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Expression of galectins on microvessel endothelial cells and their involvement in tumour cell adhesion

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Lactoside-binding lectins (galectins) with molecular weights of about 14.5 kDa (galectin-1) and 29–35 kDa (galectin-3) bind preferentially to polylactosaminoglycan-containing glycoconjugates and have been found on the surface of tumour cells and implicated in cell-cell and cell-extracellular matrix adhesion and metastasis. We have demonstrated by immunoblotting that both galectin-1 and galectin-3 are present in extracts of endothelial cells cultured from bovine aorta, rat lung, mouse lung and mouse brain microvessels, whereas mouse hepatic sinusoidal endothelial cells expressed primarily galectin-1. These galectins were also localized by indirect immunofluorescent labelling on the surface of the different endothelial cells in culture and by immunohistochemical staining in human tissues *in vivo*. Anti-galectin-1 antibodies inhibited the adhesion of liver-preferring murine RAW117-H10 large-cell lymphoma cells to hepatic sinusoidal endothelial cells or lung microvessel endothelial cells *in vitro*. The data indicate that galectin-1 is expressed on the extracellular surface of endothelial cells and can mediate in part the adhesion of RAW117-H10 cells to liver microvessel endothelial cells.

Keywords: galectin; lectin; cell adhesion; endothelial cell; metastasis

Introduction

Blood-borne metastasis is a complex multi-step process that is initiated by the growth and invasion of malignant cells at primary sites, enzymatic degradation of the extracellular matrix and basement membrane, migration and penetration of blood vessels, transport in the blood circulation, adhesion of tumour cells to endothelial cells in distant organs, invasion, growth, and evasion of host immune and non-immune defences [1, 2]. Certain tumours tend to metastasize to particular organs, and this nonrandom process is, at least in part, the result of specific interactions between tumour cells and endothelial cells in distinct organs [3–6]. Tumour cell adhesion to endothelial cells is complex because numerous tumour cell and endothelial cell surface adhesion molecules have been implicated in this process. The adhesion molecules implicated in metastasis include integrins [7, 8], selectins [9–11], cadherins [8, 12]; CD-44 hyaluronate receptor [8], N-CAM [13], annexins [14, 15], and Lu-ECAM-1 [16].

Carbohydrates have been suggested to play a role in adhesive interactions in metastasis [17–20] and in particular in the adhesion of tumour cells to endothelial cells [17, 18, 21]. These interactions are mediated by carbohydrate-binding proteins (lectins) that bind specific oligosaccharide structures on cell surface or extracellular matrix glycoconjugates [17, 18, 21–27].

Vertebrate lectins that bind galactose-containing glycoconjugates but prefer polyacetosaminoglycan chains are prevalent in various species and tissues [28–31]. These lectins constitute a family of molecules with related amino acid sequences and they have been recently renamed galectins [32]. The two most prominent galectins have apparent molecular weights of 14.5 kDa (galectin-1) and 29–35 kDa (galectin-3). Galectins expressed on the surface of tumour cells may be involved in cell adhesion and metastasis [17, 18]. However, galectins are also present on the surface of certain normal cells, where they may participate in the adhesion of tumour cells [17]. In this study, we examined the expression of galectins in cultured microvessel endothelial cells and analysed the role of galectin-1 in tumour cell-endothelial cell adhesion.

Materials and methods

Endothelial cells

Hepatic sinusoidal endothelial cells from BALB/c mice (C57-HSE); brain endothelial cells from C57B1/6 mice

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(MBE-12); lung microvascular endothelial cells from Fisher 344 rats (RLE); lung microvascular endothelial cells from C57B1/6 mouse (C57-LE-1); and bovine aortic endothelial cells (BAE) were isolated as described previously [33, 34]. Briefly, microvessel trees from various organs were isolated, digested with collagenase, and cultured on gelatin-treated tissue culture plates (Corning, Corning, NY). Colonies of cells having endothelium-like morphology were isolated and characterized biochemically, histologically, enzymatically, and morphologically (by electron microscope). Typical cultures exhibited intercellular tight junctions, lack of platelet binding, presence of Von Willebrand factor antigen and angiotensin converting enzyme, binding of acetylated low-density lipoprotein, and synthesis of a subendothelial matrix containing laminin and type IV collagen [33]. Endothelial cells were cultured in 1:1 (v/v) Dulbecco's modified Eagle's minimal essential medium (DMEM) and Ham's/F12 (GIBCO, Grand Island, NY) containing 2% horse serum (Hyclone Laboratories, Logan, UT), 5% fetal bovine serum (FBS, Hazelton, Lenexa, KS), non-essential amino acids (GIBCO), 1 mM sodium pyruvate, 0.1 mM L-glutamine, and 0.1 mg ml^{-1} endothelial mitogen (ECGF; Biomedical Technologies Inc., Stoughton, MA) in gelatin-coated 24-well plastic tissue culture dishes (Costar, Cambridge, MA) at 37 °C in a humidified 5% CO_2: 95% air atmosphere. After the cultures reached confluence, they were maintained in medium without ECGF.

