Transendothelial migration of lymphocytes across high endothelial venules into lymph nodes is affected by metalloproteinases

Christelle Faveeuw, Graham Preece, and Ann Ager

The migration of lymphocytes from the bloodstream into lymph nodes (LNs) via high endothelial venules (HEVs) is a prerequisite for the detection of processed antigen on mature dendritic cells and the initiation of immune responses. The capture and arrest of lymphocytes from flowing blood is mediated by the multistep adhesion cascade, but the mechanisms that lymphocytes use to penetrate the endothelial lining and the basement membrane of HEVs are poorly understood. Matrix metalloproteinases (MMPs) control the metastatic spread of tumor cells by regulating the penetration of blood vessel basement membranes. In this study, synthetic and natural inhibitors were used to determine the role of MMPs and MMP-related enzymes in regulating lymphocyte extravasation in mice. Mice were treated systemically with the hydroxamate-based MMP inhibitor Ro 31-9790 and plasma monitored for effective levels of Ro 31-9790, which block shedding of L-selectin. The total numbers of lymphocytes recruited into LNs were not altered, but L-selectin levels were higher in mice treated with Ro 31-9790. A reduced number of lymphocytes completed diapedesis and there was an increase in the number of lymphocytes in the endothelial cell lining, rather than the lumen or the basement membrane of HEVs. Lymphocyte migration and L-selectin expression in the spleen were not altered by Ro 31-9790 treatment. Two MMP inhibitors, TIMP1 and Ro 32-1541, did not block L-selectin shedding and had no effect on lymphocyte migration across HEVs. These results suggest that metalloproteinase activity is required for lymphocyte transmigration across HEVs into LNs and provide evidence for the concept that metalloproteinases are important players in some forms of transendothelial migration. (Blood. 2001;98:688-695) © 2001 by The American Society of Hematology

Introduction

The constitutive trafficking of naïve lymphocytes in and out of lymphoid organs is a prerequisite for the detection of processed antigen on mature dendritic cells and the initiation of immune responses. Lymphocytes enter lymph nodes (LNs) from the bloodstream by migrating across the walls of high endothelial venules (HEVs), which are postcapillary venules structurally adapted to support lymphocyte trafficking. Although lymphocyte migration from HEVs does not depend on exogenous inflammatory stimuli, it has features in common with the migration of leukocytes to inflammatory sites. The capture and arrest of lymphocytes from flowing blood onto the inner, endothelial surface is controlled by the multistep adhesion cascade. In peripheral LNs of mice, L-selectin mediates rolling and lymphocyte function–associated antigen 1 (LFA-1) integrin, the chemokine-dependent arrest of lymphocytes in HEVs. The subsequent migration of lymphocytes across the HEV wall into the LNs is thought to be directed by a chemotaxtractant produced within the LN paracortex, which establishes a gradient across the HEV wall. Secondary lymphoid tissue chemokine (SLC; also known as 6Ckine, eotaxin-2, or TCA-4) is produced by endothelial cells lining HEVs and mediates chemokine-dependent arrest of rolling lymphocytes in HEVs. Gene ablation studies have confirmed that the expression of CCR7, a receptor for SLC, is required for normal trafficking of lymphocytes into LNs; however, the chemokines that direct lymphocyte migration across the HEV wall to the LN paracortex have not been identified.

