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Expressió diferencial determinant del fenotip metastàtic en un model d'adenocarcinoma de pulmó humà

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CAPÍTOL I

L'expressió incrementada dels gens de les Fucosiltransferases del tipus α(1,3) es correlaciona amb l'adhesió depenent d'E-selectina i el potencial metastàtic de les cèl·lules d'adenocarcinoma de pulmó humà

Els oligosacàrids fucosilats en posició α(1,3) o α(1,4) del tipus sialil-Lewis^x (sialil-Le^x) i sialil-Lewis^a (sialil-Le^a) participen en l'adhesió de les cèl·lules tumorals a l'endoteli. Hem analitzat mitjançant citometria de flux l'expressió de Le^x, sialil-Le^x, sialil-Le^x dimeric, Le^a i sialil-Le^a a la superfície de dues línies cel·lulars d'adenocarcinoma de pulmó humà amb diferent potencial colonitzador de pulmó: HAL-8Luc (metastàtiques) i HAL-24Luc (no metastàtiques). Les cèl·lules HAL-8Luc expressen nivells elevats de tots aquests antigens mentre que les cèl·lules HAL-24Luc només expressen sialil-Le^a i sialil-Le^x dimèric i a uns nivells inferiors que a les cèl·lules metastàtiques. La síntesi d'aquests antigens està controlada per Fucosiltransferases del tipus α(1,3) [α(1,3)-Fuc-T] i actualment n'hi ha cinc d'identificades (Fuc-TIII - Fuc-TVII). Hem demostrat, mitjançant anàlisi per Northern blot, que l'expressió dels gens que codifiquen per tots cinc enzims és més elevada a les cèl·lules metastàtiques que a les no metastàtiques.

Els assajos d'adhesió *in vitro* indiquen que només les cèl·lules metastàtiques s'adhereixen de forma significativa a cèl·lules endotelials humanes que expressen E-selectina. Aquesta unió s'inhibeix en bloquejar l'E-selectina amb un anticòs monoclonal contra aquesta molècula. Els experiments d'inhibició de l'adhesió, utilitzant anticossos monoclonals contra els diferents oligosacàrids fucosilats i sialilats expressats a les cèl·lules tumorals, fan palesa la implicació d'aquests antigens en les unions cèl·lula tumoral-cèl·lula endotelial. Amb l'anticòs anti-sialil-Le^x, CSLEX-1, s'obté l'efecte inhibitori més pronunciat, un 85%.

Aquests resultats suggereixen que la sobreexpressió dels gens de les Fuc-Ts del tipus α(1,3) a les cèl·lules metastàtiques HAL-8Luc, quan es compara amb la de les cèl·lules HAL-24Luc, resulta en un increment de l'expressió a la superfície d'aquestes cèl·lules d'oligosacàrids fucosilats, la qual cosa contribueix a la capacitat d'adhesió d'aquestes cèl·lules a l'endoteli activat i es correlaciona amb el seu potencial colonitzador de pulmó.

Enhanced expression of α(1,3)-fucosyltransferase genes correlates with E-selectin-mediated adhesion and metastatic potential of human lung adenocarcinoma cells

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ABSTRACT

α(1,3)- and α(1,4)-fucosylated oligosaccharides such as sialyl-Lewis^x (sialyl-Le^x) and sialyl-Lewis^a (sialyl-Le^a) have been reported to participate in tumor cell adhesion to activated endothelium. We examined by cytofluorometry the expression of Le^x, sialyl-Le^x, sialyl-Le^x dimeric, Le^a and sialyl-Le^a on the surface of two human lung adenocarcinoma cell lines with different lung colonization potential. High expression levels of all these antigens were detected in the metastatic HAL-8Luc cells while the closely related nonmetastatic HAL-24Luc cells only expressed the sialyl-Le^a and sialyl-Le^x dimeric antigens, both at lower level than in HAL-8Luc cells. Five α(1,3)-fucosyltransferases (α(1,3)-Fuc-T) controlling the synthesis of these molecules have been identified to date (*Fuc-TIII-Fuc-TVII*). The expression of these five genes was also higher in the metastatic cells than in the nonmetastatic counterparts as was shown by Northern blot analysis.

In vitro adhesion assays showed that only the metastatic cell line adheres significantly to E-selectin-expressing human endothelial cells. Moreover, monoclonal antibody (mAb) blockade of E-selectin completely abolished tumor cell binding. Adhesion inhibition experiments using mAbs against sialylated fucosylated oligosaccharides expressed on tumor cells indicated that these antigens are involved in the binding. Anti-sialyl-Le^x mAb (CSLEX-1) inhibited adhesion by 85%, having the most pronounced inhibitory effect.

These findings suggest that the overexpression of α(1,3)-Fuc-T genes in the metastatic HAL-8Luc cells, compared with HAL-24Luc cells, results in an enhanced surface display of fucosylated oligosaccharides, that contributes to the adhesive capacity of these cells to the activated endothelium and correlates with their lung colonization potential.

INTRODUCTION

Lung cancer is the most prevalent form of cancer worldwide being adenocarcinoma a major form of this pathology (Gazdar, 1994). Metastatic spread of primary tumor cells is the main cause of death in oncologic patients. A major step in the metastatic process is the extravasation of tumor cells through the capillary endothelium at the target organ (Fidler, 1990). Tumor cell adhesion leading to extravasation is governed by the expression of a repertoire of adhesive molecules on tumor cells specific for receptors expressed by

endothelial cells (Lafrenie *et al.*, 1994).

L-, P- and E-selectins form a family of calcium-dependent receptors involved in leukocyte adhesion to vascular endothelium, regulating leukocyte recruitment to inflammation sites (Nelson *et al.*, 1995). E-selectin is transcriptionally activated on endothelial cells upon inflammatory stimuli such as interleukin-1, tumor necrosis factor and lipopolysaccharide (Bevilacqua & Nelson, 1993). This receptor recognizes sialylated fucosylated lactosaminoglycans such as sialyl-Lewis^x (sialyl-Le^x), sialyl-Lewis^a (sialyl-Le^a)[4] and related oligosaccharides associated with both glycolipids and glycoproteins, on the surface of various leukocyte subsets, thus facilitating their arrest and extravasation to sites of injury (Varki, 1994). E-selectin has been proposed to promote attachment of tumor cells to endothelium in similar fashion (Chirivi *et al.*, 1996; Daneker *et al.*, 1996; Tozeren *et al.*, 1995; Ye *et al.*, 1995). Moreover, carcinoma cells are often enriched with sialyl-Le^x and/or sialyl-Le^a structures (Fukushi *et al.*, 1984; Hanski *et al.*, 1995; Ikeda *et al.*, 1996), a fact that may contribute to their metastatic behavior (Furukawa *et al.*, 1994; Inufusa *et al.*, 1991; Kishimoto *et al.*, 1996; Matsushita *et al.*, 1991; Nakamori *et al.*, 1993; Saitoh *et al.*, 1992).

