Glycoconjugate Journal (1996) **13:** 33–43

Selectin ligands on human melanoma cells

NICHOLAS MILLER[1]*, RICHARD G. VILE[1] and IAN R. HART[2]

[1]*Imperial Cancer Research Fund and* [2]*Richard Dimbleby Department of Cancer Research, The Rayne Institute, St Thomas' Hospital, Lambeth Palace Road, London, SE1 7EH, UK*

Received 26 June 1995, revised 6 August 1995

Twelve established human melanoma lines were screened for surface expression of the carbohydrate antigens Lewis^a (Le^a), sialyl Lewis^a (SLe^a), dimeric sialyl Lewis^a (diSLe^a), sialyl Lewis^X (SLe^X) and dimeric sialyl Lewis^X (diSLe^X). None of the lines expressed SLe^X, but 11/12 were positive for diSLe^X and 7/12 were positive for SLe^a. Although both diSLe^X and SLe^a have been reported to bind to E-selectin, none of the melanoma lines exhibited E-selectin-dependent adhesion to activated human umbilical vein endothelial cells (HUVECs). Three melanoma lines infected with a retroviral vector carrying the cDNA for the human Lewis fucosyltransferase (FucT-III) subsequently expressed SLe^X at their cell surface and exhibited E-selectin-dependent adhesion to activated HUVECs. Treatment of these transduced cells with inhibitors of O-linked or N-linked protein glycosylation significantly inhibited E-selectin-mediated adhesion, though fluorescence-activated cell sorter analysis indicated no decrease in cell surface expression of SLe^X, SLe^a or diSLe^X. This suggests that the majority of SLe^X/SLe^a-type glycans endogenously produced by human melanoma cells are not protein-associated and do not mediate E-selectin-dependent adhesion. These results support the hypothesis that E-selectin-dependent adhesion requires presentation of SLe^X-type moieties on appropriate glycoproteins.

Keywords: melanoma, E-selectin, Lewis antigens, Lewis fucosyltransferase, metastasis, cell adhesion

Introduction

Extravasation of tumour cells is a fundamental component of cancer metastasis, and much effort has been directed at identifying the cell surface molecules involved in this step. There is evidence that some of these tumour-associated molecules are identical to those known to be important in the recruitment of leukocytes to sites of inflammation. For instance, many epithelial carcinoma lines produce carbohydrate moieties typical of the selectin ligands found on leukocytes [1, 2]. In some cases, production of such moieties has been shown to correlate with metastatic potential [3, 4] tumour progression [5–7] and poor prognosis [8]. Furthermore, these moieties appear to support tumour cell adhesion to E-selectin expressed on activated endothelial cell monolayers *in vitro* [9–16]. It is possible therefore that the selectin-glycan interaction has a direct role in the arrest and extravasation of certain types of tumour cells [17]. The leukocyte integrin VLA-4 ($\alpha 4\beta 1$), normally expressed on leukocytes, directs adherence to VCAM-1 on activated endothelium [18]. Expression of VLA-4 on the surface of melanoma lines has been demonstrated [19], and it has been shown that some integrins can display Lewis antigens and thereby facilitate both selectin-dependent and ICAM-dependent adhesion [20]. Therefore the ability of melanoma cells to utilize selectins for endothelial adhesion requires investigation, particularly in view of a recent report [21] indicating that at least one melanoma line can bind E-selectin. We have analysed twelve human melanoma lines for expression of various selectin-binding carbohydrates and related structures (Fig. 1) and examined the capacity of these cells to adhere to human endothelium via an E-selectin-dependent process.

*To whom correspondence should be addressed at: Glaxo Institute for Molecular Biology, 14 Chemin-les-Aulx, 1228 Plan-les-Ouates, Geneva, Switzerland.

Materials and methods

Reagents

Phleomycin, tunicamycin, gelatin, sodium azide, bovine serum albumin (BSA), heparin and endothelial cell growth supplements (ECGs) were obtained from Sigma Chemical

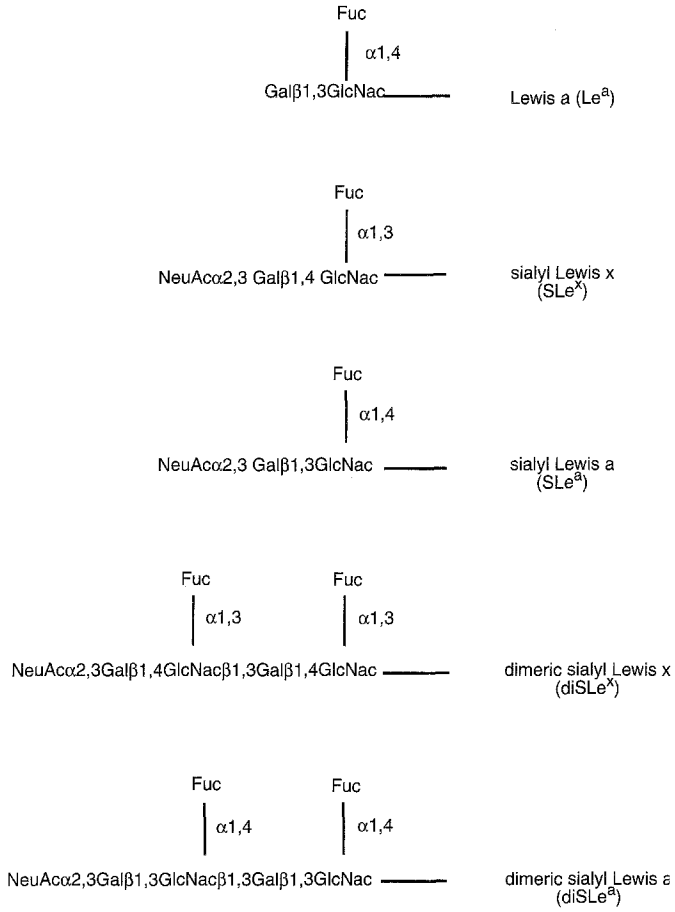

Figure 1. Carbohydrate sequences of Lewis antigens recognized by the monoclonal antibodies used in this study. Some of these oligosaccharide structures have affinity for E-selectin (see text). Fuc, fucose; Gal, galactose; GlcNAc, *N*-acetylglucosamine; NeuAc, sialic acid.

Co., Poole, Dorset, UK. Calcium phosphate transfection kits were obtained from ProMega, Madison, WI, USA; fetal calf serum (FCS) from Gibco, Paisley, UK; collagenase from Boehringer-Mannheim UK, Lewes, Sussex, UK; benzyl-α-GalNAc from Oxford GlycoSystems Ltd, Abingdon, Oxford, UK; polyclonal FITC-labelled rabbit antimouse immunoglobulins from DAKO, Denmark; [^{51}Cr]-sodium chromate isotope (1 mCi ml^{-1}) from Amersham Life Science, Bucks, UK; TNFα from Genzyme, Cambridge, MA, USA; and Citifluor mountant from City University, London, UK.

Monoclonal antibodies used in this study were CSLEX-1 (Becton-Dickinson, UK) which recognizes SLe^X; KM231 (gift from Professor N. Hanai, Kyowa Hakko Kogyo Ltd, Tokyo) and B67.4 (gift of Dr David Miles) which recognize SLe^a; BC9-E5 (supernatant from American Type Culture Collection (ATCC) hybridoma CRL1670) which recognizes Le^a; FH6 (ATCC hybridoma HB8073) which recognizes diSLe^X; and FH7 (ATCC hybridoma HB8861) which recognizes diSLe^a. Blocking antibodies were BBA2 (anti-ELAM-1) and BBA6 (anti VCAM-1) from British Biotechnology, Abingdon, UK.

