000 live cells was determined using a Becton Dickinson FACSort (Mountain View, CA) and CellQuest software. Baseline staining was obtained by adding cell sorter medium to the cells instead of primary antibody. Flow cytometry scores for selectin ligand expression were calculated as described previously (13). The scores represent the multiplication of the mean fluorescence by the percent positive cells $\times 10^{-4}$. Scores for each selectin ligand were divided into three categories: high, medium, and low. For sLe-a, high = 8.2 to 19.7, medium = 5.7 to 7.6, and low = 1.8 to 0.2; for Le-b, high = 8.7 to 11.6, medium = 2.2 to 5.6, and low = 0.1 to 1.3; for sLe-x, high = 7 and medium = 2.3 to 4.9.

RNA Preparation and Northern Blot Analysis. Total RNA was prepared, and Northern blotting was performed as described by Eshel et al. (14) in our lab.

Western Blotting. Colorectal cancer cells were lysed with Laemmli sample buffer (21). Lysates were boiled for 10 minutes, centrifuged, and applied on a miniprotean II system (Bio-Rad, Hercules, CA) for SDS-PAGE using a 12% slab gel as described by Laemmli (21). Electrophoretic transfer of proteins from the polyacrylamide gel to nitrocellulose (Schleicher & Schull, Dassel, Germany) was performed by a mini-transblot electrophoretic cell (Bio-Rad) at 250mA for 2 hours. After transfer, the nitrocellulose membrane was incubated at room temperature with 3% BSA in TBS-Tween for 30 minutes to block free binding sites on the membrane. The blocked nitrocellulose membrane was incubated over night with anti-FX 3G10/12 monoclonal antibody, diluted 1:16 in 1% BSA in TBS-Tween with 0.02% sodium azide or with rabbit polyclonal antihuman FX antibody 701(2), diluted 1:1000 in 5% milk in TBS-Tween with 0.02% sodium azide, then washed three times for 5 minutes with TBS-Tween and incubated for 50 minutes with horseradish peroxidase-conjugated secondary goat antibody against mouse or rabbit IgG diluted 1:10,000 with 5% milk in TBS-Tween at room temperature. Finally, the nitrocellulose membrane was washed five times for 5 minutes with TBS-Tween. The bands were visualized by chemiluminescence-enhanced chemiluminescence reaction (Amersham, Buckinghamshire, United Kingdom) and autoradiography by exposure to Kodak XAR5 film (Eastman Kodak Co., Rochester, NY) for 1 to 5 minutes.

The quantification of protein in the lanes was determined in reference to the amount of glyceraldehyde-3-phosphate dehydrogenase in the lanes. This was performed by incubating the membrane with anti-glyceraldehyde-3-phosphate dehydrogenase diluted 1:1000 with 5% milk in TBS-Tween with 0.02% sodium azide.

Lectin Blot. The first steps of blotting were performed as described above for Western blotting. After transfer of the proteins to nitrocellulose, the membrane was incubated at room temperature for 60 minutes with 3% BSA in PBS-0.05% Tween- 20 to block free binding sites on the membrane. The blocked nitrocellulose membrane was incubated for 2 hours with horseradish peroxidase-conjugated Ulex europaeus agglutinin lectin (Sigma), which binds to L-fucose (22), diluted 1:2000 in PBS containing 3% BSA and 0.1% Tween-20, then washed four times for 10 minutes with PBS containing 0.1% Tween-20. The bands were visualized by the chemiluminescence-enhanced chemiluminescence reaction (Amersham) and autoradiographed by exposure to Kodak X-AR5 film (Eastman Kodak Co.) for 5 seconds.

Transfection. SW620 cells were transfected with pSUPER-FX siRNA (205 cells) or with pSUPER-FX-mutated siRNA oligonucleotides (360 control cells) by electroporation using the Electro cell manipulator 830 (BTX-Genetronics, San Diego, CA). Thirty micrograms of pSUPER and 3 μg of the pBabe-puro plasmid were used for transfection. Forty-eight hours after electroporation, the cells were selected with 1 μg/mL puromycin. Individual clones were then picked, expanded and analyzed for FX protein levels. To increase transfection yield, cells were re-transfected using the pSUPER coupled with pcDNA3.1-zeo vectors. In this case, 500 μg/mL zeocin was used as a selection marker. The transfected cells were grown in the presence of 25 mmol/L fucose. SW480 cells were cotransfected either with FX-cDNA pRc/CMV vector or

Table 1 *Expression levels of selectin ligands by colorectal cancer cell lines*

	Selectin ligand								
Cell line	sLe-a	Le-b	Le-a	sLe-x	Le-x	Le-y	CD24	VIM2	PSGL-1
HT-29	6.5	5.6	4.4	2.3	0.6	0.4	3	0.1	>0.1
044	17.1	0.9	1.4	>0.1	>0.1	0.2	0.1	>0.1	>0.1
1086	7.6	1.3	1.7	>0.1	0.6	>0.1	0.5	0.4	>0.1
474	8.2	5.6	0.2	4.7	2.2	0.7	0.5	>0.1	0.8
0485	5.7	2.5	>0.1	>0.1	1.6	1.1	1.6	>0.1	>0.1
427	19.7	11.6	13.5	7	8.2	>0.1	0.6	>0.1	0.2
1203	9.7	8.7	3.1	4.9	16.3	0.2	0.2	2.7	>0.1
SW480	0.2	2.2	0.1	>0.1	4.3	2.8	>0.1	7.5	0.1
SW620	5.9	0.1	2.3	>0.1	7.3	2	1.1	0.5	>0.1
KM12C	0.2	0.1	0.1	>0.1	>0.1	>0.1	4.2	0.3	>0.1
KM12L4	0.7	0.2	0.1	0.1	0.3	0.1	3.4	2	0.1
KM12SM	1.8	0.3	0.1	0.9	>0.1	0.2	4.1	0.1	0.4

NOTE. Selectin ligand expression was determined by flow cytometry. Values represent expression scores obtained by the multiplication of mean fluorescence values by the percent of positive cells ($\times 10^{-4}$) as described previously (13).

with pRc/CMV vector without insert together with pEGFP-C vector by electroporation.

