Changes in the invasive and metastatic capacities of HT-29/M3 cells induced by the expression of fucosyltransferase 1

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Lewis antigens are terminal fucosylated oligosaccharides synthesized by the sequential action of several glycosyltransferases. The fucosyltransferases are the enzymes responsible for the addition of terminal fucose to precursor oligosaccharides attached to proteins or lipids. These oligosaccharides, defined as cell surface markers, have been implicated in different types of intercellular interactions and in adhesion and invasion processes. Transfection of HT-29/M3 colon cancer cells with the full length of human fucosyltransferase (FUT1), induces the synthesis of H type 2 and Lewis y antigens, associated with a decrease of sialyl-Lewis x. The capacity to develop primary tumors when cells were injected intrasplenically was similar in parental and FUT1-transfected cells, but the capacity to colonize the liver after spleen removal was significantly reduced in M3/FUT1 transfected cells. These results indicate that the expression of FUT1 induces changes in the metastatic capacity of HT-29/M3 colon cancer cells, as a consequence of the altered expression pattern of type 2 Lewis antigens. Also, an association between MUC5AC expression and the degree of gland differentiation in both primary splenic tumors and hepatic metastases was detected. (*Cancer Sci* 2007; 98: 1000–1005)

Lewis antigens are fucosylated oligosaccharides carried by glycoproteins and glycolipids in the terminal position of the carbohydrate chains. These sugar structures are synthesized by the sequential action of specific glycosyltransferases. Between them, the family of α-1,2-fucosyltransferases catalyzes the addition of fucose in α-1,2-linkage to the galactose of type 1 (Galβ1,3GlcNAc-R) and type 2 (Galβ1,4GlcNAc-R) disaccharide to form H type 1 and H type 2 antigens, respectively. FUT1,[(1)] and FUT2,[(2)] are the human α-1,2-fucosyltransferases cloned, that are also named H and Secretor genes, respectively. α2,3-sialyltransferases, ST3Gal III, ST3Gal IV and ST3Gal VI, catalyze the addition of sialic acid to the same positions in competition with α-1,2-fucosyltransferases. The addition of α-1,3/1,4-fucose to GlcNAc residues in type 1 and type 2 precursors is catalyzed by several human α-1,3/1,4-fucosyltransferases: FUT3, FUT4, FUT5, FUT6, FUT7 and FUT9 (reviewed by de Vries *et al*.[(3)] Among them, FUT3 is the Lewis gene, expressed in epithelial tissues with α-1,3 and α-1,4-fucosyltransferase activity.

The Lewis structures are expressed in many cell types and their presence in the surface of epithelial cells contributes to several processes of intercellular adhesion and immunological recognition. In this sense, it has been reported the implication of sialyl-Lewis a (s-Le a) and sialyl-Lewis x (s-Le x) in interaction with selectins.[(4)] The specific recognition of the carbohydrate structure s-Le x, associated to glycoproteins and glycolipids expressed in the surface of endothelial cells and leukocytes, promotes the adhesion of neutrophils to the vascular endothelium in the first stage of the extravasation to the sites of inflammation.[(5)] The sialylated Lewis structures, s-Le a and s-Le x, are frequently expressed in human cancer cells acting as ligands for E-selectin expressed on vascular endothelial cells, contributing to the adhesion and extravasation of tumor cells and facilitating the process of metastasis. This process of adhesion is enhanced by the secretion of humoral factors by the tumor cells that induce the expression of E-selectin on the surface of endothelial cells.

