Endothelial L-Selectin Ligands Are Likely to Recruit Lymphocytes into Rejecting Human Heart Transplants

Sanna Toppila,* Timo Paavonen,[†]
Markku S. Nieminen,[‡] Pekka Häyry,[§] and
Risto Renkonen*

From the Department of Bacteriology and Immunology,* the
Department of Pathology,[†] and the Transplantation Laboratory,[§]
Haartman Institute; and the Department of Internal Medicine,[‡]
University of Helsinki and The Helsinki Universital Central
Hospital, Helsinki, Finland

L-selectin-dependent lymphocyte extravasation is a hallmark of acute heart allograft rejection in rats. On screening over 600 endomyocardial biopsies (EMBs), taken at different time points after heart transplantation in man, we identified 91 samples with histological signs of acute rejection. Rejection and nonrejection EMBs were analyzed for the presence of properly glycosylated, ie, sulfated sialyl Lewis-x (sLex) decorated L-selectin ligands. Two anti-sLex (2F3 and HECA-452) and one anti-6- or 6'-sulfated and/or 6,6'-bisulfation (MECA-79) monoclonal antibodies were used. Nonrejecting heart endothelium did not express, or expressed only weakly, sulfated and or sLex decorations of L-selectin ligands. On the contrary, these epitopes were readily detectable on endothelium of capillaries and venules at the onset and during acute rejection episodes. The more intense the sulfated sLex expression was, the more severe the rejection episode was in histological grading. The endothelial expression of L-selectin ligands decreased to background levels as the rejection resolved. Our data demonstrate a complete correlation between the level of expression of the sulfated sLex-decorated ligands on the one hand and the histological severity of acute heart allograft rejection on the other hand. These data suggest that functionally active endothelial L-selectin ligands are instrumental in lymphocyte extravasation into the human heart allografts at the onset and during acute rejection episodes. (*Am J Pathol* 1999, 155:1303-1310)

Acute heart allograft rejection is characterized by heavy infiltration of lymphocytes.^{1,2} To infiltrate the graft and to promote rejection, the lymphocytes extravasate from blood through the vascular endothelial layer into the graft parenchyma.³⁻⁵ Extravasation of lymphocytes is initiated

by an interaction of members of the selectin family and their oligosaccharide-containing ligands. L-selectin is expressed on leukocyte surfaces and recognizes its endothelial counterreceptors, such as GlyCAM-1, CD34, and MAdCAM-1, provided they are decorated with α 2,3-sialylated, α 1,3-fucosylated, and sulfated lactosamines.⁶⁻⁸ We and others have shown that high endothelial cells in lymph nodes express glycans fulfilling all or most of the above-listed requirements, the prototype decoration being sulfated sialyl Lewis x (sLex) for L-selectin.⁹⁻¹² Importantly, endothelial cells under normal conditions in other locations do not express proper glycoforms of L-selectin ligands. However, proinflammatory stimuli in *in vitro* and animal studies have shown that endothelium can be induced to express these glycans *de novo* and to promote leukocyte extravasation.^{2,13,14} Likewise, the endothelial E- and P-selectins have been shown to be inflammation-inducible molecules, both in animal models and in human patients.¹⁵⁻¹⁹ A novel approach to increase immunosuppressive efficacy, without targeting activation and proliferation of T cells, would be to inhibit the carbohydrate-dependent entry of lymphocytes into the graft.

Experimental animal work has demonstrated that free sLex-containing glycans can prevent short-term selectin-dependent inflammation, for example, after reperfusion injury.²⁰⁻²³ These glycans target mainly the endothelial P-selectin-dependent extravasation of granulocytes.²⁰ To investigate the relevance of inducible, properly decorated endothelial L-selectin ligands on lymphocyte extravasation to the sites of inflammation, we initiated experiments in allografts where the inflammatory event is composed of lymphocytes and monocytes but not of granulocytes. With rat heart and kidney allograft models, we have shown a strong induction of sLex-decorated L-selectin ligands on only microvascular endothelium occurring concomitantly with the accumulation of lymphocytes and mononuclear phagocytes into the grafts.^{2,24} Furthermore, the L-selectin-dependent leukocyte-endo-

---

Supported by grants from the Academy of Finland, the Technology Development Center of Finland, the Emil Aaltonen Foundation, the Helsinki University Central Hospital Research Fund, the Duodecim, and the Orion-Farmos Foundations of Helsinki.

Accepted for publication May 11, 1999.

Address reprint requests to Dr. Risto Renkonen, Haartman Institute, Department of Bacteriology and Immunology, P. O. Box 21 (Haartmaninkatu 3) SF-00014 University of Helsinki, Helsinki, Finland. E-mail: risto.renkonen@helsinki.fi.

Table 1. Summary of Antibody Reactivities of Glycans on L-Selectin Ligands

Name	Structure	Antibody	References
Sialyl Le _x		2F3* HECA-452	27,28 27,29
6-sulfo sialyl Le _x		2F3 HECA-452 MECA-79	30,31
Sulfated lactosamine		MECA-79†	

*Antibodies 2F3 and HECA-452 recognized sialyl Le_x always, regardless of whether it was nonsulfated, 6- or 6'-sulfated, or 6,6'-bissulfated.