Adhesion of Colorectal Cancer to HUVECs. Ninety-six-well culture plates were coated with 50 μL of 20 μg/mL fibronectin per well for 30 minutes at 37°C. After one wash with PBS, 3×10^4 HUVECs in a volume of 100 μL/well were cultured on the plate for 16 hours to form a confluent monolayer. Endothelial cells were stimulated for 4 hours with human recombinant IFN-γ (100 units/mL) and recombinant tumor necrosis factor α (100 units/mL) and washed. A total of 2×10^6 colorectal cancer cells were washed twice with PBS and suspended in 1 mL of cold PBS, labeled with the CFDA-SE reagent (250 nmol/L; Molecular Probes, Eugene, OR) for 5 minutes, and then washed with 2 mL of FCS. After an additional wash with PBS, the labeled cells (1×10^5/100 μL) were added to a stimulated HUVEC monolayer in TBS containing 2 mmol/L $CaCl_2$ (for selectin-depended adhesion) and incubated for 30 minutes at 37°C. The total fluorescence signal was that of labeled cells added to the well before removing the nonadherent cells, which were removed by three washes with PBS. Total fluorescence and that of adhering cells was measured by a fluorescent ELISA reader (Bio-Tek FL500; Bio-Tek Instruments, Inc., Winooski, VT) at wavelength of 490/530. The results are presented as percent adhesion. The number of adherent green fluorescent protein (GFP)-transfected cells was determined by counting fluorescent cells in several fields with the aid of a fluorescence microscope (magnification ×100, FITC filter, Olympus IX70; Olympus, Hamburg, Germany).

Adhesion of Colorectal Cancer to rE-Selectin, Extracellular Matrix (ECM), or Fibronectin. Nontissue culture plates (Nunc-Immuno plate NUNC; Nunc International, Roskilde, Denmark) were coated for 60 minutes at room temp with 100 μL/well of 2 μg/mL recombinant E-selectin (R&D Systems, Minneapolis, MN) diluted in TBS-Tween containing 2 mmol/L $CaCl_2$. Three wells were not coated for detection of nonspecific binding. Supernatants were aspirated from the wells, and 200 μL of blocking medium (1% BSA in TBS-Tween containing 2 mmol/L $CaCl_2$) were added for at least 30 minutes at room temperature. Cells were labeled with CFDA-SE reagent as described above. Labeled cells (1×10^5/100 μL) suspended in 1% BSA in TBS-Tween, 2 mmol/L $CaCl_2$ were added to rE-selectin or uncoated wells for 45 minutes at 37°C. The rest of the assay was performed as described above. A similar procedure was performed with plates coated with a mixture of matrix proteins (E-TCMT-F NOVAmed; Jerusalem, Israel) or with fibronectin in the presence of 5 mmol/L $MgCl_2$.

Statistical Analysis. Significance was calculated using the two-way Student's *t* test.

RESULTS

An Expression Profile of Fucosylated Glycans by Colorectal Cancer Cell Lines

Table 1 shows the expression profile of several fucosylated glycans by 12 colorectal cancer cell lines. This profile was derived from several repetitions of flow cytometry experiments. All lines expressed sLe-a as well as Le-b. High levels of sLe-a were expressed by four cell lines and medium levels by four lines. Four cell lines expressed low levels of sLe-a. High levels of Le-b were expressed by cells of two lines (427 and 1203, which also expressed high levels of sLe-a). Four lines expressed medium levels of Le-b, and six lines expressed low levels of this fucosylated glycan. In all, it seems that the expression pattern of sLe-a by the various cell lines was similar to that of Le-b and to some extent to Le-a. The expression levels of sLe-x, a dominant selectin ligand on activated lymphocytes, were rather low. With the exception of four cell lines, of which, one expressed high and three medium levels of sLe-x, all other lines expressed either low levels of this selectin ligand or did not express it at all.

Taken together, these results indicated that sLe-a is the dominant selectin ligand in all cell lines tested. Because of its relative high expression by most of the colorectal cancer cell lines assayed, we consider sLe-a to be the representative selectin ligand of colorectal cancer cells.

In view of the possibility that in colorectal cancer, as in HNSCC (14), the fucose-generating FX enzyme functions as a limiting factor in sLe-a biosynthesis, we studied, in the following series of experiments, its contribution to sLe-a expression.

Expression Levels of the FX Enzyme Correlate with Those of sLe-a

Fig. 1 shows a regression curve correlating the expression of FX protein to that of sLe-a in several colorectal cancer cell lines. A

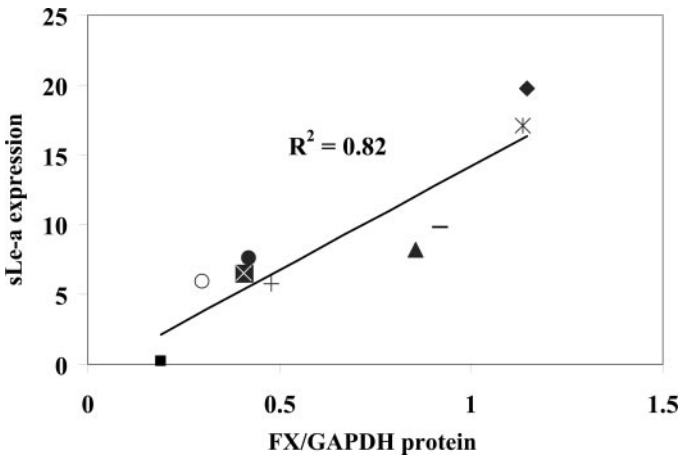

Fig. 1. Correlation between the expression of the FX protein and sLe-a by colorectal cancer cells. Lysates of colorectal cancer cells were assayed for FX protein by Western blot analysis using anti FX antibodies prepared in our lab. Anti- glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies were used as control. Colorectal cancer cells were assayed by flow cytometry for sLe-a expression. The values of sLe-a expression were calculated as for Table 1. HT-29 ⊠, 044 ✳, 1086 ●, 474 ▲, 0485 +, 427 ◆, 1203 −, SW480 ■, and SW620 ○.