Changes in the glycosylation patterns of the cell surface glycoproteins have been correlated with an altered invasiveness capacity of cancer cells. In epithelial cells, most of the Lewis antigens expressed are carried by the mucin molecules that cover and protect the epithelial surfaces. Alterations in their glycosydic compartment can imply the partial or complete loss of certain functions or even the acquisition of new roles. In this sense, certain tumor cells display long carbohydrate structures attached to the mucin core in their surface that can increase their invasive and metastatic potential.[(6,7)] Other cell surface glycoproteins, as CD44, are implicated in adhesion and invasion processes. The binding of CD44 to hyaluronic acid is regulated by glycosylation in at least four different ways.[(8)] In this sense, Goupille has reported that the expression of α(1–2) fucosylated antigens in rat colon carcinoma cells contributes directly to the aggressiveness of these cells probably by altering a function of CD44 variants.[(9)]

In the synthesis of type 2 Le antigens the glycosyltransferases, FUT1 and ST3Gal IV and ST3Gal VI, can compete for the same substrate, the precursor structure Galβ1–4GlcNAc-R. The action of FUT1 and FUT3 will catalyze the synthesis of H type 2 antigen and Le y, whereas ST3Gal IV, ST3Gal VI and FUT3 are the responsible for the synthesis of s-Le x. In this context, the ectopic expression of FUT1 can alter the relation between the amounts of both antigens. Recently, we described that the expression of FUT1 in HT-29/M3 colon cancer cells determines the expression of type 2 Le structures, H type 2 antigen and Le y, and induces changes in the glycosylation patterns of MUC1 and MUC5AC apomucins.[(10)] In the present work, we have analyzed in these cell lines the expression pattern of Lewis antigens, as well as their metastasic behavior induced by the expression of FUT1.

Materials and Methods

Cell lines. HT-29/M3 human colon adenocarcinoma cells are derived from the HT-29 colon cancer cell line treated with 10^{-3}M of methotrexate.[(11)] HT-29/M3 cell monolayer is mainly constituted by absorptive cells with a low percentage of mucus-secreting cells (<5%). M3/FUT1 clones were obtained by transfecting HT-29/M3 with the cDNA of human FUT1. C50/FUT1 is one of the selected M3/FUT1 clones. M3-4b and C50-6a/FUT1 cell lines were obtained by the culture of the tumor cells derived from the primary tumors grown after the subcutaneous injection of HT-29/M3 and C50/FUT1 cells in BALB/c nude mice.[(10)]

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Cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 1% L-glutamine, nonessential amino acids, penicillin and streptomycin (Invitrogen, Carlsbad, CA, USA) at 37°C in 5% CO_2 atmosphere. Stable transfectants were maintained in complete DMEM supplemented with 0.5 mg/mL G418. Cells were routinely checked for *Mycoplasma* contamination (Stratagene, La Jolla, CA, USA).

Flow cytometry. Cultured cells were trypsinized and counted. A total of 5×10^5 viable cells were incubated for 30 min at 4°C with antibodies diluted in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA). Cells were rinsed in PBS-BSA 1% and incubated with the secondary antibody Alexa Fluor 488 (Invitrogen) for 30 min at 4°C. After washing, fluorescent analysis was carried out using a FACScan (Becton Dickinson, Franklin Lakes, NJ, USA).

Phenyl-β-D-galactoside treatment. Phenyl-β-D-galactoside (Sigma-Aldrich, St Louis, MO, USA) was added at different concentrations (5–40 mM) to the culture medium and C50-6a/FUT1 cells were cultured under standard conditions. At day 4 of culture, the number of viable cells was counted and C50-6a/FUT1 cells were processed for flow cytometry.

E-Selectin binding assay. Ninety-six-well microplates were coated with 5 μg/mL recombinant human E-Selectin (R & D Systems, Minneapolis, MN, USA) or 1% BSA in PBS and incubated at 4°C for 24 h. Plates then were blocked with PBS containing 1% BSA. Cells were trypsinized and counted. 10^5 viable cells were added to the wells and incubated at room temperature for 1 h. In selected experiments, cells were previously incubated with specific antibodies for 30 min at 4°C. After washing twice with PBS, adherent cells were estimated by adding 0.5 mg/mL Thiazolyl Blue (Sigma-Aldrich) in phenol red-free medium for 2 h. Formazan crystals were solubilized with 50 μL of Dimethyl Sulfoxide. Optical density was measured at 550 nm. All the experiments were carried out in triplicate, and four independent assays were undertaken.