RAW117-H10 large-cell lymphoma cells

Highly liver-metastatic murine large-cell lymphoma cell line RAW117-H10 cells [35, 36] were grown as suspension cultures in plastic Petri dishes (Falcon, Lincoln Park, PA) in DMEM supplemented with 10% heat-inactivated FBS (Hyclone) and 2.2 mM D-glucose without antibiotics. Cells were tested for *Mycoplasma* contamination by Gen Probe™ (Gen Probe, San Diego, CA) and were found to be negative.

Polyacrylamide gel electrophoresis and immunoblotting

Cells were scraped off the surface of tissue culture dishes in Dulbecco's phosphate-buffered saline (DPBS), pH 7.4 and pelleted by centrifugation. Cell pellets were solubilized in a lysis buffer containing 0.5% Nonidet P-40, 0.25 M sucrose, 0.05 mM $CaCl_2$, 0.1 mM phenylmethylsulfonylfluoride, in 5 mM Tris-HCl buffer, pH 7.4, and samples containing 100 μg cell extract protein were resolved by SDS-PAGE (14% polyacrylamide) as described previously [37]. The proteins were then transferred electrophoretically to a nitrocellulose membrane (0.1 μm, Micron Separation Inc., Westboro, MA) at 150 V for 2 h. The nitrocellulose membrane was then immersed in 0.5% Ponceau S in 1% acetic acid to evaluate sample loading and transfer efficiency. After incubating overnight in a blocking solution containing 3% bovine serum albumin (BSA), 0.9% NaCl, 0.02% sodium azide, in a 10 mM Tris-HCl buffer, pH 7.4, the nitrocellulose membranes were incubated for 3 h with the appropriate antibodies (see below) in 10 ml blocking buffer. The membranes were then washed (0.9% NaCl, 10 mM Tris HCl, pH 7.4) to remove excess unbound antibodies and incubated for 2 h with ^{125}I-goat anti-rabbit IgG (12.7 μCi μg^{-1}, ICN Radiochemicals, Irvine, CA) [1.5×10^6 cpm in 10 ml blocking buffer], washed in the blocking buffer supplemented with 0.1% Tween-20, air dried, and exposed to X-ray film at −70 °C for autoradiography. The following anti-galectin antibodies were utilized: (a) a polyclonal antiserum raised in rabbits against galectin-1 purified from rat lung (kindly provided by Dr Samuel H. Barondes, University of California, San Francisco, CA); and (b) a polyclonal antiserum raised in rabbit against mouse galectin-3 (CBP35) (kindly provided by Dr John Wang, Michigan State University, MI).

Indirect immunofluorescence

Cells grown on glass coverslips (Corning Glass, Corning, NY) were washed with PBS and blocked in 10% normal goat serum (NGS) in PBS for 20 min at 23 °C. After washing with PBS, the cells were incubated with the anti-lectin antibodies diluted 1:50 in 10% NGS in PBS for 1 h at 23 °C. After washing with PBS, the cells were incubated with FITC conjugated goat anti-rabbit IgG (diluted 1:25; Sigma Chemical Co., St. Louis, MO). After washing with PBS three times, the cells were fixed with 3.5% formaldehyde in PBS for 30 min at 23 °C and washed three times with PBS and once with distilled water. The coverslips were mounted on microscope with a mounting solution (70% glycerol and 5% propylgallate in PBS). The cells were observed and photographed using a Nikon fluorescence microscope. Controls included preimmune rabbit serum provided by Dr J. Wang and normal rabbit serum diluted 1:50 instead of the anti-galectin antibodies and second antibody only. These controls did not show any fluorescence staining of the endothelial cells. In some experiments, cell monolayers were prefixed with 3.5% formaldehyde for 30 min at 23 °C before incubation with the anti-galectin antibodies.