†It is not known whether MECA79 recognizes 6- and/or 6'-sulfated and/or 6,6'-bissulfated glycans.

thelial recognition could be inhibited by enzymatically synthesized multivalent sLex glycans.^{2,25} We therefore wanted to investigate whether the expression of endothelial L-selectin ligands in human heart allograft rejection follows the same patterns as in the rat. In this study we used a large selection of endomyocardial biopsies (EMBs) from human heart transplants at different stages of rejection to demonstrate the point.

Our results show that although normal heart endothelium does not practically express sulfated sLex decorations, these epitopes are readily detectable on capillaries and venules during acute rejection. Furthermore, although the level of endothelial L-selectin ligand expression increased at the onset and during progression of the rejection, it decreased as the rejection episode resolved. There was a significant correlation between the intensity of expression of endothelial sulfated sLex-decorated L-selectin ligands and the histological severity of the rejection episodes. These data suggest that the strong up-regulation of the endothelial L-selectin ligands is a key factor in the initiation of lymphocyte extravasation to the heart allografts, and therefore also provide a putative target for new immunosuppressive and anti-inflammatory agents in organ transplantation.

Materials and Methods

Endomyocardial Biopsies

The study plan was approved by the Review Board of the Helsinki University Central Hospital (HUCH). Searching through over 600 EMBs from the archives of the Transplantation Laboratory of the University of Helsinki's Haartman Institute from 1992 to 1997, we identified 91 biopsies as having unequivocal histological signs of acute rejection of various degrees. These, together with the nonrejection control biopsies, were further analyzed with immunohistochemistry for the presence of endothelial L-selectin ligands (see below). The tissue specimens were formalin-fixed, paraffin-embedded, and processed for routine histological diagnosis.

Transplant recipients received triple drug immunosuppressive therapy consisting of cyclosporine, methylpred-

nisolone, and azathioprine; acute episodes of rejections were treated with a high dose of methylprednisolone, either alone or, in the most severe cases, combined with antithymocyte globulin. All recipients also received acyclovir prophylaxis against herpesvirus infections for the first 3 months. The histological evidence of rejection was diagnosed by one pathologist and graded according to the Working Formulation of the International Society for Heart and Lung Transplantation.²⁶ Normal histology did not present any signs of rejection. The diagnosis of an intermediate rejection (ie, a borderline or a resolving rejection) was referred to small perivascular lymphocytosis, lymphocyte activation, edema, and fibrosis, ie, signs of very mild acute rejection that did not fulfill the criteria of either grade 1A or normal histology. Grade 1A rejection represented a focal lymphocytic infiltrate; grade 1B was characterized by diffuse, but sparse infiltrate; grade 2 consisted of myocyte necrosis in association with a focal infiltrate; grade 3A possessed multifocal infiltrates with myocyte necrosis; grade 3B had diffuse infiltrate with myocyte necrosis; and grade 4 represented diffuse infiltrate with vasculitis, hemorrhage, and edema. No EMBs with a grade 4 rejection were found in our material.

Fifty-four biopsies represented normal histology, 27 biopsies represented intermediate diagnoses, and 91 cases had various grades of rejection (grade 1A, *n* = 49; 1B, *n* = 26; 2, *n* = 3; 3A, *n* = 7; 3B, *n* = 6). For further analysis the most severe rejections with myocyte necrosis, ie, grades 2 through 3B, were grouped together due to the small number of specimens.

Antibodies

The glycan epitopes on L-selectin ligands identified by the monoclonal antibodies (mAbs) used in the present study are described in Table 1.²⁷ Both mAbs 2F3 (5 μg/ml,²⁸ kindly provided by R. Kannagi, Aichi Cancer Center, Nagoya, Japan) and HECA-452 (15 μg/ml,²⁹ kindly provided by S. Jalkanen, University of Turku, Turku, Finland) are anti-sLex mAbs, requiring both the presence of α2,3 sialylation and α1,3 fucosylation of the lactosamine, whereas MECA-79 (1: 100 culture supernatant,^{30,31} also from S. Jalkanen) recognized 6-sulfation of

the GlcNAc-residue of N-acetyllactosamine on a family of endothelial proteins for proper recognition. Polyclonal anti-murine Fuc-TVII antibody shown to cross-react with the human α 1,3 fucosyltransferase VII (Fuc-TVII) was a generous gift from J. B. Lowe (1:10 dilution,³² Howard Hughes Medical Institute, Ann Arbor, MI). Isotype-matched mouse and rat IgMs (15 μ g/ml, Pharmingen, San Diego, CA) as well as rabbit IgG (15 μ g/ml) were used as control reagents.

