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Dual roles of sialyl Lewis X oligosaccharides in tumor metastasis and rejection by natural killer cells

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Aberrant expression of cell surface carbohydrates such as sialyl Lewis X is associated with tumor formation and metastasis. In order to determine the roles of sialyl Lewis X in tumor metastasis, mouse melanoma B16-F1 cells were stably transfected with α1,3-fucosyltransferase III to express sialyl Lewis X structures. The transfected B16-F1 cells, B16-FTIII, were separated by cell sorting into three different groups based on the expression levels of sialyl Lewis X. When these transfected cells were injected into tail veins of C57BL/6 mice, B16-FTIII·M cells expressing moderate amounts of sialyl Lewis X in poly-*N*-acetyllactosamines produced large numbers of lung tumor nodules. Surprisingly, B16-FTIII·H cells expressing the highest amount of sialyl Lewis X in shorter *N*-glycans died in lung blood vessels, producing as few lung nodules as B16-FTIII·N cells which lack sialyl Lewis X. In contrast, B16-FIII·H cells formed more tumors in beige mice and NK cell-depleted C57BL/6 mice than did B16-FTIII·M cells. B16-FTIII·H cells bound to E-selectin better than did B16-FTIII·M cells, but both cells grew at the same rate. These results indicate that excessive expression of sialyl Lewis X in tumor cells leads to rejection by NK cells rather than tumor formation facilitated by attachment to endothelial cells.

Keywords: NK cells/selectin/sialyl Lewis X/tumor cell rejection/tumor metastasis

Introduction

Cell surface carbohydrates are characteristic of different stages of differentiation, and distinct carbohydrates are expressed in tissue- and cell-specific manners during development and in adulthood (Feizi, 1985; Rademacher *et al.*, 1988; Fukuda, 1996). Although the physiological roles for these carbohydrates are not understood, many of these carbohydrates are thought to play a role in cell–cell interaction. Among various cell type-specific carbohydrates, sialyl Lewis X, NeuNAcα2→3Galβ1→4(Fucα1→3)GlcNAc→R, is expressed on neutrophils, monocytes and certain T lymphocytes (Fukuda *et al.*, 1984; Mizoguchi *et al.*, 1984) and plays a key role in the recruitment of leukocytes. E- and P-selectin expressed on activated endothelial cells capture these leukocytes, allowing them to roll, which leads to extravasation of leukocytes (Lowe, 1994; Rosen and Bertozzi, 1996). When lymphocytes recirculate from the intravascular compartment to the lymphatic compartment, circulating lymphocytes are captured to high endothelial venules. This process is initiated by binding of L-selectin on lymphocytes to sulfated sialyl Lewis X present in receptors that are restricted to high endothelial venules (Imai *et al.*, 1993; Tsuboi *et al.*, 1996). These results clearly indicate that sialyl Lewis X and its sulfated form play critical roles in the interaction between leukocytes and endothelial cells (Lowe, 1994; Rosen and Bertozzi, 1996).

In parallel with these studies, several laboratories have reported that expression levels of sialyl Lewis X are increased substantially in tumor cells such as carcinomas and leukemias (Fukushima *et al.*, 1984; Fukuda *et al.*, 1985; Saitoh *et al.*, 1992; Shimodaira *et al.*, 1997). In many carcinoma cells, the amount of sialyl Lewis A, NeuNAcα2→3Galβ1→3(Fucα1→4)GlcNAc→R, is also increased (Magnani *et al.*, 1982; Itzkowitz *et al.*, 1988). In breast and colonic carcinoma patients, the presence of sialyl Lewis X and sialyl Lewis A was correlated with poor prognosis (Nakamori *et al.*, 1993; Renkonen *et al.*, 1997; Shimodaira *et al.*, 1997). A recent study demonstrated that expression of sialyl Lewis X and sialyl Lewis A in mucin-type *O*-glycans is highly correlated with lymphatic and venous invasion (Shimodaira *et al.*, 1997). Considering that sialyl Lewis A is also a ligand for E- and P-selectin (Berg *et al.*, 1991; Takada *et al.*, 1991), these results strongly suggest that blood-borne tumor cells may utilize selectin–carbohydrate interaction when tumor cells adhere to the endothelia distant from the site of primary tumor growth. In fact, we and others have shown previously that highly metastatic tumor cells adhere more strongly to E-selectin than do their poorly metastatic counterparts (Sawada *et al.*, 1994; Martín-Satué *et al*, 1998). Tumor formation in lung was diverted to livers by making transgenic mice expressing E-selectin in livers (Biancone *et al.*, 1996). These results strongly suggest that carbohydrates on tumor cell surfaces may be recognized by selectins on the endothelium. However, no studies have determined how different amounts of sialyl Lewis X play different roles in tumor metastasis.

Sialyl Lewis X is formed at termini of *N*- and *O*-glycan precursor carbohydrates that were pre-assembled. In particular, poly-*N*-acetyllactosamines are often modified to express sialyl Lewis X (Lee *et al.*, 1990; Saitoh *et al.*, 1992; Wilkins *et al.*, 1996). Poly-*N*-acetyllactosamine is composed of *N*-acetyllactosamine repeats and the increased amount of poly-*N*-acetyllactosamine is parallel with the increase of side chain synthesized by *N*-acetylglucosaminyltransferase V (GnT-V) (Sasaki *et al.*, 1987; Van den Eijnden *et al.*, 1988). Because of this biosynthetic relationship, it has been observed repeatedly that transformed cells and metastatic tumor cells contain more