Primary tumors and metastasis assays. 10^6 cells (M3-4b and C50-6a/*FUT1*cell lines) suspended in 50 μL of serum-free DMEM were injected intrasplenically into BALB/c nude mice under sterile conditions. Spleens were removed 8 days later, observed macroscopically, formalyn fixed and embedded in paraffin to microscopically analyze the formation of intrasplenic tumors. Eight to ten weeks later, livers from splenectomyzed mice were removed and analyzed macroscopically. Then they were carefully sliced for microscopic detection of tumors. Sections from spleens and livers were hematoxylin-eosin and periodic acid Schiff (PAS)-diastase stained, and used for immunohistochemistry.

A total of 10^6 cells (M3-4b and C50-6a/FUT1cells) were also administered by intracecum injection in three mice for each of the two cell lines. Animals were killed between 9 and 13 weeks later and the presence of tumors was analyzed in the cecum and liver.

Animal protocols, ref. B-990010, were approved by the Dep. Agricultura Ranaderia I Pesca (DARP) (Generalitat de Catalunya) with the number 2189.

Antibodies and immunohistochemical assays. Monoclonal antibodies (MoAb) M8,[12] CLH2,[13] T-218,[14] and 77/180,[15] that recognize MUC1, MUC5AC, Le b and Le y, respectively, were used as undiluted hybridoma supernatant. MoAb 57/27 (anti-s-Le a),[15] and Cslex-1 (anti-s-Le x),[16] were used at 1:2 dilution, and MoAb 19–0LE to detect H type 2 antigen,[17] was used as ascites diluted at 1/1000. MoAb KM93 (anti-s-Le x) (Chemicon International, Temecula, CA, USA) was diluted following the manufacturer's instructions. B12 MoAb (Dr Castro, Barcelona, Spain) that recognizes a synthetic dextran molecule was used as a negative control for the monoclonal antibodies. Rabbit polyclonal serum LUM5.1,[18] detecting MUC5AC was used at 1/1000 and preimmune rabbit serum was used as a negative control. An indirect immunoperoxidase technique was carried out on paraffin-embedded sections as described.[19]

Sections were visualized in an Olympus AX70 microscope and images were acquired using Studio Lite 1.0 software.

Statistical analysis. For statistical analysis the *t*-test for Equality of Means was used. Statistical significance was defined as $P \leq 0.05$.

