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Synthesis of an endogeneous lectin, galectin-1, by human endothelial cells is up-regulated by endothelial cell activation

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The pattern of expression of an endogenous lectin, galectin-1, was examined in human lymphoid tissue. Galectin-1 was detected in the endothelial cells lining specialized vessels, termed high endothelial venules, in activated lymphoid tissue, but not in a resting lymph node. Cultured endothelial cells (human aortic and umbilical vein endothelial cells (HAECs and HUVECs)) expressed galectin-1. Activation of the cultured endothelial cells increased the level of galectin-1 expression, as determined by ELISA, Northern blot analysis and high throughput cDNA sequencing. These results suggest that galectin-1 expressed by endothelial cells may bind to and affect the trafficking of cells emigrating from blood into tissues.

Keywords: lectin; galectin-1, endothelial cells, high endothelial venules, lymph node

Introduction

The galectins are a family of β-galactoside-binding lectins which are found in species ranging from sponges to humans [1-6]. Despite the high degree of structural conservation among the members of this family of lectins, and the broad distribution of these molecules in a variety of tissues, the precise function of the galectins in any specific tissue is not fully understood. A number of workers have shown that galectins play a role in cell adhesion and cell migration [7–9]. In addition, there are data demonstrating that galectins are immunosuppressive in animal models of autoimmune disease [10, 11]. We have recently found that human galectin-1, a 14.5 kDa member of the family, is expressed by thymic epithelial cells and appears to mediate the adhesion of immature T cells to thymic epithelial cells (Baum *et al.*, J Exp Med, in press). We have also found that galectin-1 binds to circulating mature T cells and activated T cell blasts [12].

The ability of galectin-1 to bind to T cells, and the immunosuppressive properties of galectin-1, prompted us to examine whether galectin-1 was expressed in peripheral lymph nodes, the site of T cell interaction with antigen-presenting cells [13]. The present study demonstrates that galectin-1 was present in specialized vessels, the high endothelial venules, in activated lymphoid tissue. Moreover, we found that cultured endothelial cells expressed galectin-1, and that expression increased when the endothelial cells were activated. These results are discussed in relation to the trafficking of normal leukocytes and of metastasizing tumour cells.

Materials and methods

Antibodies and reagents The polyclonal rabbit antiserum against galectin-1 was prepared as described in [14]. Control antiserum for immunohistochemistry and ELISA was prepared by incubating the anti-galectin-1 antiserum with recombinant human galectin-1 conjugated to Sepharose beads overnight at 4°C. After removal of the beads by centrifugation, the remaining material was used as adsorbed antiserum. Purified recombinant galectin-1, expressed in *E. coli*, and galectin-1 cDNA were obtained as described in [14].

Tissues and cells Human tonsil was obtained at the time of surgery. Unstimulated human lymph nodes were obtained at autopsy. HAECs were isolated and grown as previously described [15]. For some studies, HAECs were treated for 4 h with 100 μg ml^{-1} of minimally oxidized low density lipoprotein (MM-LDL), prepared as described previously [16], or with 2 ng ml^{-1} of lipopolysaccharide.

Immunohistochemistry Tonsil and lymph node specimens were fixed in 4% paraformaldehyde for 24–72 h at 4°C, and embedded in paraffin. For immunohistochemistry, 6 μm sections were incubated with the polyclonal rabbit antiserum to galectin-1, diluted 1:1000 in 0.01 M sodium phosphate, 0.15 M NaCl, 1% bovine serum albumin, pH 7.5 (PBA) overnight at

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4°C. Control sections were incubated with adsorbed immune rabbit serum at a final dilution of 1:1000 in PBA. Sections were washed three times with PBA at room temperature, and incubated with a goat anti-rabbit reagent conjugated to horseradish peroxidase (HRP) (Bio-Rad), diluted 1:1000 in PBA, for 2 h at room temperature. After washing with PBA, bound antibody was detected by the addition of the chromogenic substrate AEC (Peroxidase Chromogen kit, Biomeda), and slides were counterstained with Haematoxylin.