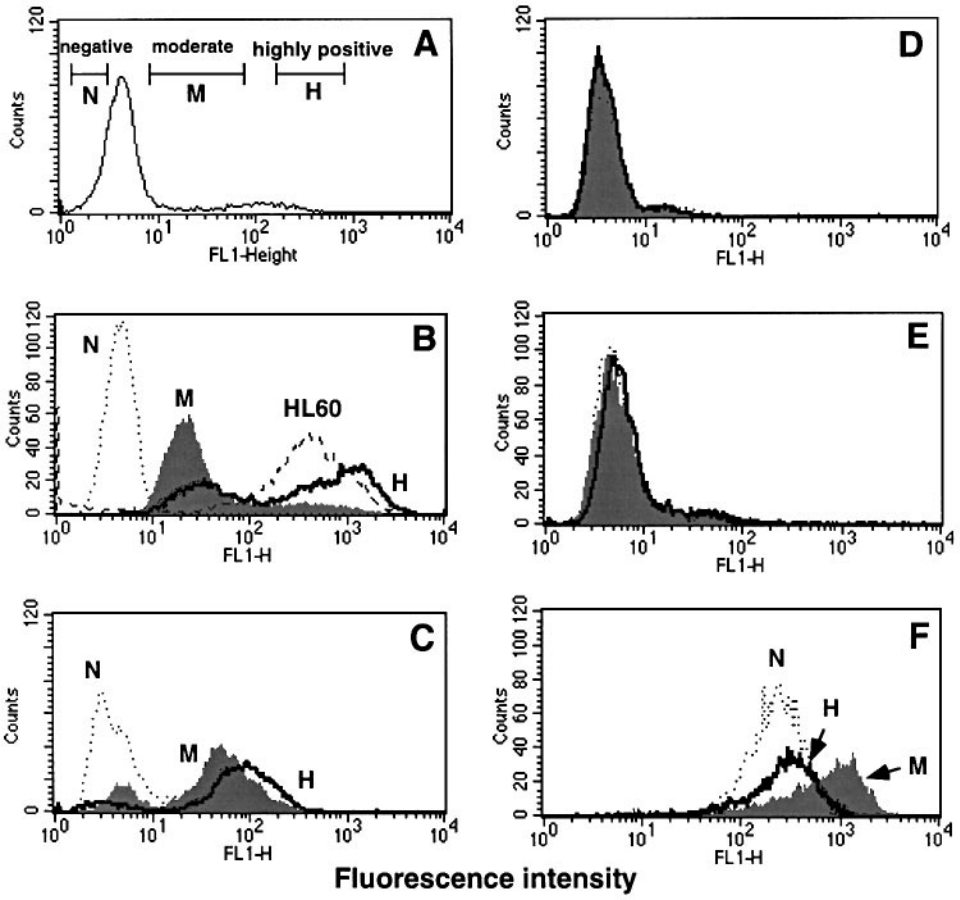

Fig. 1. Cell sorting and flow cytometric analysis of B16-FTIII cells. (**A**) B16-FTIII cells were stained with anti-sialyl Lewis X antibody followed by FITC-conjugated secondary antibody and sorted by FACStar. Cells indicated by open bars were pooled and designated B16-FTIII·N (negative), B16-FTIII·M (moderate) and B16-FTIII·H (highly positive), respectively. (B–F) Cultured B16-FTIII·N (N), B16-FTIII·M (M) and B16-FTIII·H (H) cells were subjected to flow cytometry after staining with anti-sialyl Lewis X (**B**), anti-sialyl Lewis A (**C**) or anti-Lewis X antibody (**D**), followed by FITC-conjugated secondary antibody, or FITC-conjugated SNA (**E**) or FITC-conjugated LEA (**F**). HL-60 cells were also stained in (B). The staining by FITC-conjugated MAA gave the same results as FITC-conjugated SNA.

poly-*N*-acetyllactosamine and side chains synthesized by GnT-V than the parent cells or low metastatic counterparts (Yamashita *et al.*, 1984; Saitoh *et al.*, 1992; Demetriou *et al.*, 1995). These results indicate that it is important to understand the formation of terminal structures such as sialyl Lewis X in conjunction with the synthesis of underlying backbone carbohydrates (Fukuda, 1994).

To determine whether sialyl Lewis X and underlying carbohydrates play a role in tumor metastasis, we tested the ability of mouse melanoma B16 cells (Fidler, 1973) to form tumors after injection into a tail vein. We reasoned that adhesion of tumor cells to the endothelium in this system plays a primary role in establishing tumor nodules, since tumor cells are already in the bloodstream and no escape from the primary tumor site is involved. B16-FI cells were thus transfected with a cDNA encoding α1,3-fucosyltransferase III (Fuc-TIII) (Kukowska-Latallo *et al.*, 1990), which is distributed widely in various tissues and directs the synthesis of both sialyl Lewis X and sialyl Lewis A. Transfected B16-FI cells expressing a moderate amount of sialyl Lewis X in long *N*-glycans (B16-FTIII·M) produced significantly increased lung tumor nodules in C57BL/6 mice. On the other hand, those transfected cells expressing the highest amount of sialyl Lewis X in short *N*-glycans (B16-FTIII·H) scarcely produced lung nodules as did those expressing no sialyl Lewis X in wild-type and SCID mice. B16-FTIII·H cells were found to undergo apoptosis in those mice. In contrast, B16-FTIII·H cells produced more lung nodules in beige mice or C57BL/6 mice depleted of natural killer (NK) cells than did B16-FTIII·M cells. These results demonstrate for the first time that acquisition of sialyl Lewis X leads to lung tumor formation or rejection by NK cells, depending on the amount of sialyl Lewis X and underlying glycans expressing sialyl Lewis X.

Results

Isolation of B16-FTIII cells expressing different amounts of sialyl Lewis X

Since Fuc-TIII is distributed widely in various tissues and directs expression of both sialyl Lewis X and sialyl Lewis A, B16-F1 cells were transfected with Fuc-TIII. Stably transfected B16-FTIII cells were subjected to cell sorting with anti-sialyl Lewis X (CSLEX1) antibody. As shown in Figure 1A, B16-FTIII cells can be sorted into three groups: those highly positive for sialyl Lewis X (B16-FTIII·H), those moderately positive for sialyl Lewis X (B16-FTIII·M) and those negative for sialyl Lewis X (B16-FTIII·N). As shown in Figure 1B, B16-FTIII·H cells

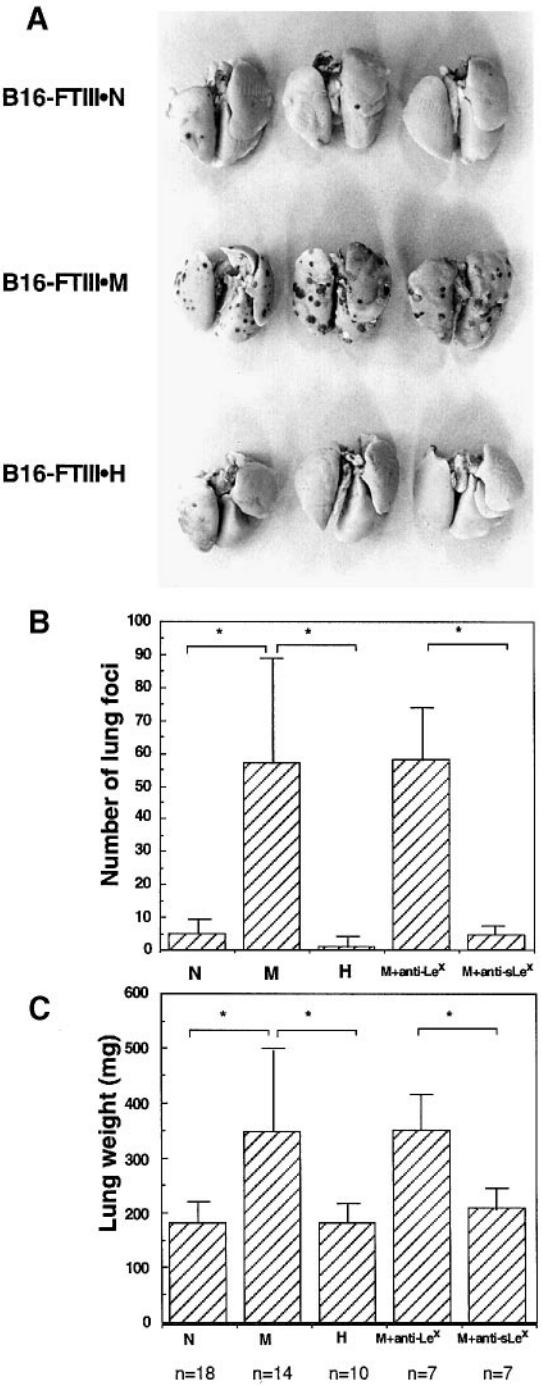

Fig. 2. Representative lungs and number and weight of lung tumor nodules in BALB/c mice after intravenous injection of B16-FTIII·N, B16-FTIII·M or B16-FTIII·H cells. (**A**) The lungs were examined 3 weeks after injection. (B and C) B16-FTIII·N (N), B16-FTIII·M (M) and B16-FTIII·H (H) cells were injected intravenously, and tumors formed after 3 weeks were counted (**B**) and the weight of lungs was measured (**C**). B16-FTIII·M cells were incubated with anti-sialyl Lewis X antibody (anti-sLe^x) or anti-Lewis X antibody (anti-Le^x) at 4°C for 30 min and injected intravenously under the same conditions. The differences in both tumor nodule number and lung weight, denoted by asterisks, are statistically significant by the Mann–Whitney *U*-test ($p < 0.01$ and $p < 0.05$, respectively).