Immunohistochemical analysis

Paraffin-embedded blocks of formalin-fixed surgical specimens of head and neck and lung carcinomas were obtained from Dr Adel El-Naggar (Department of Pathology, M. D. Anderson Cancer Center), and 5 μm sections were prepared using a microtome and placed on microscope slides. The sections were deparaffinized in xylene, then rehydrated with decreasing concentrations of ethanol. After two washes with PBS, endogenous peroxidase activity was blocked by incubating the slides in a solution of methanolic hydrogen peroxide for 10 min, followed by three PBS washes. Nonspecific binding was blocked by incubation in NGS for 30 min at 23 °C. The sections were then incubated with anti-galectin antibodies diluted 1:100 in PBS for 2 h at room temperature. After two washes with PBS, the sections

were incubated with a biotinylated goat anti-rabbit antibody or biotinylated goat anti-rat antibody for 30 min followed by avidin peroxidase complex and a peroxidase substrate (Vectastain ABC kit, Vector Labs, Burlingame, CA). The sections were washed in PBS, counterstained with Mayer's Hematoxylin and covered with a glass coverslip using mounting solution. Negative control slides were prepared for each separate specimen group using normal goat serum, or second antibody alone.

Adhesion assays

Adhesion assays were performed, in general, as described previously [4, 14, 38] Confluent monolayers of endothelial cells cultured in 24-well tissue culture dishes were washed once with 1 ml of serum-free medium and incubated for 3 h in 1 ml of serum-free medium. The medium was then removed, and 1 ml of fresh serum-free medium was added. RAW117-H10 cells (2×10^6) in 30 ml of complete medium were labelled overnight with 0.5 μCi ml^{-1} of [^{35}S]methionine (ICN, Irvine, CA; specific activity 400 Ci $mmol^{-1}$), washed in serum-free medium three times, and added to wells containing confluent endothelial cell monolayers (approximately 2×10^5 cells per well) in HEPES-buffered DMEM plus 1% BSA (adhesion medium). The endothelial cell monolayers were fixed with 0.0125% glutaraldehyde in DPBS for 45 min with rocking at room temperature, to prevent the endothelial monolayer from detaching during subsequent repeated washes. Excess glutaraldehyde was removed, and the fixed monolayers were washed three times with DPBS, incubated with 1% BSA in DPBS for 1 h, and then stored at −70 °C or used as described above in the adhesion assays. The tumour cells were incubated on the endothelial monolayers at 37 °C on an orbital shaker (60 rpm). At various times the nonadherent tumour cells were removed by gentle aspiration, followed by addition of serum-free medium. This was repeated four times, and the remaining adherent tumour cells were solubilized with 150 μl M NaOH, neutralized with 150 μl 1 M acetic acid, mixed with ScintiVerse II scintillation cocktail (Fisher Scientific, Pittsburgh, PA), and radioactivity was quantitated with a Beckman Model LS 7500 scintillation counter.

Inhibition of RAW117-H10 cell adhesion by anti-galectin-1 antibodies was carried out as follows. Fixed endothelial cell monolayers were incubated for 30 min at room temperature with 10 μg ml^{-1} of polyclonal rabbit anti rat lung galectin-1 that had been purified by affinity-chromatography using immobilized human placenta galectin-1 in DPBS. The endothelial cell monolayers were rinsed three-times with DPBS and used in adhesion assays with radiolabelled RAW117 cells. Control samples contained normal rabbit serum diluted 1:20 in DPBS. The number of adherent tumour cells was calculated using the equation: No. bound cells per well = (Total CPM bound per well) ÷ (CPM per cell), and the percentage of attachment was then calculated taking the input cell number as 100%. The assays were performed in triplicate, and the standard deviation was calculated for each experiment. ANOVA statistical analysis was performed on various time points in the assays.

Results

Expression of galectin-1 and galectin-3 by endothelial cells

The analysis of extracts from endothelial cells isolated from different organs by immunoblotting, which detects both intracellular and cell surface-associated lectin, revealed that all cell types expressed galectin-1, albeit in differing amounts. BAE cells expressed the lowest amounts of galectin-1, whereas murine lung endothelial cells expressed the highest amounts. All but the murine hepatic sinusoidal endothelial cells also expressed galectin-3, and the murine lung endothelial cells expressed the highest amount of galectin-3 (Fig. 1).