The common feature of these carbohydrates is a lactosamine core of either type 1 (Galβ1-3GlcNAc-R) or type 2 (Galβ1-4GlcNAc-R). These epitopes are generated either by fucosylation alone or by sialylation followed by the addition of one or more fucosyl residues. α(1,4)-fucosylation at the penultimate GlcNAc of type 1 acceptors generates Le^a antigen whereas Le^x is generated by α(1,3)-fucosylation of type 2 acceptors at the same position. In both cases a previous terminal α(2,3)-sialylation can occur, thus yielding sialyl-Le^a and sialyl-Le^x respectively. Dimerization of sialyl-Le^x takes place through a type 2 chain elongation followed by α(2,3)-sialylation and α(1,3)-fucosylation (Hakomori, 1989).

Sequentially acting glycosyl-transferases are responsible for the synthesis of these fucosylated glycans (Kuijpers, 1993). The final step in this pathway is controlled by one or more specific α-(1,3)-fucosyltransferases (α-(1,3)-Fuc-Ts) and to date, five α(1,3)-Fuc-T genes have been cloned and characterized: Fuc-TIII (Kukowska-Latallo *et al.*, 1990), Fuc-TIV (Goelz *et al.*, 1990; Lowe *et al.*, 1991), Fuc-TV (Weston *et al.*, 1992a), Fuc-TVI (Weston *et al.*, 1992b) and Fuc-TVII (Natsuka *et al.*, 1994; Sasaki *et al.*, 1994). The five Fuc-T enzymes utilize type 2 oligosaccharides as acceptors to generate Le^x-related molecules, but Fuc-TIII (the Lewis type enzyme) can also efficiently utilize type 1 carbohydrates, thus yielding Le^a and sialyl-Le^a determinants (Kukowska-Latallo *et al.*, 1990). Elevated levels of Fuc-T activities have been found in various human tumor cell lines (Asao *et al.*, 1995; Wang *et al.*, 1995; Yago *et al.*, 1993) and poor prognosis of lung cancer has been correlated with Fuc-TIV and Fuc-TVII expression (Ogawa *et al.*, 1996).

HAL-8 and HAL-24 are human lung adenocarcinoma cell lines, derived from a common parental cell line, KUM-LK-2, that show different lung colonization potential.

HAL-8 cells developed lung colonies when i.v. injected in athymic mice while HAL-24 cells did not metastasize under the same experimental conditions (Inufusa *et al.*, 1991). For the present study we have used the HAL-8Luc and HAL-24Luc sublines, originated by transfection of HAL-8 and HAL-24 cells respectively with the luciferase gene. This phenotypic labeling was introduced as an aid for future studies of tumor cell spread (work in progress). Here we demonstrate that the transfected cells retain the different lung colonization potential reported for the original HAL-8 and HAL-24 cell lines and further characterize this model for lung adenocarcinoma by generating i.m. primary tumors in athymic mice.

The main purpose of the present work was to analyze the expression of several fucosylated side-chain oligosaccharides on the surface of HAL-8Luc and HAL-24Luc cells, and to determine their role in the adhesion of tumor cells to the endothelium. We have also studied the expression of the α-(1,3)Fuc-T genes responsible for the synthesis of these molecules.

MATERIALS AND METHODS

Cell Lines

Human lung adenocarcinoma cell lines, HAL-8 (metastatic) and HAL-24 (nonmetastatic), were kindly provided by Dr. O. Matsuo (Kinki University School of Medicine, Osaka, Japan). Cells were cultured in RPMI 1640 medium (Bio-Whitaker, Verviers, Belgium) suplemented with 10% (v/v) heat-inactivated foetal bovine serum (FBS) (Bio-Whitaker, Belgium) and 2 mM L-glutamine (Bio-Whitaker, Belgium) in a humidified atmosphere of 5% CO_2 at 37°C.

Both cell lines were estably transfected with the *Photinus pyralys* luciferase cDNA as previously described (Rubio & Blanco, 1996). After limiting dilution culture, the clones having the highest luciferase activity from each cell line were selected and termed HAL-8Luc and HAL-24Luc respectively. These transfected cell lines were used for all the experiments reported here.

HUVECs were kindly donated by Dr. C. de Castellarnau (Lab. Atherothrombosis and Vascular Biology, Institut de Recerca, Hospital Santa Creu i Sant Pau, Barcelona, Spain). HUVECs were isolated by collagenase digestion from multiple segments of normal-term umbilical cords and cultured as previously described (Lopez *et al.*, 1993). All adhesion assays were performed using endothelial cells within three passages.

Metastasis Assay

Metastasis assays were performed on six-week-old female BALB/c homozygous nude (nu/nu) mice. Animals were purchased from CRIFFA S.A. (Barcelona, Spain) and

mantained in a specific pathogen-free environment.

HAL-8Luc and HAL-24Luc cells ($5x10^5$) suspended in 0.1ml serum-free RPMI medium were i.m. injected into the right thigh of mice to generate primary tumors and determine spontaneous metastatic ability. Alternatively, $5x10^5$ cells were injected in the lateral tail vein to assess for lung colonization potential. Experiments were done in triplicate and five animals were used for each replica. I.m. injected mice were sacrified in two equal groups at 10 and 20 weeks after inoculations. I.v. injected animals were sacrified 10 weeks after injections. Following post-mortem examination, primary tumors were excised and various organs (lungs, kidney, spleen, liver, brain) as well as lymph nodes were collected for histopathological examination.

Samples were fixed in 4% (w/v) paraformaldehyde in 0.1 M PBS for 2 hours at room temperature, and then postfixed at 4°C with the same fixative supplemented with 2.5% glutaraldehyde until processed. After routine dehydratation, the samples were embedded in Spurr resin. 1 μm-thick sections were stained with methylene blue and observed and photographed with a Reichert-Jung Polyvar2 optical microscope.

Flow Cytometry

Exponentially growing HAL-Luc cells were trypsinized, washed twice with 0.1M PBS pH 7.4, 0.1% bovine serum albumin (BSA), 0.01% sodium azide and incubated for 20 min at room temperature individually with the following anti-human mAbs: FH6, an IgM anti-sialyl-Le^x dimeric, kindly provided by Otsuka Pharmaceutical (Osaka, Japan); CSLEX-1, an IgM anti-sialyl-Le^x and Leu-M1, an IgM anti-Le^x both purchased from Becton Dickinson (San Jose, CA); LE-1, an IgM anti-Le^a (Ortho Diagnostic, Neckargemünd, Germany); CA19.9, an IgG_1 anti-sialyl-Le^a (Novocastra Laboratory, Ltd., Newcastle, UK). These specific antibodies were used at saturating concentrations determined by titration.