Cell lines and tissue culture

Promyelocytic leukaemia HL60 cells were grown in Roswell Park Memorial Institute-1640 (RPMI) medium supplemented with 10% (v/v) FCS and 4 mM L-glutamine. Human colonic carcinoma HT29 cells, AM12 amphotropic packaging cells and the human melanoma lines HMB2, LT5, DX3, MeWo, MJM, XP44, SK23, T8, Mel8, A375M, Mel17 and TXM13, were grown in Eagle's minimal essential medium supplemented with 10% (v/v) FCS and 4 mM L-glutamine (DMEM) as detailed previously [22]. All hybridomas were grown according to ATCC instructions. Melanoma lines infected with the pBabe Bleo-based retroviral vector were selected in DMEM containing 20 μg ml^{-1} phleomycin. Human umbilical vein endothelial cells (HUVECs) were grown in M199 medium, (supplemented with 20% (v/v) FCS, 0.09 mg ml^{-1} heparin, 0.35 mg ml^{-1} glutamine, 0.12 mg ml^{-1} ECGS, 550 U ml^{-1} penicillin, 55 μg ml^{-1} streptomycin) in tissue culture flasks that had been pre-coated with 1% gelatin. HUVECs were used within passages 1–4. All cells were maintained at 37 °C in a humidified atmosphere of 90% air, 10% CO_2.

HUVEC isolation

Human umbilical cords were collected from hospital labour wards, washed in PBSA and endothelial cells were harvested by a 10 min incubation with 0.5 mg ml^{-1} collagenase in M199. Cells were washed in HUVEC culture medium and seeded onto gelatin-coated tissue culture flasks.

Plasmids

The plasmid pBabe Bleo, encoding a retroviral vector derived from Moloney murine leukaemia virus, was a gift from Dr Hartmut Land, Imperial Cancer Research Fund, London, UK. The plasmid pCDM7-α(1,3/1,4)FT [23] was a gift from Dr J.B. Lowe, Howard Hughes Medical Institute, Ann Arbor, MI, USA, and encodes a cDNA for the human Lewis fucosyltransferase (Fuc-TIII). Our full cloning protocol is available on request. Briefly, bacteria of strain MC1061/P3 were transformed with pCDM7-α(1,3/1,4)FT and plasmid DNA prepared by standard methods [24]. We PCR-amplified a 1.2 kb fragment of this cDNA (bp −65 to bp +1129) and ligated it into the *Eco* RI site of pBabe Bleo in both forward and reverse orientations giving plasmids named pBFTIII and pBIIITF respectively (Fig. 2). These were bulk-prepared in JM109 bacteria.

Construction and use of retroviral vectors

Plasmids pBFTIII and pBIIITF were transfected into the amphotropic retrovirus packaging line AM12, which had

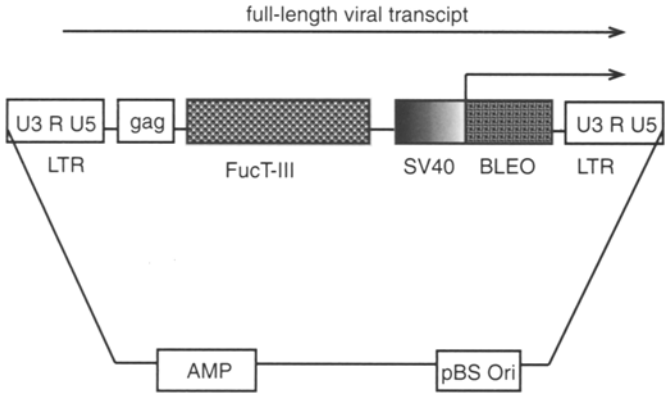

Figure 2. The pBFTIII plasmid. This was constructed by inserting the cDNA for the human Lewis fucosyltransferase (FucT-III) into the *Eco* RI site of the retroviral vector encoded by the pBabe Bleo plasmid. Transcription of the FucT-III cDNA is driven by the 5′ viral LTR. The plasmid pBIIITF contains FucT-III cDNA in the antisense orientation.

been seeded at 5×10^5 cells per T25 flask 24 h beforehand. The ProMega 'Profectin' (calcium phosphate coprecipitation) kit was used according to the manufacturer's instructions. AM12 cells were selected in phleomycin-containing medium, and resistant colonies pooled. Virus-containing supernatant from these cells were mixed wtih polybrene (final concentration 4 $\mu g\,ml^{-1}$) and filtered through a 0.45 μM filter (Nalge (UK) Ltd, Rotherham, UK). This was then used to infect melanoma cells at 2 ml per T25 flask. After overnight incubation the cells were washed and refed with DMEM and 48 h later were split into phleomycin-containing medium. Phleomycin-resistant colonies were pooled. Cells were tested for the presence of replication-competent virus by serial infection assay [25].

Flow cytometry and immunohistochemistry

Cells from semiconfluent cultures were harvested with trypsin and left to recover for 30 min in DMEM at 37 °C. Cells were then pelleted and resuspended at 4×10^6 cells ml^{-1} in PBSA/0.1% azide, and left for 40 min at 4 °C to react with the primary antibodies CSLEX-1 (5 $\mu g\,ml^{-1}$), FH6 (1:1 mixture of cell suspension:supernatant), KM231 (5 $\mu g\,ml^{-1}$) BC9-E5 (1:1 mixture of cells:supernatant) and FH7 (1:1 mixture of cells:supernatant), in a total volume of 100 μl. For a negative control, PBSA/azide alone was used. Cells were then washed and left for 40 min at 4 °C to react with FITC-labelled rabbit-antimouse antibodies in a final volume of 100 μl. Finally cells were washed, fixed in 10% formaldehyde/PBSA (v/v) and kept at 4 °C until analysis, which was done with a Becton-Dickinson FACScan.

For immunofluorescence/light microscopy, cells were seeded onto glass coverslips in 24-well plates at 1×10^6 cells ml^{-1}. After 24 h cells were washed in PBSA and fixed in 10% (v/v) formaldehyde (room temperature). Coverslips were then washed in PBSA/0.1% (w/v) BSA (PBSA/BSA) and placed cell-side down on 30 μl volumes of monoclonal antibody solution in PBSA/BSA, or PBSA/BSA alone (negative control). After 30 min at room temperature, coverslips were washed in PBSA/BSA and then placed on 30 μl aliquots of FITC-labelled rabbit anti-mouse antibodies diluted in PBSA/BSA. After 30 min at room temperature, coverslips were washed in PBSA/BSA and mounted in 30 μl of Citifluor mountant.

Treatment of cells with glycosylation inhibitors

Melanoma cells seeded 24 h beforehand at a density of 5×10^5 cells per T25 flask had their medium replaced with DMEM containing either benzyl-α-D-GalNAc (2 mM) or tunicamycin (0.5 $\mu g\,ml^{-1}$) or normal medium (control); HL60 cells were pelleted and resuspended in RPMI containing the above concentrations of inhibitors. Cells were expanded for 72 h prior to being harvested for FACS analysis or adhesion assay.