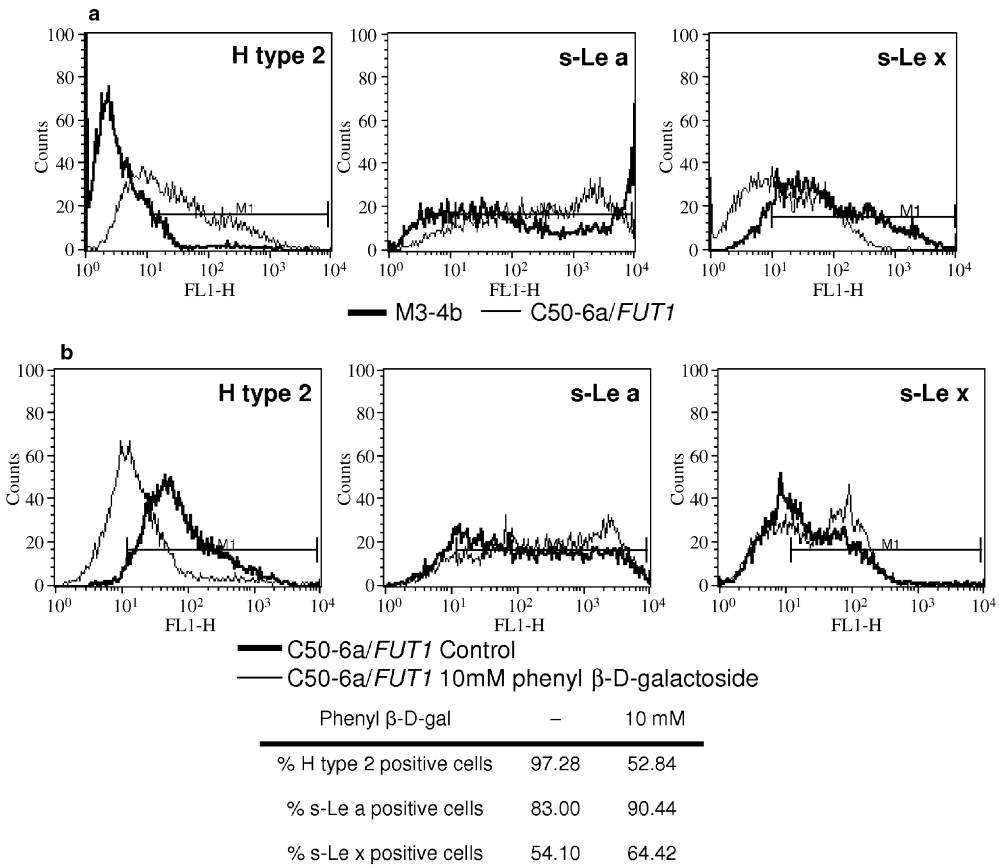
Results

Transfection of FUT1 cDNA induces the expression of non-sialylated type 2 Le antigens and lower levels of s-Le x. Treatment of M3-FUT1 cells with phenyl β-D-galactoside. The expression pattern of non-sialylated Lewis antigens in these cell lines has been reported previously.[10] Briefly, non-significant differences in Le a and Le b expression levels were detected, whereas significant changes in the type 2 antigens, H type 2 and Le y, were found to be induced by the expression of FUT1. Regarding the sialylated Lewis antigens, analyzed by flow cytometry, the M3-4b cells expressed s-Le a (61.1%) and s-Le x (58.0%) and only very low levels of H type 2 antigen (4.4%) were detected. By contrast, C50-6a/FUT1 cells expressed higher amounts of H type 2 (69.9%), similar levels of s-Le a (70.0%) and lower levels of s-Le x (38.9%) (Fig. 1a). These results were obtained in four independent FACS experiments and indicate that the induced expression of the type 2 precursor is at the expense of the level of s-Le x expression, probably by the enzyme-competition for the same precursor structure as substrate. Moreover, no changes in the expression of the sialyltransferases ST3Gal III and ST3Gal IV were detected (data not shown).

To determine that the expression of H type 2 antigens is specifically associated to the transfection of FUT1, cells were treated with phenyl β-D-galactoside. This molecule has been reported to be a specific substrate for α-1,2 fucosyltransferases,[12,20] and acts as a specific inhibitor for the synthesis of Lewis antigens. C50-6a/FUT1 cells were treated with 5, 10, 20, and 40 mM phenyl β-D-galactoside during 4 days and the expression of the H type 2 structure, s-Le *x* and s-Le a antigen were detected by FACS analysis. Results shown in Figure 1(b) indicate that the higher inhibition rate of H type 2 antigen expression (44.44%) is achieved by the 10 mM concentration, whereas a 10.32% and a 7.44% increase in s-Le x and s-Le a is detected. When the cells were treated at higher doses the viability was significantly reduced.