The HEVs comprise a layer of high endothelial cells (HECs) supported by a pericyte-containing basement membrane. It has been proposed that lymphocytes and other leukocytes need to degrade blood vessel basement membranes to complete diapedesis, and proteinases are, therefore, attractive candidates for regulating this step in leukocyte migration. A family of zinc-dependent endoproteinases, the matrix metalloproteinases (MMPs), plays a potentially important role in basement membrane penetration by leukocytes. MMP2 (gelatinase A) and MMP9 (gelatinase B) degrade type IV collagen, a major constituent of basement membranes, and these enzymes regulate tumor cell invasion of blood vessel basement membranes in vivo. T lymphocytes express MMP9, and both MMP2 and MMP9 expression can be upregulated by cellular activation or integrin-dependent adhesion. MMP inhibitors block T-cell migration across synthetic basement membranes in vitro suggesting that MMPs may regulate T-cell diapedesis in vivo. Until recently, proteinases had not been implicated in leukocyte-endothelial interactions; however, MMP-related enzymes have been shown to regulate L-selectin shedding and the rolling velocity of neutrophils on L-selectin ligands. Direct demonstration of roles for MMPs in biologic systems has depended on a combination of approaches including overexpression of activated MMPs, gene ablation, and the use of selective inhibitors. Naturally occurring MMP inhibitors are the tissue inhibitors of matrix metalloproteinases (TIMPs). Of the 4 TIMPs...
known all are broad-spectrum inhibitors of secreted MMPs and all inhibit the membrane-associated MMP (MT-MMP),20 apart from TIMP1, which does not inhibit some MT-MMPs.21 In addition some TIMPs, notably TIMP3,22 inhibit members of a related family of zinc-dependent endopeptidases, the metalloproteinase-disintegrins or ADAMs.23 Manipulation of MMPs in the clinical setting has been attempted using synthetic inhibitors. A number of low-molecular-weight MMP inhibitors have been synthesized, which are peptide analogues of collagen with a hydroxamic acid side chain to bind the zinc ion in the active site.24 Structurally related compounds differ in efficacy against individual MMPs and some inhibit members of the ADAMs family, but they show little activity against unrelated zinc-dependent endopeptidases.25 Hydroxamic acid–based MMP inhibitors prevent or reduce the spread and growth of a number of different malignant tumors in animal models,26,27 and some of these inhibitors are in the advanced stages of clinical testing for efficacy against solid tumors.28 Anti-inflammatory effects of synthetic MMP inhibitors as well as the TIMPs have been reported,29 but their effects on leukocyte extravasation were not determined.

In this study, we have used hydroxamate-based MMP inhibitors and TIMPs to determine the role of metalloproteinases in regulating lymphocyte extravasation in mice. We show that L-selectin is down-regulated on lymphocytes migrating into LNs, metalloproteinase inhibitors arrest lymphocytes in the endothelial lining of HEVs, and the effect on transendothelial migration correlates with inhibition of L-selectin shedding. These results suggest that metalloproteinases regulate transendothelial passage of lymphocytes and provide evidence for a novel regulatory step in lymphocyte diapedesis.

Materials and methods

Animals and inhibitors

Ro 31-9790 and Ro 32-1541 were kindly provided by Roche Research Products (Welwyn Garden City, United Kingdom) and suspensions were prepared for administration to mice by vigorous shaking overnight in gelofusine (B. Braun, Medical Ltd, Emmenbrucke, Switzerland) at 62.5 g/L. For in vitro analysis, the compounds were dissolved at 30 mM in dimethyl sulfoxide (DMSO) and stored at −20°C. Human recombinant TIMP130 in endotoxin-free sterile phosphate-buffered saline (PBS) was generously given by Professor G. Murphy (University of East Anglia, Norwich, United Kingdom). Eight- to 12-week-old male BALB/c mice bred and maintained under specific pathogen–free conditions at the National Institute for Medical Research were injected intraperitoneally with 0.2 to 0.4 mM inhibitors or vehicle alone (gelofusine for Roche compounds; PBS for TIMP1) 30 minutes prior to intranasal injection of lymphocytes labeled with 5-(and -6)-carboxyfluorescein diacetate succinimidyl ester (CFSE). Mice were anesthetized using Sagatal (80 mg/kg) after 30, 60, and 120 minutes and up to 1.0 mL heparinized (0.1 U/mL) blood collected by cardiac puncture. Mice were then killed for collection of LNs and spleen. In some experiments anesthetized mice were perfused via the left ventricle with 20 mL 2% formalin in PBS to maintain patency of blood vessels and remove loosely bound blood cells prior to removal of LNs. Total levels of human TIMP1 (free and complexed) in mouse plasma were determined by enzyme-linked immunoabsorbent assay (ELISA) using species-specific antibodies (Amersham Pharmacia Biotech, Little Chalfont, United Kingdom; RPN2611).