Expression of galectin-1 and galectin-3 on the surface of cultured endothelial cells

Using indirect immunofluorescence the endothelial cells examined bound anti-galectin-1 and anti-galectin-3 antibodies on their surface (Fig. 2). The binding of the antibodies was detected at the cell border as well as in a fibrillar pattern that was very similar to the binding of anti-laminin antibodies in endothelial cell cultures (data not shown). The staining intensity was variable among the cell types and did not correlate precisely with the immunoblotting results. For example, BAE cells expressed higher amounts of galectin-1 on their surface then the other cell lines, but they contained relatively less galectin-1 by immunoblotting. This may reflect differences in the distribution of lectin in the different cell types. Although galectin-3

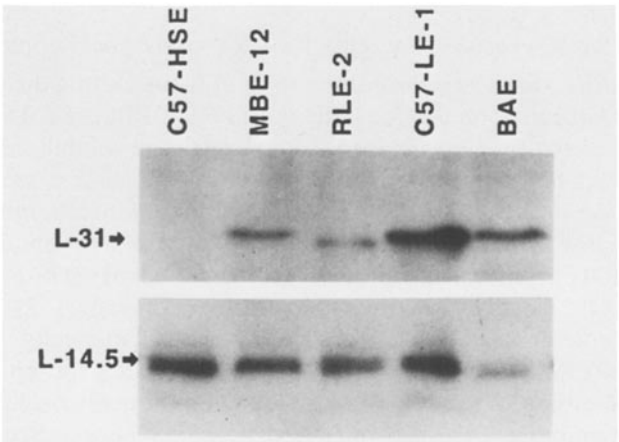

Figure 1. Expression of galectins in endothelial cells. Extracts prepared from endothelial cells isolated from different organs were analysed by immunoblotting using antibodies against galectin-1 (L-14.5) or galectin-3 (L-31) as described in Materials and methods.