Different binding to E-Selectin induced by the expression of FUT1. Binding assays were carried out to analyze if the different pattern of the Lewis antigens expression in these cell lines was able to induce changes in the adhesion to E-Selectin. As shown in Fig. 2, the capacity of the M3-4b cells to bind E-Selectin was high, whereas the C50-6a/FUT1 cells, expressing lower levels of s-Le x, attached in a 40.67% less to E-Selectin. When the cells were previously incubated with the antis-Le x antibody, the attachment to E-Selectin of the M3-4b cells was clearly inhibited (55.93%), whereas the incubation with the anti-s-Le a antibody inhibits the binding at lower levels (30.50%). In C50-6a/FUT1 cells, treatment with the anti-s-Le *x* antibody also produced a clear inhibition in E-Selectin binding (40.00%); whereas after s-Le a incubation the inhibition rate was lower (22.85%). When statistically compared, the only significant differences were found between M3-4b and C50-6a/FUT1l cells and these cells incubated with anti-s-Le *x* ($P = 0.05\%$). These results demonstrate that the s-Le *x* structure is the major one responsible for the binding to E-Selectin, and that the expression of FUT1 in HT-29/M3 cells induces a decrease in the *in vitro* binding to E-Selectin.

FUT1 expression does not induce changes in the tumorigenicity of the cells. 10^6 cells (M3-4b and C50-6a/FUT1) were injected in the spleen of BALB/c nude mice ($n = 7$ and $n = 20$, respectively). In the case of the animals injected with C50-6a/FUT1 cells, 12 animals died in the first 24 h by porta vein thrombosis. Spleens from M3-4b and C50-6a/FUT1 cell-injected mice, developed



Phenyl β-D-gal	–	10 mM
% H type 2 positive cells	97.28	52.84
% s-Le a positive cells	83.00	90.44
% s-Le x positive cells	54.10	64.42

Fig. 1. (a) Expression of H type 2, Sialyl-Lewis a (s-Le a), and s-Le x antigens in M3-4b parental cells and C50-6a/fucosyltransferase (FUT1)-transfected cells detected by flow cytometry. (b) Treatment of C50-6a/FUT1-transfected cells with phenyl β-D-galactoside: Flow cytometry detection of H type 2 antigen, s-Le a, and s-Le x after 4 days of treatment. Marker 1 (M1) indicates the positive cells.

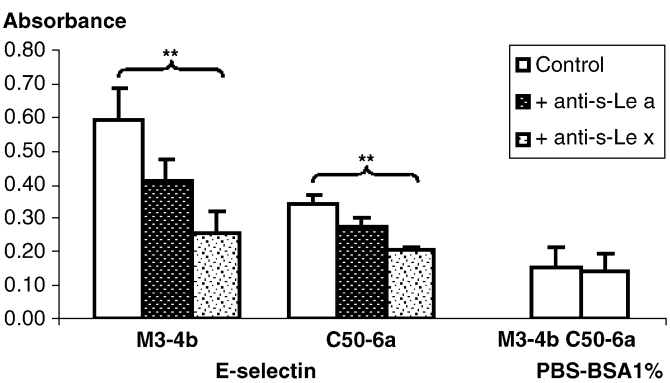


Fig. 2. Binding of M3-4b parental cells and C50-6a/ fucosyltransferase (FUT1)-transfected cells to E-Selectin. The two cell lines were also incubated with anti-s-Le x (KM93) and anti-s-Le a (57/27) antibodies. $^{**}P = 0.05$.

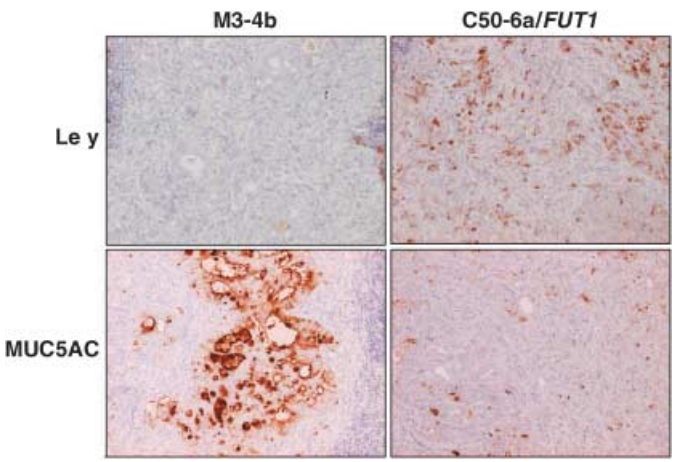


Fig. 3. MUC5AC, and Le y expression in primary splenic tumors after the injection of M3-4b parental cells and C50-6a/FUT1-transfected cells, detected by immunohistochemistry. Original magnification × 200.