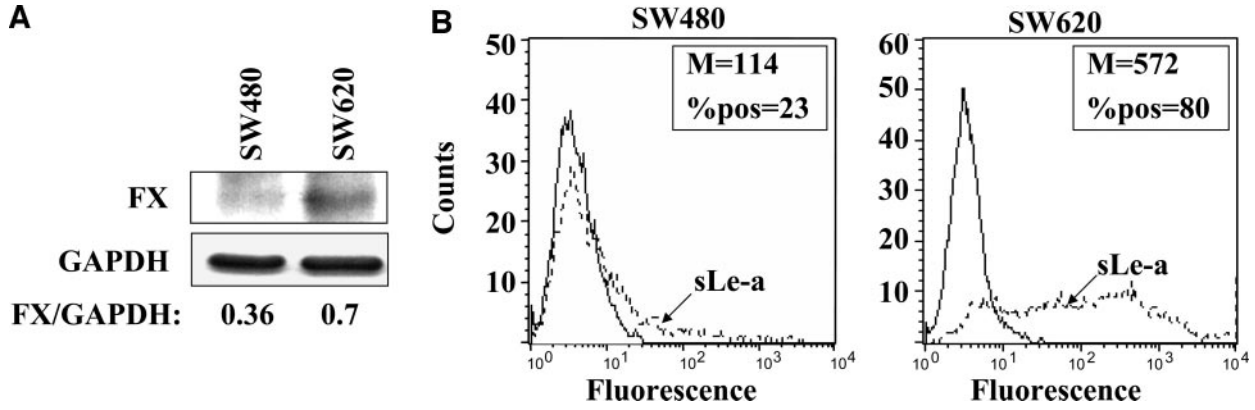

Fig. 2. Expression of FX protein and of sLe-a by SW480 and SW620 cells. *A*, expression of FX protein. Lysates of SW480 and SW620 cells were assayed for FX expression by Western blot analysis using anti FX antibodies. Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies were used as control. Values represent the ratio between the signal of FX protein in each cell and the signal of GAPDH in the same cell. *B*, expression of sLe-a. SW480 and SW620 cells were assayed by flow cytometry for sLe-a expression. (M = mean fluorescence, %pos = percent of positive cells). A representative experiment (of three performed) is presented.

significant correlation between these two parameters was seen. A similar correlation between FX expression levels and other selectin ligands was not found (results not shown).

Metastatic Colorectal Cancer Cell Line Variants Express Higher Levels of the FX Enzyme and of sLe-a Than the Corresponding Nonmetastatic Variants

To assess the contribution of the FX enzyme to various malignancy associated characteristics, we compared cell lines having an identical genetic background but that differ in their metastatic phenotype. High and low metastatic variants originating from two patients were used. SW480 and SW620 cells originated in one patient, and KM12C, KM12SM, and KM12L4 originated from another patient.

SW480 and SW620 are colorectal cancer cell lines derived, correspondingly, from the primary tumor and from a lymph node metastasis of a single patient. This cell pair was recently validated as an appropriate model to study differences between primary and secondary tumors (23).

Fig. 2A demonstrates that the metastatic SW620 cells express higher levels of the FX enzyme than the nonmetastatic SW480 cells. These cells also express higher levels of sLe-a (Fig. 2B).

The KM12C cell line was established from a primary colorectal cancer, and the KM12SM and KM12L4 lines are metastatic variants of KM12C derived from nude mouse xenotransplants. These three cell lines thus have the same genetic background (19). The KM12SM cells are more metastatic than the KM12L4 cells (19). Fig. 3A demonstrates that the metastatic KM12SM variant expresses higher levels of the FX enzyme than the primary KM12C cells. The more metastatic KM12SM variant expresses twice as much FX enzyme than the less metastatic KM12L4 variant in which FX expression was only marginally higher than that by the primary KM12C cells. Fig. 3B shows that the expression of sLe-a in the three cell lines correlated with the FX expression by these cells.

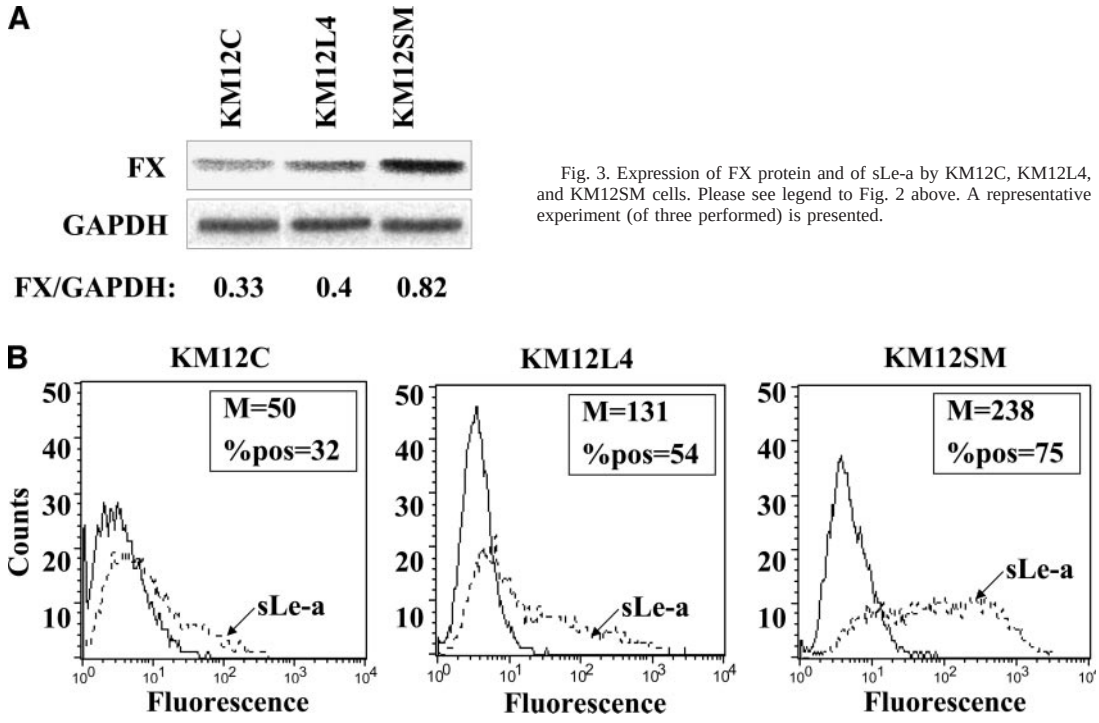

Fig. 3. Expression of FX protein and of sLe-a by KM12C, KM12L4, and KM12SM cells. Please see legend to Fig. 2 above. A representative experiment (of three performed) is presented.