Cells were washed with 0.1M PBS, 0.1% BSA, 0.01% sodium azide and then stained with fluorescein-conjugated goat anti-mouse IgG (DAKO, A/S, Denmark) at a 1:20 dilution for 20 min at room temperature. Following the final washing, cell fluorescence intensity was determined by flow cytometry on an Epics XL-MCL (Hyaleah, FL).

Cells labeled with secondary antibody alone and appropriate isotypic irrelevant murine mAb were used as controls. Mean channels of fluorescence were recorded for every analysis and the mean channels of fluorescence ratio for each mAb and control pair was also calculated.

Northern blots

3.5 μg poly(A)$^+$ RNA from HAL-8Luc and HAL-24Luc cells as well as 2 μg of RNA molecular weight markers (United States Biochemical, Cleveland, Ohio) were fractionated by electrophoresis in a 1.2% denaturing agarose gel containing 1.8% (v/v)

formaldehyde and transferred to nylon membranes (Hybond-N^+; Amersham, UK). The membranes were hybridized with ^{32}P-labelled cDNA probes in a solution containing 50% (v/v) formamide, 5x SSC, 50mM sodium phosphate buffer pH 6.5, 250 µg/ml sheared salmon sperm DNA, 10x Denhart's solution and 10% dextran sulphate overnight at 42°C. Following hybridization the membranes were washed twice for 10 min at RT in 2x SSC, 0.1% SDS and once in 0.2x SSC, 0.1% SDS for one hour at 68°C. Finally the membranes were autoradiographed by exposure to Hyperfilm MP (Amersham, UK). Blots were stripped and reprobed with human β-actin cDNA as a control for RNA integrity and loading consistency. Band intensity was analyzed with the Molecular Analyst/PC 1.4 software (BioRad, CA) and calibrated by comparison with the β-actin bands.

Fuc-TIII, IV, V and VI cDNA probes were obtained by RT-PCR using mRNA from the HAL-Luc cells and the Fuc-TVII cDNA probe was obtained from U937 cell mRNA, using published primer sequences (Cameron *et al.*, 1995; Sasaki *et al.*, 1994; Weston *et al.*, 1992b). Amplified cDNAs were cloned using the TA-Cloning kit (Invitrogen, San Diego, CA) and full-length sequenced with the T7 Sequenase 2.0 DNA sequencing Kit (Amersham, UK). Nucleotide positions for these sequences were as follows: 328-909 for Fuc-TIII (Kukowska-Latallo *et al.*, 1990), 1257-1691 for Fuc-TIV (Goelz *et al.*, 1990), 139-949 for Fuc-TV (Weston *et al.*, 1992a), 411-907 for Fuc-TVI (Weston *et al.*, 1992b), 394-888 for Fuc-TVII (Natsuka *et al.*, 1994). The human β-actin probe used as control (nucleotide position 992-1721 of GenBank accession number X00351) was also obtained in our laboratory.

Endothelial Cell Adhesion Assays

HUVEC were cultured in fibronectin-coated 96-well microtiter plates. Confluent endothelial cells were induced for E-selectin expression by incubating with 10 U/ml interleukin-1β (IL-1ß) (donated by Dr. C. de Castellarnau) for 4 hours at 37°C previous to the experiment.

For adhesion assays, HAL-8Luc and HAL-24Luc cells were detached by trypsinization, labeled with 200µCi sodium [^{51}Cr] chromate (Amersham International, Amersham, UK) and resuspended in RPMI medium with 2% FBS. Labeled tumor cells were then added to both stimulated and non-stimulated HUVEC monolayers at a nominal density of $1x10^5$/well and finally incubated for 30 min at 4°C. Nonadherent cells were removed by aspiration and washing with 2% FBS medium. Bound cells were lysed with 25mM NaOH, 0.1% SDS and radioactivity determined using a gamma counter (1261 multigamma, WALLAC Oy, Turku, Finland). Percentage of adhesion was calculated as the ratio of bound cell radioactivity to total radioactivity from $1x10^5$ cells. Statistical analysis was performed using Student's *t*-test.

For E-selectin blockade, IL-1β-stimulated HUVEC monolayers were incubated with

10μg/ml anti-human E-selectin mAb, P2H3 (Chemicon International Inc., Temecula, CA) for 30 min at 4°C previous to the binding assay.

For inhibition assays, labeled HAL-8Luc cells were first incubated with the above described mAbs: Leu-M1, CSLEX-1, FH6, LE-1 or CA19.9 at a saturating concentrations of 50μg/ml for 30 min at room temperature and then added to HUVEC monolayers.

Alternatively, for competition assays, endothelial cells were incubated with either N-acetyl neuraminic acid, α-L(-)-Fucose or N-acetyl-D-glucosamine for 30 min at 4°C previous to the adhesion assay, also performed in the presence of the competitor. These monosaccharides were purchased from Sigma (St. Louis, MO) and were assayed at different concentrations up to 800mM. For sialidase treatment, tumor cell suspensions were incubated for 5 min at 37°C with 0.2 U/ml *Clostridium perfringens* sialidase (Boehringer Mannheim, Germany) before the adhesion assay.

For desulfatation experiments HAL-8Luc cells were detached with 1mM EDTA in 0.1M PBS pH 7.4, ^{51}Cr-labeled and incubated with 25 U/ml arylsulfatase (Boehringer Mannheim, Germany) in 0.1M acetate buffer pH 6.2 for 30 min at 37°C. The treated cells were then washed, resuspended in RPMI medium with 2% FBS and used for adhesion assays as described above.

RESULTS

Metastatic Assays and Histopathological Examination

HAL-24Luc cells generated no metastases when either i.v. or i.m. inoculated in athymic mice, although those i.m. injected developed primary tumor at the injection site. In contrast, 7-22 pulmonary metastases per animal were observed in 13 out of 15 mice i.v. inoculated with HAL-8Luc cells. The same proportion of mice with pulmonary metastatic foci was produced by inoculation of nude mice with untransfected HAL-8 cells, showing that transfection of HAL-8 cells with the luciferase cDNA had no effect on the lung colonization potential of these cells (data not shown). I.m. injection of HAL-8Luc cells induced metastases in 9 out of 15 animals. Moreover, these mice developed primary tumors 3-fold larger than those originated by HAL-24Luc cells. No metastases were detected in the rest of the organs or tissues analyzed in either experimental group of mice.

Primary tumors showed infiltrated fibroblasts from perimysium and endomysium. Infiltrated fibroblasts secreted abundant extracellular matrix, mainly constituted by banded collagen fibrils. Tumors were supplied by blood capillaries and some lymphatics, but were not innervated. The inner zone of the tumors consisted of tumor cell islets surrounded by randomly oriented collagen fibers while the outer region was formed by a well-developed and highly vascularized capsule of connective tissue.