Immunohistochemistry

EMB sections 5 μ m thick were deparaffinized and rehydrated by rinsing with xylene (5 minutes $\times$ 5), 100% ethanol (5 minutes $\times$ 2), 96% and 70% ethanol (5 minutes each), and aqua (5 minutes $\times$ 2). The slides were then incubated for 60 minutes at 37°C with 0.5% trypsin in phosphate buffered saline (PBS), pH 7.5. The trypsin was rinsed quickly with PBS and then washed 3 times for 10 minutes each time. We used a Histostain-plus kit (Zymed Laboratories Inc, San Francisco, CA) for immunoperoxidase staining according to instructions.

The levels of reactivity of mAbs 2F3, HECA-452, MECA-79, and rabbit anti-mouse Fuc-TVII antibody were scored by two observers who had no knowledge of the previously given pathological diagnosis of the specimens. The analysis was done by scoring the staining intensity of arterioles, capillaries, and venules with a semiquantitative staining score: normal, 0; mild, 1; moderate, 2; and intense, 3. At the site of a given rejection the intensity of endothelial staining was homogeneous and all identified vessels (from 4 to >20) within the rejection focus were included in the analysis.

Statistical Analysis

We used the nonparametric Kruskal-Wallis test to determine the differences in staining intensities between groups of different histological diagnosis (normal, intermediate, 1A, 1B, and 2-3B). *P* values <0.05 were considered significant. This test is basically a comparison of the medians of several unpaired groups and the null hypothesis is that the medians are all equal. We used also the nonparametric Spearman rank correlation test to determine whether the rank of the staining intensity was correlated with the rank of the groups of histological diagnosis. *P* values <0.05 were considered significant. Unpaired Student's *t*-test was used to analyze the significance between the endothelial staining intensity and different time groups during the progression of rejection and *P* values <0.05 were considered significant.

Results

High endothelium in lymph nodes express specific glycoforms of L-selectin ligands such as CD34, fulfilling most or all of the requirements (ie, a prototype decoration being a 6-sulfo sialyl Lewis x) for L-selectin recognition. Even though CD34 protein is abundantly present in en-

Figure 1. Expression of L-selectin ligands on capillary endothelium during acute heart allograft rejection episodes. The reactivity of different specimens and the various endothelial staining intensities with mAbs 2F3, HECA-452, and MECA-79 are correlated to the histological severity of rejection. The *P* values, obtained with Kruskal-Wallis test and marked on the upper right corner of each diagram, indicate that the distributions of staining patterns differ statistically between different histological groups (ie, between normal, intermediate, and grades 1A, 1B, and 2-3B).

dothelium of essentially all tissues and organs, it putatively participates in L-selectin-dependent traffic only into lymph nodes in a normal animal, because only there could the ligand carry the above-mentioned glycodecorations.³³ There is not a single antibody available recognizing all known decorations of the L-selectin ligands. To probe the differently decorated L-selectin ligands presumably responsible for the lymphocyte recruitment through the activated (high) endothelium in rejecting organ allografts, we had to use a panel of different mAbs. As shown in Table 1, 2F3 and HECA-452 mAbs detect α 2,3 sialylation and α 1,3 fucosylation of lactosamine and do not distinguish between sulfation and nonsulfation.^{29,30} On the other hand, MECA-79 needs sulfation of its epitope for proper recognition.^{31,32} The best ligands, ie, ligands with highest relative binding affinity toward L-selectin, would possess all these decorations, as shown in lymphatic tissue.⁴

First we performed immunohistochemical analysis of the EMBs with the three mAbs detecting different decorations of L-selectin ligands and correlated the intensity of endothelial staining, given as a semiquantitative score of 0 to 3, with the histological severity of acute graft rejection, as judged by quantitative histology from normal to grade 2-3B. The analysis was performed separately from all vascular endothelia present in endomyocardial biopsies, ie, from arterioles, intramuscular capillaries, and venules. Arterioles never reacted with the tested antibodies. The expression of L-selectin ligands was observed in the intermuscular capillaries as well as venules during rejection episodes. As these compartments of the vasculature gave similar results, only the results of stainings in the capillaries are presented for clarity in this study.

The anti-sLex mAb 2F3 reacted with normal nonrejection biopsies; approximately 60% of the biopsies were positive. However, the staining intensity was weak (Figure 1). Along with increasing histological severity of rejection, the percentage of positively reactive specimens increased from 60% to >90%, and over half of the specimens gave intermediate or strong staining reaction.

Only 10% of all specimens of nonrejecting normal biopsies stained with mAb HECA-452 and the reactivity

Figure 2. Correlation between the expression of functionally active endothelial L-selectin ligands, detected by the mAbs 2F3, HECA-452, and MECA-79, and the histological severity of rejection. The Spearman rank correlation test, marked in the upper left hand corner of the graphics, demonstrates correlation between the intensity of endothelial staining in capillaries with different mAbs and various histological severity grades (P values were corrected for ties). For clarity, the single observations in the scattergrams are removed; the solid line represents the mean and the dotted lines represent the 95% confidence limits of the mean.

was weak (Figure 1). The proportion of positively reactive specimens increased up to 30% in intermediate grade rejections and to 60 to 75% in grade 1A, 1B, and 2–3B rejection biopsies. Concomitantly, an increased intensity of staining was observed, and in grades 2–3B rejections up to 60% of the samples demonstrated intermediate or strong staining intensity.