intrasplenic tumors (5/7 and 6/8, respectively) 8 days after the injection of the cells. In the mice injected with control M3-4b cells, tumors were only visible microscopically. In animals injected with the FUT1-transfected cells, the presence of macroscopically visible tumors was detected in 2/6 spleens. The intrasplenic tumors from control M3-4b cells were very differentiated with glandular architecture and mucin droplets into the glands lumen, even though the size of the tumor was small. In contrast, intrasplenic tumors from spleens injected with C50-6a/FUT1 cells showed a poorer degree of differentiation when compared with the tumors obtained from control cells, giving rise to the formation of solid tumors without gland formation. PAS-diastase staining was used to confirm the presence of intraglandular mucin in intrasplenic tumors from control cells. In contrast, only focal PAS-diastase staining was detected in the case of tumors from transfected cells. No differences were detected regarding the MUC1 expression in

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Table 1. H type 2, Sialyl-Lewis a (s-Le a) and Sialyl-Lewis x (s-Le x) expression in tumors from M3-4b and C50-6a/fucosyltransferase (FUT1) injected mice

Case	Intrasplenic primary tumors			Liver metastasis		
	H type 2	s-Le a	s-Le x	H type 2	s-Le a	s-Le x
M3-4b						
I	≤10%[†]	40–50%	20%	≤10%	50%	30%
II	NT	NT	NT	≤5%	70%	30–40%
III	<5%	60%	10%	≤5%	70–80%	60%
IV	<5%	40–50%	10%	–	80%	50–60%
V	NT	NT	NT	–	70%	50%
VI	–	<10%	40%	–	80–90%	70%
VII	–	30%	30%	–	60–70%	50%
C50-6a/FUT1						
I	50%	–	–	NT	NT	NT
II	NT	NT	NT	NT	NT	NT
III	NT	NT	NT	40%	40–50%	30%
IV	30–40%	20%	–	NT	NT	NT
V	30–40%	30%	<5%	NT	NT	NT
VI	30%	20%	–	30–40%	70%	25%
VII	30–40%	20%	–	NT	NT	NT
VIII	50–60%	20%	–	NT	NT	NT

[†]Percentage of positive cells; NT, no tumor.

intrasplenic tumors, and the pattern of MUC5AC expression was associated to the gland formation and the mucus-secreting differentiation degree. As instrasplenic tumors from C50-6a/FUT1 cells were less differentiated, the level of MUC5AC expression was reduced (Fig. 3).

Regarding the expression of Lewis antigens, no changes were detected in type 1 Le antigens (s-Le a, Le b) expression between M3-4b and C50-6a/FUT1 tumors. The presence of type 2 Le antigens was also analyzed by immunohistochemistry. M3-4b tumors showed expression of s-Le *x* (22.00 ± 13.04% of positive cells), whereas H type 2 and Le y were undetected or detected at very low levels (2.50 ± 3.06% positive cells). By contrast, in C50-6a/FUT1 primary tumors H type 2 was detected (40.00 ± 10.00% positive cells) whereas s-Le *x* was not expressed. Statistically significant differences were only found when the expression of H type 2 structures and s-Le *x* antigen were compared ($P \leq 0.05\%$). The expression patterns of Lewis antigens detected in the tumors were equivalent to those observed in the original cell lines. Summaries of these results are shown in Table 1 and Fig. 3.