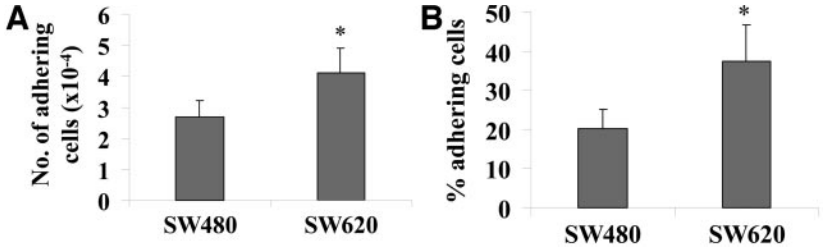

Fig. 4. Adhesion of SW480 and SW620 cells. *A*, adhesion to HUVECs. SW480 and SW620 cells were labeled with CFDA-SE reagent and incubated on activated HUVECs for 30 minutes as described in Materials and Methods. Nonadherent cells were removed, and the number of adherent cells was determined by a fluorescence ELISA reader in reference to a standard curve. The *bars* represent mean ± SD of values obtained in four independent experiments. *, $P < 0.03$. *B*, adhesion to rE-selectin. CFDA-SE–labeled SW480 and SW620 cells were incubated on rE-selectin–coated plates for 45 minutes as described in Materials and Methods. Percentage of adhering cells was calculated as the ratio between fluorescence signal of adhering cells and that obtained from the total number of plated cells. Fluorescence of the cells was determined as described above. The *bars* represent mean ± SD of values obtained in three independent experiments. *, $P < 0.02$.

The results presented above established, thus, a correlation between the metastatic phenotype of colorectal cancer and expression levels of the FX enzyme and of sLe-a.

The Adhesive Capacity of Colorectal Cancer Cells to Activated Endothelial Cells and to E-Selectin Is Linked to the Expression Levels of Endogenous FX and sLe-a

Fig. 4A demonstrates that the metastatic SW620 cells expressing higher levels of FX and sLe-a than SW480 cells (Fig. 2), adhere better to activated HUVEC ($P < 0.03$).

To test whether the differential adhesion of SW620 and SW480 cells to activated HUVECs reflects their adhesion capacity to E-selectin, we measured the adhesion of these cells to recombinant E-selectin. The results presented in Fig. 4B show that the metastatic SW620 cells adhere significantly better to rE-selectin than SW480 cells ($P < 0.02$), suggesting that the adhesion of the cells is mediated by a selectin-selectin ligand interaction.

Similar results were obtained when comparing the adhesive capacity of primary KM12C cells with that of the metastatic KM12SM cell variant. The highly metastatic KM12SM cells, expressing high levels of the FX enzyme and of sLe-a (Fig. 3), adhered better to activated HUVECs ($P < 0.03$; Fig. 5A) and to rE-selectin ($P < 0.02$; Fig. 5B) than the KM12C cells, which express a low metastatic behavior and low levels of FX and sLe-a.

The FX Enzyme Directly Regulates the Expression of sLe-a and the Selectin-Mediated Adhesion of Colorectal Cancer Cells

FX cDNA Transfection. The results presented in the previous sections show a positive correlation between expression levels of the fucose-generating FX enzyme, expression levels of the selectin ligand sLe-a, and adhesion of colorectal cancer cells to activated HUVECs and rE-selectin. The next set of experiments was aimed to determine if the FX enzyme directly regulates the expression of sLe-a on colorectal cancer cells and their adhesion to activated HUVECs and rE-selectin. It was assumed that if fucose is indeed a limiting factor in selectin ligand biosynthesis in colorectal cancer cells and thus of their E-selectin–dependent adhesion to activated HUVECs, then overexpressing the FX enzyme in low FX-expressing cells would result in higher levels of selectin ligand expression and in an up-regulated adhesive capacity of FX cDNA-transfected cells. By the same token, down-regulating the levels of FX by siRNA should decrease the expression levels of selectin ligands. The FX siRNA-transfected cells should, as a result, express a down-regulated adhesive capacity. The experiments described below demonstrate that this is indeed the case.

We first overexpressed the FX enzyme in SW480 cells, which express very low levels of endogenous FX and sLe-a (Fig. 2). These cells were transiently cotransfected with FX and GFP cDNA (in a 10:1 ratio). These cells expressed higher levels of FX protein than control cells transfected with GFP alone (Fig. 6A). Fig. 6B shows that these transfectants adhered better to activated HUVECs than cells transfected with GFP cDNA alone ($P < 0.001$) and shows that the adhesion of the FX-transfectants was reduced to background levels (i.e., adhesion to nonactivated HUVECs) by antibodies directed against E-selectin ($P < 0.001$).

Adhesion experiments in which rE-selectin was used instead of activated HUVECs (Fig. 6C) yielded similar results ($P < 0.001$). Antibodies against E-selectin reduced the adhesion of the FX-transfectants to rE-selectin also in this case ($P < 0.001$).

FX siRNA Transfection. The next step was to down-regulate the expression of the FX enzyme in SW620 cells by stably transfecting them with FX siRNA (205 cells; ref. 20). SW620 cells transfected with a mutated FX siRNA sequence in the pSuper vector (360 cells) served as controls. Northern blotting indicated that the FX siRNA-transfected 205 cells expressed lower levels of FX mRNA than the control-transfected 360 cells (Fig. 7A). The FX siRNA-transfected 205 cells expressed also lower levels of the FX protein than the control transfected 360 cells (Fig. 7B). It is interesting to note that the down-regulation of FX protein was more remarkable at the protein level than at the mRNA level. No explanation for this observation is available at this stage. Fig. 7C demonstrates that the siRNA-transfected 205 cells express less sLe-a than control transfectants.