express as much sialyl Lewis X as promyelocytic HL-60 cells. B16-FTIII·M cells express a moderate amount of sialyl Lewis X, showing five times the intensity of anti-sialyl Lewis X antibody staining compared with B16-FTIII·N (Figure 1B). The expression levels of sialyl Lewis X in different B16-FTIII cells were stable even after five passages (see also Discussion). To ensure that the phenotype was maintained, fluorescence-activated cell sorting (FACS) analysis was always carried out before further experiments.

Metastatic properties of B16-FTIII cells expressing different amounts of sialyl Lewis X

To determine if the metastatic properties of these B16-FTIII cells differ, the three cell populations were injected into tail veins of C57BL/6 mice. We found that only a few lung nodules were formed 3 weeks after injection of wild-type B16-F1 cells or B16-FTIII·N cells (Figure 2A, top). In contrast, B16-FTIII·M cells produced a large number of lung nodules under the same conditions (Figure 2A, middle). These results, reproduced more than five times using different B16-FTIII·M clones, indicate that the metastatic potential of B16-F1 cells was remarkably increased after transfection of Fuc-TIII. To determine if sialyl Lewis X plays a critical role in this process, B16-FTIII·M cells were incubated with anti-sialyl Lewis X antibody and then injected into tail veins of mice. Tumor formation was suppressed completely by this pre-treatment, while it was not suppressed by pre-incubation with an antibody specific to Lewis X (Figure 2B and C), which is a negligible ligand for selectins (Lowe, 1994; Rosen and Bertozzi, 1996).

Surprisingly, B16-FTIII·H cells, which were highly positive for sialyl Lewis X, produced as few tumor nodules as did B16-FTIII·N or the parent B16-F1 cells (Figure 2A, bottom). This observation was confirmed by comparing the number of lung nodules and weight of the lungs (Figure 2B and C). The same experiments also showed that melanin synthesis was not influenced by transfection of Fuc-TIII, since a good correlation exists between the number of lung nodules, determined by counting pigmented B16-FTIII cells, and the weight of lungs. These results indicate that B16-FTIII·M cells became highly metastatic after expressing a moderate amount of sialyl Lewis X, while B16-FTIII·H cells expressing the highest levels of sialyl Lewis X were poorly metastatic.

Expression of sialyl Lewis X does not increase cell growth

It is possible that B16-FTIII·M cells are more metastatic because they grow faster than B16-FTIII·N or B16-FTIII·H cells. To address this question, the cells were plated at low density and the growth rate was determined. The results clearly demonstrate that expression of sialyl Lewis X did not alter the rate of tumor cell growth when assayed by a cell proliferation assay (Figure 3). These results exclude the possibility that B16-FTIII·M cells grow faster than the other two cell lines, demonstrating that a difference other than growth rate contributed to the formation of lung tumor nodules.

Binding of an E-selectin chimeric protein to different B16-FTIII cells

To determine whether these different B16-FTIII cells differ in E-selectin binding, cells were incubated with an E-selectin–IgM chimeric protein and the binding of E-selectin was assayed by flow cytometry.

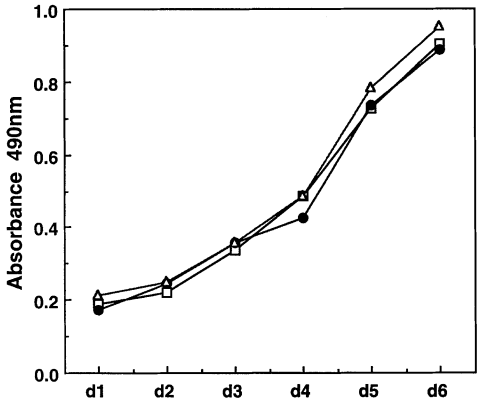

Fig. 3. Comparison of tumor cell growth. Growth kinetics of B16-FTIII·N (□), B16-FTIII·M (●) and B16-FTIII·H (△) cells are shown. Living cells were measured each day by the MTT assay.

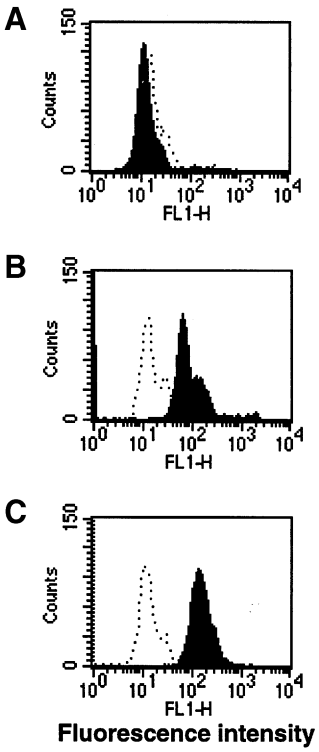

Fig. 4. Flow cytometric analysis of E-selectin binding to different B16-FTIII cells. Monodispersed different B16-FTIII cells were incubated with E-selectin–IgM chimeric protein followed by FITC-conjugated goat anti-human IgM, and then subjected to flow cytometric analysis (closed figures). The open figures indicate control experiments in the presence of 10 mM EDTA. (**A**) B16-FTIII·N; (**B**) B16-FTIII·M; (**C**) B16-FTIII·H.

As shown in Figure 4A and B, much more E-selectin chimeric protein was bound to B16-FTIII·M cells than to B16-FTIII·N cells. The highest amount of E-selectin binding was, however, observed in B16-FTIII·H cells (Figure 4C). When E-selectin–IgG chimeric protein (Sueyoshi *et al.*, 1994) was used, the difference in E-selectin binding was less significant, but greater binding was observed to B16-FTIII·H cells than to B16-FTIII·M cells (data not shown). These results indicate that binding of E-selectin chimeric proteins was proportional to the amount of sialyl Lewis X as estimated by anti-sialyl Lewis X antibody staining.

Characterization of cell surface oligosaccharides of different B16-FTIII cells

The results above indicate that B16-FTIII·M and B16-FTIII·H cells may differ in glycan structures and that such a difference may contribute to different metastatic potentials.

To address this question, B16-FTIII·N, B16-FTIII·M and B16-FTIII·H cells were stained with anti-sialyl Lewis A antibody or various lectins. As shown in Figure 1B and C, the expression profiles of sialyl Lewis X and sialyl Lewis A in different cell lines were similar, and B16-FTIII·H showed the strongest expression of sialyl Lewis A. These results exclude the possibility that sialyl Lewis A may be more abundant in B16-FTIII·M cells, contributing to higher metastatic potentials.

