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*

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The GPI-linked Ly-6 antigen E48 controls the expression of FX. By differential display we demonstrated that antibody-mediated ligation of the GPI-linked protein product of E48, a newly discovered human Ly-6 gene, up-regulates the expression of the FX enzyme in 3 lines of head and neck squamous carcinoma cells. FX is responsible for the last step in the synthesis of GDP-L-fucose. The up-regulation of FX was E48 ligand-specific. 22AWT head and neck squamous carcinoma cells expressing high levels of E48 expressed significantly higher levels of FX than the E48 antisense transfected 22AWT cells (8–3 cells). The former cells also expressed higher levels of two major fucosylated glycans (the selectin ligand, Sialyl Lewis a, and VIM-2) than the E48 antisense transfec-
tants. Conversely, transfection of cells from the 14CWT line expressing very low levels of E48 with E48 cDNA caused an up-regulated expression of FX and of the two fucosylated glycans in the 14C-CMV16 transfectants. Moreover, the expression levels of Sialyl Lewis a was significantly up-regulated on HNSSC upon ligation of E48 by anti-E48 antibodies. The functional significance of the E48-mediated up-regulation of Sialyl Lewis a was demonstrated in rolling experiments on E-selectin bearing surfaces under physiological conditions of shear flow and on tumor necrosis factor α-activated human umbilical venous endothelial cells. Only high E48/FX/Sialyl Lewis a expressing 14C-CMV16 cells could roll on purified E-selectin or establish E-selectin dependent rolling on the activated human umbilical venous endothelial cells. Low E48/FX/Sialyl Lewis a expressing 14CWT cells did not roll. These results show that E48 controls the expression of the FX enzyme and of certain fucosylated E-selectin ligands by HNSSC. E48 may thus function as a key regulator of the adhesiveness of these tumor cells to inflamed vessel walls expressing E-selectin.

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1 The abbreviations used are: mAb, monoclonal antibody; GPI, glycosyl-phosphatidylinositol; HNSSC, Head and Neck Squamous Carcinoma Cells; HUVEC, Human umbilical venous endothelial cells; LAD, Leukocyte adhesion deficiency; SLea, sialyl Lewis a; SLeα, sialyl Lewis x; TNF, tumor necrosis factor; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; MOPS, 4-morpholinepropanesulfonic acid; EC, endothelial cells.
E48 levels were determined by flow cytometry. \( n_p \) is the percentage of E48 expressing cells. m is the mean fluorescence of E48 positive cells. The horizontal line represents the cell population gated for E48 expression (see "Experimental Procedures").

protein (10), a key enzyme in fucose biosynthesis (11) was up-regulated in HNSCC treated with antibodies against E48. Furthermore, E48 ligation by anti-E48 antibodies on HNSCC also up-regulated expression levels of the selectin ligand sialyl Lewis a (SLea).

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Tissue Culture**—The HNSCC lines UM-SCC-22A, UM-SCC-22B, and UM-SCC-14C (12) were kindly provided by Dr. T. E. Carey (Ann Arbor, MI) (12). Below are the designations and the descriptions of the cells utilized in this study: Fig. 1 shows the E48 expression profile of these cells: 1) 22AWT cells are the original UM-SCC-22A cells. These cells express E48. 2) 22BWT cells are the original UM-SCC-22B cells. These cells express E48. 3) 22A-E48hi cells were obtained by flow cytometry sorting of 22A-WT cells for a high E48 expression. These cells express significantly higher levels of E48 than the parental 22AWT cells. 4) 14CTWT cells are the original UM-SCC-14C cells. These cells express E48. 5) 14C-MV16 cells were obtained by a stable transfection of 14CTWT cells with E48 cDNA. These cells express high levels of E48. 6) 22BWT cells are the original UM-SCC-22B cells. These cells express E48. 7) 22B-E48hi cells were obtained by flow cytometry sorting of 22BWT cells for a high E48 expression. These cells express significantly higher levels of E48 than the parental 22BWT cells.

Cells were routinely cultured in humidified air with 5% CO\(_2\) at 37 °C in DMEM (Biological Industries, Beit-Ha’Emek, Israel), 5% FCS (HyClone, Logan, UT), 2 mM l-glutamine, 1% penicillin/streptomycin (Biological Industries, Beit-Ha’Emek, Israel).