L-selectin shedding assay

Doubling dilutions of plasma from control and inhibitor-treated mice were tested for their ability to block phorbol myristate acetate (PMA)–induced L-selectin shedding. Lymphocytes (2.5 × 10^6) were incubated in 50 μL plasma for 20 minutes at 37°C, prior to addition of 5 μM PMA and incubated for a further 30 minutes. Excess PMA was removed by washing and L-selectin expression determined by flow cytometry following staining with MEL-14 monoclonal antibody (mAb).31 The plasma level of Ro 31-9790 was measured from a standard curve constructed using 0.01 to 100 μM exogenous Ro 31-9790 in plasma from control mice.

Angiotensin-converting enzyme assay

Hydrolysis of the synthetic substrate furylacryloylphenylalanylglycylglycine (FAPGG) by angiotensin-converting enzyme (ACE) was measured as described previously.32 FAPGG (ACE reagent, Sigma, Poole, United Kingdom) and ACE from porcine kidney (ACE calibrator, Sigma) were reconstituted in water and used according to manufacturer’s instructions. The synthetic ACE inhibitor pGlu-Trip-Pro-Arg-Pro-Glu-Leu-Pro-Pro-Ser (Sigma A0773) was dissolved at 12 mM in water, Ro 31-1541 and Ro 31-9790 were dissolved at 30 mM in DMSO and stored at −20°C in aliquots. ACE reagent (1.0 mL) and ACE calibrator (0.1 mL) were preincubated with inhibitors (final concentration of ACE inhibitor at 24 μM and Ro 31-9790/Ro 32-1541 at 100 μM) or equivalent amounts of vehicle (DMSO or water) for 15 minutes at 37°C before mixing. Absorbanse at 340 nm was measured immediately and following 30 minutes incubation at room temperature using a Shimadzu UV mini-1240 spectrophotometer (Shimadzu Europa, Milton Keynes, United Kingdom). The decrease in absorbance was calculated and the results are average change in absorbance calculated for triplicate samples ± SD.

Migration of CFSE-labeled lymphocytes

Lymphocytes were labeled with CFSE as described previously.33 For each experiment, 5 to 10 × 10^6 spleen cells were labeled at 5 × 10^6 cells/mL for 15 minutes at 37°C with 1 μM CFSE (Molecular Probes, Eugene, OR). 5 mM stock solution in DMSO stored at −20°C in PBS and excess CFSE removed by washing in PBS containing 1% fetal calf serum (FCS). CFSE labeling did not affect cell viability (determined by vital dye exclusion); expression of L-selectin, CD44, LFA-1, α4 integrins, or ICAM-1; proliferation to mitogens or anti-CD3; or lymphocyte adhesion to cultured endothelial cells (data not shown and reference 33). CFSE-labeled cells (40 × 10^6) in 0.1 mL PBS were injected intravenously into mice (groups of 3 or 4) pretreated for 30 minutes with inhibitors or vehicle alone and the number of CFSE-labeled lymphocytes in blood, LNs, and spleen determined at different times by flow cytometric analysis of cell suspensions. The localization of CFSE-labeled lymphocytes in blood vessels of LNs and spleen was determined by fluorescence microscopy of tissue sections.