Western analysis Tonsil stroma, obtained after removal of lymphocytes by teasing the tissue and straining through a steel mesh, was extracted by homogenization in lysis buffer (10 mM TRIS, pH 7.3, 130 mM NaCl, 5 mM $CaCl_2$, 1 mM PMSF, 0.5% NP-40). Cultured HAECs were lysed by brief vortexing in lysis buffer. Nuclei were removed by centrifugation in the cold for 2 min at top speed in an Eppendorf 5415 microcentrifuge. Protein concentrations of the supernatants were assayed using the BCA protein assay kit (Pierce). Fifty μg of protein were loaded on SDS-PAGE gels, along with 4 μg of purified recombinant galectin-1, and electrophoresed under reducing conditions. Western blot analysis was performed exactly as described in [14], using the polyclonal rabbit anti-galectin antiserum diluted 1:1000 in PBS containing 1% nonfat dry milk.

Enzyme linked immunosorbant assay HAECs were cultured in 96 well dishes and processed as previously described [16]. For non-permeabilized cells, the wells were rinsed with medium 199, and anti-galectin-1 antibody was added at a 1:1000 dilution. For permeabilized cells, the cells were fixed for 30 min with 4% paraformaldehyde, washed three times with PBS and permeabilized for 15 min with 0.5% Triton X-100 at room temperature. After permeabilization, the cells were washed with PBS, and anti-galectin-1 antibody was added as described above. The cells were incubated with the primary antibody for 2 h on ice. The wells were rinsed with PBS, and a goat anti-rabbit secondary antibody, conjugated to alkaline phosphatase, was added at a 1:1000 dilution. The cells were incubated for 1 h on ice. After washing with PBS, bound antibody was detected by the addition of para-nitrophenyl phosphate. The absorbance at 405 nm was determined, and values were expressed as optical density (OD) units at 405 nm. For control samples, secondary antibody alone was added to the cells in the wells. The control values were subtracted from the experimental values to determine the relative amount of antibody bound. Six replicate samples were assayed in each experiment.

Northern analysis Total RNA was prepared from cultured HAECs by the guanidinium/thiocyanate method [17]. Analysis was performed as previously described [18], using a 311 bp *Eco* R1 restriction fragment of human galectin-1 [14] labelled with [α-^{32}P] dCTP by random priming (Amersham). The α-tubulin probe was identical to that described in [16]. Autoradiographs of the Northern blots were densitometrically scanned to determine the relative amounts of galectin-1 and tubulin mRNA.

High throughput cDNA sequencing An aliquot of 2×10^8 primary HUVEC cells (passage 5) (Cell Systems Inc.) was treated at confluence with 1 U ml^{-1} interleukin-1β and 100 ng ml^{-1} *E. coli* lipopolysaccharide (LPS) for 5 h, and combined with a second aliquot treated with 4 U ml^{-1} tumor necrosis factor–α and 2 U ml^{-1} interferon γ for 96 h. A control aliquot (passage 1-3) was untreated. The cells were harvested and cytoplasmic RNA was prepared by differential sedimentation to remove nuclei, followed by direct phenol extraction and precipitation [19]. Poly(A^+) RNA was prepared from each sample using the PolyATtract mRNA Isolation System (Promega Corp.). The mRNA was used to construct a cDNA library using the lambda insertion vector Uni-ZAP XR (Stratagene). Following bulk *in vivo* excision of the pBluescript Phagemid from the Uni-ZAP vector, colonies were picked at random for DNA preparation. Isolated plasmid DNA was sequenced on a 373A sequencer (Applied Biosystems) using the T3 primer. The resulting DNA sequences were used to identify each clone as to its gene of origin using the Inherit search program (Applied Biosystems). Clones corresponding to galectin-1 were scored by virtue of their identity to the published sequence [14] and were expressed as a percentage of total genes sequenced.

Results

Immunohistochemical detection of galectin-1 in activated lymphoid tissue Activated lymph nodes have a distinctive morphological appearance, marked by the presence of secondary lymphoid follicles with germinal centres. In contrast, unstimulated or resting lymph nodes have only primary follicles with no germinal centres [13]. We examined an activated lymphoid tissue, tonsil, and a resting lymph node for expression of galectin-1 by immunohistochemical analysis.