Human umbilical cord vein endothelial cells (HUVEC) were isolated from umbilical cord veins according to the method of Jaffe et al. (13). The cells were pooled and established as primary cultures in M199 containing 10% FCS, 8% pooled human serum, 50 \( \mu g/ml \) endothelial cell growth factor (Sigma Israel Chemicals Ltd., Rehovot, Israel), porcine intestinal heparin (10 units/ml) (Sigma Israel Chemicals Ltd.) and antibiotics. Primary cultures were serially passaged (1:3 split ratio), and passages 3 and 4 were taken for adhesion experiments.

**Antibodies**—The mouse mAb against E48 (aE48) has been described previously (1). Mouse mAb against human VIM-2 (aVIM-2) (14) was kindly supplied by Dr. V. Knapp (Institute of Immunology, Vienna University, Austria). Mouse mAb against human NCA (aNCA) was kindly supplied by Dr. D. Goldenberg and Dr. H. Hansen, Immunomedics, Palo Alto, Calif. This antibody is also cross reactive with the GPI linked 50/90 antigen, which is expressed on activated granulocyte (16). Mouse mAb against human SLEx (aSLEx, clone 2D3) has been described previously (15). Mouse mAbs against human SLEx (aSLEx, clone Km-93), human CD59 (clone YTH53.1), and ICAM-1 (clone 84H10) and against human CD62E (E-selectin, clone 1.2B6) were purchased from Serotech (Oxford, UK). A rabbit antibody against human actin was purchased from Sigma. Polyclonal rabbit antibodies directed against FX were generated in our laboratory, using two peptides from the native human FX protein (11): 1) H2N-CNGPPMNSNFGYS (amino acids 133–144) and 2) H2N-SLEXNLKRLTPFRFP (amino acids 284–298).

**Generation of Sense and Antisense E48-cDNA Transfected HNSCC Cells**—The E48 encoding cDNA in \( \phi \)CDM8 (4) was inverted to antisense orientation by EcoRI cleavage and religation. The isolated inserts of the sense and antisense cDNAs were excised by HindIII/NotI digestion and inserted in the HindIII/NotI sites of pRC-CMV (Invitrogen, Leek, The Netherlands). The cell lines were transfected by Lipofectin (Life Technologies, Inc.). In short, cells were plated in a 6-well plate (2 \( \times \) 10\(^5\) cells/well) and cultured overnight. A mixture of Lipofectin and DNA was prepared: 10 \( \mu g/ml \) Lipofectin and 10 \( \mu g/ml \) DNA for UM-SCC-14C cells or 50 \( \mu g/ml \) Lipofectin and 10 \( \mu g/ml \) DNA for UM-SCC-22A cells. The Lipofectin and DNA were mixed in a small volume of serum-free DMEM (20% of the final transfection volume) and incubated at room temperature for 15 min. The solution was adjusted to the final volume with serum-free DMEM. The cultured cells were washed twice with serum-free medium before the addition of the transfection solution (0.75 ml/well). After 24 h of incubation, the transfection solution was replaced by regular tissue culture medium. After an incubation period of 72 h, selection medium was added containing 1 mg/ml G418 (Life Technologies, Inc.). Surviving clones were tested for E48 expression by immunocytochemical staining. From transfecants with a heterogenous expression or a down-regulated expression of E48, clones with a homogeneous E48 expression were obtained by limiting dilution.

**Sorting of 22AWT and 22BWT Cells for High E48 Expression**—3 \( \times \) 10\(^6\) cells were incubated for 60 min at 4 °C with anti-E48 mAb. Following two washes with DMEM supplemented with 5% FCS, the cells were incubated with a fluorescein isothiocyanate-conjugated secondary mouse antibody against human IgG. After two more washes, cellular populations expressing high levels of E48 (22A-E48\(^{hi}\) or 22B-E48\(^{hi}\)) were obtained by using a FACS-IV sorter (Becton Dickinson, Mountain View, CA). Several cycles of sorting were required to obtain cells with a significantly higher E48 expression than the parental cells.

**E48-mediated Signal Transduction and Differential Gene Expression Analysis**—E48-mediated signals were transduced to HNSCC by incubating cells with anti-E48 mAb for 1 h at 37 °C. Control cells were incubated under the same conditions without antibody. The cells were supplemented, washed, and was isolated (17). Differentially expressed genes were determined by differential display polymerase chain reaction (17) using the Delta\(^{TM}\) RNA Fingerprinting kit (CLONTECH Laboratories, Inc.). The following primers were used: 5’-CAT-TAT-GCTGATGATATCCTTTTTTGTGC-3’ and 5’-ATATACCCCT-ACTAATGAGGACTCGG-3’.