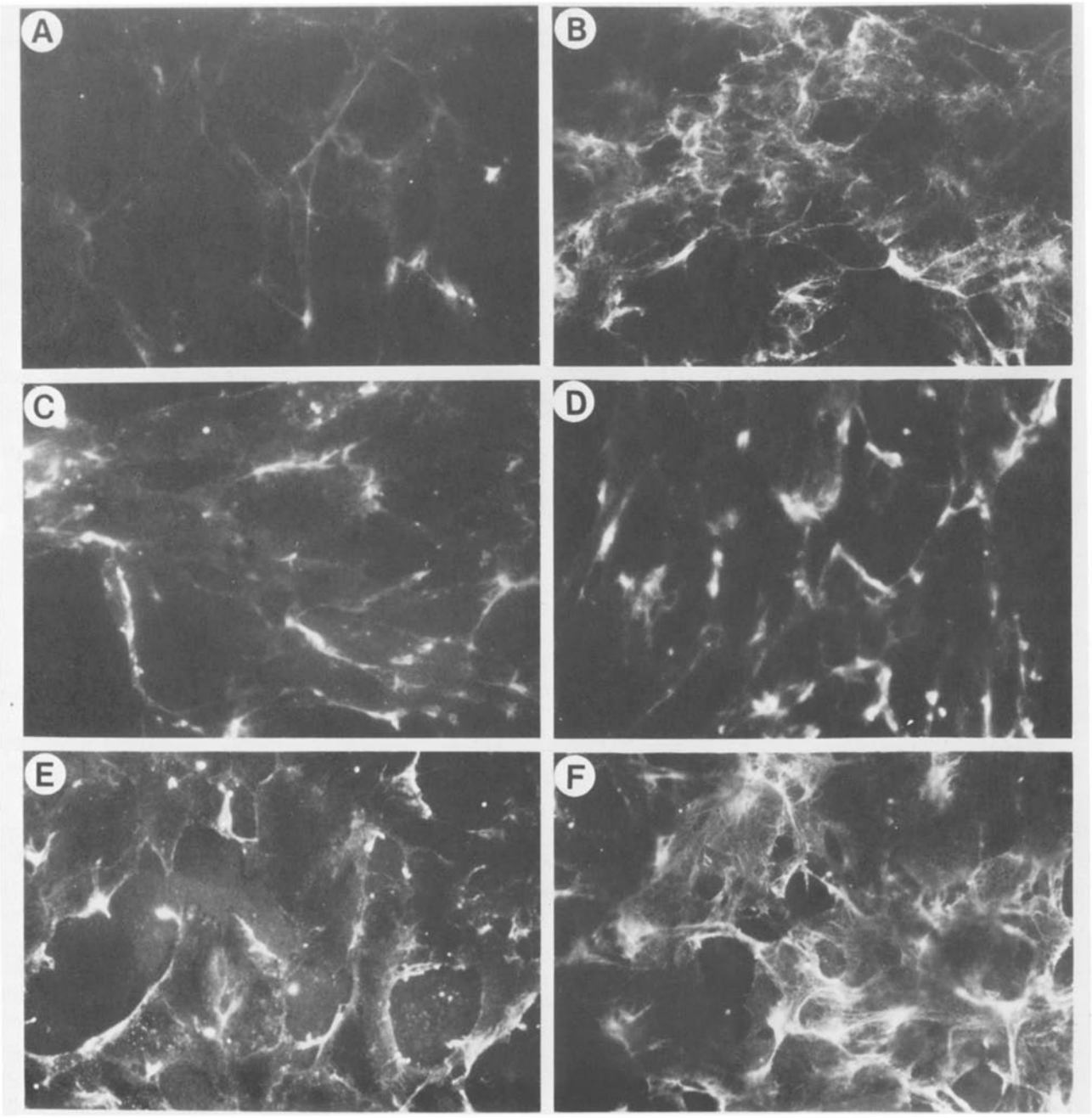

Figure 2. Detection of galectins on the surface of endothelial cells. Cells grown on glass cover slips were labelled by indirect immunofluorescent antibody technique as described in Materials and methods using anti-galectin-3 antibodies (A, C, and E) or anti-galectin-1 antibodies (B, D, and F). A, and B: HSE cells; C and D, RLE-1 cells; and E, and F, BAE cells.

was not detected in C57-HSE by immunoblotting, the cells stained faintly with anti-galectin-3 antibodies (Fig. 2A). This may indicate that the immunofluorescence labelling method is more sensitive than immunoblotting. The native distribution of galectins revealed by indirect immunofluorescence labelling on the surface of cells that had been prefixed was quite similar to that shown in Fig. 2 for cells that had not been prefixed.

Expression of Galectins in Microvessel Endothelial cells in vivo

Immunohistochemical staining of microvessel endothelial cells present in adjacent normal tissue in carcinoma specimens showed that both galectins are expressed in these cells *in vivo* (Fig. 3). Thus, there is a concordance between the *in vitro* and the *in vivo* galectin expression.