Flow cytometric analysis of lymphocyte suspensions

Spleen and axillary, brachial, and inguinal LNs were collected separately from each recipient mouse into PBS (calcium and magnesium free) at 4°C. The spleen was cut in half and cell suspensions prepared from one piece of spleen and from one of each LN pair. Red blood cells were lysed with 3 to 5 mL lysis buffer (150 mM NH4 Cl in 10 mM KHCO3/0.1 mM EDTA, pH 7.4) for 10 minutes at room temperature and the cells washed twice with PBS. Heparinized blood (300 μL) was also analyzed following lysis of red blood cells. CFSE-labeled lymphocytes were analyzed on a FACScan Vantage (Becton Dickinson, Oxford, United Kingdom) using the fluorescein isothiocyanate–labeled antibody (Joephep, Scripps Institute, La Jolla, CA). The percentage of CFSE-labeled cells recovered in the spleen, blood, and each group of LNs was determined and compared between control and treated animals. Expression of L-selectin on CFSE-labeled cells was determined following incubation with biotinylated MEL-14 and detection of bound antibody using phycoerythrin (PE)–conjugated streptavidin (Southern Biotechnology, Birmingham, AL). The percentage of CFSE-labeled cells expressing L-selectin and the mean fluorescence intensity of cells positive for L-selectin were determined.
Fluorescence microscopy

One inguinal LN, one brachial LN, and 5-mm slices of spleen from each mouse were fixed in 2% formaldehyde/5% sucrose in PBS for 2 hours, transferred to 20% sucrose in PBS for 2 hours, snap frozen in liquid nitrogen, and stored at −70°C for up to 2 years. Cryostat sections of 8 μm were cut and stored at −70°C for up to 1 year. HEVs in LN sections were stained with MECA-79 (American Type Culture Collection, Bethesda, MD) and Texas Red (TXRD)–conjugated goat anti-rat immunoglobulins (Igs; Molecular Probes) to detect bound antibody. The position of CFSE-labeled lymphocytes (green) in relation to HEVs (red) was determined by fluorescence microscopy using a Biorad MRC 600 confocal microscope (Biorad, Hemel Hempstead, United Kingdom) and a 20× objective. For each LN, 10 to 15 images containing complete cross-sections through 30 to 55 HEVs (average HEV size 2500 μm²) were collected and analyzed using the “NIH-Image” image analysis software for the Macintosh (public domain software downloadable from the NIH Image FTP site, http://rsbweb.nih.gov). The total cross-sectional area of HEVs and the remaining area within each image were calculated. CFSE-labeled cells “inside HEVs” were those attached to the luminal surface of the vessel wall and within the HEV wall (Figure 3; arrows). The remaining CFSE-labeled cells were scored as “outside HEVs” (Figure 3; arrowheads). The numbers of CFSE-labeled cells inside and outside HEVs were not significantly different in inguinal and brachial LNs of individual mice or between animals in each experimental group. However, there were differences between experimental groups, which reflect variation in the absolute number of CFSE-labeled cells injected and the exact time of LN collection. The results have been pooled from inguinal and brachial LNs of mice within each experimental group and, where the results were similar, between experimental groups and are expressed as means ± SEM CFSE-labeled cells/mm² inside and outside HEVs. Laminin in the HEV basement membrane was stained using rabbit anti–mouse laminin34 (generously provided by C. Streuli, Manchester, United Kingdom) and bound antibody detected with Cy5-conjugated goat anti–rabbit Ig (Jackson Immunoresearch, West Grove, PA) (Figure 4). LN sections were examined using an Olympus IX70 inverted microscope, and digital images of 40 to 50 HEVs were acquired using a Photometrics CH135 liquid-cooled CCD camera and DeltaVision deconvolution software (Applied Precision, Issaquah, WA). The length of laminin around each HEV was measured using the NIH-Image software. The number of CFSE-labeled cells between the endothelial lining and laminin and within the laminin layer were counted. Results are expressed as number of CFSE-labeled cells/mm of laminin (mean ± SEM). CFSE-labeled lymphocytes in the spleen were localized to the white pulp or the surrounding marginal zone (MZ). Sections were incubated with rat anti–mouse sialoadhesin 3D6 (generously provided by P. Crocker, Dundee, United Kingdom), which is expressed by MZ macrophages35 and bound antibody detected using TXRD-conjugated goat anti–rat Ig. Images were collected using the confocal microscope and a ×10 objective, the areas of MZ and white pulp were calculated using NIH-Image software, and the numbers of CFSE-labeled cells in these locations counted. The numbers of CFSE-labeled cells in the MZ and white pulp were not significantly different between animals and the results have been pooled from 6 mice. Results are expressed as mean ± SEM CFSE cells/mm² of MZ or white pulp.