Differentially expressed cDNA bands in antibody-stimulated cells as compared with control cells were eluted from the gel and reamplified by polymerase chain reaction using the same primers according to the manufacturer’s instructions. A higher expression of mRNA corresponding to the cDNA identified in the above assays was confirmed by using Northern blotting of RNA from untreated or from anti-E48 mAb-treated cells. Differentially expressed cDNA bands were isolated from the sequencing gel, radiolabeled, and used as a probe in RNA blot analysis. The corresponding cDNA fragment that generated a specific hybridization pattern on RNA blots was sequenced in both directions, and the nucleotide sequence obtained was compared with known sequences by searching the GenBank\(^{TM}\) with the FASTA program (Genetic Computer Group software (Madison, WI)).

**RNA Isolation and Northern Blotting**—Total RNA was isolated from antibody-treated or control cells using RNAzol solution (Bio Labs, Jerusalem, Israel). A total of 20 \( \mu g \) of RNA was loaded on an 1% agarose formaldehyde gel and electrophoresed in MOPS buffer as described by Sambrook et al. (18). The RNA was subjected to Northern blotting by capillary transfer in 20\( \times \) SSC onto nitrocellulose filters (Bio-Rad Laboratories, Inc.). The filters were prehybridized in hybridization buffer for 2 h at 65 °C. A 250 bp probe specific for the E48 cDNA was prepared by polymerase chain reaction using the same primers according to the manufacturer’s instructions and labeled with \( ^{32} \)P-dCTP (Amersham). The filters were exposed to X-ray film (Kodak). When necessary, the filters were stripped in 0.1% SDS and rehybridized with another probe.

**Sequence Analysis**—The corresponding cDNA fragment that generated a specific hybridization pattern was sequenced in both directions. Sequencing was performed at the Genetics Core Facility at the UCI School of Medicine. The DNA sequence was analyzed with the Genetic Computer Group program (Madison, WI). The sequence was compared with the GenBank databases for similarity.
between the signal of FX mRNA in each cell (untreated or treated with anti-E48 antibodies), and the signal of rRNA in the same cell was exposed to the same treatment as above.

**Probes**—Differentially expressed cDNA bands were excised from the differential display sequencing gel and labeled with [γ-^32^P]dCTP (3000 Ci/mmol, Amersham Biosciences). Primers for reverse transcription (Roche Molecular Biochemicals) and hybridized overnight at 42 °C to filters. The filters were washed once in 2 × SSC/0.1% SDS at room temperature for 30 min followed by two washes in 0.1% SSC/0.1% SDS for 30 min each at 50 °C and autoradiographed for 1-3 days.

**Flow Cytometry**—1 × 10^5 cells were incubated for 60 min at 4 °C with the different mAbs (diluted 1:80 to 1:100). Following two washes with DMEM supplemented with 5% FCS and 0.05% sodium azide, the cells were resuspended from the bottom of the flask, and SLea expression was determined using biotin-conjugated IgM mouse antibody directed against human SLea (clone 2D3) (Seikagaku Co., Tokyo, Japan) and as secondary Ig antibody Phycoerythrin-conjugated Streptavidin (Jackson ImmunoResearch Lab. Inc., West Grove, PA). Following two more washes, the pattern of antigen expression was determined using a Becton Dickinson FACSort (Mountain View, CA) and the CellQuest software. Mean Fluorescence was determined for antigen-positive cells only.

**Statistical Evaluation**—Student’s t test was used to evaluate the statistical significance of differences between FX and SLea expression on HNSCC before and after treating the cells with anti-E48 or anti-NCA antibodies.

**SDS-Polyacrylamide Gel Electrophoresis and Western Blotting**—HNSCC monolayers were cultured in to confluence and lysed on their culture dishes with Laemmli sample buffer (19). Lysates were boiled for 10 min, centrifuged, and applied on a miniprotein II system (Bio-Rad) for SDS-polyacrylamide gel electrophoresis using a 12% slab gel as described by Laemmli (19). Electrophoretic transfer of proteins from the polyacrylamide gel to nitrocellulose (Schleicher & Schull) was performed by a mini-transblot electrophoretic cell (Bio-Rad) at 100 V for 1.5 h. After transfer, the nitrocellulose membrane was cut into strips and incubated overnight at 4 °C with rabbit FX polyclonal antibodies raised in our laboratory, diluted 1:2000, then washed three times for 10 min with Tween-20 buffer, and blocked free binding sites on the membrane.

