Tumor Cell Surface β1-4-Linked Galactose Binds to Lectin(s) on Microvascular Endothelial Cells and Contributes to Organ Colonization

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Abstract. Cell surface carbohydrate structures acting as ligands for tissue specific mammalian lectins have been implicated in cell-cell interactions during embryogenesis, lymphocyte homing, and tumor cell metastasis. In this report, we provide evidence that β1-4 linked galactose (Gal) residues in N-linked oligosaccharides on the surface of blood born tumor cells serve as a ligand for binding to microvascular endothelial cells. D36W25, a class 1 glycosylation mutant of the MDAY-D2 lymphoreticular tumor cell line, lacks sialic acid and Gal in cellular glycans due to a defect in the Golgi UDP-Gal transporter. Using UDP-Gal and bovine galactosyltransferase in vitro, β1-4 Gal was restored to the surface of the cells and 70% of the galactosylated glycans persisted for 8 h in vitro at 37°C. Compared to mock-treated D36W25 cells, galactosylated D36W25 cells showed an 80% increase in binding to microvascular endothelial cell monolayers in vitro. The enhanced binding of galactosylated D36W25 cells to endothelial cell was inhibited by the addition of lactosamine-conjugated albumin to the assay. Consistent with these observations, swainsonine and castinospermine, two inhibitors of N-linked processing that result in loss of lactosamine antennae inhibited the binding of wild-type MDAY-D2 cells to endothelial cells in vitro. Injection of radiolabeled tumor cells into the circulation of syngeneic mice, showed that galactosylation of D36W25 cells resulted in 2-3 more tumor cells retained in the lungs and livers. In addition, galactosylation of D36W25 cells increased by 30-fold the number of visible liver metastases on inspection 4 wk after tumor cell injection. These results suggest that β1-4Gal-binding lectins on microvascular endothelial cells can contribute to retention and secondary tumor formation of blood born tumor cells. With the increasing availability of purified glycosyltransferases, reconstruction of a variety of carbohydrate sequences on the surface of class 1 mutants provides a controlled means of studying carbohydrate-lectin interactions on viable cells.

Cell surface carbohydrates on lymphocytes and endothelial cells have been implicated in lymphocyte homing to specific tissues, presumably due to their recognition by mammalian lectins (Stoolman et al., 1984; Gallatin et al., 1986; Aizawa and Tavassoli, 1988). For example, mannose-6-P has been shown to inhibit lymphocyte homing to lymph node and attachment to high endothelium of postcapillary venules (Stoolman et al., 1984). Gal-BSA and asialofetuin inhibited homing of murine lymphocytes to the bone marrow (Aizawa and Tavassoli, 1988). Galactose (Gal)[1]-binding lectins have been identified on bone marrow endothelial cells (Kataoka and Tavassoli, 1985) and may be important in organ retention and colonization by blood born tumor cells. In this regard, intravenous infusion of Gal or arabinogalactan has been shown to inhibit organ colonization by murine tumor cells (Beuth et al., 1987). Antibodies specific for Galα1-3Gal, a sequence that is often expressed in human breast carcinomas, have been shown to reduce tumor cell adhesion to human cord endothelium (Castronovo et al., 1989). A variety of Gal-binding lectins has also been isolated from mammalian tissues (Sparrow et al., 1987; Hinek et al., 1988; Raz, 1987). More recently, the determination of primary sequences of several mammalian lectins and their carbohydrate recognition domains has lead to the identification of other putative lectins based on sequence homologies (Drickamer, 1988). Two cell adhesion receptors designated ELAM-1 and GMP-140 have recently been identified in endothelial cells, and both show sequence homology with the C-type animal lectins (Bevilacqua et al., 1989; Johnston et al., 1989). However, the carbohydrate-binding specificity of these receptors and the importance of these domains for lymphocyte and tumor cell adhesion remain to be determined.

We have used a glycosylation mutant of the highly metastatic lymphoreticular tumor cell line MDAY-D2 (Dennis, 1986*a*,*b*) as well as N-linked processing inhibitors, swainso-

1. *Abbreviations used in this paper*: BCE, bovine capillary endothelial; BSII, *Bandereia simplicifolia*; FACS, fluorescence-activated cell sorter; Gal, D-galactose; Gal-T, galactosyl transferase; HSA, human serum albumin; L-PHA, leucoagglutinin; WGA, wheat germ agglutinin.

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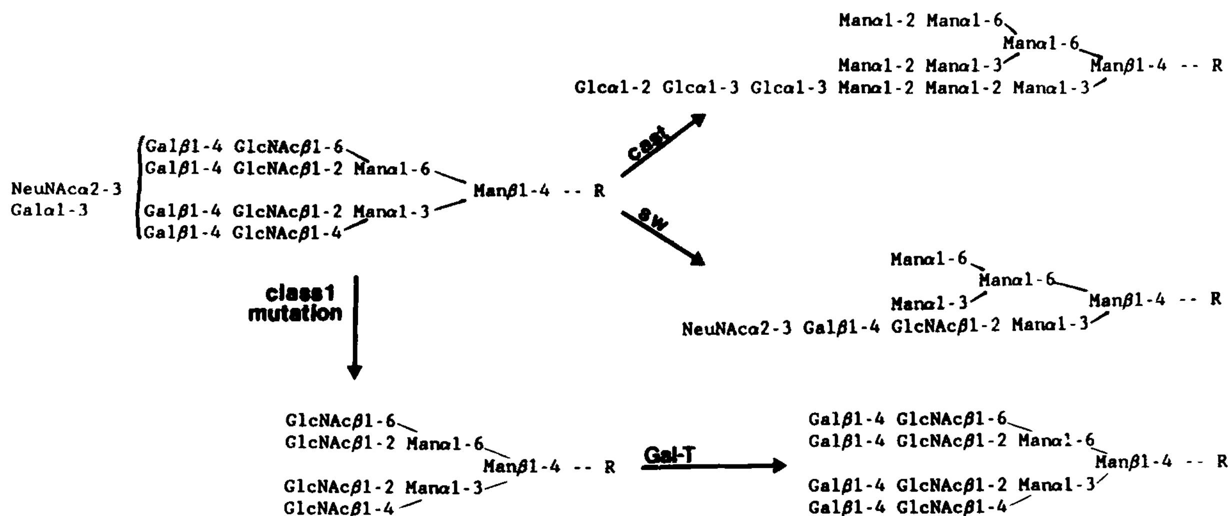