Liver invasion and colonization of tumor cells expressing type 2 Le antigens. A total of 7/7 spleen-removed mice from M3-4b cells developed macroscopically visible liver metastases 8–10 weeks later. Instead, only 2/8 C50-6a/FUT1 injected mice developed focal liver metastases. As in the primary tumors, these tumors were poorly differentiated, and in no case gland formation was detected, whereas tumors from M3-4b cells were more differentiated and PAS-diastase positive. All tumors from liver metastases maintained the expression of MUC1 and MUC5AC as the primary splenic tumors.

In the analysis of Lewis antigens expression, the liver tumors maintained the expression patterns of the primary tumors, for both type 1 Le and type 2 Le: hepatic metastases from M3-4b cells expressed s-Le a (70.71 ± 11.34% of positive cells) and s-Le x (50.00 ± 5.23% cells) and did not express H type 2 antigen; whereas in the two tumor metastases from the C50-6a/FUT1 cells, higher levels of H type 2 antigens (37.50 ± 2.78 positive cells), equivalent levels of s-Le a (57.50 ± 17.68% of the cells), and lower levels of s-Le x (27.50 ± 3.54% of positive cells) were detected. Statistically significant differences were only obtained when H type 2 antigen levels were statistically compared ($P = 0.05$). Results are shown in Table 1 and Fig. 4.

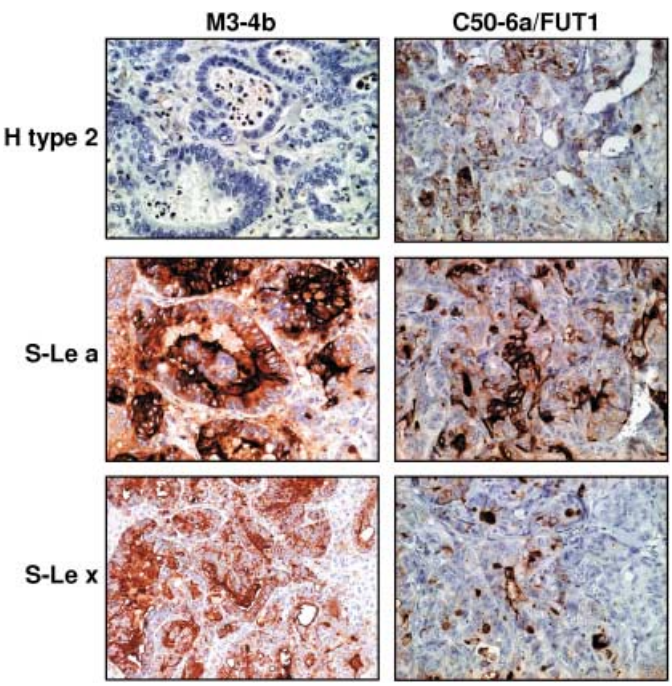

Fig. 4. H type 2 antigen, Sialyl-Lewis a (s-Le a), and s-Le x expression in liver metastases after the intrasplenic injection of HT-29/M3 and C50-6a/FUT1 cells, detected by immunohistochemistry. Original magnification × 200.

Similar results were obtained when the cells were injected ortotopically in the cecum ($n = 3$ for each cell line). All of the animals developed macroscopically visible primary colonic tumors, whereas all of the mice injected with the HT-29/M3-4b parental cells developed hepatic metastases (3/3) and only in 1/3 C50-6a/FUT1-injected mice one micrometastasis was detected.

In conclusion, in the two mice models, the parental HT-29/M3 cells are able to induce microscopic primary tumors and macroscopic visible liver metastases, whereas the transfected C50-6a/FUT1 cells form larger primary tumors and either do not produce, or only produce, microscopic metastases.