The background staining of mAb MECA-79 in normal biopsies was negligible, ie, below 5%. The proportion of positive specimens increased to 15% in intermediate grade biopsies and to 50% in grade 1A, 1B, and 2–3B rejection biopsies. Virtually all specimens reacting with MECA-79 displayed a strong staining intensity (Figure 1).

The statistical correlation between the intensity of endothelial L-selectin ligand expression and the severity of graft rejection was shown using two separate and independent tests. The Kruskal-Wallis test was used to analyze the distribution of the mAb staining intensity in different histological groups, ie, normal, intermediate and grades 1A, 1B, and 2–3B, representing different histological severities of rejections. P values for all tested antibodies were <0.001 , indicating that the observed distribution of intensity of staining was significantly different between specimens of different histological severities (Figure 1).

To test whether the intensity of endothelial L-selectin ligand expression correlated with the severity of the histological grade of the rejection, we also performed the Spearman rank correlation test. The correlation curves obtained indicated a strong and significant positive correlation between the staining intensity with antibodies reacting with various glycodecorations of L-selectin ligands on one hand and the histological severity of rejection on the other hand ($P < 0.0001$, Figure 2). These observations demonstrate at the population-based level that, when more functionally decorated L-selectin ligands are expressed on the graft endothelium during rejection, the more severe is the histological severity of rejection episodes.

Finally, we wanted to investigate whether these glycomodifications also dictated the development of graft rejection in a timewise manner. To do this, we analyzed three representative cases with up to 6 consecutive EMBs taken from each individual patient before, during,

Figure 3. Mean $\pm$ SD of the intensity of expression of endothelial L-selectin ligands on human endomyocardial biopsies in three patient cases with up to six consecutive endomyocardial biopsies. The data are pooled into three categories: prerejection biopsies (Pre), biopsies obtained during early and late rejection (Rx), and postrejection specimens (Post). The consecutive endomyocardial biopsies were stained with 2F3, HECA-452, and MECA-79 mAbs and with polyclonal anti Fuc-TVII antibody. As shown, the staining intensity with all four antibodies significantly enhanced in the early rejection specimens, remained elevated throughout the episode, and decreased to background levels as the rejection resolved. The P values are comparisons with the prerejection specimens and obtained with unpaired Student's t -test; *, $P < 0.05$; ***, $P < 0.001$.

and after rejection. As shown in Figure 3, the endothelial staining intensity with 2F3, HECA-452, and MECA-79 was significantly increased when the rejection specimens were compared to prerejection specimens. The staining remained elevated throughout the rejection episode. As the rejection episode resolved, concomitantly with the intensified immunosuppressive therapy, the endothelial expression of L-selectin ligands decreased until it was close to or at background levels.

The last step in the synthesis of sLex glycans is the addition of fucose to the sialyl N-acetylglucosamine by an $\alpha 1,3$ fucosyltransferase. Previous data have indicated that of the five different human $\alpha 1,3$ fucosyltransferases, Fuc-TVII, might be the most important in the synthesis of sLex in endothelial cells.³⁴ Therefore, we also analyzed the expression pattern of Fuc-TVII with a specific polyclonal rabbit antibody during the progression of acute rejection and correlated this to the expression pattern of L-selectin ligands. The prerejection samples did not show any reactivity with Fuc-TVII, whereas the level of expression was significantly elevated in samples representing rejection. As the rejection episode resolved, the expression of endothelial Fuc-TVII also decreased to background levels (Figure 3). In Figure 4 representative microphotographs are shown from various immunohistochemical stainings.

Discussion

We show here, with a large collection of endomyocardial biopsies taken after cardiac transplantation, that the level of endothelial expression of sulfated and/or sLex-decorated (ie, lymphocyte extravasation-promoting) L-selectin ligands is increased at the onset and during histologically confirmed acute rejection episodes. The more these epitopes were expressed on the heart endothelium, the more severe histological rejection episode was observed; when the rejection resolved, the level of expression of these glycans decreased. These results hold