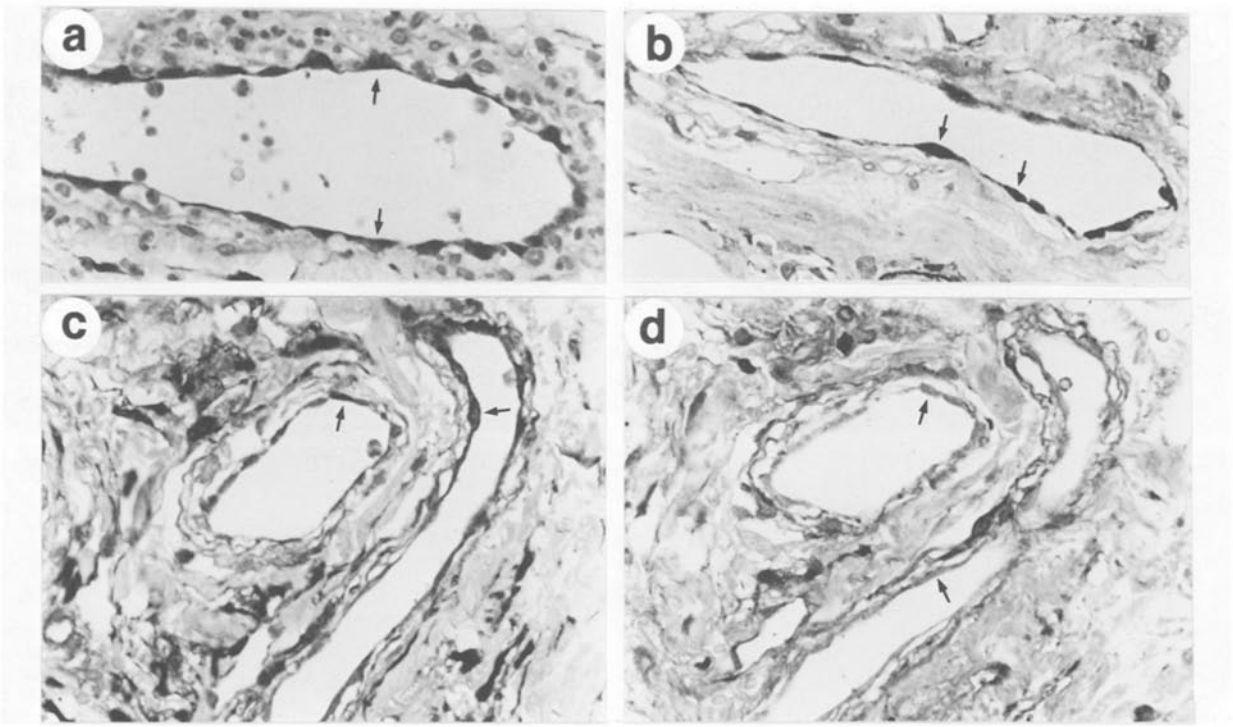

Figure 3. Detection of galectins in microvessel endothelial cells *in vivo*. Histological sections of head and neck (a and b) and lung (c and d) tissues from cancer patients were analysed by Vectastain ABC procedure using antibodies against galectin-1 (a and c) and galectin-3 (b and d) as described in Materials and methods. The arrows indicate the location of immunostained endothelial cells lining the microvessel wall.

Inhibition of attachment of large-cell lymphoma cells to endothelial cells by anti-galectin-1 antibodies

The rate of attachment of RAW117-H10 cells to liver endothelial cells was greater than to lung endothelial cells (Fig. 4), reflecting their *in vivo* organ preference for metastasis [35, 36]. When affinity-purified anti-galectin-1 antibodies were allowed to bind to the endothelial cells before incubation with the tumour cells their attachment to either type of endothelial cell was inhibited. The inhibition of attachment of leukaemia cells to liver and lung endothelial cells of about 30% and 75%, respectively by the anti-galectin-1 antibodies was significant ($p < 0.05$) after a 90 min incubation.

Discussion

This is the first direct demonstration of the expression of galectins in endothelial cells by a combination of immunoblotting and immunolocalization techniques. Previous studies suggested the presence of galactose-specific lectins on the surface of endothelial cells by indirect methods. For example, hepatic rat endothelial cells were shown to take up particles coated with lactosylated serum albumin [39, 40], and endothelial cells from gingiva were found to bind lactosylated serum albumin, presumably via galactose-specific lectins [41]. Anti-galectin-1 antibodies were found to bind to endothelial cells in histologic sections of tissue specimens and to the extracellular matrix produced by cultured endothelial cells [42].

The physiological function of galectins in endothelial cells is not clear. The identification of endothelial cell surface glycoconjugates that bind galactose-specific plant lectins *in vitro* and *in vivo* [33, 43–45], suggests that cell surface galectins may bind such molecules on adjacent neighbouring cells to mediate homotypic adhesion or initiate junctional coupling. The distribution of the galectins on the cell surface resembled the distribution of laminin. This was not surprising, because the presence of galectin-1 in the extracellular matrix synthesized *in vitro* by endothelial cells indicates that the endothelial cells secrete or externalize this lectin [42], and both galectin-1 [46] and galectin-3 [47] have been shown to bind laminin. In addition, galectin-1 secreted by fibroblasts or endothelial cells may act as an autocrine or paracrine growth stimulator for endothelial cells [48].