We then stained cells with anti-Lewis X antibody, *Maackia amurensis* agglutinin (MAA), *Sambucus nigra* agglutinin (SNA) and tomato lectin (LEA). However, different B16-FTIII cell lines did not show detectable differences in staining either by anti-Lewis X antibody (Figure 1D), SNA (Figure 1E) or MAA (data not shown). The results indicate that these different B16-FTIII cells did not vary in their expression of Galβ1→4(Fucα1→3) GlcNAc→R (Lewis X), NeuNAcα2→3Gal→R (detected by MAA; Wang and Cummings, 1988) or NeuNAcα2→ 6Gal→R (detected by SNA; Shibuya *et al.*, 1987). In contrast, B16-FTIII·M cells were stained more strongly by fluorescein isothiocyanate (FITC)–LEA than were B16-FTIII·H or B16-FTIII·N cells (Figure 1F). LEA was shown to bind preferentially to poly-*N*-acetyllactosamines (Merkle and Cummings, 1987; Lee *et al.*, 1990). It was also demonstrated that sialylation or fucosylation does not weaken this binding (Lee *et al.*, 1990; Saitoh *et al.*, 1992). These results thus strongly suggest that B16-FTIII·M and B16-FTIII·H cells differ in their amount of poly-*N*-acetyllactosamines.

To corroborate the findings above, the carbohydrate structures of glycoproteins present in different B16-FTIII cells were examined. The cells were metabolically labeled with [^{3}H]galactose, from which glycopeptides were prepared. The glycopeptides were then fractionated by serial lectin affinity chromatography. Both B16-FTIII·N and B16-FTIII·M cells produced high levels of glycopeptides that bound to and eluted from LEA–agarose (Figure 5B and E). When glycopeptides which did not bind to LEA–agarose were applied to a *Datura stramonium* agglutinin (DSA)–agarose column, a large quantity of those glycopeptides bound to DSA–agarose (Figure 5F). In contrast, glycopeptides from B16-FTIII·H cells were barely bound to either LEA–agarose or DSA–agarose (Figure 5H and I).

The glycopeptides bound to LEA–agarose (IB) and DSA–agarose (IA2) were digested by endo-β-galactosidase before and after defucosylation as described previously (Lee *et al.*, 1990; Saitoh *et al.*, 1992). The results indicate that IB and IA2 fractions from B16-FTIII·M cells contained highly sialylated glycopeptides having two to three *N*-acetyllactosamine repeats. Moreover, IB contained substantial amounts of sialyl Lewis X as determined by the method described (Sueyoshi *et al.*, 1994). IA1 contained the largest amount of radioactively labeled glycopeptides, which represent all of the *O*-glycans and the rest of the *N*-glycans. The amount of IB and IA2 glycopeptides thus constitutes a substantial portion of *N*-glycans, although

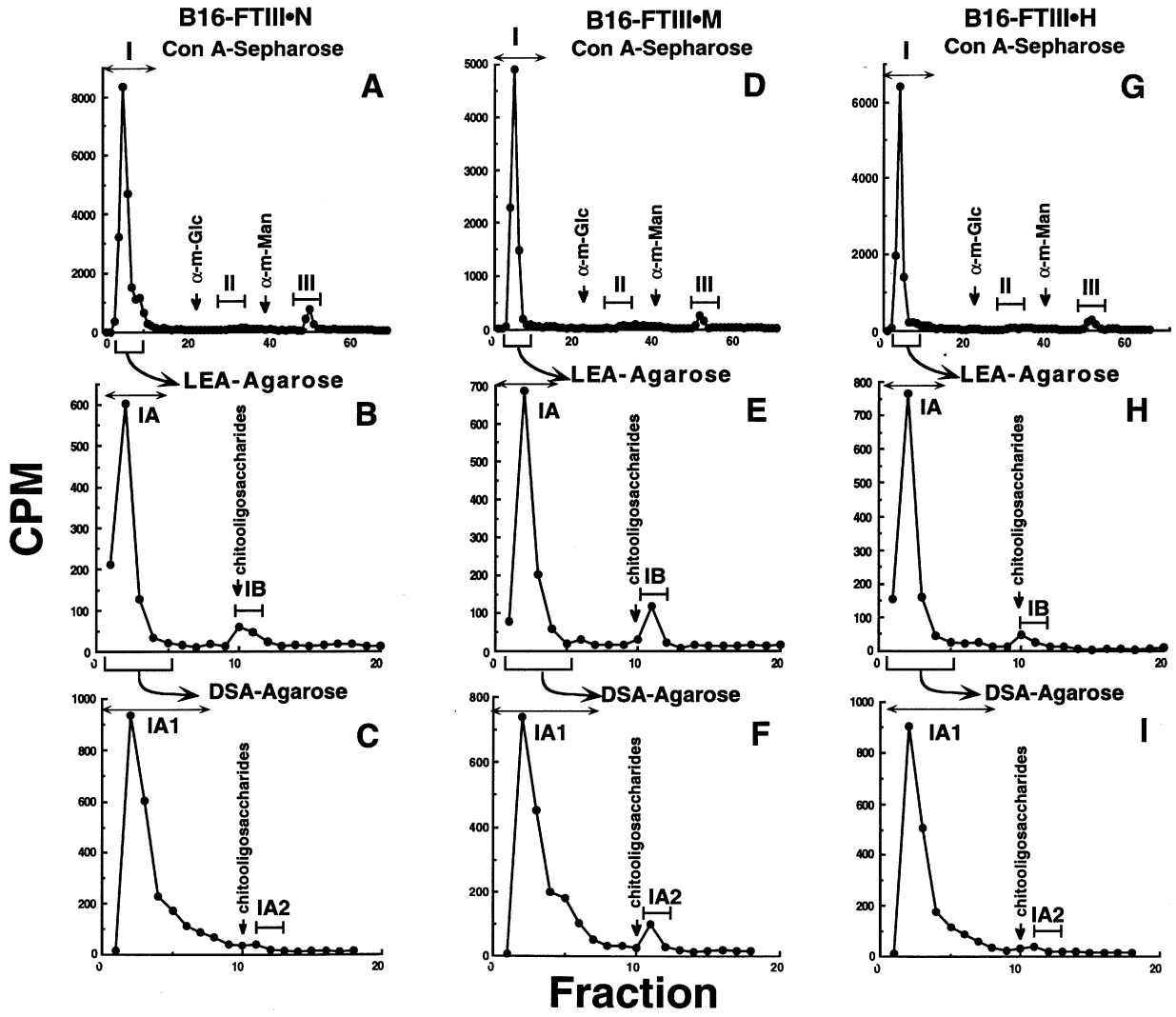

Fig. 5. Serial lectin affinity chromatography of [^{3}H]galactose-labeled glycopeptides. B16-FTIII·N (**A**, **B** and **C**), B16-FTIII·M (**D**, **E** and **F**) and B16-FTIII·H (**G**, **H** and **I**) cells were metabolically labeled with [^{3}H]galactose and glycopeptides prepared were applied sequentially to ConA–Sepharose, LEA–agarose and DSA–agarose. The glycopeptides subjected to the next step are indicated by horizontal bars. Vertical arrows indicate the initiation of elutions by a mixture of chitotriose and chitobiose.

they do not appear that way in Figure 5. These results, taken together, indicate that B16-FTIII·M cells contained more sialyl Lewis X and possibly sialyl Lewis A in poly-*N*-acetyllactosamines than did B16-FTIII·H cells. B16-FTIII·H cells, in contrast, express the highest amount of sialyl Lewis X in short *N*-glycans.