Figure 4. Expression of endothelial L-selectin ligands on human endomyocardial biopsies detected by immunohistochemistry. **A:** A biopsy from a graft having no signs of rejection showed no reactivity with an isotype-matched control murine IgM antibody. **Arrow** points to a venule; scale bar, 10 μ m. **B:** A high power field from a biopsy with normal histology with no reactivity with an isotype-matched control rat IgM antibody. **Arrows** point to a venule; scale bar, 10 μ m. **C:** This biopsy had a strong lymphocyte infiltration (grade 2 rejection), but the control murine IgM antibody showed no endothelial reactivity. **D:** A strong staining (brown color) with 2F3 identifying the sialylation and fucosylation modifications during acute rejection was noted both in small capillaries and larger venules (**arrow**). **E:** Staining for 2F3 showed clear reactivity with the small capillaries within the rejection focus, whereas the capillaries outside the rejection focus (in the lower part of the panel) showed no reactivity. **F:** HECA-452 also stained both the capillaries and venules (**arrows**) during a grade 1A rejection. **G:** A high power field of the same venules shown in **E** showed a strong reactivity with HECA-452. **H:** MECA-79 detected sulfated lactosamine structures as L-selectin ligands both on capillaries and venules (grade 2 rejection). **I:** Several venules stained strongly with MECA-79 during a grade 1B rejection. **J:** A biopsy showing no signs of rejection did not stain with a polyclonal anti-Fuc-TVII antibody. **K:** During acute rejection (grade 1A), venule with positive reaction with polyclonal anti Fuc-TVII antibody (**black arrow**) could be identified. Note that this antibody also reacted with muscle cells (**white arrows**) at the vicinity of a rejection focus. **L:** Two Fuc-TVII-positive vessels within an endomyocardial inflammation lesion in a biopsy with grade 1A rejection.

when data are analyzed in a time-dependent manner and provide a putative new strategy for site-directed immunosuppression based on closing of the selectin-dependent leukocyte extravasation to sites of inflammation.

The high correlation between the intensity of endothelial cell expression of sulfated and/or sLex decorated ligands and presence of acute rejection, suggest that endothelial cells up-regulate the expression of these in-

inflammation-promoting glycoforms of L-selectin ligands at the onset of rejection thus leading to increased lymphocytic traffic into the human heart allografts. This is in accordance with our previously published results in rodent models of heart and kidney allograft rejection.^{2,13} The *de novo* induction/up-regulation of endothelial sLex glycans at the onset of both animal and human rejection, suggest that this pattern of regulation is crucial in the initiation of site-directed lymphocyte extravasation into the allograft and, therefore, in the generation of local inflammation. Furthermore, the down-regulation of the expression of the same glycoforms at the time the rejection resolves is linked with the decrease of lymphocytic infiltrate in the allograft, suggesting that the graft endothelium has the machinery to induce the synthesis of these glycans as well as the capacity to decrease the expression by enzymatic degradation or other mechanisms.

Tethering of lymphocytes to and rolling of lymphocytes on endothelium is dependent on interactions between selectins and their glycosylated ligands and is in most cases imperative to the generation of inflammatory reactions. Thus, our data presented here suggest that the strong up-regulation of the endothelial L-selectin ligands is a key factor in the initiation of lymphocyte extravasation to heart allografts and thus provides a putative target for new immunosuppressive/anti-inflammatory drug therapy. Experimental animal work has already shown the effect of sLex glycans on rejection and reperfusion injury after lung transplantation.¹⁹

The reason for using a panel of antibodies detecting various parts of posttransplantation decoration of L-selectin ligands was that no single antibody has been shown to detect solely all these glycoforms. We selected our anti-sLex antibody panel according to the following evaluations. We first used a larger panel of mAbs including clones 2H5, CSLEX-1, 2F3, and HECA-452¹² on representative histological specimens. Although all these mAbs stained high endothelial venules (HEV) in frozen sections prepared from lymph nodes, only 2F3 and HECA-452 were able to do so in formalin-fixed and paraffin-embedded tissues, normally obtained from EMBs, and therefore we selected these antibodies for this study.

The original antigens in 2F3 and HECA-452 preparation were a sLex containing glycolipid and stromal components of human lymph nodes, respectively. The mAb 2F3 is able to inhibit *in vitro* the L-selectin-dependent adhesion of lymphocytes to human lymph node HEV, whereas HECA-452 did not block this.²⁹ The HECA-452 epitope has also been termed a cutaneous lymphocyte antigen (CLA).^{35,36} This CLA is a sLex-decorated variant of a P-selectin ligand glycoprotein, present on the skin-homing lymphocytes.³⁷ The glycans of P-selectin ligand glycoprotein-1 on a poorly differentiated myeloid leukemia cell (HL-60) have been identified, and the most attractive candidate for selectin recognition is a sialylated triLex polylectosamine.^{38,39} Whether HECA-452 detects the same or a similar glycan on the CLA-antigen on skin-directed lymphocytes is still unknown.

MECA-79 was originally defined to recognize an antigen called peripheral lymph node addressin (PNAd).^{30,31} This is actually a family of glycoproteins, most of which

are able to bind L-selectin. In humans only CD34 and podocalyxin have been identified at molecular level among the glycoprotein species reacting with MECA-79.⁴⁰ Later the recognizing epitope of MECA-79 was shown to be a sulfate group, most probably on the 6-position of GlcNAc of a lactosamine backbone.^{41,42} This antibody does not recognize α 2,3 sialylation or α 1,3 fucosylation; however, it is able to block *in vitro* and *in vivo* lymphocyte homing to murine lymph nodes.^{30,31}