The blocked nitrocellulose membrane strips were incubated overnight with anti-FX polyclonal antibodies raised in our laboratory, diluted 1:2000, then washed three times for 10 min with Tween-20 buffer, then incubated for 45 min with horseradish peroxidase-conjugated secondary goat antibody against rabbit IgG at room temp. Finally, the nitrocellulose membrane was washed three times for 10 min with Tween-20 buffer and blocked for 30 min with TBS-Tween. The bands were visualized by chemoluminescence-ECL reaction and autoradiography by exposure to Kodak X-AR5 film (Eastman Kodak Co., Rochester, NY) for 1–8 min.

The quantification of protein in the lanes was determined by Western blotting the membrane also with anti-actin antibodies diluted 1:1000. The level of FX expression is presented as the ratio between the FX protein signal (determined by densitometry) for each cell type and the actin signal in the same cell type.

**Laminar Flow Assays**—Cultured H1 cells grown as monolayers were harvested by a 10-min incubation with H/H medium (Hanks’ balanced salt solution (Sigma Israel Chemicals Ltd.), containing bovine serum albumin (2 mg/ml, fraction V, Sigma) and 10 mM HEPES, pH 7.4), supplemented with 5 mM EDTA at 37 °C. Washed cells were resuspended in the same medium at a concentration of 1 × 10^6 cells/ml and kept at room temperature until use. Cells were diluted 20-fold into binding medium (H/H supplemented with 2 mM Ca^2+ and ) and immediately perfused through the flow chamber. An E-selectin coated substrate was prepared as described (20). Briefly, protein A (20 μg/ml in coating medium; Sigma) was spotted onto a polystyrene plate, the substrate was blocked with 2% human serum albumin (Fraction V, Calvin Labs, La Jolla, CA) in phosphate-buffered saline and overlaid with culture supernatant from COS cells transfected with cDNA of human E-selectin-G1 (a kind gift of Dr. T. S. Kupper, Brigham and Women’s Hospital, Boston, MA). For control, a protein A spot was overlaid with culture supernatant from untransfected COS cells. The E-selectin coated plate or the protein-A control plate were assembled in a parallel flow chamber (260-μm gap thickness) (21) and mounted on the stage of an inverted phase-contrast microscope (Diaphot-TMD, Nikon Inc., Garden City, NY). For adhesion experiments on resting or activated EC, primary HUVEC (passage 2 or 3) were plated at confluent density for 1 h in tissue culture dishes (Becton Dickinson, Falcon Plates, Plymouth, UK) and cultivated with human fibronectin (25 μg/ml in phosphate-buffered saline). Nonadherent EC was gently rinsed out, and adherent cells were prepared on the fibronectin-coated spots for 24 h before cytokine treatment. The EC monolayers were left intact or stimulated for 18 h with heparin-free culture medium supplemented with TNFα (2 ng/ml, 50 units/ml) (R & D, Minneapolis, MN). Before assay, the various EC-coated plates were washed three times with binding medium and assayed in the lower wall of the flow chamber, whereas the bottom portion of the monolayer (5 × 30 mm) was exposed to flow. 5 × 10^6/ml cells suspended in binding medium were perfused in the flow chamber with a syringe pump (Harvard Apparatus, Natick, MA) attached to the outlet side. Cells were visualized with a 10× objective and videotaped with a long integration LIS-700 CCD video camera (AppiQtech, Holon, Israel) and a Time Lapse SVHS-Video recorder (AG-6730, Panasonic, Japan). The number of cells that accumulated in both representative fields (each 0.17 mm² in area) during 1 min of constant flow generating a wall shear stress of 1 dyn/cm² was manually quantitated by analysis of played back images directly from a monitor screen. For inhibition studies, substrates were washed with H/H medium supplemented with 5 mM EDTA. The cells were then suspended in the same medium and perfused through the chamber in a wall shear stress of 1 dyn/cm². Rolling velocities were measured for cells accumulated on the E-selectin substrate during 1 min of flow at 1 dyn/cm². Rolling of cells accumulated at low flow and then subjected to elevated shear stresses of 5, 10, and 15 dyn/cm²; each shear increment lasting for 10 s was determined thereafter.