Figure 1. Schematic of the major N-linked oligosaccharides in MDAY-D2 and D36W25 mutant tumor cells as determined previously (Dennis et al., 1986*a*). The consequences of swainsonine (*SW*)- and castinospermine (*Cast*)-treatment as well as the resulting structure on D36W25 cells after treatment with bovine Gal-T and UDP-Gal are shown. The latter assumes the complete substitution of GlcNAc-termini on complex-type oligosaccharide of D36W25 cells.

nine and castinospermine (Elbein, 1987), to assess the contribution of complex type N-linked structures in tumor cell attachment to endothelial cells in vitro. The results suggest that loss of lactosamine antennae of N-linked oligosaccharides is associated with a reduction in cell adhesion to endothelial cells in vitro. However, somatic mutations and chemical inhibitors of oligosaccharide processing affect intracellular as well as cell surface glycoproteins and may induce secondary changes affecting cell surface phenomena. Therefore, we have developed a model system to reconstruct oligosaccharide structures on the surface of viable tumor cells using purified glycosyltransferases and sugar-nucleotide donors. A similar approach has been used to define the specificity of hemagglutinins from influenza virus for sialyllactose. Neuraminidase-treated erythrocytes were reconstituted with various types of sialic acid in either α2–6 or α2–3 linkages using purified sialyltransferase (Paulson et al., 1979; Higa and Paulson, 1985). In the present study, we have used D36W25, a poorly metastatic class 1 glycosylation mutant of the MDAY-D2 tumor cell line that lacks Gal and sialic acid. The mutant cells produce truncated glycoconjugates, mainly tetra-antennary N-linked with only GlcNAc as antenna (Fig. 1). We have restored the β1-4Gal to the N-linked oligosaccharides on the surface of the mutant tumor cells using bovine galactosyltransferase (Gal-T) to determine whether the monosaccharide residue mediate tumor cell adhesion to vascular endothelial cells and increase organ colonization by blood borne tumor cells.

Materials and Methods

Cell Lines

MDAY-D2 is a highly metastatic lymphosarcoma tumor cell line of DBA/2 origin (Kerbel et al., 1980). D36W25 was isolated in a single step from MDAY-D2 cells without mutagenic treatment by selection in growth medium containing wheat germ agglutinin (WGA) as previously described (Lagarde et al., 1983). The cells were maintained in α-MEM plus 5% FCS (Gibco Laboratories, Grand Island, NY). Bovine capillary endothelial (BCE) cells, provided by Dr. J. Folkman, were obtained from the adrenal gland and were cultured on gelatin-coated plates in DME (Gibco Laboratories), supplemented with 10% FCS and 120 μg/ml endothelial mitogen (Biomedical Technologies, Inc., Cambridge, MA) in a 10% CO_2 atmosphere.

Treatments with Glycosylation Inhibitors

The cells were cultured for 1 wk in the presence of 1 μg/ml of chemically synthesized swainsonine (Toronto Research Chemicals, Toronto, Ontario) and 20 μg/ml castinospermine (Sigma Chemical Co., St. Louis, MO). These inhibitors do not reduce the plating efficiency of the tumor cells at these concentrations (VanderElst, I., and J. W. Dennis, manuscript submitted for publication). The drug concentrations and a 48-h incubation have previously been shown to be optimal for inhibition of oligosaccharide processing in MDAY-D2 cells (Dennis, 1986*a*,*b*). However, inhibition of processing has previously been shown to be incomplete (i.e., 80–90%) (Elbein et al., 1987).

Tumor Cell Adhesion to Endothelial Cells

BCE cells were seeded into gelatin-coated 24-well plates (Costar, Cambridge, MA) and cultured until the cells were confluent. Passage 15 and 18 cultures were used for adhesion assays and only after they reached confluence. Tumor cells were labeled with 2 μCi/ml [^{3}H]thymidine (25 Ci/mmol; Amersham Corp., Arlington Heights, IL) for 24 h, washed 3× in serum-free medium (DME) and adjusted to a concentration of 10^5 cells/ml in the adhesion medium made of 10 mM Hepes-buffered DME plus 1% BSA. Endothelial monolayers were washed once and incubated for 2 h in adhesion medium. One milliliter of tumor cells was added to test wells, and the cultures were placed on a rotating platform maintained at 37°C in 5% CO_2. A rotational speed of 100 rpm was used as previously described (Ally and Auerbach, 1984). After various incubation times, the wells were gently rinsed 4× with adhesion medium at 37°C, and the attached cells were lysed in 0.2 ml 1N NaOH. The lysates were neutralized with 0.2 ml 1N HCl and radioactivity was assessed in a β scintillation counter (LKB Instruments, Gaithersburg, MD). The neoglycoprotein Galβ1-4GlcNAc-coupled to HSA (provided by Dr. Y. Sommarin (Biocarb, Lund, Sweden)) 166 μg/ml was added in 0.5 ml DME, 1% BSA 15 min before adding 10^5 tumor cells in 0.5 ml of the same medium. The molar ratio of Gal to HSA was 15–20.