B16-FTIII·M but not B16-FTIII·H cells extravasate in lung tissues

The results above demonstrate that B16-FTIII·H and B16-FTIII·M cells do not differ in presentation of selectin ligands but differ in metastatic potential and glycan structures. These results suggest that both of these cell types may attach to endothelial cells in lungs but the fate of those cell types may differ.

To follow the fate of these cells, animals were sacrificed 3, 24 and 48 h after injection of different B16 cells, and lung tissue sections were examined using antibodies specific to a melanoma antigen (HMB-45) (Kapur *et al.*, 1992). As shown in Figure 6A, B16-FTIII·N cells expressing no detectable amount of sialyl Lewis X barely arrested within lung blood vessels 3 h after the injection and did not show any extravasation into the interstitial stroma 24 or 48 h after injection (Figure 6B and C). In contrast, B16-FTIII·M cells were found to arrest in lung blood vessels 3 h after injection, apparently invading parenchyma cells and extravasating into the interstitial stroma (Figure 6D, insert). The tumor cells started dividing and growing 24 and 48 h after the injection, respectively (Figure 6E and F).

B16-FTIII·H cells displayed a more complex profile. The cells appeared to arrest within lung blood vessels and extravasate into the interstitial stroma 3 h after the injection (Figure 6G). These cells, however, were apoptotic, displaying condensed nuclei, which were even broken in some cells (see the cell indicated by an arrowhead and that in the left insert, Figure 6G). The cells then disappeared 24 h after the injection (Figure 6H and I). These results strongly suggest that B16-FTIII·H cells died during or after penetration of lung tissue, while B16-FTIII·M cells established tumor growth during the same process.

To determine whether B16-FTIII·H cells underwent

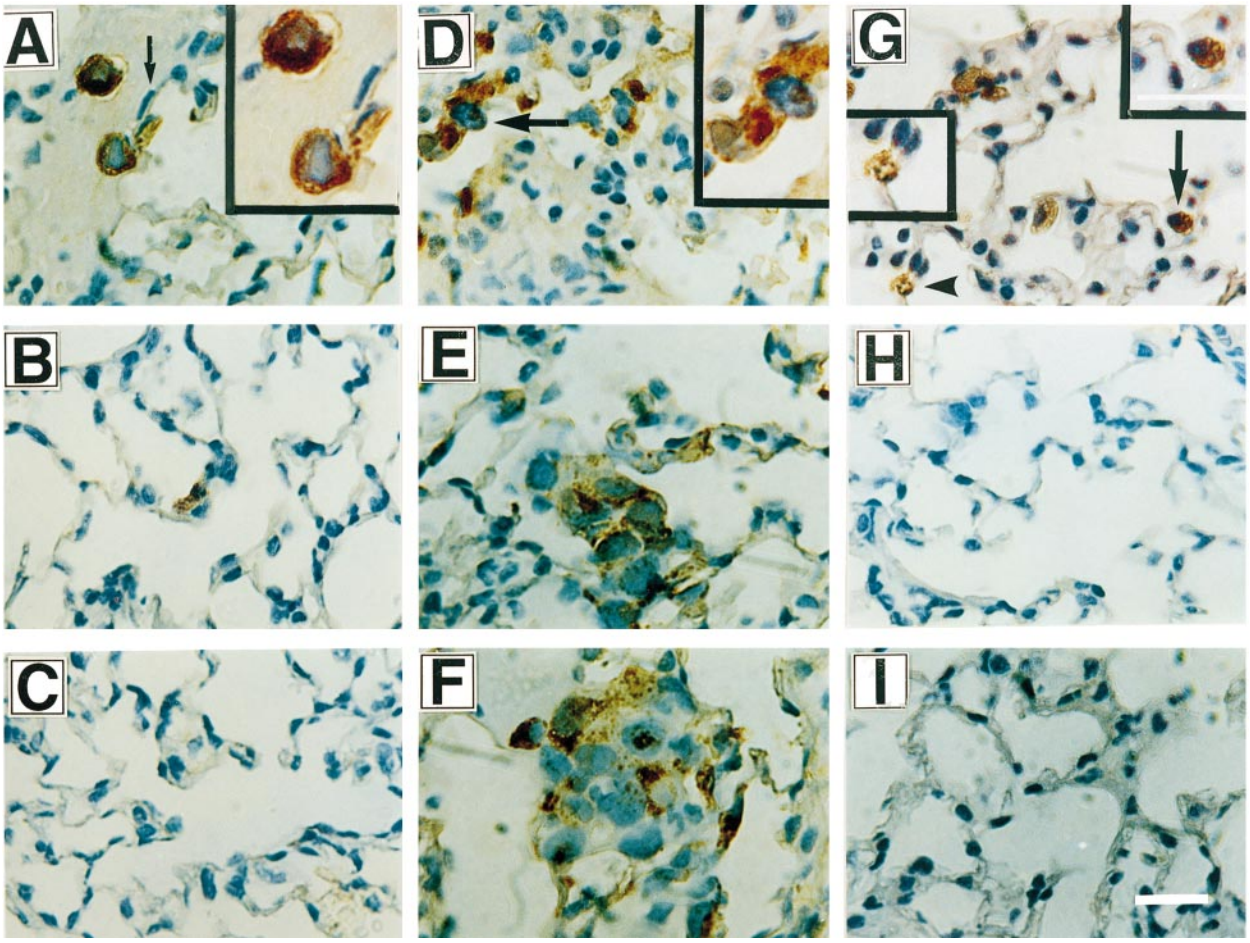

Fig. 6. Immunostaining of tumor cells in C57/BL6 mice by anti-melanoma antibody. Lungs were examined after injection of B16-FTIII·N (**A**, **B** and **C**), B16-FTIII·M (**D**, **E** and **F**) and B16-FTIII·H (**G**, **H** and **I**) cells by anti-melanoma antibody (HMB-45), followed by biotinylated anti-mouse IgG and avidin–biotin reagent. The sections were prepared at 3 (A, D and G), 24 (B, E and H) and 48 h (C, F and H) after injection. Lung endothelial cells are shown by the arrow in (A). Bar, 20 μm.

apoptosis in lung tissues, TUNEL assays were performed on tissue sections. A very small number of TUNEL-positive cells were observed 3 or 24 h after injection of B16-FTIII·N or B16-FTIII·M cells (Figure 7A–D). In contrast, a large number of TUNEL-positive cells were detected 3 h after injection of B16-FTIII·H cells (Figure 7E), but they were no longer detected after 24 h (Figure 7F). These TUNEL-positive cells were also positive for the melanoma antigen as described above. Moreover, some of those apoptotic cells were engulfed by macrophages 3 h after injection (data not shown).

B16-FTIII·H cells were eliminated due to attack by NK cells

The results above demonstrate that overexpression of sialyl Lewis X on B16-F1 melanoma cells leads to apoptosis in lung tissues where they arrest, suggesting that those cells may be attacked by immune cells. To address this question, the three different B16-FTIII transfectants were injected into SCID and beige mice in the same manner as described for injection into C57BL/6 mice.