At 10 weeks post-inoculation HAL-8Luc cells in tumors were voluminous (up to

50-60 μm maximum diameter) with abundant cytoplasm, euckromatic nuclei and one or more prominent nucleoli (Fig. 1A). Cells in islets were held together by intercellular contacts that included desmosomes (electron microscopy observations not shown). Some mitotic figures and few dead cells were also observed.

In contrast, after the same time periode HAL-24Luc cells in tumors showed a typically regressive pattern. Cytoplasm was highly vesiculated, had a spongy aspect and nuclei were predominantly pycnotic (Fig. 1B). Tumor cell islets were scarce, and few intercellular contacts were observed.

At 20 weeks post-inoculation HAL-8Luc tumors showed a thick capsule with circularly oriented fibroblasts and collagen fibers. Tumor capsule and outer zone were supplied by blood vessels derived from the surrounding muscle vascularization. This outer zone was composed of loose connective tissue heavily infiltrated by blood cells, mainly lymphocytes (Fig. 1C). The capsule capillaries, which had no associated pericytes, showed angiogenic bouttons that developed in a radial pattern branching profusely into the tumor. Tumor capillaries, also having no pericytes, showed courses close to cell islets (Fig.1D). After the same time period tumors from HAL-24Luc inoculated mice showed no tumor cells and the connective tissue formed a scar (data not shown).

Lymph nodes from mice injected with HAL-8Luc cells had enlarged sinuses, the most noticeable being the subcapsular one. The lymphatic space was occupied by numerous lymphocytes, dead cells and heterogeneous round-shaped cells, some of them having macrophage characteristics (Fig. 1E). On the contrary, the lymph nodes from the HAL-24Luc injected mice showed a normal pattern with no enlarged sinuses (Fig. 1F).

Lung metastases from HAL-8Luc tumors were associated with the basal lamina of terminal bronchioles and were organized forming islets in which individual tumor cells were surrounded by a poorly developed extracellular matrix. Metastases were supplied by numerous blood vessels, which lined the cell islets. Metastatic cells showed similar morphology as primary tumor cells (Fig.1G).

Surface Antigen Characterization By Flow Cytometry

The expression of several fucosylated lactosaminoglycans: Le^x, sialyl-Le^x, sialyl-Le^x dimeric, Le^a and sialyl-Le^a on the surface of HAL-8Luc and HAL-24Luc cells was examined by flow cytometry using a panel of mAbs specific for each epitope (Fig. 2).

HAL-24Luc cells were characterized by a consistently lower reactivity than HAL-8Luc cells with all the mAbs used for this study. In HAL-8Luc cells, reactivity against Le^x-related antigens was stronger than that against Le^a-related molecules. The ratios of mean fluorescence intensity were 75, 34.5 and 24 for the anti-Le^x, -sialyl-Le^x and -sialyl-Le^x dimeric mAbs respectively and 2.9 and 3.6 for those recognizing Le^a and sialyl-Le^a respectively.

MAbs recognizing Le^x and Le^a antigens did not react with HAL-24Luc cells and the sialyl-Le^x epitope was barely detected. MAbs against sialyl-Le^x dimeric and sialyl-Le^a were the most reactive with these cells, althought the resulting ratios of mean fluorescence intensity (6.5 and 2.4 respectively) were lower than those of the same mAbs reacting with HAL-8Luc cells.

Northern Blot Analysis of Fucosyltransferase genes

The different expression level of the above reported fucosylated antigens on HAL-8Luc and HAL-24Luc cells suggested the existence of a corresponding difference in the expression levels of Fuc-T genes.

Northern blot analysis of poly(A)$^+$ RNA from HAL-8Luc and HAL-24Luc cell lines using Fuc-TIII, IV, V, VI and VII cDNA probes shows that both cell lines express the five Fuc-T genes (Fig. 3). The transcripts detected were, in all cases, more abundant in the metastatic HAL-8Luc cells than in the nonmetastatic HAL-24Luc cells. Differences in expression ranged from 1.4-fold in the case of Fuc-TIII probe to 6-fold for the Fuc-TIV probe.

Single transcripts with sizes 2.5, 2.4 and 1.5 kb were detected by the Fuc-TIII, V and VII probes, respectively. However, the expression level of Fuc-T VII mRNA in HAL-24Luc cells was barely detectable. Fuc-TIV and Fuc-TVI probes detected several mRNA species expressed at different intensity levels including small-size transcripts of 1.3 and 1.4 kb respectively. Although the sizes of the other transcripts detected correspond to previously described messages for Fuc-Ts, we are not aware of a previous description of these small-size mRNA species in lung.

Endothelial Cell Adhesion Assays

The differential expression of surface antigens in HAL-24Luc and HAL-8Luc cell lines pointed to the possible existence of a corresponding difference in the adhesive character of both cell lines to endothelial cells. To test this, isotopically labeled tumor cells were incubated, under various experimental conditions, with HUVEC monolayers that had been stimulated or not with IL-1ß.

Metastatic HAL-8Luc cells adhered to IL-1ß-stimulated HUVEC monolayers but did not detectably bind to non-activated endothelial cells. On the contrary, nonmetastatic HAL-24Luc cells were unable to bind to HUVEC monolayers, regardless of their activation state. We had previously confirmed by flow cytometry that only the IL-1ß-stimulated-HUVEC expressed E-selectin (data not shown). Incubation of endothelial cells with the anti-human E-selectin P2H3 mAb completely abolished binding of tumor cells. Normalized values for the mean and SD of these binding results are illustrated in Figure 4A.

MAbs reacting with Le^x- and Le^a-related molecules were used to explore the

involvement of these carbohydrate ligands in the binding of HAL-8Luc cells to endothelial monolayers. All of the mAbs used inhibited adhesion to a different extent. Blocking of sialyl-Le^x oligosaccharides with the CSLEX-1 mAb resulted in 85% inhibition of binding. Lower but significant binding inhibitions (22% and 30%) were obtained with anti-Le^x and sialyl-Le^x dimeric mAbs respectively. Finally, mAbs againts Le^a and sialyl-Le^a inhibited binding by 37% and 27% respectively (Fig. 4B). Irrelevant mAbs used as controls (mouse anti-human IgG_1 and mouse anti-human IgM from Becton Dickinson, San Jose, CA) had no effect on adhesion (data not shown).