Galactosylation of Viable D36W25 Cells

Galactosylation was performed using conditions similar to those previously described for lymphocytes (Torres and Hart, 1984). D36W25 cells were washed twice in PBS and once in the reaction buffer (10 mM Hepes [pH 7.3], and 150 mM NaCl). The optimized conditions for galactosylation were 10^7 cells/ml in 5 mM D-galactose, 2.5 mM $MnCl_2$, 25 mM Hepes (pH

7.0), 120 mM NaCl, 1.25 mM AMP, 150 μM UDP-Gal, and 900 mU of bovine galactosyltransferase (Boehringer Mannheim Biochemicals, Indianapolis, IN) for 20 min at 37°C. The enzyme was stored in 2.5 mM $MnCl_2$ and 25 mM Hepes. In experiments designed to optimize the reaction conditions, 5 μCi of radioactive UDP-[^{3}H]galactose (17.3 Ci/mmol; Amersham Corp.) was added to a 100-μl reaction mixture. The reaction was terminated by placing the tubes on ice and washing the cell pellet 3× in cold PBS. The cell pellets were lysed in 0.2 ml 1 M NaOH, neutralized with 0.2 ml 1 M HCl and counted with a β scintillation counter. Mock-treated D36W25 cells were incubated in the same reaction buffer with the omission of Gal-T. Omission of Gal-T or UDP-Gal produced the same results.

SDS Gel Electrophoresis

SDS-PAGE separation of glycoproteins was performed with 12.5% polyacrylamide gels (Laemmli, 1970). Cells were lysed in 0.5% Triton X-100, 2 mM PMSF and 0.1% tranylol and an equivalent of 2×10^5 [^{3}H]-galactosylated cells was loaded onto the gel that was subsequently fixed, stained with Coomassie blue, treated with enhancer (Biotechnology System), dried, and exposed to x-ray film for 4 d.

Lectin Binding and Quantitation by Fluorescence-activated Cell Sorter (FACS)

Cells were suspended at 5×10^5/50 μl of PBS, 1% BSA and 50 μl of lectin in the same buffer was added. The final lectin concentrations used were 2 μg/ml for L-PHA (Sigma Chemical Co.) and 4 μg/ml for BSII lectin (Sigma Chemical Co.). The cells were incubated for 1 h at 4°C in the presence of lectin, washed 3× in PBS-BSA, and resuspended with rabbit antilectin antibodies (1/1,000) prepared in our laboratory (Dennis et al., 1989). After 30-min incubation at 4°C, the cells were washed twice in PBS-BSA and incubated in a 1:100 dilution of FITC-conjugated anti-rabbit antibody (Cappel Laboratories, Malvern, PA) for 30 min. The final washes were performed in PBS, the cells were then fixed in 1% buffered paraformaldehyde and were subjected to cytofluorimetry analysis using a fluorescence-activated cell sorter (EPICS-C; Coulter Electronics, Hialeah, FL). To estimate the half-life of galactosylated cell surface glycoproteins, galactosylated D36W25 cells were cultured in DME with 1% BSA at 37°C and at intervals the cells were washed in cold PBS-BSA and reacted with BSII lectin at 4°C as described above and analyzed by FACS.

Organ Retention Assay

Tumor cells were labeled with 2 μCi/ml of [^{125}I]UdR (5 Ci/mg; Amersham Corp.) for 24 h. The cells were then galactosylated, and 10^5 control or galactosylated D36W25 cells were injected into the lateral tail vein of syngeneic male DBA/2 mice. At different intervals, three mice per group were killed and their lungs and livers were collected, washed for 48 h in three changes of 70% ethanol to remove soluble ^{125}I. The remaining ethanol insoluble radioactivity was associated with the DNA of viable tumor cells in the organs (Hart and Fiddler, 1980) and was measured in a γ counter (LKB Instruments, Inc., Gaithersburg, MD).

Organ Colonization Assay

Tumor cells (10^5/mouse) in 0.5 ml of PBS were injected into lateral tail vein of DBA/2 mice. Mice injected with galactosylated and control D36W25 cells were sacrificed and autopsied at 4 wk. Mice injected with MDAY-D2 cells were sacrificed at 3 wk because of the more aggressive nature of these tumor cells. In all experiments, the quality of the galactosylation was confirmed by BSII lectin binding and FACS analysis.

Results

Adhesion of MDAY-D2 to Endothelial Cells Is Reduced by the Class 1 Mutation, Swainsonine, and Castinospermine

Initial experiments comparing the adhesion of MDAY-D2 cells and the class 1 mutant to confluent monolayers of bovine microvascular endothelial cells showed that the mutant cells D36W25 were ~9× less adhesive after 25 min (Fig. 2). Visual examination of the cultures during the endothelial cell adhesion assay indicated that homotypic aggregation of tumor cells did not occur. Similar results were obtained for adhesion to human cord endothelial cells (data not shown). The class 1 mutation results in almost complete loss of Gal-containing oligosaccharides in glycoproteins and glycolipids. A more selective loss of carbohydrate sequences in the antennae of N-linked complex type oligosaccharides can be induced by growing the MDAY-D2 tumor cells in the presence of the glycosylation inhibitors swainsonine and castanospermine. Swainsonine inhibits α-mannosidase II resulting in the synthesis of hybrid type oligosaccharides rather than complex type. Castanospermine inhibits glucosidase I and cells grown in the presence of the drug produce oligosaccharides of the high mannose type (Fig. 1). MDAY-D2 cells grown in the presence of either drug for 48 h before the endothelial cell adhesion assay showed a two- to threefold loss in adhesiveness after 25 min (Fig. 2). Visual examination of the cultures during the endothelial cell adhesion assay indicated that homotypic aggregation of tumor cells did not occur. These results suggest that a significant component of the adhesive interaction between MDAY-D2 cells and endothe-