Adhesion assays

Confluent HUVEC monolayers in 96 well plates were treated with fresh M199 medium, either with or without 20 ng ml^{-1} TNF-α, for 4–6 h prior to addition of melanoma and HL60 cells. Melanoma cells were harvested with trypsin and allowed to recover for 30 min as described above, prior to labelling with ^{51}Cr (1 h at 37 °C) by conventional means [26]. Labelled cells were washed thoroughly with PBS containing 100 $\mu g\,ml^{-1}$ concentration each of Ca and Mg (PBSABC) at 4 °C, resuspended at 2×10^5 cells ml^{-1} and 50 μl volumes added to each well of HUVECs. HUVECs were washed twice in cold PBSABC prior to addition of labelled cells; during addition of cells, HUVECs were kept on ice. Adhesion of labelled cells to HUVECs were allowed to proceed for 12 min, at 37 °C, after which non-attached cells were flicked off. The wells were gently washed twice in cold PBSABC prior to addition of lysis solution (0.1% Triton-X/dH_20) which was then harvested and subjected to gamma counting.

Blocking antibodies were diluted from 1 mg ml^{-1} H_20 stock solutions into HUVEC culture medium (either with or without TNF-α), to a final concentration of 50 $\mu g\,ml^{-1}$, and HUVEC supernatant was replaced with these solutions. HUVECS were incubated with blocking antibodies for 40 min. Quantitative assays of ^{51}Cr counts per well were performed using a Wallac 1261 'Multi-gamma' counter, and adhesion expressed as a percentage of total counts added per well. For each treatment the mean was taken of four individual wells.

Results

Expression of Lewis antigens on human melanoma cells

As determined by antibody binding, none of the melanoma lines produced SLe^X (Table 1), yet 11/12 produced $diSLe^X$, which is an internally fucosylated form of SLe^X (Fig. 1). None of the lines produced $diSLe^a$, and only one line produced low levels of Lea, but 7/12 lines were found to produce SLe^a using mAb KM231, and 5/12 using mAb B67.4. The positive control colon carcinoma line HT29 produced all the above Lewis antigens.

It appears that in the melanoma cells the common pattern of expression of fucosyltransferases and their acceptor and donor substrates is such that Type II chains bearing Le^X structures are common and are exclusively difucosylated, not monofucosylated, at their termini. Type I chains bearing Le^a structures are less common but, where present, are exclusively monofucosylated at their termini and not difucosylated. For Type I chains at least terminal sialylation appears to be complete, as indicated by the absence of Le^a structures on melanoma cells. Evidently human melanoma cells produce types of Lewis antigen that would predict an ability to adhere via E-selectin.

Adhesion of human melanoma cells to activated HUVEC monolayers

Each of the twelve melanoma lines was tested for its ability to adhere via E-selectin to activated and non-activated human endothelial cells, in the presence and absence of blocking antibodies, in three or more independent experiments. For 8/12 melanoma lines, no increased affinity for activated HUVECs over non-activated HUVECs was demonstrated. The other four lines (TXM13, MJM, LT5, DX3) showed enhanced binding to activated endothelium (a slight increase for TXM13 and MJM cells, larger increases for DX3 and LT5 cells). Data from representative experiments are shown only for HMB2, DX3 and LT5 cells (Fig. 3). DX3 cells consistently adhered to HUVECs at higher levels than any other melanoma cell line. Blocking antibodies showed however, that in all cases the increased adhesion to activated HUVECs was mediated primarily through endothelial VCAM-1 and not endothelial E-selectin. Thus anti-VCAM-1 antibody could abolish this increased adhesion almost entirely whereas anti-E-selectin antibody could not (Fig. 3). That endothelial cells were producing significant amounts of functional E-selectin only after activation was demonstrated by the behaviour of the positive control HL60 cells, which adhered to activated but not non-activated endothelium, and exhibited significantly decreased enhancement of binding in the presence of anti-E-selectin antibody (Fig. 3). (HL60 cells also commonly showed some decrease in enhancement of binding after treatment of HUVECs with anti-VCAM-1 antibody, in agreement with other studies [15].) Although these experiments were designed to maximize the possibility of E-selectin interactions, it is not surprising that there should be some interference from VCAM-1, since E-selectin expression on HUVECs peaks at 4–6 h of cytokine stimulation, and VCAM-1 also reaches very high levels at 6 h [27].

Table 1. Production of Lewis antigens on the surface of human melanoma cells. None of the melanoma lines produce SLe^X, but 11/12 produce $diSLe^X$ and 7/12 (mAb KM231) or 5/12 (mAb B67.4) produce SLe^a. Each cell line was tested with each antibody in three or more independent experiments. ++, fluorescence shift of about 2 logs; +, fluorescence shift of about 1 log; +/–, variable fluorescence shift of up to 1 log; –, no fluorescence shift. The HT29 colon carcinoma line (positive control) reacts with all the monoclonal antibodies used in this study.

	Cell line												
Lewis antigen (mAb)	*A375M*	*MEL17*	*MEW0*	*MJM*	*SK23*	*T8*	*MEL8*	*XP44*	*DX3*	*HMB2*	*LT5*	*TXM13*	*HT29*
SLE^X (CSLEX1)	–	–	–	–	–	–	–	–	–	–	–	–	+
$DISLE^X$ (FH6)	+	+	++	+	++	+	+	–	+	+	+	++	+
SLE^A (KM231)	–	+	–	+	–	+	+	–	+	–	+	+	+
SLE^A (B67.4)	–	+	–	–	–	–	±	–	+	–	+	±	+
$DISLE^A$ (FH7)	–	–	–	–	–	–	–	–	–	–	–	–	+
LE^A (BC9-E5)	–	–	–	–	–	±	–	–	–	–	–	–	+