The results shown in Figure 8A illustrate that B16-FTIII·H and B16-FTIII·N cells barely formed lung tumor nodules when injected into SCID mice (deficient in both B and T cells), while B16-FTIII·M cells produced a large number of lung tumor nodules. In contrast, B16-FTIII·H cells produced a large number of lung tumor nodules in beige mice which are deficient in NK cells and macrophages (Figure 8A and C). Finally, B16-FTIII·H cells produced a large number of lung tumor nodules in C57BL/6 mice, which had been depleted of NK cells by anti-asialo–GM1 (Figure 8A and D). In beige mice and NK cell-depleted mice, the number of tumor nodules produced by different B16-FTIII cells was proportional to the amount of sialyl Lewis X expressed on the cell surface. These results strongly suggest that B16-FTIII·H cells underwent apoptosis subsequent to attack by NK cells in C57BL/6 mice and SCID mice.

Discussion

This study demonstrates that B16-FI melanoma cells become highly metastatic by acquisition of sialyl Lewis X through expression of fucosyltransferase III (Figures 1 and 2). This increase of metastatic capability was due to expression of the E-selectin ligand, but not due to increased growth rate (Figures 3 and 4). Such expression of E-selectin ligand resulted in the arrest in lung blood vessels (Figure 6). Moreover, tumor formation was inhibited by pre-incubation of those cells with anti-sialyl Lewis X antibody but not by incubation with a control antibody specific to Lewis X. These results demonstrate that acquisi-

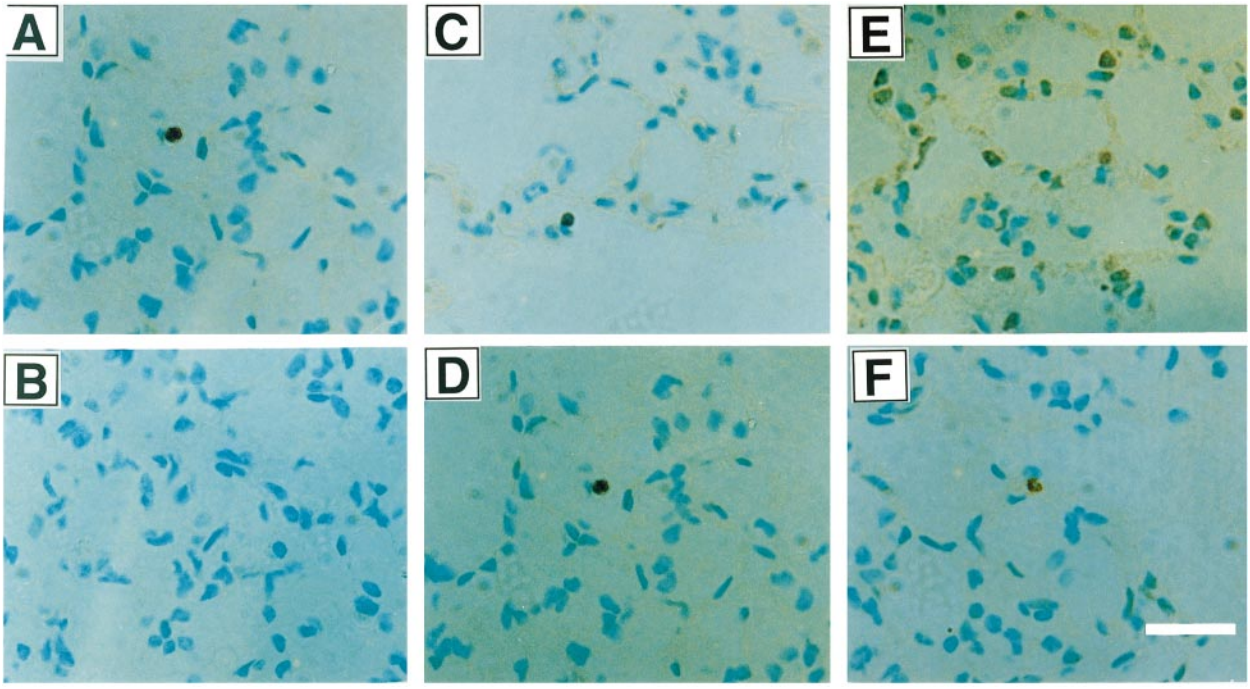

Fig. 7. Apoptosis of B16-FTIII cells after injection into mice. B16-FTIII·N (**A** and **B**), B16-FTIII·M (**C** and **D**) and B16-FTIII·H (**E** and **F**) cells were injected into tail veins of C57BL/6 mice, and lung sections were prepared 3 (A, C and E) and 24 h (B, D and F) after injection. Apoptotic cells were detected by Frag EL DNA Fragmentation Detection Kits. Bar, 20 μm.

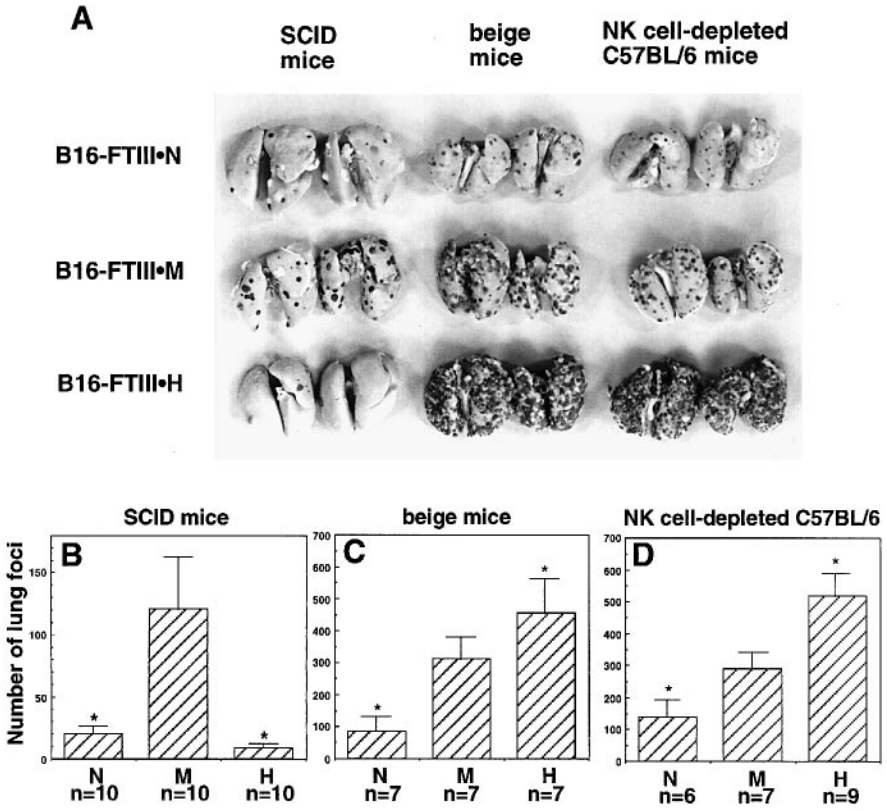

Fig. 8. Lung tumor nodules formed in various immunodeficient mice. (**A**) B16-FTIII·N (N), B16-FTIII·M (M) and B16-FTIII·H (H) cells were injected into tail veins of C57BL/6 SCID mice (SCID), C57BL/6 bg/bg mice (beige) and C57BL/6 mice that had been depleted of NK cells by anti-asialo-GM1 antibody (NK^-). (B–D) The number of lung tumor nodules formed in SCID (**B**), beige (**C**) and NK cell-depleted C57BL/6 mice (**D**).

tion of sialyl Lewis X resulted in the increased metastatic capability of B16-F1 melanoma cells.