As to the putative pathways on which the glycoforms detected by 2F3 and HECA-452 would be synthesized by endothelial cells in an inflammation-dependent manner, we analyzed the expression of α 1,3 fucosyltransferase Fuc-TVII enzyme in these specimens.^{43,44} The addition of fucose with an α 1,3 linkage to the sialylated lactosamine is the last reaction generating sLex glycans. Previous work has shown that this enzyme is present in lymph node high endothelium, which is the site of lymphocyte extravasation into the lymphatic tissue.³² Work carried out in Fuc-TVII-null mice showed that the absence of endothelial α 1,3 fucosylated glycans leads to a marked lymphocytosis in blood and impaired leukocyte rolling and to decreased extravasation to sites of inflammation.⁴² Taken together, these results suggest a role for Fuc-TVII enzyme in the synthesis of functionally glycosylated endothelial ligands for L-selectin in mice. To investigate whether the same enzyme is involved in the synthesis of sLex in human endothelium at sites of inflammation, we used a rabbit polyclonal anti-murine Fuc-TVII antibody³² that had been shown to cross-react with human Fuc-TVII. Using this antibody on consecutive EMBs ranging from prerejection biopsies and extending to biopsies after rejection was resolved, we could show that also the expression of Fuc-TVII enzyme increased at the onset of rejection and decreased as the rejection episode resolved. Our data support, indirectly, the suggestion that endothelial Fuc-TVII participates in the synthesis of functional fucosylated L-selectin ligands. The endothelial specific 6-sulfotransferase(s) involved in the induction of the MECA-79 reacting epitopes have not yet been identified.

These observations broaden the previous experimental analysis from our laboratory^{2,13} and others,¹⁴ and demonstrate that a similar strong induction of properly decorated endothelial ligands for L-selectin, as previously found in rodent models of inflammation, can be found in man, particularly in acute rejection of human heart allografts. Our findings are in complete agreement with previous work with L-selectin-deficient mice, which demonstrated the importance of L-selectin in targeting the lymphocyte traffic to sites of inflammation.⁴⁵

Selectin-dependent leukocyte extravasation seems to be a crucial step in the initiation of lymphocytic inflammatory reactions such as acute allograft rejection. The data presented here suggest that not only the inducible expression of endothelial E- and P-selectin, but also the inducible endothelial glycoforms of L-selectin ligands, guide lymphocyte traffic to the sites of inflammation, thus providing a new target for glycan-based immunomodulatory interventions.

Acknowledgments

We thank Tuula Kallioinen and Kaisa Karvo for excellent technical help, Prof. Seppo Sarna for help with statistical assays, and Sirpa Jalkanen, Reiji Kannagi, and John B. Lowe for providing the antibodies.