Statistical analysis

Pooled data were computed as means ± SEM and were compared using Student t test.

Results

Ro 31-9790 inhibits the major classes of secreted MMPs, including interstitial collagenase (MMP1), the gelatinases (MMP2 and MMP9), stromelysin (MMP3), and neutrophil collagenase (MMP8), as well as the membrane-associated ectoenzymes, MT1-MMP (MMP14) and MT4-MMP (MMP17), with inhibitory concentration of 50% (IC50) values ranging from 2 to 200 nM.21,36,37 Ro 31-9790 also inhibits ADAM1722 and phorbol ester-induced shedding of L-selectin from the surface of lymphocytes.31 In contrast, Ro 32-1541, another hydroxamate MMP inhibitor,16 and the endogenous MMP inhibitor TIMP1 did not inhibit L-selectin shedding (Figure 1A,B).16 Ro 31 9790 and Ro 32-1541 did not inhibit ACE (Table 1), a zinc-dependent endopeptidase belonging to the thermolysin family.23 We used the broad-spectrum MMP inhibitor, Ro 31-9790, in the first instance, to determine the potential role of metalloproteinases in regulating lymphocyte extravasation. Mice were treated systemically with inhibitor and its effect was determined locally by studying the migration of lymphocytes from the blood into lymphoid organs. The effect of plasma from mice treated with Ro 31-9790 was compared with an
optimal dose of Ro 31-9790 (30 μM\textsuperscript{39}) on L-selectin shedding in vitro (Figure 1B,C). Sufficient Ro 31-9790 to inhibit L-selectin shedding by more than 90% was found in the bloodstream of mice 60 minutes following a single intraperitoneal injection of at least 100 mg/kg, but Ro 31-9790 levels were suboptimal after 120 minutes (Figure 1C, solid bars, 60 minutes; hatched bars, 120 minutes). Plasma from mice given a similar dose of Ro 32-1541 or 33mg/kg TIMP1 had no effect on L-selectin shedding (Figure 1C).

Lymphocytes migrate from the bloodstream into LN and spleen during normal trafficking to survey the body for invading pathogens.\textsuperscript{1} Fluorescent (CFSE)-labeled lymphocytes were injected 30 minutes following a single intraperitoneal injection of 100 mg/kg Ro 31-9790 and the localization of lymphocytes in LNs, spleen, and blood determined 30, 60, and 120 minutes following intravenous injection of cells. Plasma levels of Ro 31-9790 at each time point were measured using exogenous Ro 31-9790 in mouse plasma as a standard and confirmed by high-performance liquid chromatography analysis (E. Worth, C.F., A.A., unpublished observations, 1998). Circulating levels of Ro 31-9790 sufficient to completely inhibit L-selectin shedding (> 30 μM) were found at 30 and 60 minutes, but levels were suboptimal (< 30 μM) at 120 minutes (Table 2). The recovery of CFSE-labeled lymphocytes from LNs, spleen, or blood of Ro 31-9790–treated mice was not significantly different from control mice (Figure 2A). The kinetics of lymphocyte migration were similar to published studies using different labeling protocols.\textsuperscript{38} The number of lymphocytes in the blood stabilized after 30 minutes, whereas the number of lymphocytes entering LNs and spleen increased between 30 and 60 minutes (Figure 2A); splenic localization was higher than LN localization at both time points. Blood pressure and vascular permeability were not affected, as reported previously using similar treatments of Ro 31-9790\textsuperscript{39} or other hydroxamate MMP inhibitors.\textsuperscript{18} Ro 31-9790 was not cytotoxic or proapoptotic to lymphocytes and histologic analysis of lymphoid organs showed no adverse effects on tissue architecture (Figure 4B). The numbers of lymphocytes positive for L-selectin recovered from LNs, spleen, and blood were analyzed for L-selectin expression. The total number of L-selectin–positive lymphocytes in each tissue was not affected by Ro 31-9790. However, the level of L-selectin was higher on lymphocytes harvested from LNs, but not the spleen or blood, of mice treated with Ro 31-9790. Bars are mean percent CFSE-labeled cells ± SEM and the number of mice analyzed is given below each bar. *P < 0.005.