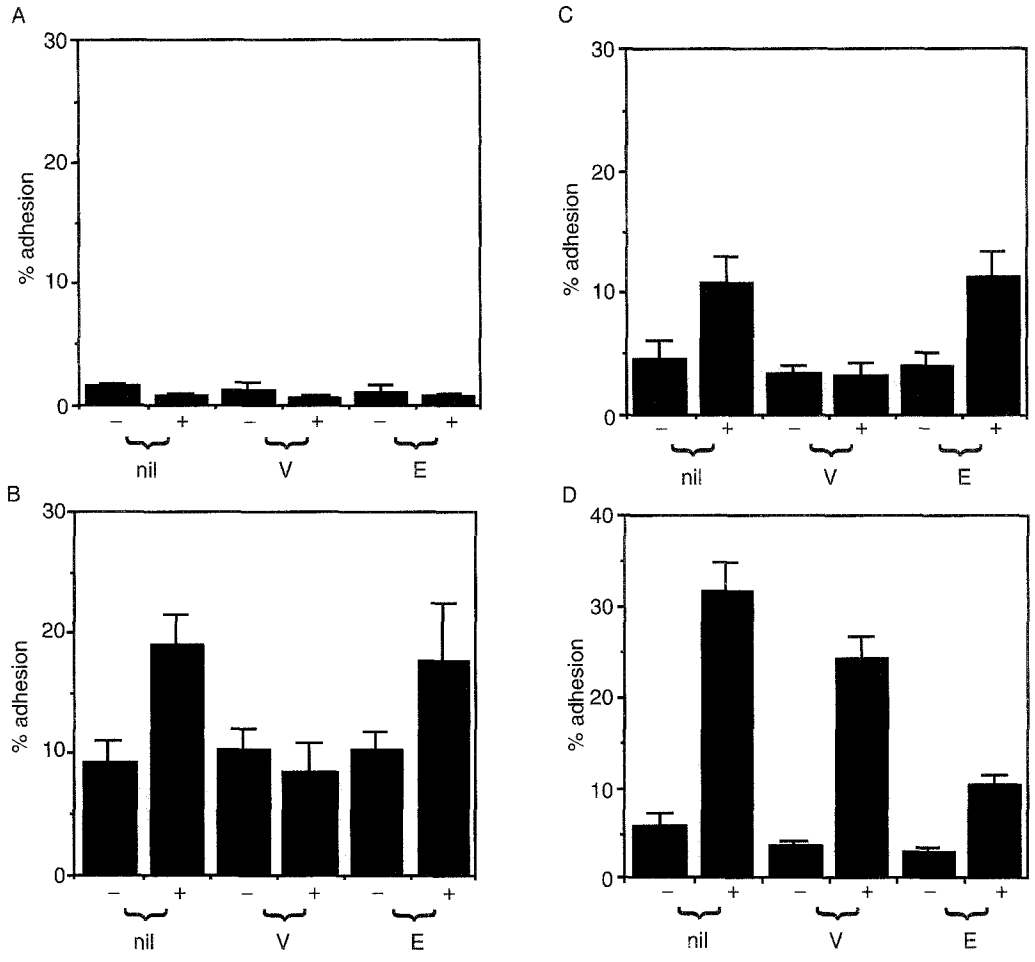

Figure 3. Adhesion of human melanoma lines to cultured monolayers of human endothelial cells (HUVECs). This shows the ability of HMB2 (A), DX3 (B) and LT5 (C) melanoma cells to adhere to non-activated (−) and activated (+) endothelial cells, after pre-incubation of endothelial cells with no antibody (nil), with blocking antibody BBA6 (V), or with blocking antibody BBA2 (E) as detailed in the text. The positive control cell line HL60 (D) shows significantly enhanced adhesion ($p < 0.05$) to activated as compared with non-activated endothelium both in the absence of antibody and after pretreatment with BBA6 and BBA2. There is a significant ($p < 0.05$) inhibition of adhesion after pretreatment with BBA6, but there is a significantly greater ($p < 0.05$) inhibition of adhesion after pretreatment with BBA2. DX3 and LT5 cells show significantly enhanced adhesion ($p < 0.05$) to activated endothelium which is abolished by pretreatment with BBA6 but not significantly affected by pretreatment with BBA2. HMB2, which does not express integrin $\alpha 4\beta 1$ [22], shows no increased affinity for activated over non-activated endothelium. Data presented for each cell line are taken from an individual experiment, and are representative of at least three independent repeats.

Clearly human melanoma cells do not adhere to E-selectin on activated endothelium under our experimental conditions, in spite of expressing, at their cell surface, high levels of carbohydrate moieties typical of E-selectin ligands.

Human melanoma cell adhesion via E-selectin after retrovirally-mediated transfer of a human Lewis fucosyltransferase cDNA

The human Lewis $\alpha(1,3/1,4)$ fucosyltransferase cDNA has been shown to mediate production of both SLe^X and SLe^a [23] and to confer E-selectin-dependent adhesion on transfected Cos cells [28]. We infected the HMB2, DX3 and LT5 lines with retroviral vectors derived from the plasmids pBFTIII or pBIIITF (Fig. 2). Pooled populations of phleomycin-resistant melanoma cells were analysed for Lewis antigen production by FACS and immunofluorescence microscopy and for E-selectin-dependent adhesion to activated HUVECs. FACS analysis showed that cells infected with the vector BFTIII, but not with the control BIIITF, expressed high levels of SLe^X (Fig. 4) and this was confirmed by immunofluorescence microscopy (Fig. 5). The infectants all showed significantly increased binding to activated HUVECs (Fig. 6) relative to control BIIIFT-infected lines (data not shown). Some component

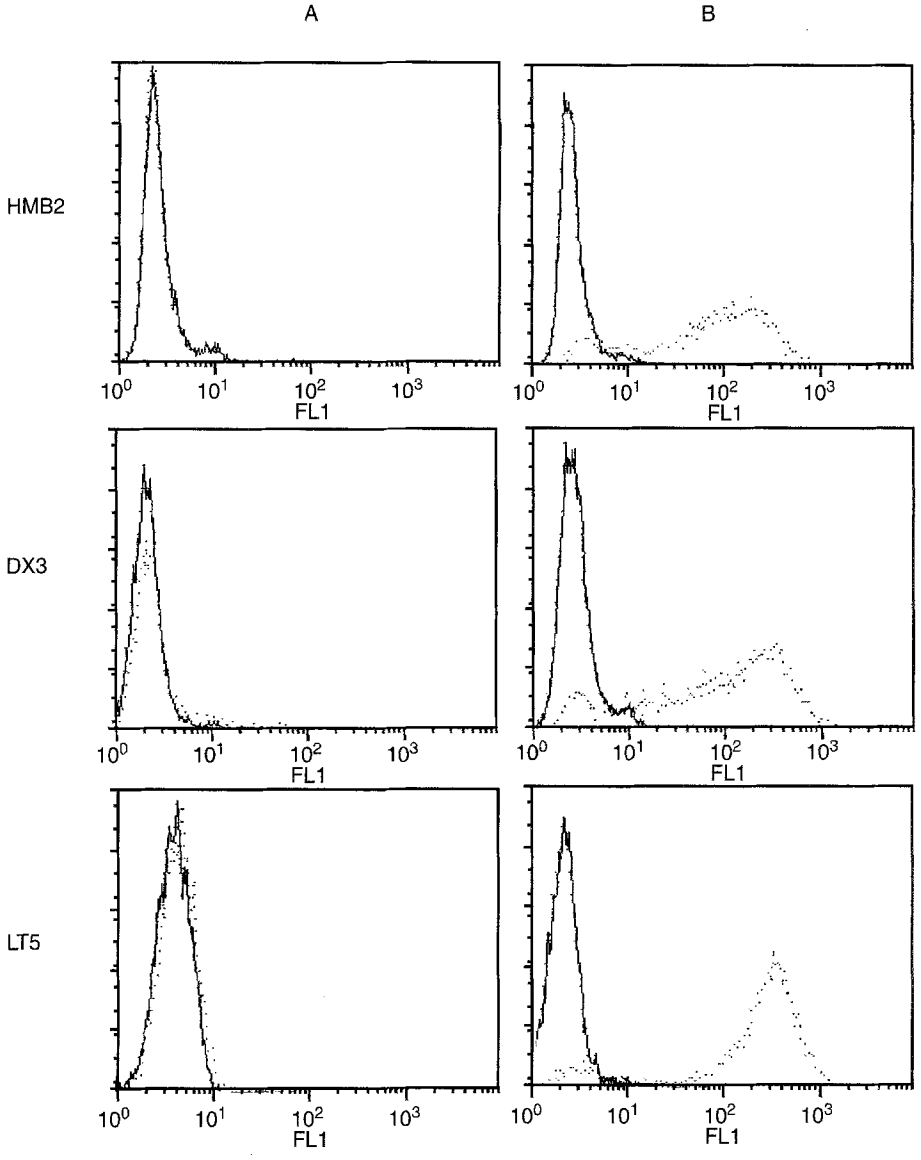

Figure 4. Production of SLe^X at the surface of phleomycin-resistant HMB2, DX3 and LT5 cells after infection by retroviral vectors encoded by pBIIITF (A) or pBFTIII (B), as determined by FACS analysis. Cell lines infected with BIIITF, which contains FucT-III cDNA in the antisense orientation, did not produce SLe^X (panel A), whereas those infected with BFTIII (panel B) all exhibited a 2-log shift.

of the enhanced adhesion to activated endothelium is again VCAM-1-mediated for DX3 and LT5, but not for HMB2 (Fig. 6) but most of the enhanced adhesion is blocked by antibodies to E-selectin (Fig. 6).