This study also demonstrates that highly metastatic B16-FTIII·M cells express more sialyl Lewis X in poly-*N*-acetyllactosamines than do poorly metastatic B16-FTIII·H cells (Figures 1 and 5). B16-FTIII·H cells, however, contain more sialyl Lewis X in short *N*-glycans than do B16-FTIII·M cells. Similarly, it was shown previously

that highly metastatic colonic carcinoma cells express more sialyl Lewis X in poly-*N*-acetyllactosamines than do their poorly metastatic counterparts, when both were derived from the same patient (Saitoh *et al.*, 1992). Moreover, the total amount of sialyl Lewis X does not differ between these carcinoma cells, and sialyl Lewis X in short *N*-glycans is more abundant in poorly metastatic colon carcinoma cells (Saitoh *et al.*, 1992). These findings are in agreement with those obtained in the present study.

Our preliminary studies indicate that almost identical results were obtained using human MeWo melanoma cells. Cells expressing high levels of sialyl Lewis X, similar to B16-FTIII·H cells, were not metastatic, while those corresponding to B16-FTIII·M cells produced a large number of lung tumor nodules. Under the same conditions, the parent MeWo cells barely formed tumors. Structural characterization of *N*-glycans attached to MeWo transfectants also showed a similar difference among those three different cells as for the different B16-FTIII cells.

The present study provides direct evidence that sialyl Lewis X plays a critical role in tumor metastasis. Preferential arrest of sialyl Lewis X-expressing tumor cells on lung epithelium is probaby due to their binding to E- and possibly P-selectin, although this mechanism has not been shown in the present study. In support of this hypothesis, it has been demonstrated that sialyl Lewis X-expressing B16-F10 cells lodged in liver that expresses an E-selectin transgene, while the same cells formed tumors in the lungs of wild-type mice (Biancone *et al.*, 1996). In breast carcinoma patients, carcinoma cells expressing sialyl Lewis X were found to arrest to endothelial cells expressing E-selectin (Renkonen *et al.*, 1997). These results, together with the results obtained in the present study, strongly suggest that the interaction between sialyl Lewis X and selectin plays a key role in tumor metastasis.

One of the striking discoveries presented in our study is that overexpression of sialyl Lewis X on tumor cells, as seen in B16-FTIII·H cells, can be unfavorable to tumor metastasis in wild-type and SCID mice (Figures 6 and 7). In contrast, B16-FTIII·H cells formed tumor nodules more efficiently in beige mice or NK cell-depleted mice than did B-FTIII·M cells (Figure 8). As discussed above, B16-FTIII·H cells express significant quantities of sialyl Lewis X in short *N*-glycans. These results, taken together, indicate that overexpression of sialyl Lewis X in short *N*-glycans may be recognized by NK cells. Our histochemical analysis demonstrated that the mice used in the present study express small but detectable amounts of sialyl Lewis X as determined by mouse monoclonal 2H5 antibody (Sawada *et al.*, 1993) (data not shown). We thus conclude that NK cells do not recognize low levels of sialyl Lewis X as foreign and suggest that only sialyl Lewis X overexpressed in short *N*-glycans is recognized by NK cells. This conclusion is corroborated by the experiments employing B16-FTIII·H cells after >15 passages of culture. Those cells tend to lose sialyl Lewis X expression and became stable once they expressed a moderate amount of sialyl Lewis X, which corresponds to B16-FTIII·M cells. Such a change in sialyl Lewis X expression was also accompanied by a conversion from poorly to highly metastatic potentials in wild-type mice. These results, taken together, strongly suggest the proposal that the difference in metastatic potentials in these populations is caused solely by the difference in sialyl Lewis X content.

It has been shown that NK cells express the C-type lectin-like receptor Ly-49A (Phillips *et al.*, 1996; Matsumoto *et al.*, 1998). C-type lectin is the collective name for lectins dependent on Ca^{2+} for their activity which share a conserved domain structure (Weis *et al.*, 1992). Selectins are also members of this gene family (Lowe, 1994; Rosen and Bertozzi, 1996). The C-type lectin domain of Ly-49A was shown to bind to MHC class I antigen in a carbohydrate-independent manner (Matsumoto *et al.*, 1998). The same domain also bound well to fucoidin, an inhibitor of L-selectin, and such binding was shown to inhibit the binding of Ly-49A to H-2d, triggering an attack by NK cells (Matsumoto *et al.*, 1998). Crystallographic studies on the tertiary structure of mannose-binding protein and E-selectin demonstrated that these two C-type lectins share conserved tertiary structures (Weis *et al.*, 1992; Graves *et al.*, 1994). It is thus likely that the NK cell receptor Ly-49A also shares a tertiary structure with other C-type lectins and binds to sialyl Lewis X structures. This hypothesis is supported by a report that an NK cell lectin-like receptor recognizes sialyl Lewis X (Bezouska *et al.*, 1994), although its avidity is not as strong as originally reported (Bezouska *et al.*, 1996). It is tempting to speculate that binding of Ly-49A to sialyl Lewis X interferes with its recognition of MHC class I antigen and that such interference triggers an attack on tumor cells by NK cells.

Previously, it was demonstrated that T cells were involved in the rejection of B16-F10 melanoma cells when interleukin-12 (IL-12) was administered (Brunda *et al.*, 1993). In contrast, IL-10-stimulated tumor rejection was found to be due to an NK cell-mediated attack (Zheng *et al.*, 1996). Moreover, it was demonstrated that NK cells together with CD8-positive cells attack tumor cells (Iwanuma *et al.*, 1997). These findings are similar to the results presented in this study and indicating a critical role for NK cells in tumor rejection. Moreover, the results obtained in this study provide an insight into the mechanism of NK cells recognition of tumor cells. The present study, as a whole, demonstrates that the amount of sialyl Lewis X and the structure of backbone glycans containing sialyl Lewis X determine whether tumor cells form tumor metastasis or are rejected by NK cells, pointing toward an exciting possibility that tumor metastasis may be inhibited by carbohydrate-based antagonists or up-regulation of an α1,3-fucosyltransferase.