Fig. 8 shows that the FX siRNA-transfected 205 cells adhered less well to rE-selectin than control 360 cells ($P < 0.01$). In conformity with the results reported in Fig. 6C, antibodies directed against E-

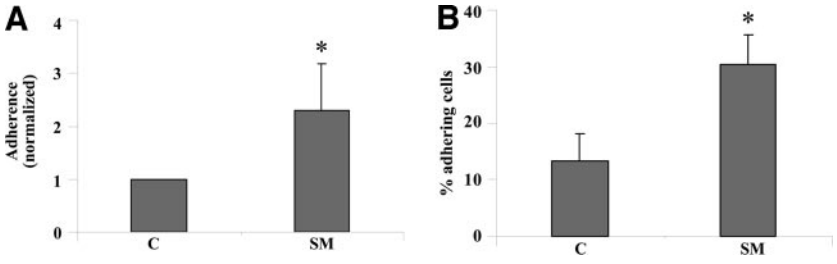

Fig. 5. Adhesion of KM12C and KM12SM cell lines. *A*, adhesion to HUVECs. KM12C and KM12SM cells were labeled with CFDA-SE and incubated on activated HUVECs for 30 minutes as described in Materials and Methods. The percentage of adherent KM12C was given a value of 1.0. Normalized adherence values were obtained by dividing percentage of adhesion of KM12SM cells by percentage of adhesion of KM12C cells. The bars represent mean ± SD of values obtained in five independent experiments. *, $P < 0.03$. *B*, adhesion to rE-selectin. Please see legend to Fig. 4*B* above. The bars represent mean ± SD of values obtained in three independent experiments. *, $P < 0.02$. (C = KM12C, SM = KM12SM).

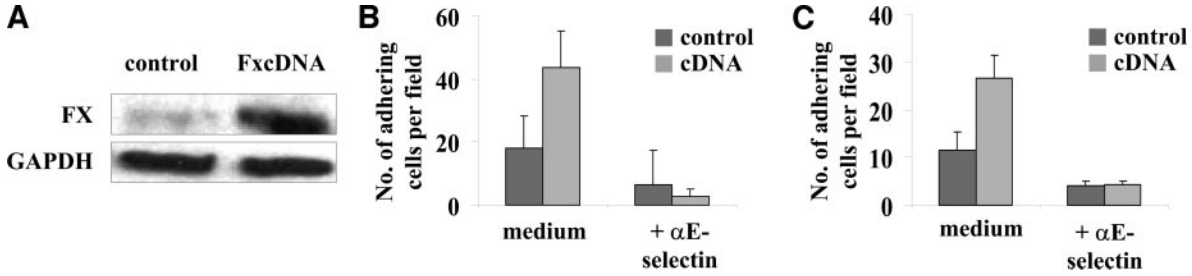

Fig. 6. Adhesive properties of SW480 cells transiently overexpressing FX cDNA. *A*, expression of FX protein. SW480 cells were cotransfected with FX cDNA and GFP. Controls were transfected with GFP alone. Lysates were subjected to Western blot analysis as described in the legend for Fig. 2*A*. *B*, adhesion to HUVECs and its blocking by antibodies against E-selectin. The transfected cells were incubated on activated HUVECs, as described in the legend for Fig. 4*A*. The *bars* represent mean ± SD of values obtained from five independent experiments. $P < 0.001$ for the difference between FX cDNA and control transfectants. Similar experiments were performed in the presence of antibodies against E-selectin. Two independent experiments were performed. $P < 0.001$ for the difference between the adhesion of FX cDNA transfectants in the presence or absence of the antibody. *C*, adhesion to rE-selectin and its blocking by antibodies against E-selectin. The transfected cells were incubated on rE-selectin–coated plates, as described in the legend for Fig. 4*B*. The *bars* represent mean ± SD of values obtained by counting several fields in two independent experiments. Adhesion was blocked, in two independent experiments, by anti-E-selectin antibodies (α E-selectin; $P < 0.001$). The adhesion presented in *B* and *C* was determined by counting adherent, GFP-expressing cells under a fluorescence microscope (the average number of cells per field was obtained by counting several fields). Control = SW480 cells cotransfected with control pRc/CMV vector and GFP; FX cDNA = SW480 cells cotransfected with FX cDNA and GFP; see Materials and Methods.

selectin blocked to background levels the adhesion of the control 360 cells to rE-selectin ($P < 0.001$; Fig. 8). The low adhesion of the FX siRNA-transfected 205 cells to rE-selectin was also blocked to background levels by these antibodies ($P < 0.001$; Fig. 8). An isotype control antibody used in these experiments had no blocking activity whatsoever.

The transfection experiments described above provide conclusive evidence that a FX→selectin ligand→adhesion pathway operates in colorectal cancer. However, the involvement of other fucosylated adhesion molecules controlled by the fucose-generating FX enzyme in the adhesion of colorectal cancer cannot be ruled out.

The siRNA transfectants adhered less well also to ECM ($P < 0.002$; Fig. 9A). To identify the ECM protein to which adhesion was reduced by down-regulating the fucose-generating FX enzyme, we compared the adhesion of FX siRNA transfectants (205 cells) and of control transfectants (360 cells) to collagen, laminin, and fibronectin. Whereas the adhesion of both types of transfectants to collagen and laminin was similar, the FX siRNA-transfected cells adhered significantly less well to fibronectin than the controls ($P < 0.04$; Fig. 9B). This finding led us to tentatively conclude that cellular adhesion to fibronectin may be fucose dependent.

We also tested if knocking down FX levels by FX siRNA transfection would affect adhesion to uncoated tissue-culture plastic vessels under normal culture conditions. Three independent experiments showed a reduced survival of SW620 cells in which levels of FX were knocked down by FX siRNA transfection, as compared with control cells transfected with mutated FX siRNA. At the time point of 96 hours after the initiation of culture, the average number of viable-adherent control cells was 1.96-fold higher than that of the FX siRNA transfectants ($P < 0.02$).

The above results suggest that FX knockdown may influence global fucosylation of colorectal cancer glycomolecules. To test this possibility, we evaluated levels of fucoconjugates in FX siRNA transfectants (205 cells) and in control 360 cells. Fig. 10 shows a lectin blot