As shown in Fig. 1, the small vessels in the activated lymphoid tissue demonstrated strong reactivity with the polyclonal rabbit antiserum to human galectin-1 (Fig. 1A–C). These small vessels, located in the interfollicular zone of the lymph node, are called high endothelial venules (HEV), and are composed only of a layer of specialized endothelial cells with an underlying basal lamina, with no subendothelial layer of smooth muscle. The precipitated material, indicating the presence of bound antibody, completely lined the lumens of the small vessels. Staining was seen within the endothelial cell cytoplasm (Fig. 1C), consistent with the intracellular localization previously describe for galectin-1 [2, 20, 21]. In contrast to the reactivity of the HEV seen in the activated tonsil, the HEV in the resting lymph node demonstrated minimal staining with the antiserum to galectin-1 (Fig. 1G, H). In both activated and resting lymphoid tissue, the small lymphocytes surrounding the HEVs were detected by nuclear staining with haematoxylin. These results indicated that galectin-1 could be

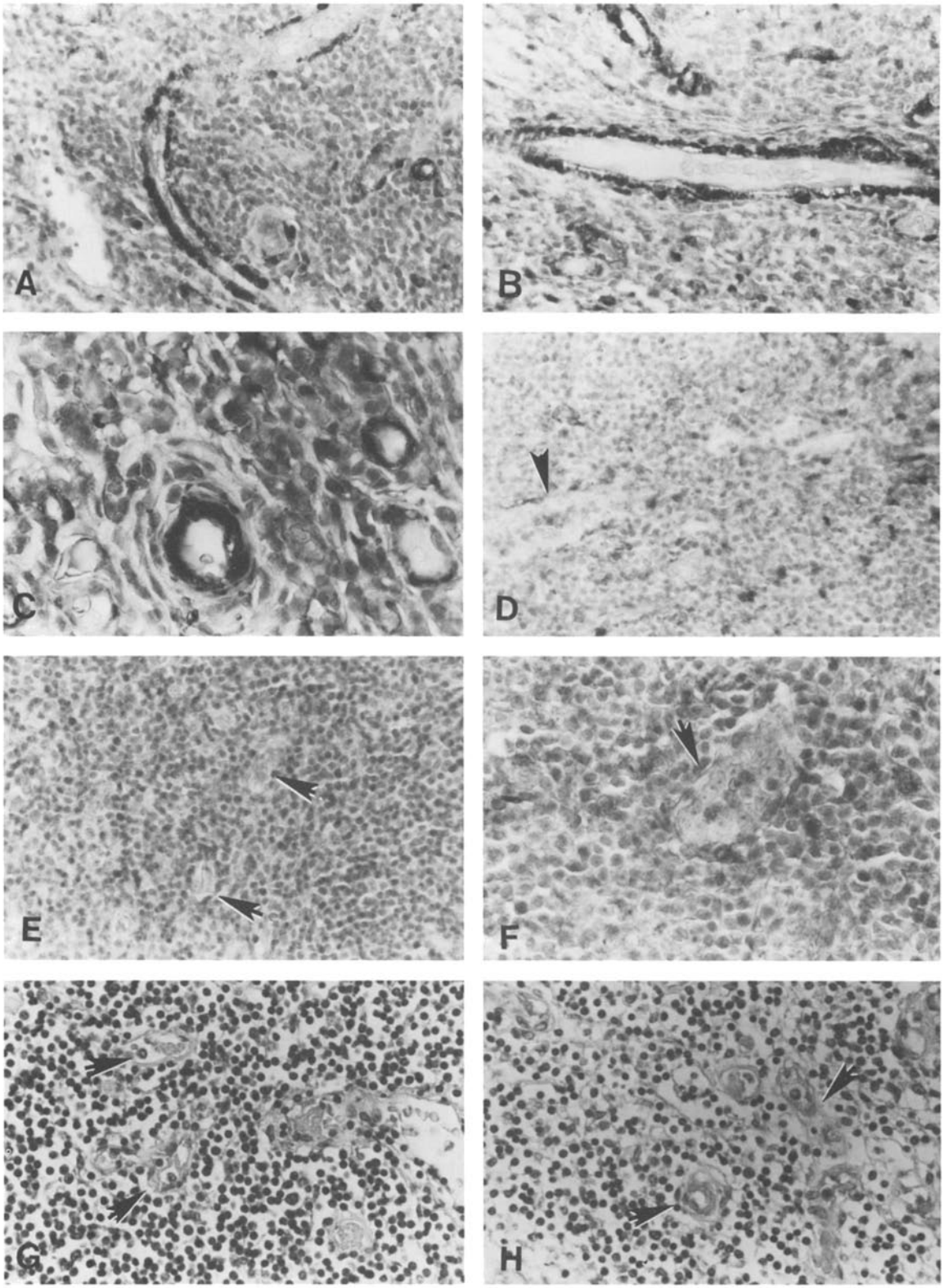

Figure 1. Expression of galectin-1 in HEV of stimulated and resting peripheral lymphoid tissue. Sections of human tonsil (A–F) or abdominal lymph node (G, H) were incubated with rabbit anti-galectin-1 antiserum (A–C, G) or with antiserum which had been adsorbed on galectin-1 beads (D–F, H). Antibody binding was detected by addition of goat-anti-rabbit-HRP reagent, and chromogenic substrate. Positive staining is seen as the dark precipitate lining the vessels in the tonsil (A–C), but not in the resting lymph node (G, vessels identified by arrowheads). In the control samples, where no staining of vessels is seen, the vessels are also indicated by arrowheads (D–F, H). Magnification: A, B, D, E, G, H 250×; C, F, 400×.

expressed by endothelial cells, in blood vessels which lack a layer of smooth muscle cells. In addition, the presence of galectin-1 in HEV of activated, but not resting, lymph nodes suggested that activation of endothelial cells resulted in increased galectin-1 expression.