The possible role of galectins in adhesion of tumour cells as suggested by our findings has been proposed previously on the basis of less direct evidence. First, the expression of galactose on the tumour cell surface has been correlated with the degree of colorectal cancer metastasis to liver [47]; second, experimental metastasis was prevented by D-galactose [49, 50]; third, modification of blood borne arrest

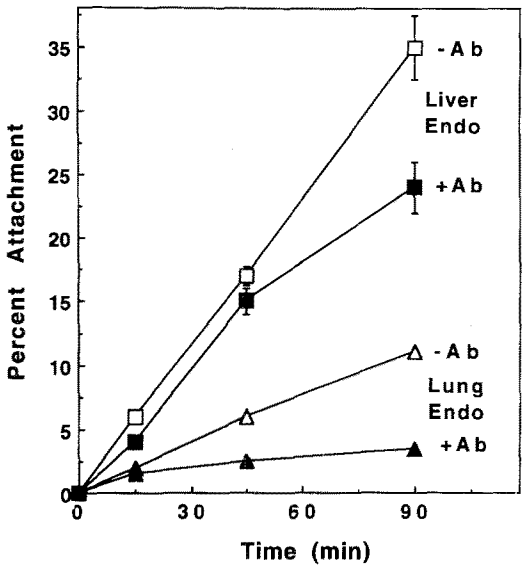

Figure 4. Attachment of large cell lymphoma cells to endothelial cell monolayers and inhibition by anti-galectin-1 antibodies. Confluent monolayer cultures to either mouse C57-HSE hepatic sinusoidal endothelial cells (liver endo) or mouse C57-LE-1 lung microvessel endothelial cells (lung endo) were incubated with control normal rabbit serum diluted 1:20 (−Ab) or with affinity-purified anti-galectin-1 antibodies (10 μg ml^{-1}) (+Ab) and then washed prior to addition of radioactively-labelled RAW117-H10 lymphoma cells. Cell attachment was determined after each of the indicated incubation times as described in Materials and methods. The difference in cell attachment in the presence of anti-galectin-1 antibodies and controls were significant ($p < 0.05$) after 90 min of attachment to either type of endothelial cell.

properties of tumour cells by inhibitors of glycosylation suggested the existence of endogenous lectins [39, 51]; and fourth, the strongest indication for galectin's involvement in tumour cell adhesion was that a glycosylation-deficient mutant lacking sialic acid and galactose derived from a lymphoreticular tumour adhered poorly to microvascular endothelial cell monolayers, whereas properly glycosylated parental cells were highly adherent to the endothelial cells. Further, galactosylation of cell surface glycoconjugates on the surface of the mutant tumour cells by galactosyl transferase and UDP-galactose increased the ability of the cells to adhere to the endothelial cells *in vitro* and to form liver metastases after i.v. injection [21]. Other indications for galectin-1-mediated adhesion include the adhesion of human ovarian carcinoma cells to immobilized, cross-linked galectin-1 [22, 52], which mimics galectin-1-mediated adhesion of lymphoma cells to the endothelial cell surface or to the matrix produced by the cells. Likewise, soluble galectin-1 was found to enhance the adhesion of embryonal carcinoma cells to laminin-coated substratum [53].

The rate of adhesion of liver-metastatic RAW117-H10 cells to liver endothelial cells was significantly higher than to lung microvessel endothelial cells as observed previously [4]. The mechanism underlying this differential rate of adhesion cannot be explained on the basis of galectin expression, because the lung endothelial cells expressed considerably more galectin-3 and similar amounts of galectin-1 than the liver sinusoidal endothelial cells. Nonetheless, our finding that anti-galectin-1 antibodies decreased the adhesion of RAW117-H10 tumour cells to endothelial cell monolayers strongly suggests that galectin-1 may mediate, in part, the adhesion process. The fact that the antibodies did not cause complete inhibition of RAW117-H10 tumour cell adhesion to endothelial cells was expected in view of our previous findings that the adhesion of these cells to liver endothelial cells involves several other adhesion molecules. Specifically, the involvement of integrins in RAW117 cell-endothelial-cell adhesion was indicated by the ability of RGD-containing peptide polymers to inhibit the adhesion of RAW117 tumour cells to liver sinusoidal endothelial cells by about 40% [7]. In addition, specific antibodies against annexin II inhibited RAW117 cell adhesion to liver sinusoidal endothelial cells by 35–50%, suggesting that annexins may also be involved in RAW117 cell-endothelial cell adhesion [14, 38]. Both integrins and annexin require calcium ions for activity in contrast, galectins can mediate calcium independent adhesion.

In conclusion, we have demonstrated that both galectin-1 and galectin-3 are expressed in various endothelial cells and that galectin-1 may contribute to the adhesion of tumour cells to endothelial cells.

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