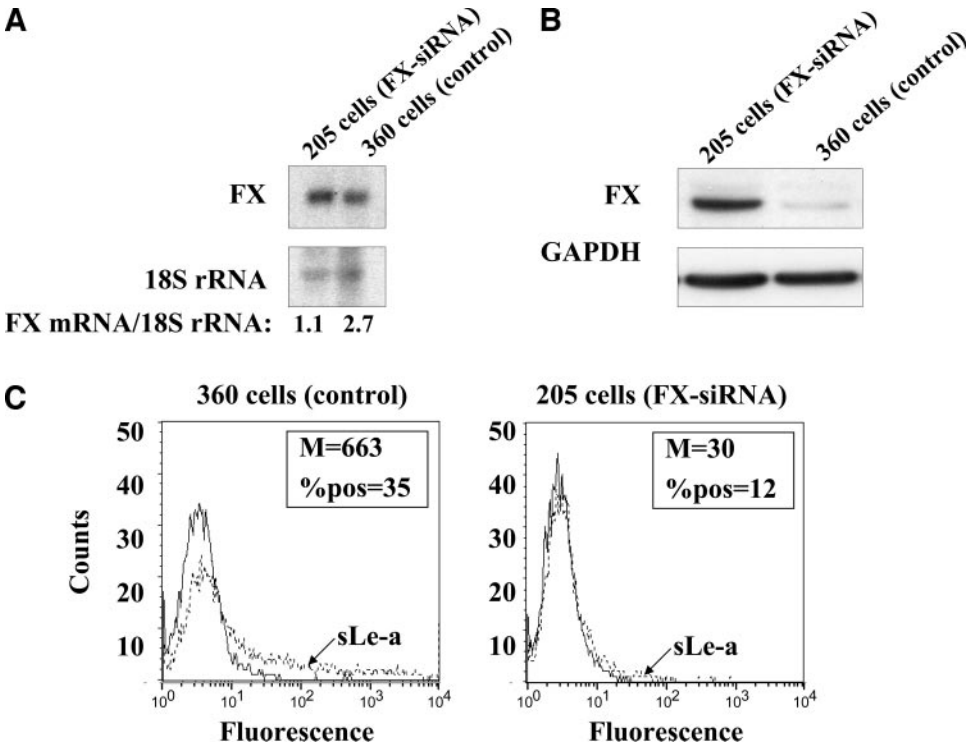

Fig. 7. Expression of FX mRNA, FX protein and of sLe-a by SW620 cells stably transfected with FX siRNA. *A*, FX mRNA. Expression was determined by Northern blot analysis. Values represent the ratio between the signal of FX mRNA in the cells and the signal of 18S rRNA in the same cell sample. *B*, FX protein. Expression was determined as in Fig. 2*A*. *C*, sLe-a. Expression was determined as in Fig. 2*B*. A representative experiment (of three performed) is presented (360 cells = control cells; 205 cells = FX siRNA transfectants). M = mean fluorescence; %pos = % positive cells.

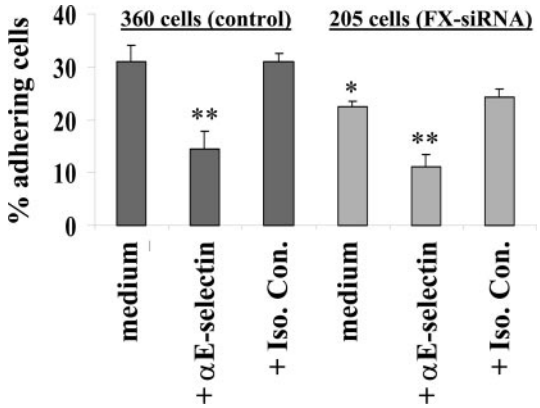

Fig. 8. Adhesion of SW620 cells transfected with FX siRNA to rE-selectin. 205 and 360 cells were labeled with CFDA-SE reagent and incubated on rE-selectin in the presence of antibodies against E-selectin (α E-selectin) or of irrelevant antibodies of the same isotype (iso.con). For details, please see legend to Fig. 4*B*. The bars represent mean ± SD values obtained in four independent replicates (*, $P < 0.01$). 360 cells = control cells; 205 cells = FX siRNA transfectants.

of extracts derived from control or FX siRNA-transfected cells developed with the Ulex europaeus agglutinin 1 agglutinin, a fucose-specific lectin (22). A clear reduction in global fucosylation is indicated in the FX siRNA transfectants.

DISCUSSION

The attachment of selectin ligand-expressing tumor cells to selectin-expressing endothelial cells is crucial event in the initial step of tumor cell-endothelium interactions, including extravasation (24–29). Therefore, understanding the regulation of selectin ligand synthesis in tumor cells is crucial for the development of metastasis-targeting manipulations.

The proper functioning of selectin ligands requires their fucosylation (1, 30–34), mainly by the fucose-generating FX enzyme (1, 13, 14, 33, 34). The present study documents a functional relationship between expression levels of the FX enzyme and those of the selectin ligand sLe-a by colorectal cancer cells. This conclusion was based on a direct and positive correlation between expression levels of FX and those of sLe-a by several colorectal cancer cell lines. Furthermore down-regulating FX expression by FX siRNA transfection decreased sLe-a expression.

We also documented a functional axis linking FX and sLe-a expression to the capacity of colorectal cancer cells to adhere to E-selectin. It was thus found that the FX enzyme is a limiting factor for the capacity of at least those colorectal cancer cells used in this study to adhere to endothelium. This conclusion is supported both by correlative evidence, as well as by direct evidence provided by transfection experiments.

It is assumed that highly metastatic tumor variants extravasate more efficiently than cells with a low metastatic phenotype (18, 26, 29). This implies that the former variants would adhere better to endothelial cells than the latter ones. Indeed, the highly metastatic variants SW620 (23) and KM12SM (19) adhered better to endothelial cells than the corresponding low metastatic variants SW480 and KM12C. Each of these variant pairs originated from a single patient. The highly metastatic variants expressed also higher levels of the FX enzyme and of sLe-a than the variants expressing a low metastatic phenotype. It was interesting to note that KM12SM, the most highly metastatic variant of the KM12C primary tumor (19), expressed higher levels of the FX enzyme and of sLe-a than KM12L4, the less metastatic variant from the same tumor. It seems, therefore, that the degree of malignancy correlates positively with expression of the fucose-generating FX enzyme and of its selectin ligand product sLea.

Taken together, these results lead us to hypothesize that the FX enzyme controls, by regulating selectin ligand biosynthesis, the interaction of at least certain colorectal cancer cells with endothelium and thus the capacity to extravasate and form metastasis. If this hypothesis will be confirmed by testing additional high and low metastatic cell pairs, the FX enzyme could become a target molecule for prevention of metastasis.