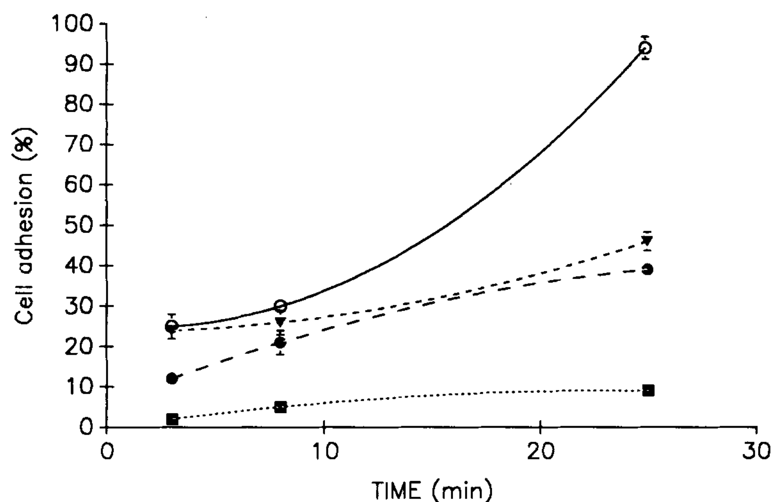

Figure 2. Tumor cell adhesion to bovine capillary endothelial monolayers in vitro. (○) MDAY-D2 cells; (●) swainsonine-treated MDAY-D2 cells; (▼) castanospermine-treated MDAY-D2 cells; and (■) D36W25. Values are expressed as a percent of total cells added per well. Error bars represent SD of triplicates.

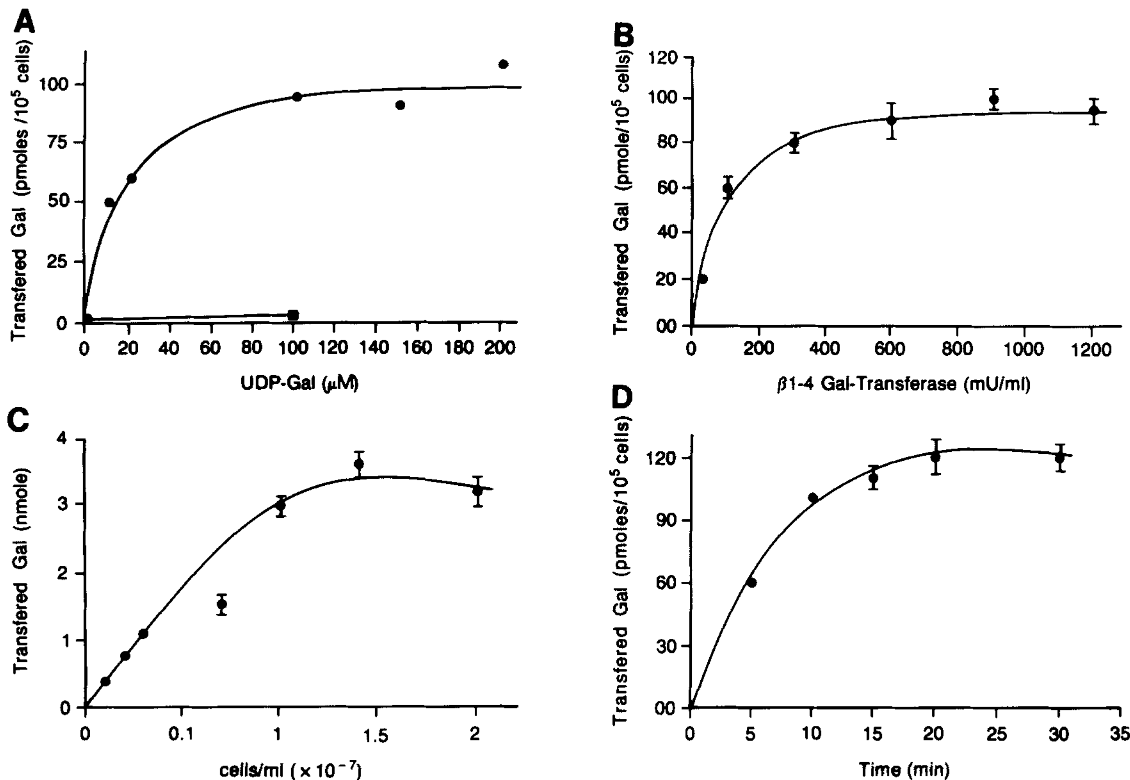

Figure 3. Optimization of the conditions for galactosylation of D36W25 cells. (*A*) UDP-Gal concentration. 2 × 10^5 D36W25 cells (●) or MDAY-D2 cells (■) were incubated with 5 μCi UDP-[^{3}H]Gal, 0–200 μM cold UDP-Gal, and 900 mU/ml bovine Gal-T for 30 min. (*B*) Gal-T concentration. 2 × 10^5 D36W25 cells were incubated with the optimal 150 μM UDP-galactose concentration in presence of 5 μCi UDP-[^{3}H]Gal and 0–1,200 mU/ml Gal-T for 30 min. (*C*) Cell number. 0–2 × 10^6 D36W25 cells were incubated with 5 μCi UDP [^{3}H]Gal, 150 μM UDP-Gal, and 900 mU/ml Gal-T for 30 min. (*D*) Incubation time. 2 × 10^5 cells were incubated with 5 μCi UDP-[^{3}H]Gal, 150 μM UDP-Gal, and 900 mU/ml Gal-T for 5–30 min.

lial cells requires the expression of complex type N-linked oligosaccharides.