**RESULTS**

**Ligation of E48 by αE48 mAb Up-regulates the Expression of FX**—To find out whether E48 is capable of altering the expression of cellular genes in HNSCC by transducing signals to such cells, αE48 mAb (1) was added to 22AWT cells as a surrogate ligand (the physiological ligand of E48 has not been identified thus far). The method used to detect altered gene expression was differential display polymerase chain reaction of mRNA (17). 22AWT cells treated with αE48 mAb for 60 min at 37 °C were used in the differential display assay. 22AWT cells incubated under the same conditions but without antibody served as controls. The density of several cDNA bands was increased in antibody-stimulated cells. Multiple repetitive experiments yielded the same results. One of these cDNA bands was then eluted from the gel and amplified. A higher expression of a mRNA species corresponding to the cDNA species identified in the above assays was confirmed using Northern blotting of RNA from untreated or αE48 mAb-treated 22AWT cells. The corresponding cDNA was then cloned and sequenced. GenBank™ analysis showed that this cDNA species had a 98.2% homology to FX (Ref. 22; accession number U58766). Fig. 2 shows that compared with control cells FX mRNA is up-regulated in 22AWT cells by αE48 mAb ligation. Similar results were obtained with 22AWT cells incubated with αE48 mAb conjugated to polystyrene 6-μm microparticles (Polysciences Inc., Huntsville, AL). An exposure of cells to E48 mAb for 60 min at 37 °C yielded a maximal up-regulation of FX mRNA expression in antibody-stimulated cells. Multiple repetitive experiments yielded the same results. One of these cDNA bands was then eluted from the gel and amplified. A higher expression of a mRNA species corresponding to the cDNA species identified in the above assays was confirmed using Northern blotting of RNA from untreated or αE48 mAb-treated 22AWT cells.

Eight different ligation experiments with 22AWT cells were performed. A different RNA preparation was analyzed in each experiment. The average increase in FX mRNA expression in E48 ligated cells compared with control cells was 1.7 ± 0.06-fold (p < 0.005) (Fig. 2). To confirm these data we performed three ligation experiments with E48 antisense-transfected 8-3 cells. These cells expressed significantly lower levels of E48 than the parental 22AWT cells (Fig. 1). A different RNA preparation was used in each experiment. The average increase in FX mRNA in E48-ligated 8-3 cells as compared with nonligated 8-3 cells was 2.5 ± 0.09-fold (p < 0.005) (Fig. 2). Cells from a
highly significant in all experiments (see "Results"). The Northern blot shown is a representative of intensity of the 28 S/18 S RNA intensity in the same lane (see "Experimental Procedures"). The increase in FX mRNA expression was calculated by dividing the intensity of the FX protein signal in each of the lanes by the intensity of the actin signal in the same lane (see "Experimental Procedures"). The Western blot shown is a representative of two experiments.

The up-regulation of FX in 22AWT cells by E48 ligation was also demonstrated at the protein level. Western blotting of lysates from cells treated with E48 mAb for 60, 120, and 180 min as well as from control cells showed an increased expression of FX protein in antibody-treated cells. Two independent experiments using different lysates in each were performed. The results demonstrated that the transfection of the 14CWT (E48-negative) cells with E48 cDNA concomitantly increased E48 (Fig. 1) and FX levels (Fig. 5). Complementary experiments in which FX mRNA levels were measured in E48-negative 14CWT and in E48-positive 14C-CMV16 cells were performed. The results demonstrated that the transfection of the 14CWT (E48-negative) cells with E48 cDNA concomitantly increased E48 (Fig. 1) and FX levels (Fig. 5) in the 14C-CMV16 transfectants.

The linkage between the expression of E48 and that of FX was also demonstrated at the protein level. Fig. 6 shows Western blots of lysates from the two sets of E48- and E48b cells described above. Transfection of E48 antisense significantly decreased FX protein levels in the 8-3 transfectant compared with FX protein levels in the 22A-E48hi cells. On the other hand, the transfer of E48 cDNA to 14CWT cells significantly increased FX protein levels in the 14C-CMV16 transfectants compared with FX protein levels in control 14CWT cells. These results support the possibility that a constitutive signaling through E48 takes place in HNSCC cultures. Additional support for this hypothesis was provided by the results reported above (Fig. 2) showing that FX up-regulation following experimental ligation of E48 was more pronounced in cells expressing low basal levels of E48 than in cells expressing high basal levels of E48.