Materials and methods

Transfection of B16-Fl cells with pcDNA3-Fuc-TIII

The cDNA encoding Fuc-TIII was excised by *Hin*dIII and *Xba*I digestion from pcDNAI-Fuc-TIII (Kukowska-Latallo *et al.*, 1990; Sueyoshi *et al.*, 1994) and was cloned into the same sites of pcDNA3(*neo*), resulting in pcDNA3(*neo*)-Fuc-TIII. B16-F1 cells, completely negative for sialyl Lewis X expression as assessed by immunostaining, were transfected with pcDNA3(*neo*)-Fuc-TIII by electroporation as described previously (Fukuda *et al.*, 1995). B16-F1 cells were purchased from the American Type Culture Collection. Transfected cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) and G418 (1 mg/ml, Gibco-BRL) for 2 weeks and several single colonies were selected for screening. The isolated clones were dissociated into monodispersed cells using an enzyme-free cell dissociation solution (Hank's based, purchased from Cell and Molecular Technologies, Lavellette, NJ). Monodispersed cells were incubated with 10 μg/ml of

mouse anti-sialyl Lewis X antibody (CSLEX1, Becton Dickinson), followed by FITC-conjugated goat affinity-purified $(Fab')_2$ fragment specific to mouse IgM (Cappel). The cells were then separated by FACS using FACStar (Becton Dickinson) into cells negative for sialyl Lewis X expression (B16-FTIII·N), cells expressing moderate amounts of sialyl Lewis X (B16-FTIII·M) and cells strongly positive for sialyl Lewis X (B16-FTIII·H).

Tumor formation in mice

The above cell populations were cultured for ~1 week to obtain sufficient numbers of cells. The cloned cells were dissociated into monodispersed cells as described above. A total of 1×10^5 of cells exhibiting >90% viability were suspended in 100 µl of serum-free DMEM and injected into a tail vein of C57BL/6, SCID (C.B.-17 *scid*) and beige (C.B.-17 *scid-beige*) mice (6–8 weeks, female). After 2 weeks (for beige and NK cell-depleted mice, see below) or 3 weeks (for C57BL/6 and SCID), mice were sacrificed, lung, liver, spleen and lymph nodes were fixed with Bouin's solution, and tumor nodules were counted under a dissecting microscope. Since the majority of tumors were formed in lung, the weight of the lung was also measured. In this protocol, wild-type B16-F1 cells or mock-transfected B16-F1 cells barely formed tumors. C57BL/6 mice were purchased from B&K Universal, Fremont, CA, and SCID and beige mice were from Taconic, Germantown, NY.

Monoclonal antibody treatment of B16-FTIII·M cells and NK cytotoxic activity depletion

B16-FTIII·M cells were pre-incubated with CSLEX 1 (10 µg/ml) for 30 min at 4°C before tail vein injection into C57BL/6 mice. For control, 10 µg/ml of anti-Lewis X monoclonal antibody (CD15 monoclonal antibody, Immunotech) was used. For experiments with mice depleted of NK cells, C57BL/6 mice were injected intraperitoneally with 25 µl of anti-asialo GM1 1 day before tumor injection and every 5 days during the experiment, as described previously (Brunda *et al.*, 1993). Anti-asialo GM1 antibody was purchased from Wako Chemicals.

Cell proliferation assay

B16-FTIII·N, B16-FTIII·M and B16-FTIII·H cells were seeded in 96-well plates at 10^5 cells/ml in DMEM containing 10% FCS and 1 mg/ml of G418, and cultured for various times. The number of living cells was measured each day using Cell Titer 96® Aqueous One Solution Cell Proliferation Assay (Promega). Triplicate cultures were used for each sample (Qian *et al.*, 1994).

Binding of E-selectin to different B16-FTIII cells

A cDNA encoding a chimeric protein of mouse E-selectin fused with hinge and constant regions of human IgM was kindly provided by Dr John Lowe (Maly *et al.*, 1996). This vector, pcDNAI-E-selectin–IgM, was transfected into COS-1 cells using Lipofectamine™. Three days after transfection, the culture medium was used directly without dilution as a source of E-selectin–IgM. B16-FTIII·N, B16-FTIII·M and B16-FTIII·H cells were dissociated using the cell dissociation solution (see above) and incubated with E-selectin–IgM at 4°C for 30 min followed by FITC-conjugated goat anti-human IgM (Pierce). After washing the cells in DMEM, labeled cells were analyzed by flow cytometry as described using a FACScan flow cytometer (Maly *et al.*, 1996).

FACS analysis of carbohydrate antigens expressed on different B16-FTIII cells

In order to determine the expression levels of carbohydrate antigens on different B16-FTIII cells, the same cell samples used for tumor assays were dissociated and incubated with FITC-conjugated SNA, FITC-conjugated MAA or FITC-conjugated LEA. Analyses were carried out using FACSort flow cytometry using the CellQuest program (Becton-Dickinson) as described (Tsuboi and Fukuda, 1997). Similarly, the cells were incubated with CSLEX-1, anti-Lewis X antibody or mouse anti-sialyl Lewis A antibody (CSLEA1, UCLA Tissue Typing Laboratory) followed by FITC-conjugated secondary antibodies, and analyzed by flow cytometer. FITC-conjugated lectins were purchased from EY Laboratories.

Lectin affinity chromatography and characterization of glycopeptides

B16-FTIII·N, B16-FTIII·M and B16-FTIII·H cells were metabolically labeled with [^{3}H]galactose (20 µCi/ml, DuPont-NEN) in glucose-free DMEM containing 10% dialyzed FCS supplemented with 2% regular DMEM containing 10% FCS for 24 h as described (Saitoh *et al.*, 1992). The glycopeptides prepared by pronase digestion of [^{3}H]galactose-labeled cells were desalted in a small column of Sephadex G-25 equilibrated with water and then fractionated sequentially by serial lectin affinity chromatography employing ConA–Sepharose, LEA–agarose and DSA–agarose (Saitoh *et al.*, 1992). Briefly, the glycopeptides were first applied to a column of ConA–Sepharose, and unbound glycopeptides containing tri-antennary and tetra-antennary *N*- and *O*-glycans (Fraction I) were separated from the others. Fraction I was then applied to a column of LEA–agarose and separated into unbound (IA) and bound fractions directly (IB). The unbound fraction (IA) was fractionated further by a column of DSA–agarose into unbound (IA1) and bound (IA2) fractions. LEA–agarose binds to glycopeptides containing three or more *N*-acetyllactosamine units (Merkle and Cummings, 1987; Lee *et al.*, 1990; Saitoh *et al.*, 1992). DSA–agarose binds to glycopeptides containing two or more *N*-acetyllactosamine units (Cummings and Kornfeld, 1984; Lee *et al.*, 1990; Saitoh *et al.*, 1992). Highly fucosylated *N*-glycans, however, are not bound to DSA–agarose even when they contain poly-*N*-acetyllactosamines (Yamashita *et al.*, 1987; Lee *et al.*, 1990; Saitoh *et al.*, 1992). IB and IA2 glycopeptides were subjected to endo-β-galactosidase digestion (Fukuda, 1981) before and after defucosylation as described previously (Saitoh *et al.*, 1992).

Immunocytochemical detection of tumor cells and apoptotic cells

The lungs of mice injected with tumor cells were removed 3, 24 and 48 h after injection and fixed. Tissue was embedded in paraffin and 5 µm thick sections were cut and mounted on glass slides. After deparaffination and rehydration, the sections were incubated with mouse anti-melanoma antibody (HMB-45, Dako) followed by biotinylated anti-mouse IgG and avidin–biotin reagent (Vectastain ABC Kit, Vector) as described (Shimodaira *et al.*, 1997). HMB-45 antibody was shown to bind to a melanoma-specific antigen (Kapur *et al.*, 1992). TUNEL assays were performed on paraffin sections of lungs using Frag EL DNA Fragmentation Detection Kits (Oncogene Research Products) as described previously (Yu *et al.*, 1997).

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