It was also demonstrated that the FX siRNA-mediated decrease of FX expression by colorectal cancer cells down-regulated the ability of the siRNA-transfected cells to bind to the ECM protein fibronectin. This raises the possibility that fucose is also involved in the interac-

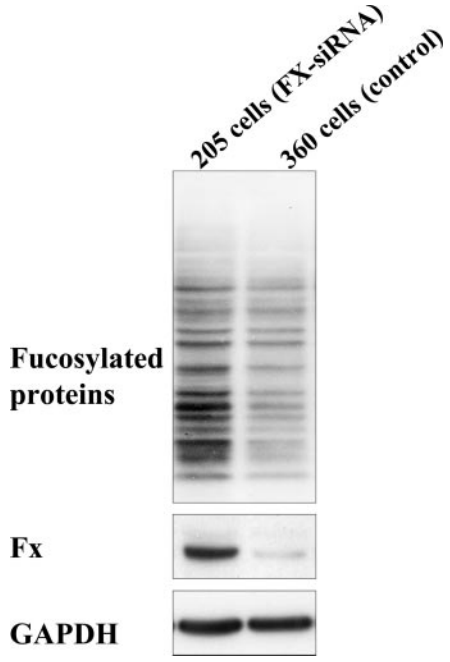

Fig. 10. Expression of fucosylated proteins by SW620 cells stably transfected with FX siRNA. Lysates of control (360) and FX siRNA (205) transfected cells were assayed for the expression of fucosylated proteins by Western blot analysis using horseradish peroxidase-conjugated Ulex europaeus agglutinin 1. Expression of FX protein in the two cell populations was determined as in Fig. 2*A*. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as loading control.

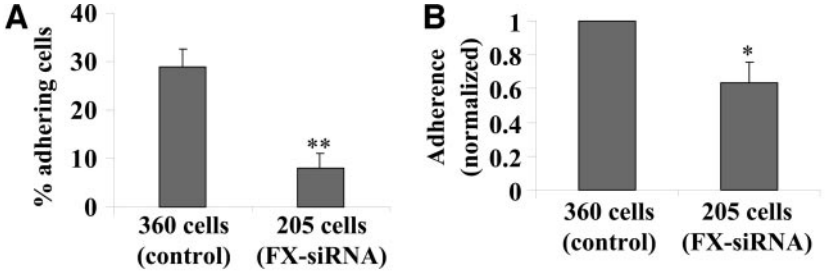

Fig. 9. Adhesion of SW620 cells transfected with FX siRNA to ECM. 205 and control 360 cells were labeled with CFDA-SE reagent and incubated on ECM (*A*) or on fibronectin (*B*). For details, please see legend to Fig. 8. The *bars* represent mean ± SD of values obtained in three independent experiments. *, $P < 0.04$; **, $P < 0.002$. 360 cells = control cells; 205 cells = FX siRNA transfectants.

tion between tumor cells and ECM. Indeed it was demonstrated that fucosylation of integrins, e.g., $\alpha_3\beta_1$, is essential for a correct assembly of the integrin α and β subunits and for the binding to fibronectin (35).

Fucose participates not only in controlling adhesive properties but also in other functions such as modifying signal transduction events. For example, Fringe, a fucose-specific glycosyltransferase initiates elongation of O-linked fucose residues attached to epidermal growth factor-like sequence repeats of Notch (6). This glycosylation modulates Notch-mediating signaling (5). Another example is the O-fucose modification of Cripto. This modification is essential for Nodal-dependent signaling (7). Because both Notch as well as Nodal-mediated signaling bear importance with respect to tumor progression (9, 36–39), it would be of interest to compare these signaling pathways in colorectal cancer variants expressing high or low levels of FX.

Taken together, the results of the present study, as well as those of other studies cited above, suggest that altering expression levels of the FX enzyme, thereby altering global fucosylation of colorectal cancer cells, could have far reaching effects on the survival and phenotype of these cells.

The FX enzyme supplies ~90% of the cellular fucose while the rest is supplied by the salvage pathway (1, 40). In this pathway, extracellular fucose is taken up by the cell, phosphorylated by a fucose-kinase, and subsequently converted to GDP L-fucose by GDP-fucose-pyrophosphorylase. The GDP L-fucose generated by the salvage pathway undergoes an identical biosynthetic route as the GDP L-fucose generated by the FX enzyme (34, 40). To fully assess the role of the FX enzyme in regulating various fucose-dependent reactions in colorectal cancer cells such as adhesion, Notch, or Cripto-mediated signaling, it would be important to determine the relative contribution of the salvage pathway to the cellular fucose supply.