The control slides in Fig. 1 were reacted with the polyclonal antiserum to galectin-1 which had been adsorbed with galectin-1-Sepharose, to demonstrate the specificity of the staining (Fig. 1D–F). However, since galectin-1 is a member of a family of highly homologous molecules which often demonstrate antibody cross-reactivity, it was possible that the positive staining we saw in the tonsil HEV was due to expression of a member of the galectin family other than galectin-1. To establish that galectin-1 was present in the tonsil tissue, we performed an immunoblot analysis on an extract of tonsil stroma. As shown in Fig. 2, the tonsil stroma extract contained an immunoreactive band which co-migrated on SDS-PAGE with recombinant human galectin-1. No other cross-reactive bands were detected with the anti-galectin-1 reagent in the tonsil stroma. These results indicated that the immunoreactive material detected in the HEV by immunohistochemistry was galectin-1.

Increased expression of galectin-1 in activated cultures of human endothelial cells We examined cultured human aortic endothelial cells (HAECs) for the expression of galectin-1. While HAECs are not identical to the specialized endothelial cells lining HEV, it has been demonstrated that, upon activation, these cells express adhesion molecules such as E-selectin and VCAM-1, and increase expression of ICAM-1 [22], and are thus useful model cells to study molecules involved in endothelial cell adhesion.

As shown in Fig. 2, we first examined whether HAECs expressed galectin-1. Immunoblot analysis of HAEC cell extract demonstrated a single band which co-migrated with recombinant human galectin-1. To examine the effect of endothelial cell activation on galectin-1 expression, we performed an ELISA assay to quantitate the amount of galectin-1 protein produced by the HAECs. In this assay, the anti-galectin-1 antibody was added to intact, unpermeabilized HAEC monolayers, in which only the apical surface of the cells is available to react with the reagent. In unstimulated HAECs, the amount of galectin-1 on the cell surface was low (approximately 5% of the total cellular galectin-1), compared with the total amount of galectin-1 detected in permeabilized cells. Stimulation of the HAECs with MM-LDL resulted in a 47–79% increase in the amount of galectin-1 on the apical surface of the cells (Table 1). The observed increase in galectin-1 on the unpermeabilized cells was not due to disruption of the cells by MM-LDL, since we have previously demonstrated that MM-LDL does not alter endothelial cell integrity [22]. Thus, the increased expression of galectin-1 on the stimulated cells resulted either from externalization of cytoplasmic galectin-1, or from increased synthesis of galectin-1.