The Expression of Fucosylated Glycans on HNSCC Increases Following E48 cDNA Transfection and Decreases Following E48 Antisense Transfection—The binding of selectins to fucosylated glycoconjugate ligands regulates the primary adhesion between blood-borne leukocytes and inflamed endothelium. Similarly, the interactions of E-selectin with ligands on cancer cells regulate metastasis formation (27–32). FX has been recently identified as the enzyme responsible for the last step of the metabolic pathway resulting in guanosine 5′-diphosphate GDP-L-fucose synthesis from GDP-D-mannose (22). In view of the fact that GDP-L-fucose is the key substrate of several fuco-syl transferases participating in the biosynthesis of diverse lactosamine glycoconjugates including the selectin ligands SLeα and SLeα, we compared by flow cytometry the expression of SLeα and SLeα, by the two sets of HNSCC expressing either high or low levels of E48 and FX. The results in Fig. 7 show that incubation of 22AWT cells with antibodies directed against ICAM-1, a non-GPI-anchored protein expressed on these cells, did not up-regulate FX mRNA expression in these cells, whereas aE48 mAb (serving as a positive control in these experiments) did (Fig. 4).

The Expression of FX Was Down-regulated in an E48 Antisense Transfectant and Up-regulated in an E48 cDNA Transfected HNSCC—As shown above, FX is up-regulated by an interaction between E48 and an E48 ligand. If a signaling pathway through E48 occurs constitutively (albeit at submaximal levels), it is to be expected that cells expressing relatively high levels of E48 would also express high levels of FX and vice versa. Comparing expression levels of FX mRNA from 22A-E48hi and from 8-3 cells transfected with E48 antisense showed that down-regulating E48 expression by E48 antisense transfection (Fig. 1) down-regulated in parallel also FX expression (Fig. 5). Complementary experiments in which FX mRNA levels were measured in E48-negative 14CWT and in E48-positive 14C-CMV16 cells were performed. The results demonstrated that the down-regulation of E48 and FX in 14CWT (E48-negative) cells with E48 cDNA concomitantly increased E48 (Fig. 1) and FX levels (Fig. 5) in the 14C-CMV16 transfectants.
Ligation of E48 Up-regulates the Expression of SLeα—The next set of experiments was performed to directly test the possibility that E48 ligation controls the level of SLeα expression. 22AWT cells expressing relatively high levels of E48 and 8-3 cells (E48 antisense-transfected 22AWT cells expressing relatively low levels of E48) were incubated with αE48 mAb for 2, 4, 6, and 8 h. 22AWT and 8-3 control cells were incubated either with an αNCA mAb (see above) or in medium. SLeα expression by these cells was then assayed by flow cytometry utilizing strepavidin-labeled anti-SLeα antibodies. Figure 8 indicates that an up-regulated expression of SLeα occurred in the αE48 mAb-treated cells compared with control ones. However, the up-regulation was more pronounced in the E48 antisense-transfected 8-3 cells, which expressed a relatively low basal level of SLeα (Fig. 7). In these cells the increase in SLeα expression was significant (p < 0.005). The up-regulated expression of SLeα following αE48 ligation was seen already after 2 h of incubation with the αE48 mAb. The highest up-regulation was achieved after 6 h of incubation (Fig. 8).

E48 ligation insignificantly increased in the expression levels of SLeα on 22AWT cells. This increase was repeatedly obtained, although these cells expressed relatively high basal levels of SLeα. The failure to obtain a statistically significant up-regulated expression of SLeα in 22AWT cells suggests that once a threshold of FX is produced by the tumor cell, a maximal biosynthesis/expression of this fucosylated glycan takes place. These results are also in line with the results reported above, showing that up-regulation of FX expression following E48 ligation was more pronounced in cells expressing low basal levels of E48 than in high E48 expressors (Fig. 2).