References

- Turunen JP, Mattila P, Halttunen J, Häyry P, Renkonen R: Evidence that lymphocyte traffic into rejecting cardiac allograft is CD11a- and CD49d-dependent. *Transplantation* 1992, 54:1053-1058
- Turunen J, Majuri M, Seppo A, Tiisala S, Paavonen T, Miyasaka M, Lemström K, Penttilä L, Renkonen O, Renkonen R: De novo expression of endothelial sialyl Lewis x, and sialyl Lewis X during cardiac transplant rejection: superior capacity of a tetravalent sLex-oligosaccharide in inhibiting L-selectin-dependent lymphocyte adhesion. *J Exp Med* 1995, 182:1133-1142
- Butcher EC, Picker LJ: Lymphocyte homing and homeostasis. *Science* 1996, 272:60-66
- Rosen SD, Hwang ST, Giblin PA, Singer MS: High-endothelial-venule ligands for L-selectin: identification and functions. *Biochem Soc Trans* 1997, 25:428-433
- Varki A: Selectin ligands: will the real ones please stand up? *J Clin Invest* 1997, 99:158-162
- Lasky LA, Singer MS, Dowbenko D, Imai Y, Henzel WJ, Grimley C, Fennie C, Gillett N, Watson SR, Rosen SD: An endothelial ligand for L-selectin is a novel mucin-like molecule. *Cell* 1992, 69:927-938
- Baumhueter S, Singer MS, Henzel W, Hemmerich S, Renz MR, Rosen SD, Lasky LA: Binding of L-selectin to vascular sialomucin CD34. *Science* 1993, 262:436-438
- Briskin MJ, McEvoy LM, Butcher EC: MAdCAM-1 has homology to immunoglobulin and mucin-like adhesion receptors and to IgA1. *Nature* 1993, 363:461-464
- Paavonen T, Renkonen R: Selective expression of sialyl-Lewis^x and sialyl Lewis^a, putative ligands for L-selectin, on peripheral lymph node high endothelial venules. *Am J Pathol* 1992, 141:1259-1264
- Munro JM, Lo SK, Corless C, Robertson MJ, Lee NC, Barhill RL, Weinberg DS, Bevilacqua MP: Expression of sialyl-Lewis x, an E-selectin ligand, in inflammation, immune processes and lymphoid tissues. *Am J Pathol* 1992, 141:1397-1408
- Sawada M, Takada A, Ohwaki I, Takahashi N, Tatene H, Sakamoto J, Kannagi R: Specific expression of a complex sialyl Lewis X antigen of high endothelial venules in human lymph nodes: possible candidate for L-selectin ligand. *Biochem Biophys Res Commun* 1993, 193:337-347
- Mitsuoka C, Kawakamikimura N, Kasugaisawada M, Hiraiwa N, Toda K, Ishida H, Kiso M, Hasegawa A, Kannagi R: Sulfated sialyl Lewis X, the putative L-selectin ligand, detected on endothelial cells of high endothelial venules by a distinct set of anti-sialyl Lewis X antibodies. *Biochem Biophys Res Commun* 1997, 230:546-551
- Turunen J, Paavonen T, Majuri M, Tiisala S, Mattila P, Mennander A, Gahmberg C, Häyry P, Tamatani T, Miyasaka M, Renkonen R: Sialyl-Lewisx, and L-selectin-dependent site-specific lymphocyte extravasation into the renal transplants during acute rejection. *Eur J Immunol* 1994, 24:1130-1136
- Onrust SV, Hartl PM, Rosen SD, Hanahan D: Modulation of L-selectin ligand expression during an immune response accompanying tumorigenesis in transgenic mice. *J Clin Invest* 1996, 97:54-64
- Briscoe DM, Schoen FJ, Rice GE, Bevilacqua MP, Ganz P, Pober JS: Induced expression of endothelial-leukocyte adhesion molecules in human cardiac allografts. *Transplantation* 1991, 51:537-547
- Briscoe DM, Yeung AC, Schoen FJ, Allred EN, Stavrakis G, Ganz P, Cotran RS, Pober JS, Schoen EL: Predictive value of inducible endothelial cell adhesion molecule expression for acute rejection of human cardiac allografts [erratum published, *Transplantation* 1995, 59:928]. *Transplantation* 1995, 59:204-211
- Qiao JH, Ruan XM, Trento A, Czer LS, Blanche C, Fishbein MC: Expression of cell adhesion in human cardiac allograft rejection. *J Heart Lung Transplant* 1992, 11:920-925
- Steinhoff G, Behrend M, Richter N, Schlitt HJ, Cremer J, Haverich A: Distinct expression of cell-cell and cell-matrix adhesion molecules on endothelial cells in human heart and lung transplants. *J Heart Lung Transplant* 1995, 14:1145-1155
- Brandt M, Boeke K, Phillips ML, Steinhoff G, Haverich A: Effect of oligosaccharides on rejection and reperfusion injury after lung transplantation. *J Heart Lung Transplant* 1997, 16:352-359
- Mulligan MS, Paulson JC, deFrees S, Zheng Z-L, Lowe JB, Ward PA: Protective effects of oligosaccharides in P-selectin-dependent lung injury. *Nature* 1993, 364:149-151
- Buerke M, Weyrich AS, Zheng Z, Gaeta FCA, Forrest MJ, Lefer AM: Sialyl-Lewis x-containing oligosaccharide attenuates myocardial reperfusion injury in cats. *J Clin Invest* 1994, 93:1140-1148
- Rao BNN, Abderson MB, Musser JH, Gilbert JH, Schaefer ME, Foxall C, Brandley BK: Sialyl Lewis X mimicks derived from a pharmacophore search are selectin inhibitors with anti-inflammatory activity. *J Biol Chem* 1994, 269:19663-19666
- Han KT, Sharar SR, Phillips ML, Harlan JM, Winn RK: Sialyl Lewis(x) oligosaccharide reduces ischemia-reperfusion injury in the rabbit ear. *J Immunol* 1995, 155:4011-4015
- Renkonen R, Soots A, von Willebrand E, Häyry P: Lymphoid cell subclasses in rejecting renal allograft in the rat. *Cell Immunol* 1983, 77:188-195
- Seppo A, Turunen JP, Penttilä L, Keane A, Renkonen O, Renkonen R: Synthesis of a tetravalent sialyl Lewis X glycan, a high-affinity inhibitor of L-selectin-mediated lymphocyte binding to endothelium. *Glycobiology* 1996, 6:65-71
- Billingham ME, Cary NR, Hammond ME, Kemnitz J, Marboe C, McCallister HA, Snovar DC, Winters GL, Zerbe A: A working formulation for the standardization of nomenclature in the diagnosis of heart and lung rejection: Heart Rejection Study Group. The International Society for Heart Transplantation. *J Heart Transplant* 1990, 9:587-593
- Mitsuoka C, Sawada-Kasugai M, Ando-Furui K, Izawa M, Nakanishi H, Nakamura S, Ishida H, Kiso M, Kannagi R: Identification of a major carbohydrate capping group of the L-selectin ligand on high endothelial venules in human lymph nodes as 6-sulfo sialyl Lewis X. *J Biol Chem* 1998, 273:11225-11233
- Ohmori K, Takada A, Ohwaki I, Takahashi N, Furukawa Y, Maeda M, Kiso M, Hasegawa A, Kannagi M, Kannagi R: A distinct type of sialyl Lewis X antigen defined by a novel monoclonal antibody is selectively expressed on helper memory T cells. *Blood* 1993, 82:2797-2805
- Duijvestijn AM, Horst E, Rouse BN, Steere AC, Picker LJ, Meijer CJLM, Butcher EC: High endothelial differentiation in human lymphoid and inflammatory tissues defined by monoclonal antibody HECA-452. *Am J Pathol* 1988, 130:147-155
- Streeter PR, Rouse BTR, Butcher EC: Immunohistological and functional characterization of a vascular addressin involved in lymphocyte homing into peripheral lymph nodes. *J Cell Biol* 1988, 107:1853-1862
- Michie SA, Streeter PR, Bolt PA, Butcher EC, Picker LJ: The human peripheral lymph node vascular addressin. An inducible endothelial antigen involved in lymphocyte homing. *Am J Pathol* 1993, 143:1688-1698
- Smith PL, Gersten KM, Petryniak B, Kelly RJ, Rogers C, Natsuka Y, Alford JAR, Scheidegger EP, Natsuka S, Lowe JB: Expression of the $\alpha(1,3)$ fucosyltransferase Fuc-TVII in lymphoid aggregate high endothelial venules correlates with expression of L-selectin ligands. *J Biol Chem* 1996, 271:8250-8259
- Baumhueter S, Dybdal N, Kyle C, Lasky LA: Global expression of murine CD34, a sialomucin-like endothelial ligand for L-selectin. *Blood* 1994, 84:2554-2565
- Swarte VVR, Joziassse DH, Vandeneijnden DH, Petryniak B, Lowe JB, Kraal G, Mebius RE: Regulation of fucosyltransferase-VII expression in peripheral lymph node high endothelial venules. *Eur J Immunol* 1998, 28:3040-3047
- Picker LJ, Kishimoto TK, Smith CW, Warnock RA, Butcher EC: ELAM-1 is an adhesion molecule for skin-homing T cells. *Nature* 1991, 349:769-771
- Berg EL, Yoshino T, Rott LS, Robinson MK, Warnock RA, Kishimoto TK, Picker LJ, Butcher EC: The cutaneous lymphocyte antigen is a skin lymphocyte homing receptor for vascular lectin endothelial cell-leukocyte adhesion molecule 1. *J Exp Med* 1991, 174:1461-1466
- Fuhlbrigge RC, Kieffer JD, Armerding D, Kupper TS: Cutaneous lymphocyte antigen is a specialized form of Psgl-1 expressed on skin-homing T cells. *Nature* 1997, 389:978-981