Effect of inhibitors of protein glycosylation

The effect of inhibitors of either O-linked or N-linked protein glycosylation on adhesion of BFTIII-infected melanoma lines to activated endothelium was examined. For the control cell line HL60, treatment with benzyl-α-GalNAc, an inhibitor of O-glycosylation, decreased enhancement of adhesion to activated HUVECs by up to 50%, whereas tunicamycin, an inhibitor of N-glycosylation, had little or no effect (Fig. 7). Thus, for HL60 cells, the E-selectin-binding epitopes appear to be associated with O-linked but not N-linked glycans, which is in agreement with previous work [11]. For the HMB2-BFTIII cell line, inhibition of both O-glycosylation and N-glycosylation decreased enhancement of adhesion to activated HUVECs by about 50% (Fig. 7). For both DX3-BFTIII and LT5-BFTIII cells, inhibition of O-glycosylation decreased enhancement of adhesion to activated HUVECs by about 50%, and inhibition of N-glycosylation almost completely abolished enhanced adhesion (Fig. 7). Thus for human melanoma cells the carbohydrate E-selectin-binding moieties produced subse-

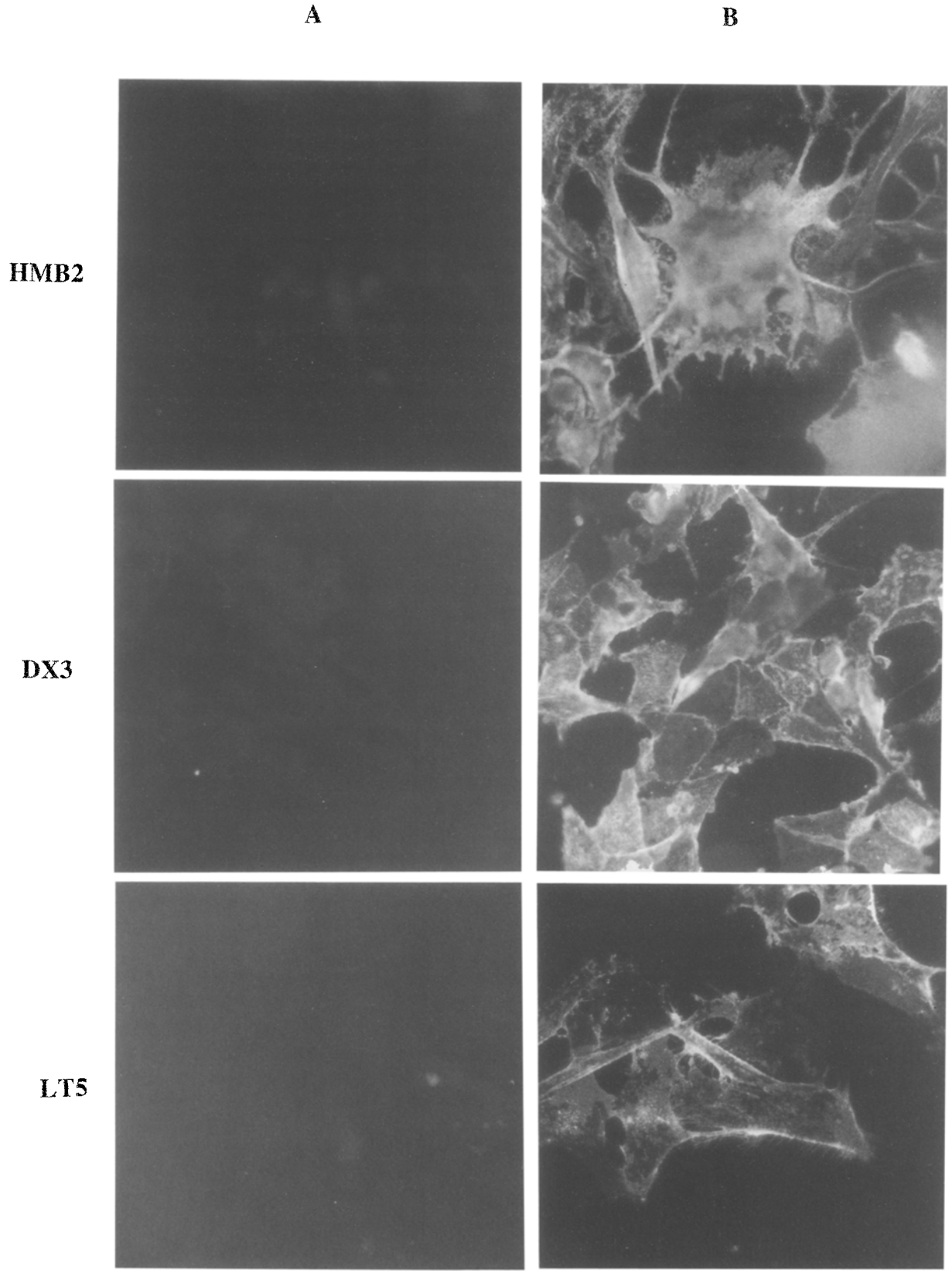

Figure 5. Production of SLe^X at the surface of HMB2, DX3 and LT5 after infection by retroviral vectors encoded by pBFTIII or pBIIITF; immunofluorescence microscopy. All three cell lines infected with BFTIII (panel B) exhibited strong cell surface fluorescence indicative of high levels of membrane-associated SLe^X. The SLe^X antigen appears to be generally distributed over the cell surface in all three cell lines. Cell lines infected with pBIIITF (panel A) were not fluorescent indicating no SLe^X production.