Rolling of E48/FXhi HNSCC on E-selectin and on TNFα-activated HUVEC—We next asked whether the overexpression of SLeα triggered by E48 ligation in HNSCC is physiologically relevant for the ability of these cells to interact with E-selectin, a major vascular receptor for this ligand. We therefore compared in *in vitro* flow chamber assays the ability of the E48-negative 14CWT cells and that of the E48-cDNA transfected 14C-CMV16 cells to tether to and roll on artificial substrates coated with recombinant E-selectin under physiological shear flow. When perfused over a plate coated with E-selectin at a shear stress of 1 dyn/cm², the lower range of physiological shear stress found in post capillary venules *in vivo*, only the high E48 expressing 14C-CMV16 transfecants but none of the E48-negative 14CWT cells could tether and roll on the adhesive substrate (Fig. 9A). All adhesion interactions were Ca²⁺ dependent because they were eliminated in the presence of the Ca²⁺ chelator EDTA. Roller adhesions were persistent at 1 dyn/cm² but weaker than those of neutrophils because 10-fold elevation of the shear stress enhanced the detachment of these cells but not of PMN from the E-selectin coated substrate (data not shown). Nevertheless, the majority of E48 cDNA-transfected 14C-CMV16 cells that accumulated on E-selectin at low flow remained adherent and continued to roll on the selectin at medium shear stress range of 5 dyn/cm² (Fig. 9A). The adhesive capacity of the E48 cDNA-transfected 14C-CMV16 cells correlated well with their ability to form rolling adhesions on cytokine-stimulated vascular endothelial cells expressing...
E-selectin. When perfused over a monolayer of TNFα-activated HUVEC, only the high E48 expressing 14C-CMV16 cells could tether and roll on the E-selectin expressing endothelial cells and the vast majority of these interactions could be specifically inhibited by an E-selectin blocking mAb (Fig. 9B). This result indicates that E48hi HNSCC express not only functional E-selectin ligands but that these ligands determine almost exclusively their ability to initiate primary rolling adhesions on cytokine-stimulated HUVEC. Put together, these results demonstrate that E48hi cells but not E48lo cells express functionally adhesive E-selectin ligands and successfully use these ligands to tether and roll on vascular E-selectin under physiological shear flow.

DISCUSSION

The major finding of this study is that a specific signaling function can be assigned to E48, one of the newly discovered human Ly-6 proteins (4). Ligation of E48 on HNSCC by anti-E48 antibodies caused an up-regulation of the FX enzyme in these cells. An up-regulated expression of SLea, a fucosylated lactosamine functioning as an E-selectin ligand, was also detected in E48-stimulated HNSCC. The E48 ligation-mediated up-regulation of FX expression was obtained in three HNSCC lines. Preliminary results show that ligation of E48 in a cervical squamous carcinoma cell line also induced an up-regulated expression of FX. E48-mediated up-regulation of FX may therefore be a general phenomenon occurring in E48-expressing squamous carcinoma cells.

The final steps of fucose biosynthesis are mediated by the GDP-mannose 4,6-dehydratase and by FX functioning as an epimerase and a reductase. The GDP-1-fucose product is the donor carbohydrate utilized by a variety of fucosyl-transferases that add it to various lactosamine acceptors, decorating both glycoproteins and glycolipids (33, 34). Results by Ohyama et al. (34) suggested that the transcription of FX and possibly also GDP-mannose 4,6-dehydratase is regulated by metabolites generated in the fucose synthesis pathway. The present study shows that an additional regulatory mechanism for the transcription of FX may operate in E48-expressing HNSCC. The FX enzyme can be regulated by externally delivered signals through E48. This E48-mediated signaling also controls selectin ligand biosynthesis and expression in at least one cellular system: HNSCC.

The interaction between selectins and selectin ligands is a key event in the process of extravasation of both inflammatory leukocytes (31) and premetastatic cells (30). This interaction permits the rolling of the extravasating cells on the endothelial cells and the establishment of a more firm attachment between the former and the latter cells through the up-regulation of integrins and their ligands on these cells (35). E48-mediated signaling in HNSCC could thus influence the interaction of such cells with endothelial cells through the binding of the E-selectin ligand (sialyl Lewis a) expressed on the tumor cells, by E-selectin expressed on endothelial cells. The fact that the
expression of FX, being a crucial component of the biosynthetic cascade of E-selectin ligands, can be regulated by external signals and might be exploited in attempts to modulate inflammatory reactions and metastasis formation. E48-mediated signaling is obviously limited to those cells expressing E48 molecules. It would be of interest and importance to identify the external signals, if any, regulating the expression levels of FX in non-E48 expressors. In this connection it is interesting to note that Liteplo et al. (36) reported that Ly-6 loss-variants of a murine tumor exhibited alterations in the incorporation of fucose and mannose into cellular glycoconjugates. It thus appears that E48 is not the only member of the Ly-6 superfamily to be involved in the regulation of glycoconjugate expression.