38. Wilkins PP, McEver RP, Cummings RD: Structures of the O-glycans on P-selectin glycoprotein ligand-1 from HL-60 cells. *J Biol Chem* 1996, 271:18732–18742
39. Niemelä R, Natunen J, Majuri ML, Lowe J, Renkonen O, Renkonen R: Complementary acceptor- and site-specificities of Fuc-TIV and Fuc-TVII allow effective biosynthesis of sialyl-triLex and related polylactosamines present on glycoprotein counterreceptors of selectins. *J Biol Chem* 1998, 273:4021–4026
40. Sasseti C, Tangemann K, Singer M, Kershaw D, Rosen S: Identification of podocalyxin-like protein as a high endothelial venule ligand for L-selectin: parallels to CD34. *J Exp Med* 1998, 187:1965–1975
41. Hemmerich S, Butcher EC, Rosen SD: Sulfation-dependent recognition of high endothelial venules (HEV)-ligands by L-selectin and MECA 79, and adhesion-blocking monoclonal antibody. *J Exp Med* 1994, 180:2219–2226
42. Maly P, Thall AD, Petryniak B, Rogers CE, Smith PL, Marks RM, Kelly RJ, Gersten KM, Cheng G, Saunders TL, Camper SA, Camphausen RT, Sullivan FX, Isogai Y, Hindsgaul O, von Andrian UH, Lowe JB: The α (1,3)fucosyltransferase Fuc-TVII controls leukocyte trafficking through an essential role in L-, E-, and P-selectin ligand biosynthesis. *Cell* 1996, 86:643–653
43. Sasaki K, Kurata K, Funayama K, Nagata M, Watanabe E, Ohta S, Hanai N, Nishi T: Expression cloning of a novel α 1,3-fucosyltransferase that is involved in biosynthesis of the sialyl Lewis X carbohydrate determinants in leukocytes. *J Biol Chem* 1994, 269:14730–14737
44. Natsuka S, Gersten KM, Zenita K, Kannagi R, Lowe JB: Molecular cloning of a cDNA encoding a novel human leukocyte α -1,3-fucosyltransferase capable of synthesizing the sialyl Lewis X determinant. *J Biol Chem* 1994, 269:16789–16794
45. Tang ML, Hale LP, Steeber DA, Tedder TF: L-selectin is involved in lymphocyte migration to sites of inflammation in the skin: delayed rejection of allografts in L-selectin-deficient mice. *J Immunol* 1997, 158:5191–5199