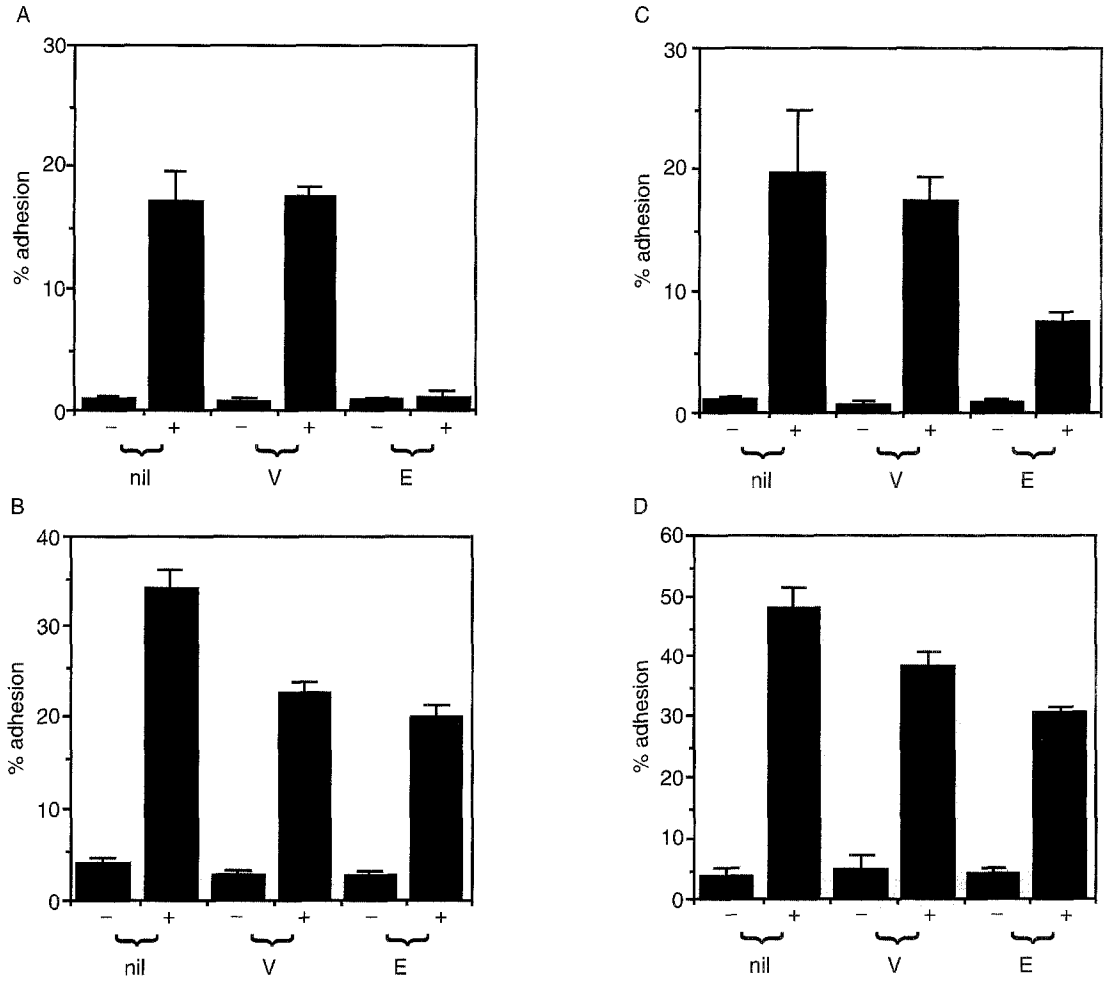

Figure 6. Adhesion of BFTIII-infected melanoma cells to cultured monolayers of human endothelial cells. HMB2-BFTIII (A) now shows significantly enhanced ($p < 0.05$) adhesion to activated endothelium which is entirely abolished by preincubation with BBA2 but not by preincubation with BBA6. For both DX3-BFTIII (B) and LT5-BFTIII (C) cells, some component of the enhanced adhesion to activated endothelium is blocked by BBA6 but a significant ($p < 0.05$) component is now blocked by BBA2. Control cell line HL60 is shown in (D). Pre-incubation with no antibody (nil), with BBA2 (E) or BBA6 (V) as detailed in the text.

quent to infection with BFTIII appear to be protein-linked, as determined by the effects of glycosylation inhibitors.

However, when the BFTIII-infected, BIIITF-infected and parental cells treated with N- or O-glycosylation inhibitors were examined by FACS in three independent experiments for surface expression of diSLe^X, SLe^X and SLe^a, no detectable effect on cell surface levels of any of these carbohydrate structures was detected for any treatment of any cell line (data not shown). These results suggest that the great majority of Lewis antigens on human melanoma cells, both constitutively expressed and BFTIII-induced, are not protein-associated.

Discussion

We show here that the carbohydrate antigens diSLe^X and SLe^a are frequently present at high levels on cultured human melanoma cell lines. The diSLe^X structure is virtually ubiquitous, being absent only from XP44 in our panel of twelve cell lines, while the SLe^a structure was present on 7/12 of the cell lines tested. These results are similar to those of a recent study [21] which found diSLe^X and SLe^a to be produced by 4/4 and 3/4 melanoma lines respectively. We used two different mAbs against SLe^a (KM231 and B67.4). It is not clear why cell lines MJM and T8 are consistently positive for SLe^a with mAb KM231 but negative with mAb B67.4. This result may be related to the phenomenon whereby recognition of a sialylated oligosaccharide by different mAbs can differ according to the type of glycoconjugates to which the oligosaccharide is attached [29] but we have no evidence to support or refute such a possibility.

The diSLe^X oligosaccharide contains SLe^X, a definitive selectin-binding carbohydrate antigen, and has been shown to be able to block E-selectin-mediated adhesion

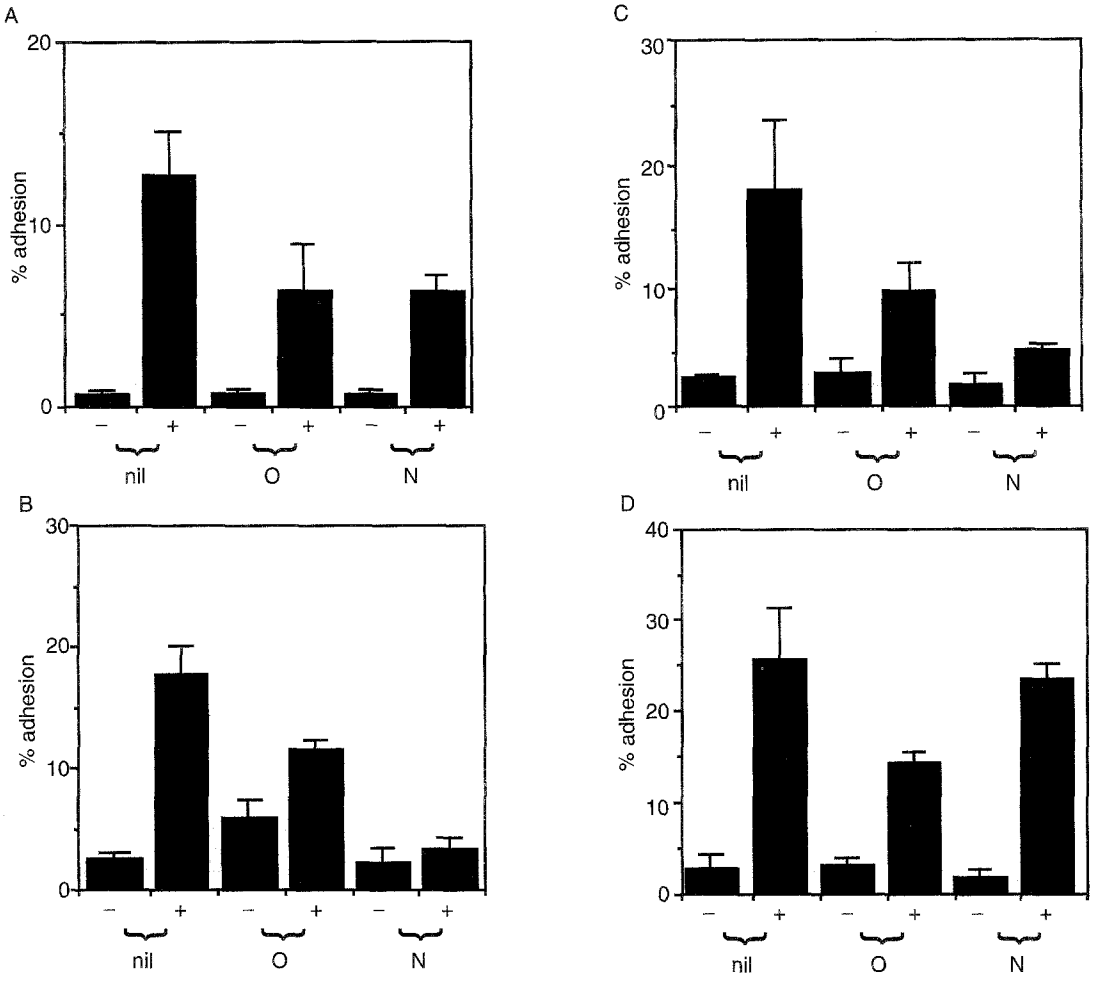

Figure 7. Effect of glycosylation inhibitors on adhesion of pBFTIII-infected melanoma cells to cultured monolayers of human endothelial cells. This shows enhancement of adhesion of BFTIII-infected melanoma cells to activated (+) as compared with non-activated (−) HUVEC monolayers, after treatment of melanoma cells with inhibitors of O-linked glycosylation (O) or inhibitors of N-linked glycosylation (N), or with no inhibitor (nil). Inhibition of O-linked glycosylation significantly inhibited the previously observed (Fig. 6) enhancement of adhesion ($p < 0.05$) to activated HUVECs for all three melanoma lines and for the positive control HL60 cells. Inhibition of N-linked glycosylation decreased enhanced adhesion almost completely for DX3 (B) and LT5 (C) but only by about 50% for HMB2 (A) and had no significant effect for HL60 adhesion (D). See text for treatment details.