Exogenous Galactosylation of Truncated N-linked Oligosaccharides on D36W25 Cell

The antennae in N-linked oligosaccharides of MDAY-D2 cells are lactosamine and polylactosamine that terminate in either SAα2-3Galβ1-4, Galα1-3Galβ1-4 or Galβ1-4 as determined previously by methylation analysis and fast atom bombardment (Fig. 1) (Dennis et al., 1986). To reconstruct the Galβ1-4 termini, the unsubstituted GlcNAc residues on viable D36W25 cells were substituted using bovine milk β1-4 galactosyltransferase and UDP-Gal (Torres and Hart, 1984). The conditions for galactosylating the cell surface were optimized using UDP-[^{3}H]Gal (Fig. 3). Saturating conditions were attained with the UDP-Gal concentrations fixed at 150 μm, Gal-T concentration at 900 mU/ml with 10^7 D36W25 cells/ml. A 20-min incubation at 37°C was sufficient to complete the galactosylation reaction. The amount of Gal added was 82 and 2 pmol/10^5 cells for D36W25 and MDAY-D2, respectively. Separation of labeled-cell lysates by SDS-PAGE showed that for D36W25, multiple cell surface glycoproteins were galactosylated, while at the same exposure none were apparent in MDAY-D2 cells treated with Gal-T (Fig. 4). Bovine Gal-T will not substitute GalNAc- or glucose (Brew et al., 1968), which are the predominant terminal residues found in O-linked and in glycolipids, respectively, of D36W25 cells (Laferté et al., 1987). Therefore, based on the specificity of the enzyme and the possible acceptors on D36W25 cells, most of the added Gal was likely present on N-linked oligosaccharides.

The efficiency of galactosylation was monitored by assessing the level of unsubstituted GlcNAc remaining on D36W25 cells after the galactosylation procedure. The cells were reacted with *Bandeiraea simplicifolia* (BSII) lectin and a fluoresceinated second antibody and analyzed by FACS. BSII lectin recognizes unsubstituted GlcNAc (Ebisu et al., 1978) and bound avidly to D36W25 cells producing a mean fluorescence intensity of 272.9 (Fig. 5 *A*). After galactosylation, this was reduced to 6.5, while for MDAY-D2 cells, the value was 1.1. This suggests that the majority (i.e., 97%) of the cell surface GlcNAc, which was accessible to BSII, had been galactosylated by Gal-T. Galactosylation of D36W25 cells increased leucoagglutinin (L-PHA) binding as indicated by a shift in fluorescence intensity from 17.8 to 42.4 (Fig. 5 *B*). L-PHA has been shown to bind to Galβ1-4GlcNAcβ1-2 (Galβ1-4GlcNAcβ1-6) Manα (Cummings and Kornfeld, 1982), and, as expected, the addition of Gal to D36W25 enhanced L-PHA binding to the cells.

The turnover rate of galactosylated glycoproteins was estimated by monitoring the progressive recovery of BSII binding sites on the surface of galactosylated D36W25 cells at 37°C (Fig. 6). The increase in BSII binding as a function of time appeared to be multiphasic with 10% recovery in 1 h,

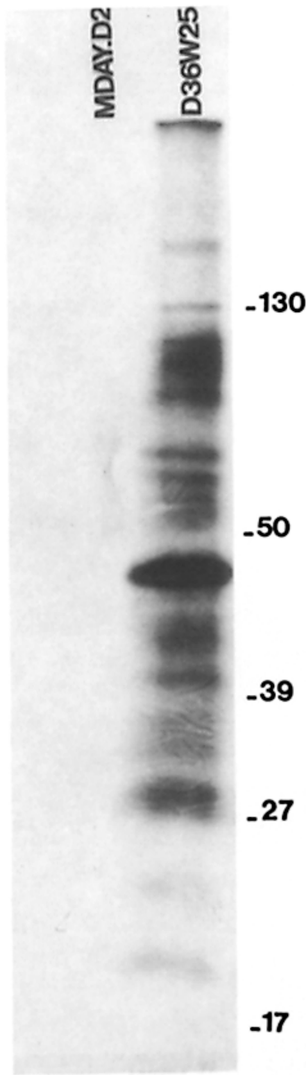

Figure 4. SDS-PAGE of D36W25 and MDAY-D2 protein lysates after exogenous galactosylation, using 5 μCi UDP-[^{3}H]Gal and optimized reaction conditions.

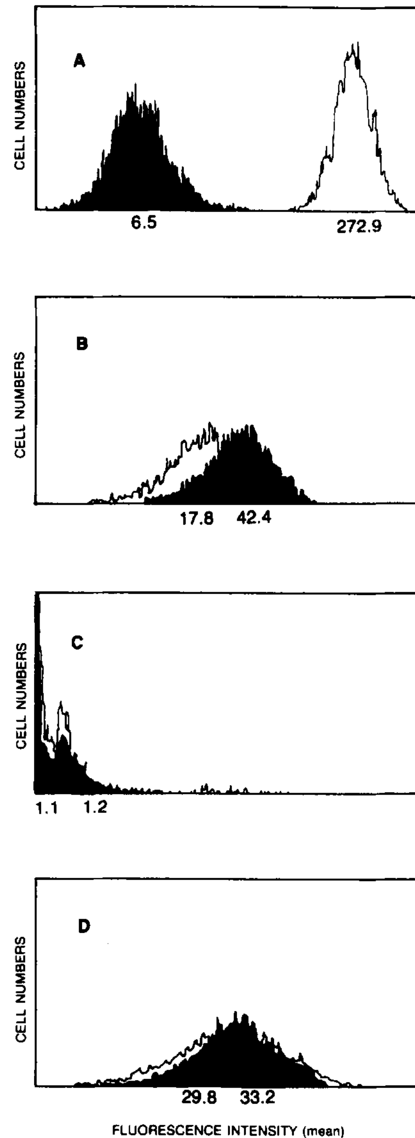

Figure 5. Flow cytometry analysis of cell surface BSII (*A* and *C*) and L-PHA (*B* and *D*) binding to D36W25 (*A* and *B*) and MDAY-D2 (*C* and *D*) before (*open*) and after (*closed*) exogenous galactosylation. The mean fluorescence intensity for each distribution has been converted to a linear scale and is indicated on the abscissa.