The phosphatidyl linkage that anchors the GPI-linked proteins to the cell surface appears to be an integral component of GPI-mediated signaling processes (5, 23). The more widely accepted mechanism for signal transduction via GPI-linked proteins is based on observations that such proteins are biochemically and/or functionally associated with intracellular kinases (24), possibly through bridging proteins (25). It is therefore not unlikely that following the interaction of the protein moiety with a ligand, conformational changes in the GPI anchor are induced. These changes may activate the associated kinases and initiate the signaling cascade. It is not known whether the GPI moieties of different GPI-linked proteins in a certain cell are all associated with the same kinases or whether the GPI moieties linked to different proteins in the cell are linked to different kinases. In the first case signaling via different GPI-linked proteins may converge into a single pathway yielding similar downstream effects. The specificity experiments reported above do not support this suggestion. Antibody-mediated ligation of CD59 and NCA, two other GPI-linked proteins expressed by HNSCC, did not up-regulate FX expression. A ligand-specific signal was thus transduced to HNSCC via E48.

It is not unlikely that a constitutive signaling pathway triggered by the binding of a microenvironmental E48 ligand to E48 is taking place in in vitro growing HNSCC. The following observations support such a possibility: 1) the occurrence of a concordant expression of E48 and FX in HNSCC that were not experimentally stimulated. 2) up- or down-regulating E48 expression levels by transfection with E48 cDNA or antisense, respectively, up- or down-regulated FX expression in these cells. 3) the increment in FX and SLea+ expression over base-line levels in E48-ligated cells, (i.e. the ratio between FX mRNA or SLea+ protein levels in E48-ligated and nonligated cells) was greater in HNSCC expressing low basal levels of E48 than in cells expressing relatively high basal levels of this protein. Low E48 expressors are thus more responsive to the E48 mAb-mediated ligation of E48 than high expressors. These results also suggest that once sufficiently high levels of FX are gener-
ated in HNSCC by constitutive E48-mediated signaling, very little additional FX can be generated through this signaling pathway by experimentally ligating E48.

Based on these arguments and on the results reported above, we propose the following hypothetic model. The binding of E48 molecules expressed by HNSCC to its microenvironmental ligand transduces a signal that up-regulates the expression of the FX enzyme in these cells. This event subsequently leads to an increase in the levels of GDP-L-fucose in the E48-stimulated cells. High levels of this donor carbohydrate, a constitutive presence of proper acceptor glycans (i.e., sialo-lactosamines), as well as the presence of an appropriate set of fucosyl transferases with specificity for these acceptors would lead to the increased expression of functional selectin ligands (37). The up-regulated expression of these ligands on proper glycoprotein or glycolipid scaffolds and their translocation to the cell surface would facilitate the interaction of cells expressing high levels of these ligands with endothelial cells expressing the proper selectins. The experiments reported above indeed demonstrated that HNSCC expressing high levels of E48, FX, and SLeα exhibited rolling on purified E-selectin or on TNFα-activated endothelial cells, whereas tumor cells expressing lower (apparently subthreshold) levels of these proteins are unable to interact with E-selectin or with the endothelial cells under physiological conditions. This interaction thus may limit the rate of extravasation and metastasis formation of E48-expressing tumor cells.

The correlative link between FX and functional E-selectin ligand expression in the tumor cells investigated in the present study is reminiscent of a congenital disorder of leukocyte adherence to vascular endothelium termed LADII. This syndrome, first described by Etzioni et al. (38), is reflected in a generalized fucose deficiency and major defects in leukocyte trafficking and function (39). Neutrophils from LADII patients lack SLeα expression and fail to adhere to activated endothelium in vitro as well as to purified endothelial selectins in vitro (40). The molecular basis of LADII is still not fully elucidated, but the physiological outcomes of impaired fucose biosynthesis are strikingly similar, whether they originate in a general defect as in the LADII syndrome or in a cell type- or activation-specific up-regulation of a key enzyme like the FX.

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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

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