when presented on liposomes [30]. The SLe^a oligosaccharide has been shown to participate in adhesion of tumour cells to E-selectin [14], and in a number of tumour cell lines E-selectin-dependent adhesion is mediated exclusively by SLe^a, even when both SLe^x and SLe^a are present [9, 13, 31]. Therefore the production of high levels of $diSLe^X$ and SLe^a by human melanoma lines led us to predict that they would bind to activated endothelium via E-selectin. However none of the twelve lines that we tested exhibited such an ability, and in lines that showed enhanced adhesion to activated endothelium, the enhancement was attributable entirely to VCAM-1 (Fig. 3). This supports previous data [32–34] demonstrating a role for VCAM-1 in attachment of other melanoma lines to activated endothelium and concurs with a study that found no correlation between $diSLe^X$ or SLe^a expression and adhesion to E-selectin in four melanoma lines [21]. Previously published values for VCAM-1 - dependent adhesion of melanoma cells to activated endothelium are similar to or slightly higher than those reported here, i.e. a four-fold [32, 34] or nine-fold [33] increase in adhesion.

It seems clear that in human melanoma lines production of carbohydrate moieties typical of E-selectin ligands is not sufficient to mediate E-selectin-dependent adhesion. It could be that (i) human melanoma cells lack 'scaffolding' molecules necessary to present these carbohydrate moieties in a manner permissive of adhesion; or (ii) the 'scaffolding' molecules are present, but the active melanoma fucosyltransferases do not participate in their

glycosylation. The carbohydrate ligands of selectins can be expressed on both glycoproteins and glycolipids, but there is a growing body of evidence that indicates that, for selectin-mediated adhesion to occur, the carbohydrate antigens must be found in the context of a particular 'scaffolding' molecule, usually a glycoprotein [7, 11, 35–44]. Also, E-selectin domains other than the lectin-like region may participate in E-selectin-mediated cell-cell adhesion, raising the possibility that in nature both glycans and protein contribute to the E-selectin ligand [45]. However, human melanoma cells clearly do not lack appropriate molecular scaffolds since infection with a retroviral vector carrying the cDNA for the human Lewis fucosyltransferase conferred upon three representative melanoma lines E-selectin-dependent adhesion to activated HUVECs (Fig. 6).

The results imply then that the endogenous melanoma fucosyltransferases are not producing selectin-binding structures on the appropriate scaffolding molecules; that is, the abundant diSLe^X and SLe^a antigens are not found on glycoconjugates that can present them in a context appropriate to E-selectin-dependent adhesion. Our data further implies that in melanoma cells which exhibit E-selectin-mediated adhesion after infection with a vector encoding the Lewis fucosyltransferase, selectin-binding carbohydrate structures are likely to be present on molecules different from those which display endogenous diSLe^X and SLe^a.

Many glycans, including Lewis-type carbohydrates, are found on both lipids and proteins. Protein-associated glycans may be linked to the peptide either via the amido-nitrogen of asparagine (N-linked glycans) or via the hydroxyl oxygen of serine or threonine (O-linked glycans). Both N-linked and O-linked glycosylation can be specifically blocked by inhibitors which do not affect lipid glycosylation, and which therefore can be used to distinguish lipid- and protein-linked glycans. We used inhibitors of protein glycosylation to examine indirectly the relative contributions of glycolipids and glycoproteins to Lewis antigen display and E-selectin-mediated adhesion in human melanoma cells. We found that treatment of BFTIII- and BIIITF (control)-infected cells with inhibitors of N- or O-linked glycosylation followed by FACS analysis, did not result in detectably lower levels of production of SLe^X, diSLe^X or SLe^a at the cell surface. This suggested that the great majority of Lewis-type antigens on human melanoma cells are present on glycolipids, not glycoproteins. However, these same treatments significantly inhibited adhesion of BFTIII-infected cells to activated endothelium, suggesting that the functional E-selectin-binding glycans mainly are protein-associated. This agrees with other studies [11, 46] which indicate that a substantial proportion of HL60 cell surface SLe^X/SLe^a is glycolipid-associated, and not able to mediate E-selectin-dependent adhesion, and that inhibition of HL60 O-glycosylation can abolish selectin interaction without significantly decreasing cell surface SLe^X as measured by FACS. In our experiments, we did not separate the effect of inhibitors of glycosylation on VCAM-1-dependent and on E-selectin-dependent adhesion for the DX3 and LT5 lines. Thus for these two cell lines it is possible that VLA-4 mediated adhesion also is affected by inhibitors of protein glycosylation and this may account for some of the observed decrease in adhesion to activated HUVECs. However, HMB2 cells exhibit no VCAM-1-dependent adhesion (Fig. 3), and indeed do not express the α4-integrin [22]; hence their ability to adhere to activated endothelium is mediated entirely by E-selectin (Fig. 6). Therefore the significant inhibition of adhesion of HMB2-BFTIII cells to activated HUVECs by inhibitors of protein glycosylation suggests that in this cell line E-selectin-binding carbohydrates are presented on glycoproteins. The inhibition of adhesion observed after treatment with benzyl-α-GalNAc was equivalent to that observed after treatment with tunicamycin (Fig. 7), suggesting that in HMB2-BFTIII the E-selectin-binding carbohydrates are produced both on N-linked and O-linked glycans. By contrast, epithelial carcinoma and HL60 cells exhibit a significant decrease in endogenous SLe^X or SLe^a-mediated E-selectin dependent adhesion only after inhibition of O-glycosylation, not after inhibition of N-glycosylation ([11, 12, 31] and Fig. 7).

In summary, most human melanoma cell lines produce abundant carbohydrate structures typical of E-selectin ligands but have no endogenous E-selectin binding capacity. These carbohydrate structures include diSLe^X and SLe^a but not SLe^X. Nevertheless, after infection with BFTIII, a retroviral vector encoding the human Lewis fucosyltransferase, three representative melanoma lines exhibit both SLe^X production and E-selectin-dependent adhesion to activated HUVECs. This adhesion to activated HUVECs is mediated entirely via E-selectin in HMB2-BFTIII cells, and probably also in DX3-BFTIII and LT5-BFTIII cells, and is significantly inhibited after treatment with inhibitors of both O- and N-linked glycosylation. The same treatments do not inhibit Lewis antigen expression on these cell lines as measured by FACS. A likely interpretation of our results is that most or all of the endogenous melanoma Lewis antigens are lipid-associated and do not participate in E-selectin-dependent adhesion; after infection with BFTIII, a minority of the total complement of melanoma Lewis antigens are produced on cell surface protein(s), and this minority then can confer significant E-selectin-dependent adhesion. This would support the hypothesis that E-selectin dependent cell-cell adhesion requires not only appropriate tetrasaccharide moieties but also presentation of these moieties on an appropriate protein 'scaffolding' molecule [17].