Figure 2. Ro 31-9790 does not affect lymphocyte migration from the blood into lymphoid organs but inhibits shedding of L-selectin during migration into LNs. (A) Mice were pretreated with 100 mg/kg Ro 31-9790 ( ), vehicle alone ( ), or 40 × 10\textsuperscript{5} CFSE-labeled lymphocytes injected. The number of CFSE-labeled cells in LNs, spleen, and blood was measured 30 minutes and 60 minutes after injection. Bars are mean percent CFSE-labeled cells ± SEM and the number of mice analyzed is given below each bar. (B) CFSE-labeled lymphocytes harvested from LNs, spleen, and blood were analyzed for L-selectin expression. The total number of L-selectin–positive lymphocytes in each tissue was not affected by Ro 31-9790. However, the level of L-selectin was higher on lymphocytes harvested from LNs, but not the spleen or blood, of mice treated with Ro 31-9790. Bars are mean fluorescence intensities of L-selectin–positive lymphocytes ± SEM, n = 3 in mice treated with Ro 31-9790 ( ) and vehicle ( ) with percent positive lymphocytes given below each bar.*P < 0.005. (C) Representative 2-color FACS profiles showing CFSE-labeled cells in lymphoid organs 60 minutes after injection (left side) and L-selectin expression on CFSE-labeled migrants (right side). Note lack of crossover of CFSE label from FL1 into FL2, which was used to measure L-selectin.

### Table 1. Effect of inhibitors on angiotensin-converting enzyme

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Absorbance 340 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.180 ± 0.004</td>
</tr>
<tr>
<td>ACE inhibitor</td>
<td>0.002 ± 0.001</td>
</tr>
<tr>
<td>Ro 31-9790</td>
<td>0.185 ± 0.003</td>
</tr>
<tr>
<td>Ro 32-1541</td>
<td>0.182 ± 0.005</td>
</tr>
</tbody>
</table>

Results are average decreases in A\textsubscript{340} values after 30 minutes of incubation at 22°C of ACE with FAPGG in the absence (none) or presence of ACE inhibitor at 24 μM and Ro 31-9790/Ro 32-1541 each at 100 μM. ACE indicates angiotensin-converting enzyme; FAPGG, furylacryloylphenylalanyl-glycylglycine.

### Table 2. Concentration of Ro 31-9790 in the plasma of mice

<table>
<thead>
<tr>
<th>Treatment of animals*</th>
<th>Time after injection of CFSE-labeled cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30 min</td>
</tr>
<tr>
<td>3 mg</td>
<td>63 ± 17</td>
</tr>
<tr>
<td>2 mg</td>
<td>nd</td>
</tr>
<tr>
<td>1 mg</td>
<td>nd</td>
</tr>
</tbody>
</table>

CFSE indicates carboxyfluorescein diacetate succinimidyl ester; nd, not determined.