30% recovery in 8 h and 80% recovery 12 h. These results suggest that the majority of the Gal added to D36W25 cells remains on the cell surface for a sufficient duration to measure its effects on tumor cell binding to endothelial cells in vitro and on organ colonization in vivo.

Exogenous Galactosylation of D36W25 Cells Increases Their Adhesion to Endothelial Cells

The galactosylated D36W25 cells were found to be nearly 2× more adhesive to endothelial cell monolayers than mock-treated D36W25 cells (Fig. 7). Moreover, addition of 83 μg/ml of lactosamine-conjugated HSA as a competitive inhibitor, reduced adhesion of galactosylated cells to that of mock-treated D36W25 cells. At this concentration, the Gal coupled to the neoglycoprotein was ~100× that added to the D36W25 by exogenous galactosylation. However, lactosamine-HSA had no effect on the basal level of D36W25 binding to endothelial cells. These results suggest that the Galβ1-4 added to D36W25 cells is a ligand for a Gal-binding lectin on the surface of endothelial cells.

Exogenous Galactosylation of D36W25 Cells Increases Their Organ Colonization Potential In Vivo

MDAY-D2 tumor cells and B16F10 melanoma cells when grown in the presence of swainsonine before their injection into the circulation of mice show loss of organ colonization potential (Humphries et al., 1986; and Dennis, 1986*b*). In addition, swainsonine-treated B16F10 cells were more rapidly cleared from the lungs than untreated cells during the first 8 h after their injection (Humphries et al., 1986). These observations suggest that complex type N-linked oligosaccharides on the tumor cells may participate in organ retention, possibly by binding to endothelial cell receptors. To determine whether β1-4Gal contributed to this phenomena, tumor cells were labeled with ^{125}I-UdR and injected into the lateral tail vein of DBA/2 mice to measure the retention or

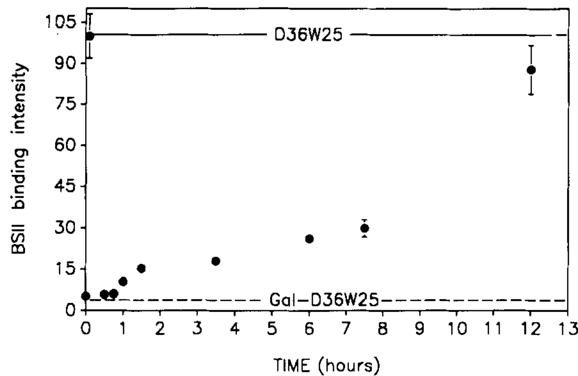

Figure 6. Turnover of galactosylated cell surface glycoconjugates monitored at 37°C by reoccurrence of BSII lectin binding sites. BSII-binding is reported as linear mean value of fluorescence intensity versus the time of incubation postgalactosylation. The initial BSII binding intensity of Gal-D36W25 (---) and the native BSII binding intensity of the untreated D36W25 (—) are represented. Error bars represent SD about the mean for triplicate determinations.

clearance rates in the lung and liver (Table I). In the first few minutes after tumor cells were injected, the majority of radioactivity was found in the lungs and ∼80–90% of the cells were lost from the lungs after 90 min. Although similar number of cells arrived in the lungs, ∼3× as many galactosylated D36W25 cells compared to mock-treated cells remained in the lung after 90 min, and this difference was maintained at 23 h (Table II). In the liver, tumor cells accumulated for the first 10 min after injection to a maximum of 20%, and were then lost over the following 23 h. Although differences between galactosylated-D36W25 and mock-treated cells were not observed until 15 h after injection, galactosylation did increase liver retention ∼1.6-fold by 23 h. The differences in liver retention between control and galactosylated cells may be less pronounced than in the lung because of the lower number of cells that reach the liver. Secondly, most of these cells have already passed through the microvasculature of the lung where selective retention based on β1-4Gal content may

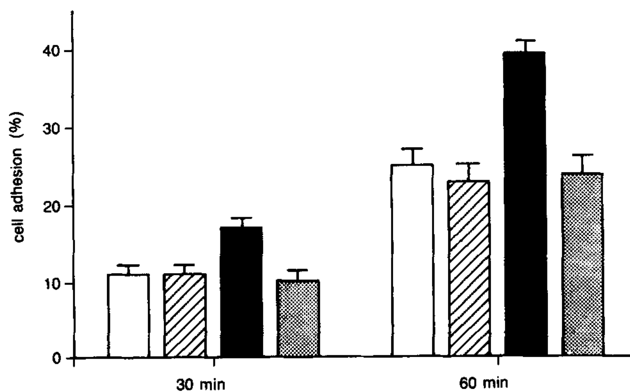

Figure 7. Inhibition of galactosylated D36W25 cells to endothelial monolayer by 100 ng/ml lactosamine-HSA. The bars are (*open bars*) mock-treated D36W25 cells; (*striped bars*) mock-treated D36W25 cells plus lactosamine-HSA; (*solid bars*) Gal-D36W25 cells; (*stippled bars*) Gal-D36W25 cells plus lactosamine HSA.