To examine the relative amount of galectin-1 expression in mock-treated vs MM-LDL stimulated cells, we performed slot blot analysis. The total amount of galectin-1 in the treated cells increased approximately 2.5-fold, relative to mock-treated cells (data not shown). This increase in galectin-1 expression in activated HAECs appeared to result from an increase in the level of mRNA encoding galectin-1. We performed Northern analysis of total RNA isolated from HAECs at various times following addition of MM-LDL. A single transcript of approximately 700 bp was detected by the galectin-1 probe in all of the samples (Fig. 3). The size of this transcript was identical to that shown to encode human galectin-1 [14]. Figure 3 demonstrates that the level of mRNA encoding galectin-1 began to increase by 2 h following cellular activation, and reached a maximum level of increase by 4 h following stimulation (approximately two-fold increase, compared with control). The increase in the level of galectin-1 mRNA persisted for 24 h following cell activation by MM-LDL. As a control, the level of mRNA encoding the housekeeping protein tubulin was determined. These results demonstrated that the increased level of expression of

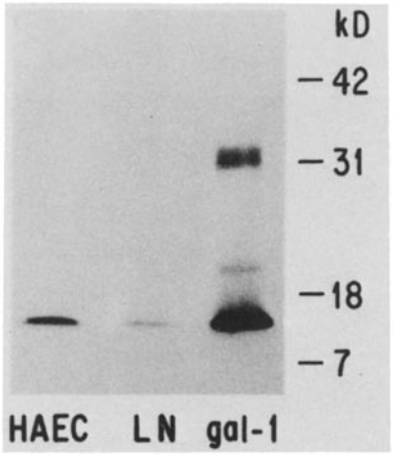

Figure 2. Galectin-1 is expressed in activated lymph nodes and in cultured HAEC. Extracts of tonsil stroma (designated LN, for lymph node) and unstimulated HAEC were analysed by Western blot, using rabbit anti-galectin-1 antiserum. In both samples, a single band of approximately 14 kDa co-migrated with purified recombinant galectin-1 (gal-1).

Table 1. Expression of cell surface galectin-1 on resting and stimulated HAECs

Treatment	*Exp. 1*[a]	*Exp. 2*	*Exp. 3*
Medium alone	0.32 +/– 0.03	0.80 +/– 0.06	0.34 +/– 0.03
MM-LDL	0.48 +/– 0.03	1.43 +/– 0.07	0.50
Percentage increase	50%	79%	47%

[a] Binding of anti-galectin-1 antibody to the surface of HAEC cultured in medium alone, or treated with MM-LDL (100 μg ml^{-1}). Data are presented as the mean +/– SD of six replicate wells in each experiment, and expressed as OD units at 405 nm.

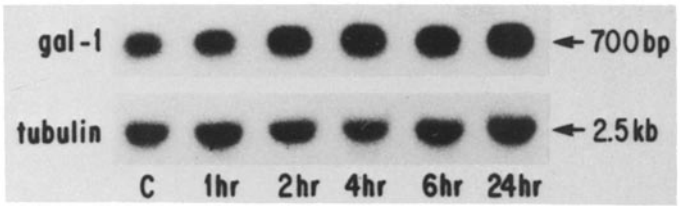

Figure 3. Effect of MM-LDL treatment of HAEC on levels of galectin-1 mRNA. HAEC were treated for the hours shown with MM-LDL (100 μg ml^{-1}), or with medium alone. Total RNA was prepared from the cells and analysed by Northern blot using a probe for galectin-1 (gal-1), or tubulin, to evaluate the amount of RNA loaded in each lane.

Table 2. Expression of mRNA encoding galectin-1, detected by high throughput cDNA sequencing, in resting and stimulated HUVECs

	Galectin-1 cDNA clones	*Clones sequenced*	*Relative frequency*
Mock-treated	0	1790	<0.06%
cytokines + LPS	12	1810	0.66%

2×10^8 primary HUVECs were treated with cytokines and LPS, or untreated. cDNA clones were prepared and sequenced as described in Materials and methods.

galectin-1 protein in activated HAECs resulted at least in part from an increase in the level of galectin-1 message.

An increase in galectin-1 message was also detected in HUVECs stimulated with cytokines and LPS (Table 2). In these experiments, cDNA clones were made from polyA$^+$RNA isolated from stimulated and mock-treated cells. Each cDNA clone was sequenced and the sequence compared with known cDNA sequences. The frequency of each sequence was scored, and this frequency reflects the relative amount of the corresponding mRNA in the cells. In the total cDNA made from mock-treated cells, no clones corresponding to galectin-1 were detected in the first 1790 clones sequenced, yielding a relative abundance of <0.06% of total cellular mRNA. In contrast, in the total cDNA made from stimulated HUVECs, 12 cDNA clones encoding galectin-1 were identified in the first 1810 clones sequenced, yielding a relative abundance of 0.66%.