Discussion

We have previously reported that HT-29/M3-FUT1 transfected cells express H type 2 and Le y antigens in the cellular membrane associated to MUC1 and MUC5AC apomucins.[10] It has been largely documented that the sialylated forms of Lewis antigens participate in the first steps of the extravasation of neutrophils to the sites of inflammation through labile interactions with Selectins, facilitating their rolling and attachment to the endothelial cells. Fuhlbrigge demonstrated that fucose is a component of leukocyte-selectin ligands because the transfection of different α-1,3-fucT induced the capacity of rolling to Jurkatt and Chinese hamster ovary (CHO) cells.[21] The aberrant expression of these structures in the surface of tumor cells is related to their metastasic capacity, and the implication of s-Le *x* in the processes of cell adhesion and migration through Selectins is well established.[22,23] The expression of sialylated Lewis antigens in epithelial tumors has been extensively reported and s-Le x and s-Le a are strongly detected in colon, breast, ovary, prostate, and uterine cancer.[24] In this sense, it has been demonstrated that the lack of expression of s-Le a induced in CX-1 colon cancer cells by the transfection of FUT3 cDNA in the antisense orientation, abolishes the adhesion of these cells to E-Selectin indicating the active role of s-Le a in the adhesion of colon cancer cells.[25] Moreover, the suppression of the sialyltransferase ST6Gal I activity by the antisense DNA approach is sufficient to reduce the invasiveness of HT-29 colon cancer cells *in vitro*.[26]

Previously, we have described similar tumorigenic capacities for HT-29/M3 parental and C50/FUT1 cells injected subcutaneously in BALB/c nude mice.[10] We show here that the expression of FUT1 in HT-29/M3 cells induces changes in the *in vivo* metastasic capacity of these cells that can be related to the induction of H type 2 and Le y expression, associated to lower levels of s-Le x in the cell membrane. Both, M3-4b and C50-6a/FUT1 cells showed similar capacities to develop primary splenic tumors, but M3-4b cells colonize the liver more efficiently than the C50-6a/FUT1 cells. These results indicate that cells expressing non-sialylated type 2 Le antigens and low levels of s-Le x, lose their ability to develop liver metastases, whereas M3-4b parental cells that express higher levels of s-Le x, form hepatic metastases in all the mice. These data suggest that the induction of type 2 Le antigens by the expression of FUT1 induces lower levels of s-Le *x* in the cellular membrane of the transfected cells that would inhibit the dissemination of these cells as a consequence of the lower adhesion capacity to E-Selectin. These data agree with recent data reporting that in the parental HT-29 cell line the expression of FUT1 induces lower levels of s-Le *x* and inhibits *in vitro* the binding to E-Selectin.[27,28]

These fucosylated terminal carbohydrate structures are attached to specific glycoproteins and glycolipids[29] in the cell surface that would condition the binding of tumor cells to E-Selectin and their implication in the metastasic process, as it has been recently pointed out for MUC1,[10] and CD44. In the colon cancer cells LS174T, the O-glycosylated CD44v isoforms function as high-affinity E-Selectin ligand specifically fucosylated and sialylated structures.[30]

Tumors from M3-4b cells were more differentiated than tumors from C50-6a/FUT1 cells, as it has been previously reported for primary subcutaneous tumors,[10] and there is a correlation between the detection of MUC5AC and the degree of differentiation in both primary splenic tumors and hepatic metastases. The higher degree of gland differentiation is associated with the highest levels of MUC5AC expression. In previous reports, we have described that MUC5AC is detected in tumors showing a high degree of glandular differentiation in gastric,[19] and bronchogenic tumors.[31] These observations suggest that MUC5AC may be a good marker for the mucus-secreting and glandular differentiation pattern of epithelial tumors.

In conclusion, C50-6a/FUT1 cells are able to form primary splenic tumors but lose the capacity to develop liver metastases in contrast to the M3-parental cells. This different behavior can be, at least in part, explained by the specific pattern of Lewis antigens expression in the cell surface induced by the expression of FUT1. These findings together with previously reported data can suggest that changes in the Lewis structures need to be considered as therapeutic strategies to inhibit the binding of tumor cells to vascular endothelium.

Acknowledgments

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