Table I.
A. Lung Retention

	D36W25		Gal-D36W25	
Time	cpm	%	cpm	%
5 min	16,580 ± 960	86.3 ± 5.0	16,218 ± 1,300	78.9 ± 6
10 min	11,246 ± 922	58.9 ± 5.2	13,354 ± 656	65.0 ± 3.2
30 min	3,820 ± 400	20.0 ± 2.1	5,790 ± 246	28.2 ± 1.2
90 min	952 ± 228	4.9 ± 1.2	3,908 ± 1,278	19.0 ± 6.2
15 h	76 ± 19.6	0.4 ± 0.1	154 ± 10	0.75 ± 0.05
23 h	32 ± 4	0.17 ± 0.02	89 ± 19.3	0.43 ± 0.1

B. Liver Retention

	D36W25		Gal-D36W25	
Time	cpm	%	cpm	%
5 min	1,315 ± 400	6.9 ± 2.1	1,765 ± 369	8.6 ± 1.8
10 min	3,622 ± 610	19.0 ± 3.2	4,106 ± 554	20.0 ± 2.7
30 min	1,601 ± 248	8.4 ± 1.3	1,765 ± 123	8.6 ± 0.7
90 min	838 ± 267	4.4 ± 1.4	1,088 ± 182	5.3 ± 0.89
15 h	78 ± 11	0.41 ± 0.06	127 ± 8.2	0.62 ± 0.04
23 h	32 ± 1.9	0.17 ± 0.01	55 ± 3	0.27 ± 0.015

Organ retention of 125IUdR-labeled D36W25 and galactosylated D36W25 cells. The inoculum of D36W25 cells contained 19,063 cpm, and, for Gal-D36W25, the inoculum contained 20,530 cpm. Background of 16.5 cpm was subtracted. The fraction of cells retained in the lung or liver were calculated from the radioactivity remaining in the organs as a percentage of the total radioactivity in the cell inoculum. The results for each time point are the mean ± SD for three mice. Analysis of variance on the log-transformed data indicated that retention of Gal-D36W25 cells in the lung and liver was significantly reduced; $P = 0.001$, and $P = 0.002$, respectively.

have already occurred. Although galactosylation of D36W25 cells improved their retention in the liver and lung, retention of wild-type MDAY-D2 cells was approximately twofold greater than that of the galactosylated D36W25 cells at 15 h (Fig. 8). This suggests that β1-4Gal is only part of the carbohydrate structure on MDAY-D2 cells that facilitates organ retention.

β1-4Gal binding lectins on the surface of some tumor cell lines have previously been shown to agglutinate tumor cells in the presence of asialofetuin (Raz, 1987). However, the galactosylated D36W25 cells did not agglutinate alone or in the presence of increasing concentrations of asialofetuin or serum suggesting that formation of tumor cell aggregates via this mechanism was not likely responsible for increased organ retention or colonization (data not shown).

Galactosylated D36W25, control D36W25, and wild-type MDAY-D2 cells were injected intravenously into DBA/2

Table II. Liver Colonization by MDAY-D2, D36W25, and Gal-D36W25 Cells

Cells	No. of mice with liver metastases/ no. injected	Mean no. of nodules/mouse (range)
MDAY-D2	18:18 (100%)	>100
D36W25	3:20 (10%)	0.26 (0–4)
D36W25 + UDP-Gal	0:10 (0)	0
Gal-D36W25	12:18 (67%)	8.6 (0–40)

Syngeneic DBA/2 mice were injected intravenously with 10^5 lymphosarcoma cells and liver nodules were counted 3–4 wk later. Mocked-treated D36W25 cells were treated with UDP-Gal alone and are designated D36W25 + UDP-Gal. Galactosylated D36W25 cells are designated Gal-D36W25.

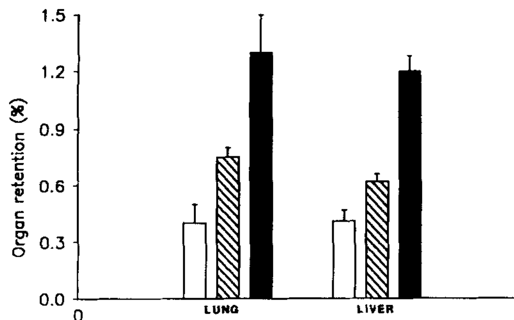

Figure 8. Organ retention of [^{125}I]UdR-labeled MDAY-D2, D36-W25, and galactosylated D36W25 cells at 15 h after intravenous injection of the tumor cells. The bars are (*open bars*) mock-treated D36W25 cells; (*striped bars*) Gal-D36W25 cells; (*solid bars*) MDAY-D2 cells. The results are the percent of ^{125}I-labeled cells retained in the lungs and liver for three mice (mean ± SD).

mice, and the mice were examined 4 wk later to determine whether added Gal would increase the number of metastatic tumor nodules in the organs (Table II). Mice injected with MDAY-D2 cells invariably showed hundreds of liver nodules, and, at much lower frequency, lung, kidney, and spleen metastases, whereas mice injected with D36W25 cells were rarely affected, as previously reported (Dennis, 1986*a*). The mean number of tumor nodules in D36W25-injected mice was 0.26 with a range of 0–4 nodules. In contrast, galactosylated D36W25 cells showed a 30-fold increase in the number of nodules found in the liver. The addition of Gal to D36W25 cells significantly increased the efficiency of liver colonization, but this was at least an order of magnitude less than that observed for the wild-type cells MDAY-D2.