More specifically, the participation of sialic acid and fucose residues in the adhesion of tumor cells to endothelium was probed by including these monosaccharides as binding competitors during the assays (Fig. 4C). N-acetyl glucosamine, an aminosugar used as control, did not affect the binding at any concentration tested. The incubation of endothelial cells with either 50mM N-acetyl neuraminic acid or 800mM α-L-(-)-fucose resulted in 95% and 50% binding inhibition respectively. Further evidence showing the requirement of sialic acid in the binding of HAL-8Luc cells to HUVEC monolayers was provided by experiments in wich sialidase pretreatment of tumor cells resulted in 95% binding inhibition.

On the other hand, tumor cell desulfatation did not affect adhesion of HAL-8Luc cells to endothelial monolayers.

DISCUSSION

We have demonstrated that the HAL-8Luc and HAL-24Luc sublines, generated by permanent transfection of the luciferase gene, retain the characteristic differential lung colonization capacity of their parental cell lines. Moreover, we have extended the analysis of this model for lung adenocarcinoma showing that i.m. injection of both HAL-8Luc and HAL-24Luc cells in athymic mice results in the formation of primary tumors at the injection sites. However, while primary tumors from HAL-8Luc cells produced lung metastases in 9 out of 15 cases, those from HAL-24Luc cells were not metastatic. These spontaneous metastasis assays revealed that both cell lines also show differences at several other levels, the most striking being the inability of HAL-24Luc cells to proliferate and maintain experimental tumors for longtime. Although primary tumors derived from both cell lines exhibit the same basic organization pattern consisting of tumor cell islets surrounded by connective tissue, HAL-24Luc tumors show a clear regression process likely the consequence of an imbalance between cell proliferation and cell death rates. These regressive phenomena, possibly related with the limited vascularization observed in the tumor mass, may contribute to the non-invasive phenotype of HAL-24Luc cells.

However the inability of circulating, i.v. injected, HAL-24Luc cells to metastasize suggests the existence of factors, other than the access to the circulatory system, present in HAL-8Luc cells, also required for success at colony formation. Since adhesion of

circulating tumor cells to the endothelium at target organs is a prerequisite to extravasate and complete the metastatic process (Fidler, 1990) we have studied the expression of several adhesion-related molecules on the surface of these two tumor cell lines.

Previous studies demonstrated that HAL-8 cells overexpress the sialyl-Le^x dimeric surface antigen when compared with HAL-24 cells (Inufusa *et al.*, 1991). Sialyl-Le^x dimeric had initialy been identified in human colonic adenocarcinoma as a cancer-associated difucoganglioside defined by the FH6 mAb (Fukushi *et al.*, 1984) and a correlation between the expression of this antigen and the metastatic behaviour of tumor cells had also been established (Matsushita *et al.*, 1991). Our results from cytometric analysis using mAbs specific for Le^x, sialyl-Le^x, sialyl-Le^x dimeric, Le^a and sialyl-Le^a surface oligosaccharides, show that the differential expression of surface carbohydrates in these cell lines is not limited to the sialyl-Le^x dimeric antigen and that the differences in the expression level of Le^x and sialyl-Le^x between HAL-8Luc and HAL-24Luc cells are even greater. Moreover these two tumor cell lines also show strong differences in the surface expression of Le^a and sialyl-Le^a epitopes.

HAL-8Luc cells were more reactive with all the mAb tested than HAL-24Luc cells. In HAL-8Luc cells reactivity was mainly associated with Le^x, sialyl-Le^x and sialyl-Le^x dimeric structures, being the highest mean fluorescence intensity associated with the Le^x-recognizing mAb. On the other hand, mAbs against Le^x and sialyl-Le^x showed a narrow cell immunofluorescence distribution pattern, in contrast with the FH6-labeled cells which present an heterogeneous distribution. On the contrary, HAL-24Luc cells only express the sialylated forms of Le^a and Le^x, both at a lower level than in HAL-8Luc cells.

The differential expression of Le^x, Le^a and related structures on HAL-Luc cell lines together with the fact that these carbohydrates share similar synthetic pathways suggested that the enzymes responsible for their synthesis may also be differentially expressed in both tumor cell lines. The final step in this synthetic pathway is controlled by one or more Fuc-Ts (Kuijpers, 1993). Previously reported RT-PCR analysis of normal human lung tissue revealed the expression of Fuc-TIII and Fuc-TVI genes, although Fuc-TVI sequences could not be detected by Northern blot analysis (Cameron *et al.*, 1995). In the same study Fuc-TV transcripts were not detected by any method assayed. Moreover high levels of Fuc-TIV and Fuc-TVII expression in the adult human lung tissue had only been associated with cancer, correlating with poor prognosis (Ogawa *et al.*, 1996). Northern blot results shown in the present work demonstrate that the two human lung adenocarcinoma cell lines HAL-8Luc and HAL-24Luc express the five known α(1,3)-Fuc-T genes. However the transcripts for these genes are differentialy expressed in both cell lines, being in all cases overexpressed in the metastatic HAL-8Luc cell line compared to HAL-24Luc cells in correlation with their differential surface antigen display. This difference in expression level might be regulated at a transcriptional level since both cell lines showed the same DNA content for these genes when analized by Southern blot (data not shown).

The Fuc-TIII message is the most abundant of the five Fuc-T studied in HAL-24Luc cells, a fact that could explain the detection by cytometry of a relatively high amount of sialyl-Le^a and sialyl-Le^x dimeric antigens on the surface of these cells, although the synthesis of the latter is not exclusive of the Fuc-TIII activity.

Northern blot analysis of Fuc-TVI products indicated the existence of a 1.4 kb transcript detected with similar intensity to the previously characterized 2.4 kb message (Kukowska-Latallo *et al.*, 1990). This 1.4 kb transcript could be the result of an additional alternative splicing in the Fuc-TVI transcripts of these cells such as that identified by Cameron, H. S. et al, 1995, who described truncated Fuc-TVI transcripts with aberrant sizes in liver and kidney, originated by additional splice events within the Fuc-TVI coding region, that did not encode functional enzymes (Cameron *et al.*, 1995). Similarly, a 1.3 kb transcript smaller than the expected full-size message, appeared as the predominant band in Northern blots hybridized with the Fuc-TIV probe. We are currently analysing these small-size Fuc-T gene products.