REFERENCES

1. Smith PL, Myers JT, Rogers CE, et al. Conditional control of selectin ligand expression and global fucosylation events in mice with a targeted mutation at the FX locus. J Cell Biol 2002;158:801–15.
2. Sakamoto S, Watanabe T, Tokumaru T, Takagi H, Nakazato H, Lloyd KO. Expression of Lewis-a, Lewis-b, Lewis-x, Lewis-y, siayl-Lewis-a, and sialyl-Lewis-x blood group antigens in human gastric carcinoma and in normal gastric tissue. Cancer Res 1989;49:745–52.
3. Homeister JW, Thall AD, Petryniak B, et al. The alpha(1,3)fucosyltransferases FucT-IV and FucT-VII exert collaborative control over selectin-dependent leukocyte recruitment and lymphocyte homing. Immunity 2001;15:115–26.
4. Carlow DA, Corbel SY, Williams MJ, Ziltener HJ. IL-2, -4, and -15 differentially regulate O-glycan branching and P-selectin ligand formation in activated CD8 T cells. J Immunol 2001;167:6841–8.
5. Bruckner K, Perez L, Clausen H, Cohen S. Glycosyltransferase activity of Fringe modulates Notch-Delta interactions. Nature (Lond.) 2000;406:411–5.
6. Moloney DJ, Panin VM, Johnston SH, et al. Fringe is a glycosyltransferase that modifies Notch. Nature (Lond.) 2000;406:369–75.
7. Haltiwanger RS. Regulation of signal transduction pathways in development by glycosylation. Curr Opin Struct Biol 2002;12:593–8.
8. Yan YT, Liu JJ, Luo Y, et al. Dual roles of Cripto as a ligand and coreceptor in the nodal signaling pathway. Mol Cell Biol 2002;22:4439–49.
9. Adamson ED, Minchiotti G, Salomon DS. Cripto: a tumor growth factor and more. J Cell Physiol 2002;190:267–78.
10. Sullivan FX, Kumar R, Kriz R, et al. Molecular cloning of human GDP-mannose 4,6-dehydratase and reconstitution of GDP-fucose biosynthesis in vitro. J Biol Chem 1998;273:8193–202.
11. Ohyama C, Smith PL, Angata K, Fukuda MN, Lowe JB, Fukuda M. Molecular cloning and expression of GDP-D-mannose-4,6-dehydratase, a key enzyme for fucose metabolism defective in Lec13 cells. J Biol Chem 1998;273:14582–7.
12. Lopez-Ferrer A, de Bolos C, Barranco C, et al. Role of fucosyltransferases in the association between apomucin and Lewis antigen expression in normal and malignant gastric epithelium. Gut 2000;47:349–56.
13. Eshel R, Besser M, Zanin A, Sagi-Assif O, Witz IP. The FX enzyme is a functional component of lymphocyte activation. Cell Immunol 2001;213:141–8.
14. Eshel R, Zanin A, Sagi-Assif O, et al. The GPI-linked Ly-6 antigen E48 regulates expression levels of the FX enzyme and of E-selectin ligands on head and neck squamous carcinoma cells. J Biol Chem 2000;275:12833–40.
15. Eshel R, Zanin A, Kapon D, et al. Human Ly-6 antigen E48 (Ly-6D) regulates important interaction parameters between endothelial cells and head-and-neck squamous carcinoma cells. Int J Cancer 2002;98:803–10.
16. Izumi Y, Taniuchi Y, Tsuji T, et al. Characterization of human colon carcinoma variant cells selected for sialyl Lex carbohydrate antigen: liver colonization and adhesion to vascular endothelial cells. Exp Cell Res 1995;216:215–21.
17. Shirahama T, Ikoma M, Muramatsu T, et al. The binding site for fucose-binding proteins of Lotus tetragonolobus is a prognostic marker for transitional cell carcinoma of the human urinary bladder. Cancer (Phila.) 1993;72:1329–34.
18. Thurin M, Kieber-Emmons T. SA-Lea and tumor metastasis: the old prediction and recent findings. Hybrid Hybridomics 2002;21:111–6.
19. Morikawa K, Walker SM, Nakajima M, Pathak S, Jessup JM, Fidler IJ. Influence of organ environment on the growth, selection, and metastasis of human colon carcinoma cells in nude mice. Cancer Res 1988;48:6863–71.
20. Brummelkamp TR, Bernards R, Agami R. A system for stable expression of short interfering RNAs in mammalian cells. Science (Wash. DC) 2002;296:550–3.
21. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond.) 1970;227:680–5.
22. Lerrer B, Gilboa-Garber N. Differential staining of Western blots of avian egg white glycoproteins using diverse lectins. Electrophoresis 2002;23:8–14.
23. Hewitt RE, McMarlin A, Kleiner D, et al. Validation of a model of colon cancer progression. J Pathol 2000;192:446–54.
24. Brandley BK, Swiedler SJ, Robbins PW. Carbohydrate ligands of the LEC cell adhesion molecules. Cell 1990;63:861–3.
25. Mannori G, Crottet P, Cecconi O, et al. Differential colon cancer cell adhesion to E-, P-, and L-selectin: role of mucin-type glycoproteins. Cancer Res 1995;55:4425–31.
26. Voura EB, Sandig M, Siu CH. Cell-cell interactions during transendothelial migration of tumor cells. Microsc Res Tech 1998;43:265–75.
27. Kim YJ, Borsig L, Han HL, Varki NM, Varki A. Distinct selectin ligands on colon carcinoma mucins can mediate pathological interactions among platelets, leukocytes, and endothelium. Am J Pathol 1999;155:461–72.
28. Burdick MM, McCaffery JM, Kim YS, Bochner BS, Konstantopoulos K. Colon carcinoma cell glycolipids, integrins, and other glycoproteins mediate adhesion to HUVECs under flow. Am J Physiol Cell Physiol 2003;284:C977–87.
29. Laferriere J, Houle F, Huot J. Regulation of the metastatic process by E-selectin and stress-activated protein kinase-2/p38. Ann NY Acad Sci 2002;973:562–72.
30. Varki A. Selectin ligands. Proc Natl Acad Sci USA 1994;91:7390–7.
31. Renkonen R, Mattila P, Majuri ML, et al. In vitro experimental studies of sialyl Lewis x and sialyl Lewis a on endothelial and carcinoma cells: crucial glycans on selectin ligands. Glycoconj J 1997;14:593–600.
32. Karsan A, Cornejo CJ, Winn RK, et al. Leukocyte adhesion deficiency type II is a generalized defect of de novo GDP-fucose biosynthesis. Endothelial cell fucosylation is not required for neutrophil rolling on human nonlymphoid endothelium. J Clin Investig 1998;101:2438–45.
33. Listinsky JJ, Listinsky CM, Alapati V, Siegal GP. Cell surface fucose ablation as a therapeutic strategy for malignant neoplasms. Adv Anat Pathol 2001;8:330–7.
34. Noda K, Miyoshi E, Gu J, et al. Relationship between elevated FX expression and increased production of GDP-L-fucose, a common donor substrate for fucosylation in human hepatocellular carcinoma and hepatoma cell lines. Cancer Res 2003;63:6282–9.
35. Litynska A, Pochec E, Hoja-Lukowicz D, et al. The structure of the oligosaccharides of alpha$_3$beta$_1$ integrin from human ureter epithelium (HCV29) cell line. Acta Biochim Pol 2002;49:491–500.
36. Allenspach EJ, Maillard I, Aster JC, Pear WS. Notch signaling in cancer. Cancer Biol Ther 2002;1:466–76.
37. Weijzen S, Rizzo P, Braid M, et al. Activation of Notch-1 signaling maintains the neoplastic phenotype in human Ras-transformed cells. Nat Med 2002;8:979–86.
38. Maillard I, Pear WS. Notch and cancer: best to avoid the ups and downs. Cancer Cell 2003;3:203–5.
39. Adkins HB, Bianco C, Schiffer SG, et al. Antibody blockade of the Cripto CFC domain suppresses tumor cell growth in vivo. J Clin Investig 2003;112:575–87.
40. Marquardt T, Luhn K, Srikrishna G, Freeze HH, Harms E, Vestweber D. Correction of leukocyte adhesion deficiency type II with oral fucose. Blood 1999;94:3976–85.