Discussion

These data demonstrated that human endothelial cells synthesize galectin-1. Activation of human aortic endothelial cells with MM-LDL, a naturally occurring metabolite of LDL which has been shown to stimulate endothelial cells *in vitro* and *in vivo* [15, 16, 22, 23], resulted in a small but significant increase in the amount of galectin-1 protein on the cell surface and galectin-1 mRNA in the cells. A more dramatic increase in the relative abundance of galectin-1 transcripts was seen in activated, compared with mock-treated, HUVECs, as shown by high throughput cDNA sequencing. As we found in the HAECs by Northern analysis (Fig. 3), stimulation of HUVECs with cytokines and LPS increased the relative abundance of galectin-1 mRNA transcripts detected by this method more than 10-fold over unstimulated cells (Table 2).

A number of investigators have performed biochemical and immunohistochemical analyses of animal and human tissues, to detect the presence of galectin-1 and related lectins [24–26]. Endothelial cell production of galectin-1 has not been previously demonstrated directly, although Allen and co-workers noted capillary wall staining with antibody to galectin-1 in tumour tissues [26]. Wasano and co-workers have identified galectin-1 in rabbits only in the smooth muscle layer of large vessels such as arteries and veins [25]. Two possible explanations exist for the lack of endothelial cell staining for galectin-1 in many previous studies. First, endothelial cells in different organs and in different types of vessels are highly specialized, and have been shown to express unique complements of adhesion molecules [27]. Since none of the previous studies directly examined HEV in lymph nodes, it is possible that galectin-1 expression is restricted to endothelial cells in vessels of a certain type or in certain tissues. Second, several of these studies examined tissues obtained from laboratory animals which were not treated with endothelial cell-activating agents. As we found in the unstimulated lymph node (fig. 1G), the level of galectin-1 expression by endothelial cells under these conditions may have been too low to be detectable by immunohistochemical analysis.

Our laboratory and others have shown that galectin-1 binds to T and B lymphoblastoid cells [12, 28]. The increase in galectin-1 expression by HEV in activated lymphoid tissue suggests that galectin-1 may play a role in the adhesion and migration of lymphocytes, or other cells bearing ligands recognized by galectin-1, into lymph nodes. An increase in the branched polylactosamine chains preferentially recognized by galectin-1 has been described in a number of types of tumour cells, and appears to relate to the ability of these cells to metastasize. Dennis and co-workers have demonstrated that murine tumour cells bearing the branched polylactosamine sequences preferentially bound to lectin on bovine endothelial cells, and predicted that this lectin or lectins would be members of the galectin family [29]. This group has also shown that treatment of human tumour cells with swainsonine, to inhibit the addition of polylactosamine sequences to *N*-linked oligosaccharides, decreased the metastatic potential of these cells in an animal model, leading to current clinical trials to determine the efficacy of swainsonine therapy in humans [30]. Since endothelial cells in tumours and in draining lymph nodes are exposed to activating agents such as TNF and IL-1, our data demonstrating increased galectin-1 synthesis in activated endothelial cells is relevant to this model of metastasis. In addition, galectin-1 synthesis by endothelial cells may be part of an autocrine process stimulating endothelial cell proliferation, since lung galaptin has been shown to stimulate proliferation of vascular cells [31].

The fact that galectin-1 expression was increased by minimally oxidized LDL suggests a potential role for this lectin in atherosclerosis as well. Oxidized lipoproteins have been shown to be present and to play a role in atherosclerotic lesion development (reviewed in [32]). Galectin-1 may contribute to the recruitment of the activated T cells that are present in atherosclerotic lesions [33, 34].

Endothelial cells have evolved a complex but efficient process to allow recruitment and immigration of specific types of effector cells to sites of inflammation in various types of tissues [27]. The importance of endogenous lectins in this process has been demonstrated. The selectins, a family of Ca^{2+}-dependent lectins, tether rapidly moving blood leukocytes to endothelial cells at sites of inflammation, and promote subsequent interactions with additional adhesion molecules which result in migration of the cells into the tissue [27, 35]. The present study indicates that a member of the galectin family may also play a role in the interaction of circulating cells with endothelium.

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