The side-chain oligosaccharides studied here had already been described as tumor-associated antigens (Hakomori, 1989). More recently these epitopes have also been characterized as E- and P-selectin counter-receptors expressed in several leukocyte subsets, participating in the recruitment of leukocytes during inflamation (Varki, 1994). Previous results had demonstrated that these side-chain oligosaccharides are *de novo* sintesized or overexpressed in tumor cells, playing a critical role in their adhesion to activated endothelium (Asao *et al.*, 1995; Kishimoto *et al.*, 1996; Majuri *et al.*, 1995; Wittig *et al.*, 1996). Here we show that HAL-8Luc cells bind efficiently to IL-1β-stimulated HUVEC monolayers while HAL-24Luc cells do not bind significantly under the same experimental conditions. We also demonstrate, using blocking mAb, that E-selectin is the main molecule expressed on endothelial cells responsible for this adhesion. Our results show that carbohydrates of types 1 and 2, both sialylated and non-sialylated, participate in the adhesion of HAL-8Luc cells to E-selectin expressing HUVEC monolayers since incubation of the tumor cells with mAb recognizing these epitopes partially inhibited the binding. The highest binding inhibitions were achieved when the mAbs used were those against type 2 related antigens, the ones expressed at a highest level in HAL-8Luc cells. Results from these *in vitro* static adhesion assays point to the relevance of the sialyl-Le^x oligosaccharide in the binding reaction. Although both Le^x and sialyl-Le^x are expressed at a high level on HAL-8Luc cells, mAb against sialyl-Le^x had a 4-fold greater binding inhibition capacity than the anti-Le^x mAb. This would indicate a higher affinity of the sialyl-Le^x epitope for E-selectin expressed on endothelial cells, supporting previous findings in other tumor cell types such as colon carcinoma (Sawada *et al.*, 1994).

Competition experiments in which either sialic acid or fucose were included at various concentrations during the binding assays further demonstrated the involvement of these sugars in the adhesion of HAL-8Luc cells to activated HUVEC monolayers. 50mM

sialic acid reduced binding by 95% while higher fucose concentrations were needed to block binding by 50%. Further evidence for the participation of sialic acid in the binding reaction was provided by experiments in which sialidase pretreatment of HAL-8Luc cells abolished binding to HUVEC.

Although P- and E-selectins show similar specificity for sialylated antigens, sulfated tyrosine residues in P-selectin glycoprotein ligand 1 are critical for P-selectin, but not for E-selectin, binding (Sako *et al.*, 1995). Desulfatation experiments described here did not alter the adhesion results indicating that sulfated proteins are unlikely to participate in the adhesion of HAL-8Luc cells to activated endothelium.

Taken together these results help us understand the process leading to the different lung colonization potential shown by HAL-8Luc and HAL-24Luc human lung adenocarcinoma cells. The low (or null) expression of putative E-selectin counter-receptors necessary for the tumor cell adhesion to the endothelium could explain the inability of HAL-24Luc cells to metastasize when i.v. injected. In contrast, the overexpression of Fuc-T genes in HAL-8Luc cells results in an increased cell surface display of fucosylated oligosaccharides and consequently in an enhanced ability to adhere to E-selectin-expressing endothelial cells.

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FIGURES

Figure 1. Light micrographs of: **(A)** 10 weeks post-inoculation HAL-8Luc tumor showing tumor cell islets surrounded by collagen fibrils; **(B)** 10 weeks post-inoculation HAL-24Luc tumor showing a regressive pattern of sparse cell islets; **(C)** HAL-8Luc cell derived tumor externally lined by a thick connective tissue capsule (*cc*), with circularly oriented collagen fibers; **(D)** HAL-8Luc cell islets supplied by a capillary network (**arrowheads**) derived from capsule (*cc*) vessels; **(E)** inguinal lymph node from a HAL-8Luc injected animal showing heavy cell infiltration of the subcapsular sinuses (*scs*); **(F)** inguinal lymph node from HAL-24Luc injected mouse showing normal appearence; **(G)** lung metastases formed by tighly packed HAL-8Luc cells having a similar pattern to that of primary tumors; these cellular masses are associated to the airways epithelium and partialy occupy the aerial spaces; **(H)** lung section from an HAL-24Luc injected mouse showing normal appearence. Scale bars = 30 μm.