References

1. Fukushi Y, Kannagi R, Hakomori SI, Shepard T, Kulander BG, Singer JW (1985) *Cancer Res* **45**: 3711–17.
2. Fukushima K, Hirota M, Terasaki PI, Wakasaki A, Togashi H, Chia D, Suyama N, Fukushi Y, Nudelman E, Hakomori S (1984) *Cancer Res* **44**: 5279–85.
3. Inufusa H, Kojima N, Yasutomi M, Hakomori S (1991) *Clin Exp Met* **9**: 245–57.
4. Irimura T, Matsushita Y, Hoff SD, Yamori T, Nakamori S, Frazier ML, Giacco GG, Cleary KR, Ota DM (1991) *Semin Cancer Biol* **2**: 129–39.
5. Matsushita Y, Clearly KR, Ota DM, Hoff SD, Irimura T (1990) *Lab Invest* **63**: 780–91.
6. Nakamori S, Kameyama M, Imaoka S, Furukawa H, Iohikawa O, Sasaki Y, Kabuto T, Iwanaga T, Matsushita Y, Irimura T (1993) *Cancer Res* **53**: 3632–37.
7. Matsusako T, Muramatsu H, Shirahama T, Muramatsu T, Ohi Y (1991) *Biochem Biophys Res Commun* **181**: 1218–22.
8. Narita T, Funahashi H, Satoh Y, Watanabe T, Sakamoto J, Takagi H (1993) *Cancer* **71**: 3044–53.
9. Iwai K, Ishikura H, Kaji M, Sugiura H, Ishizu A, Takahashi C, Kato H, Tanabe T, Yoshiki T (1993) *Int J Cancer* **54**: 972–77.
10. Sawada T, Ho JJL, Chung YS, Sowa M, Kim YS (1994) *Int J Cancer* **57**: 901–7.
11. Kojima N, Handa K, Newman W, Hakomori S (1992) *Biochem Biophys Res Commun* **182**: 1288–95.
12. Huang J, Byrd JC, Yoon WH, Kim YS (1992) *Oncol Res* **4**: 507–15.
13. Takada A, Ohmori K, Tsuyuoka K, Hasegawa A, Kiso M, Kannagi R (1993) *Cancer Res* **53**: 354–61.
14. Majuri ML, Mattila P, Renkonen R (1992) *Biochem Biophys Res Commun* **182**: 1376–82.
15. Cavenagh JD, Gordon-Smith EC, Gibson FM, Gordon MY (1993) *Br J Haemat* **85**: 285–91.
16. Zaifert K, Cohen MC (1993) *Clin Immunol Immunopath* **68**: 51–56.
17. Varki A (1994) *Proc Natl Acad Sci USA* **91**: 7390–97.
18. Elices MJ, Osborn L, Takada Y, Crouse C, Luhowskyj S, Hemler ME, Lobb RR (1990) *Cell* **60**: 577–86.
19. Albelda SM, Meite SA, Elder DE, Stewart R, Damjanovich I, Herlyn M, Buck CA (1990) *Cancer Res* **50**: 6757–64.
20. Kotovuori P, Tontii E, Pigott R, Shepherd M, Kiso M, Hasegawa A, Renkonen R, Nortamo P, Altieri DC, Gahmberg CG (1993) *Glycobiology* **3**: 131–36.
21. Kunzendorf U, Kruger-Krasagakes S, Notter M, Hock H, Walz G, Diamanstein T (1994) *Cancer Res* **54**: 1109–12.
22. Marshall JF, Nesbit SA, Helfrich MH, Horton MA, Polakova K, Hart IR (1991) *Int J Cancer* **48**: 924–31.
23. Kukowska-Latallo JF, Larsen RD, Nair RP, Lowe JB (1990) *Genes Develop* **4**: 1288–1303.
24. Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
25. Morgenstern JP, Land H (1991) In *Gene Transfer and Expression Protocols*. (Murray EJ ed.) pp. 181–206. New Jersey: Humana Press.
26. Brunner KT, Enger HD, Cerottini JC (1976) In: In Vitro *methods in cell-mediated and tumor immunity* (Bloom BR, David JHR eds) pp. 423–436. New York: Academic Press.
27. Needham L, Pearson JD, Hellewell PG (1991) In: *Vascular Endothelium: Interactions with Circulating cells*. (Gordon J ed.) pp. 61–89. Amsterdam Elsevier Science Publishers BV.
28. Lowe JB, Stoolman LM, Nair RP, Larsen RD, Behrend TL, Marks RM (1991) *Biochem Soc Trans* **19**: 649–53.
29. Saito S, Levery SB, Salyan MEK, Goldberg RI, Hakomori S (1994) *J Biol Chem* **269**: 5644–52.
30. Polley MJ, Phillips ML, Wayner E, Nudelman E, Singhal AK, Hakomori S-I, Paulson JC (1991) *Proc Natl Acad Sci USA* **88**: 6224–28.
31. Sawada R, Tsuboi S, Fukuda M (1994) *J Biol Chem* **269**: 1425–31.
32. Rice GE, Gimbrone MA, Bevilacqua MP (1988) *Am J Pathol* **133**: 204–10.
33. Rice GE, Bevilacqua MP (1989) *Science* **246**: 1303–6.
34. Martin-Padura I, Mortarini R, Lauri D, Bernasconi S, Sanchez-Madrid F, Parmiani G, Mantovani A, Anichini A, Dejana E (1991) *Cancer Res* **51**: 2239–41.
35. Sako D, Chang X-J, Barone KM, Vachino G, White HW, Shaw G, Veldman GM, Bean KJ, Ahern TJ, Furie B, Cumming DA, Larsen GR (1993) *Cell* **75**: 1179–86.
36. Lasky LA, Singer MS, Dowbenko D, Imai Y, Henzel WJ, Grimley C, Fennie C, Gillett N, Watson SR, Rosen SD (1992) *Cell* **69**: 927–38.
37. Levinovitz A, Muhlhoff J, Isenmann S, Vestweber D (1993) *J Cell Biol* **121**: 449–59.
38. Larsen GR, Sako D, Ahern TJ, Shaffer M, Erban J, Sajer SA, Gibson RM, Wagner DO, Furie BC, Furie B (1992) *J Biol Chem* **267**: 111104–10.
39. Goelz S, Kumar R, Potvin B, Sundaram S, Brickelmaier M, Stanley P (1994) *J Biol Chem* **269**: 1033–40.
40. Sawada R, Lowe JB, Fukuda M (1993) *J Biol Chem* **268**: 12675–81.
41. Picker LJ, Warnock RA, Burns AR, Doerschuk CM, Berg EL, Butcher EC (1991) *Cell* **66**: 921–33.
42. Berg EL, McEvoy LM, Berlin C, Bargatze RF, Butcher EC (1993) *Nature* **366**: 695–98.
43. Stocks SC, Kerr MA (1993) *Biochem Biophys Res Commun* **195**: 478–83.
44. Steegmaier M, Levinovitz A, Isenmann S, Borges E, Lenter M, Kocher HP, Kleuser B, Vestweber D (1995) *Nature* **373**: 615–20.
45. Li SH, Burns DK, Rumberger JM, Presky DH, Wilkinson VL, Anastaro M, Wolitzky BA, Norton CR, Familetti PC, Kim KJ, Goldstein AL, Cox DC, Huang KS (1994) *J Biol Chem* **269**: 4431–37.
46. Norgard KE, Moore KL, Diaz S, Stults NL, Ushiyama S, McEver RP, Cummings RD, Varki A (1993) *J Biol Chem* **268**: 12764–74.