Discussion

The binding of circulating tumor cells to microvessel endothelial cells has been suggested to be a significant factor in the initial retention of tumor cell in specific organs (Nicolson, 1988). Subsequent events, tumor cells invasion and growth of the cells in a particular tissue environment are also thought to be important factors controlling the establishment of metastases. In this report, we have found that a somatic mutation that blocks addition of Gal and SA to cellular glycoconjugates, as well as two chemical inhibitors of N-linked processing, impaired tumor cell adhesion to endothelial cells in vitro. A similar loss of adhesiveness to endothelial cells was also observed for the class 1 type mutation of the human MeWo melanoma cell line called 3S5 (Ishikawa et al., 1988) (data not shown). The addition of β1-4Gal to the N-linked oligosaccharides on the surface of the glycosylation mutant (i.e., D36W25) partially restored adhesion of the cells to endothelial cell monolayers. The Gal-dependent adhesion was inhibited by the addition of lactosamine-HSA, suggesting that the β1-4Gal restored to the cell surface served as a ligand for a mammalian lectin found on the endothelial cells. In vivo, the galactosylated D36W25 cells showed increased organ retention and colonization compared to mock-treated D36W25 cells. The relationship between the cell lines for endothelial cell adhesion, organ retention and organ colonization was consistent and proportionate: MDAY-D2 > Gal-D36W25 > D36W25. Swainsonine- and castinospermine-treated MDAY-D2 cells were also intermediate between MDAY-D2 and D36W25 for adhesion to endothelial cells in vitro, and, as previously shown, the drugs inhibit organ retention and colonization (Schaaf-Lafontaine, 1985; Humphries et al., 1986; Dennis, 1986*b*).

Since galactosylation of mutant D36W25 cells does not completely restore endothelial cell adhesion and organ retention to the level observed for wild-type MDAY-D2 cells, it is likely that more complex sequences containing β1-4Gal participate in MDAY-D2 adhesion to endothelium. Unlike the galactosylated D36W25 cells, a large portion of the β1-4Gal in N-linked oligosaccharides of MDAY-D2 cells is not terminal but rather found in polylactosamine sequences (i.e., Galβ1-4GlcNAc repeating sequences) and substituted (i.e., SAα2-3Galβ1-4, Galα1-3Galβ1-4 or Galβ1-4) (Fig. 1) (Dennis et al., 1986). Calf heart lectin has been shown to bind more avidly to polylactosamine sequences than to simple lactosamine antenna (Merkle and Cummings, 1988). Endothelial cells may have a lectin with similar specificity where the β1-4Gal is part of a larger ligand structure. Undersialylated glycosylation mutants of the murine B16 melanoma have also been reported to be less adhesive to endothelial cell monolayers (Tao and Johnson, 1982), suggesting that sialic acid may also be part of the ligand. Experiments are in progress to examine this possibility.

In addition to direct adhesion of tumor cells to endothelial cells, galactosylation of D36W25 cells may enhance heterotypic interactions with platelets (Gartner et al., 1978), lymphocytes (Parrish et al., 1984), and even with hepatocytes and Kupffer cells (Schirrmacher et al., 1980; Schlepper-Schafor et al., 1980) on which Gal-binding lectins have previously been detected (Ashwell and Harford, 1982). These interactions in addition to endothelial cell binding, may cumulatively contribute to the observed enhancement of organ retention and colonization in vivo by galactosylated D36W25 cells.

Since the relative difference in adhesiveness of D36W25 and galactosylated D36W25 cells was observed on both bovine adrenal microvascular and human cord macrovascular endothelial cells, the β1-4 Gal component of recognition does not appear to be organ specific. Although there may be a lack of organ specificity in the Gal-mediated adhesion to endothelium, there was a clear preference for the MDAY-D2 lines to produce colonies in the liver rather than the lungs. The growth of visible nodules may be more related to a preference for the growth environment of the liver, rather than to retention of tumor cells in a specific organ. In this regard, murine lymphosarcoma cell lines such as MDAY-D2, Esb, and Friend virus leukemia cells (Kerbel, 1980; Schirrmacher et al., 1979; Belardelli et al., 1984) appear to grow preferentially in the liver.

Somatic mutations that impair complex type N-linked oligosaccharide processing in tumor cells (Finne et al., 1982; Ishikawa et al., 1988; Dennis, 1986*a*,*b*) as well as chemical inhibitors of processing have been shown to reduce metastatic potential in several experimental tumor models. In particular, the degree of -GlcNAcβ1-6Manα1-6Manβ1-branching and the completion of these structures with SAα2-3Galβ1-4 appears to be closely associated with metastatic ability (Dennis et al., 1987; Dennis et al., 1989). Based on these experimental systems, the complex type N-linked oligosaccharides in metastatic cells appear to contribute to decreased cell adhesion

on extracellular matrix proteins and to increased invasiveness of human amnion basement membranes in vitro (Laferté and Dennis, 1988; Yagel et al., 1989). In addition, the oligosaccharides are associated with increased responsiveness to autocrine growth stimulation (VanderElst, I., and J. W. Dennis, manuscript submitted for publication). Invasion and cell proliferation are events that would not occur or be completed before the exogenously added β1-4Gal had been replaced by membrane turnover (Crissman et al., 1985). Therefore, even if exogenous glycosylation were able to restore organ retention to that of the wild-type cells, the number of organ nodules observed several weeks later might be not be restored. If the oligosaccharide sequences required for wild-type levels of endothelial cell adhesion could be restored to D36W25 cells by exogenous glycosylation with several transferases, it should be possible to test this hypothesis directly.

Finally, the class 1 glycosylation mutants lack polylactosamine that form the basis for synthesis of many of the blood group and embryonic antigens (Hakamori and Kannagi, 1983). Therefore, when the purified glycosyltransferases necessary for synthesis of these sequences become available, the mutants could be exogenously glycosylated with several monosaccharide residues to create a variety of structures. This approach should help define the carbohydrate specificity of cell surface lectins and the role of both ligand and receptor in cell-cell interactions.

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