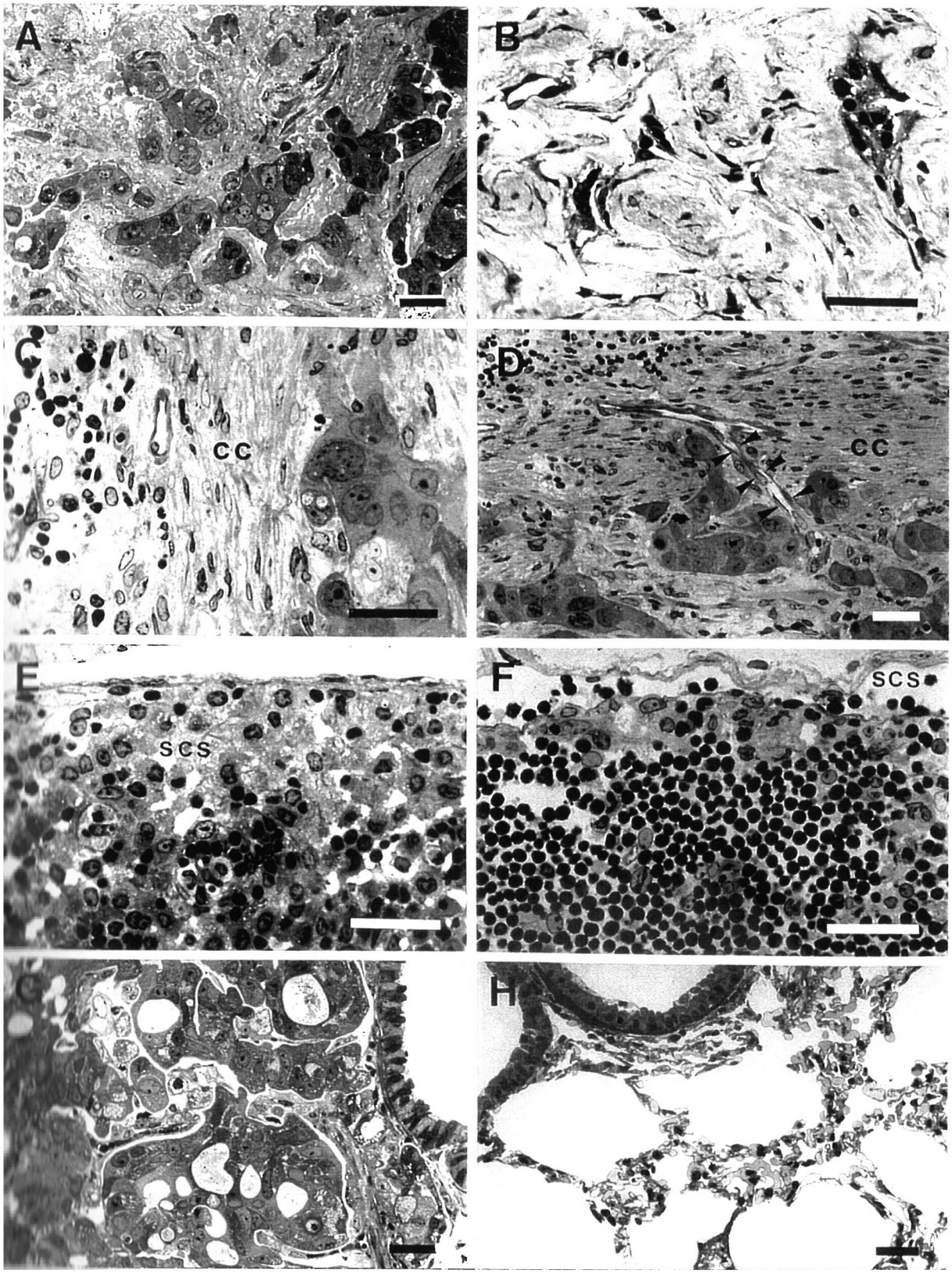

A
B
C
cc
D
cc
E
scs
F
scs
G
H

Figure 2. Fluorescence intensity histograms showing flow cytometry analysis of HAL-8Luc and HAL-24Luc cells labeled with mAb against Le^x (*Leu-M1* mAb), sialyl-Le^x (*CSLEX-1* mAb), sialyl-Le^x dimeric (*FH6* mAb), Le^a (*LE-1* mAb) and sialyl-Le^a (*CA19.9* mAb) (from left to right). Data represents one of five similar experiments. *Dotted lines*, negative controls; *solid lines*, relevant anti-oligosaccharide antibodies.

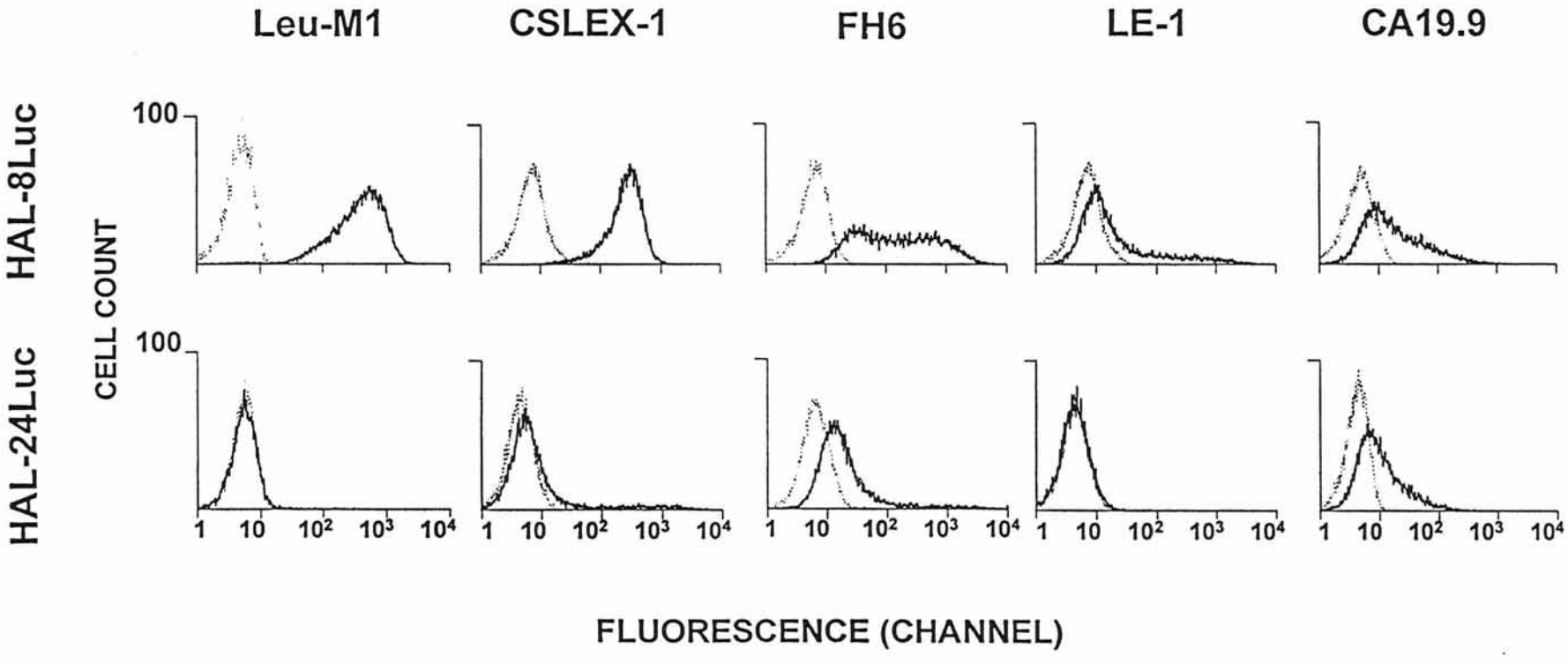

Leu-M1
CSLEX-1
FH6
LE-1
CA19.9
HAL-8Luc
HAL-24Luc
CELL COUNT
100
100
1
10
10²
10³
10⁴
FLUORESCENCE (CHANNEL)

Figure 3. Northern blot analysis of HAL-8Luc and HAL-24Luc cell mRNA. Poly(A)$^+$ RNA (3.5 μg) was electrophoresed, blotted and hybridized with the ^{32}P-labeled Fuc-T III, IV, V, VI and VII cDNA probes (from left to right) as described in "Material and Methods". After a first autoradiographic exposure the probes were stripped out and the blots rehybridized with the β-actin probe (*β-act*). Band sizes are indicated in kb at the left of each set. Numbers *8* and *24* on top of the sets refer to HAL-8Luc and HAL-24Luc mRNA respectively.

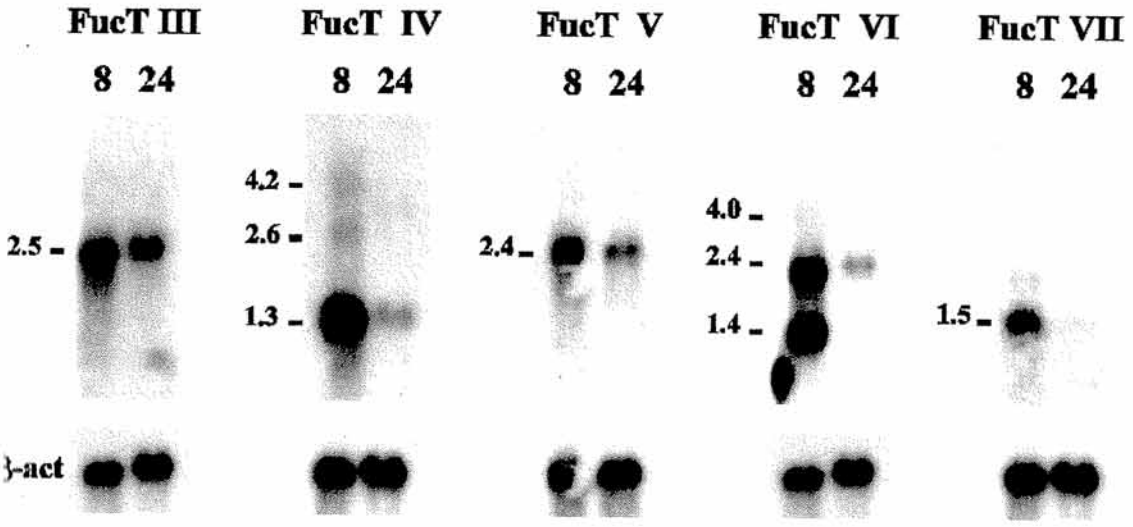
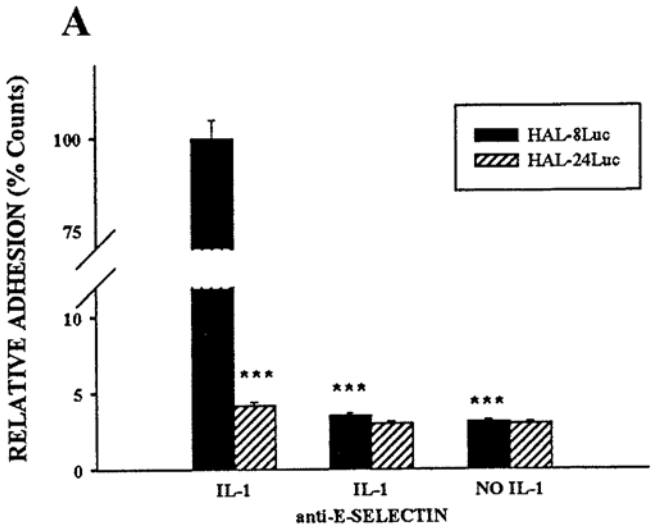
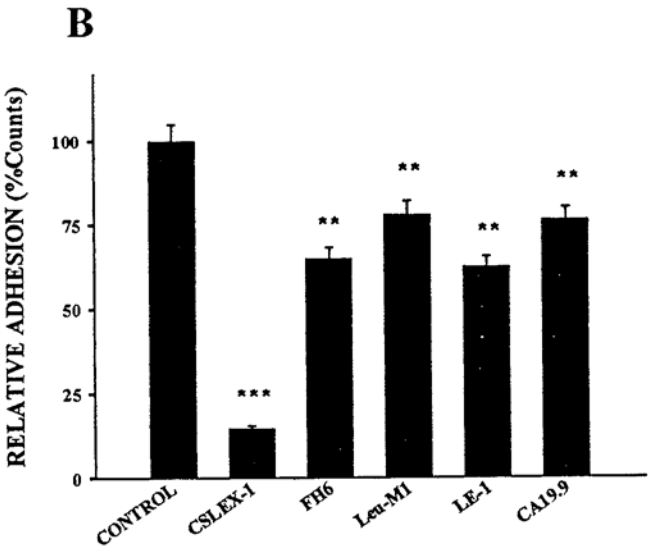
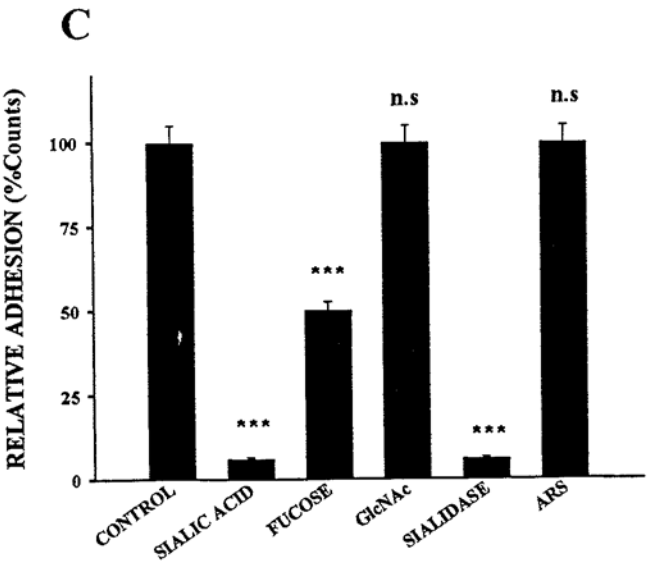

FucT III
8 24
2.5
β-act
FucT IV
8 24
4.2
2.6
1.3
FucT V
8 24
2.4
FucT VI
8 24
4.0
2.4
1.4
FucT VII
8 24
1.5

Figure 4. Adhesion of tumor cells to HUVEC monolayers. Data show normalized values corresponding to the fraction of bound cells and represent the mean ± SD of four different experiments each performed in six replicates. (**A**) Adhesion of HAL-8Luc (solid bars) and HAL-24Luc cells (striped bars) to IL-1β-stimulated (*IL-1*) and non-stimulated (*NO IL-1*) HUVEC monolayers. Alternatively, IL-1-stimulated HUVEC were incubated with *anti-E-selectin* mAb previous to the addition of tumor cells. (**B**) HAL-8Luc cell adhesion to IL-1-stimulated HUVEC monolayers after either no treatment (*CONTROL*) or treatment of tumor cells with different mAbs recognizing, from left to right: Le^x (*Leu-M1* mAb), sialyl-Le^x (*CSLEX-1* mAb), sialyl-Le^x dimeric (*FH6* mAb), Le^a (*LE-1* mAb) and sialyl-Le^a (*CA19.9* mAb). (**C**) IL-1-stimulated HUVEC were pre-incubated with either 50mM *sialic acid*, 800mM *fucose* or 800mM N-Acetyl glucosamine (*GlnNAc*); alternatively tumor cells were incubated with either *sialidase* or arylsulfatase (*ARS*) before the addition to HUVEC monolayers; *CONTROL* bar indicates adhesion of untreated HAL-8Luc cells to IL-1-stimulated HUVEC.

Significant differences between individual test adhesion values and control values (HAL-8Luc cell adhesion to IL-1-stimulated HUVEC monolayers): **, $P<0.01$; ***, $P<0.001$; **n.s**, not significant.


A
RELATIVE ADHESION (% Counts)
100
75
10
5
0
HAL-8Luc
HAL-24Luc
***
***
***
IL-1
IL-1
NO IL-1
anti-E-SELECTIN


B
RELATIVE ADHESION (%Counts)
100
75
50
25
0
***
**
**
**
**
CONTROL
CSLEX-1
FH6
Leu-M1
LE-1
CA19.9


C
RELATIVE ADHESION (%Counts)
100
75
50
25
0
***
***
n.s
***
n.s
CONTROL
SIALIC ACID
FUCOSE
GlcNAc
SIALIDASE
ARS