*1, 2, and 3 mg/mouse equivalent to 33, 66, and 100 mg/kg body weight, respectively. Results are mean plasma levels of Ro 31-9790 (μM) ± SD (n = 5). †n = 2.
the number of lymphocytes outside HEVs at 30 and 60 minutes. The effect of Ro 31-9790 was greatest at 60 minutes with a 2-fold increase in lymphocytes inside HEVs. This increase was seen in mice that had been perfused intravenously prior to collection of LNs demonstrating that it was not simply due to loosely bound lymphocytes in the HEV lumen. However, the effect of Ro 31-9790 was transient and completely reversible as shown by the normal numbers of lymphocytes accumulating outside HEVs 120 minutes after injection. The time-dependent effects of Ro 31-9790 on lymphocyte extravasation correlated with the highest plasma levels at 60 minutes and the lowest at 120 minutes (Table 2). The colocalization of CFSE labeling and MECA 79 staining (Figure 3) did not allow the exact position of lymphocytes in the HEV wall to be determined. LN sections from mice that had been intravenously perfused with fixative to maintain patency of HEVs were counterstained for laminin to determine whether CFSE-labeled cells accumulated in the subendothelial basement membrane. As reported above, the number of lymphocytes inside HEVs increased (Figure 4A). There was a slight increase in the number of lymphocytes either adjacent to or within the HEV basement membrane of mice treated with Ro 31-9790; however, it was not significant (Figure 4A), suggesting that the increased number of lymphocytes inside HEVs was not simply due to arrest at the basement membrane. Circulating Ro 31-9790 will affect injected (CFSE-labeled) and host (unlabeled) lymphocytes equally. To determine whether Ro 31-9790 affected lymphocyte binding in the lumen of HEVs, we used ultrastructural analysis without distinguishing between injected and host lymphocytes (Figure 4B). As reported previously, about 90% of lymphocytes in HEVs of control mice were within the endothelial lining, with very few bound in the lumen (<10%). Although the total number of lymphocytes inside HEVs was visibly increased in Ro 31-9790–treated mice, the distribution of lymphocytes in the lumen and endothelial lining was not altered (Figure 4B). The ultrastructural appearance of HEVs, basement membranes, and lymphocytes were unaltered in mice treated with Ro 31-9790, and other types of leukocytes were not bound to HEVs. Ro 31-9790 did not inhibit lymphocyte motility or chemotaxis per se because lymphocytes migrated normally from the MZ to the white pulp of the spleen in Ro 31-9790–treated mice (Table 3). These results suggest that Ro 31-9790 does not alter lymphocyte binding to the luminal surface of HEVs or the initiation of migration across the endothelial lining but prevents lymphocytes from successfully completing transendothelial migration or increases their transit time, thus resulting in accumulation within the endothelial lining.

To explore the potential role of metalloproteinases further, 2 MMP inhibitors that do not block L-selectin shedding were tested. The recovery of CFSE-labeled lymphocytes from LNs 60 minutes after intraperitoneal injection of a similar dose of Ro 32-1541 (100 mg/kg) was similar to that in control mice (data not shown) and the number of lymphocytes inside and outside HEVs was not altered (Table 4 and data not shown). Intraperitoneal administration of 33 mg/kg recombinant human (h)TIMP1 yielded circulating levels of hTIMP1 ranging from 310 to 400 μg/mL (11-14 μM); however, lymphocyte transendothelial migration across HEVs was not altered in TIMP1-treated mice (Table 4) and CFSE-labeled lymphocytes accumulated in normal numbers in the LN paracortex (127 ± 7 cells/mm² outside HEVs in control mice and 126 ± 8 cells/mm² in TIMP1-treated mice). Immunocytochemical staining showed that hTIMP1 was evenly distributed throughout the HEV wall and the LN paracortex (data not shown).

**Discussion**

Using the broad-spectrum MMP inhibitor Ro 31-9790 we have shown that L-selectin is down-regulated on lymphocytes migrating into LNs from the bloodstream via HEVs. In addition, although the
number of lymphocytes that enter LNs is not altered, lymphocytes accumulate in the endothelial lining of HEVs in inhibitor-treated mice. Together these results suggest that metalloproteinases regulate transendothelial migration of lymphocytes across HEVs and L-selectin shedding during migration from the blood into LNs.

The effects of Ro 31-9790 were dose dependent and the effective systemic doses and plasma levels achieved were similar to those reported by others to block L-selectin shedding from neutrophils and tumor metastasis in rodents. A structurally related hydroxamate-based MMP inhibitor, Ro 32-1541, did not block L-selectin shedding and had no effect on lymphocyte transmigration across HEVs suggesting that the effects of Ro 31-9790 were specific to this compound. Although Ro 31-9790 inhibits both MMPs and ADAMs, marimastat, which is structurally similar to Ro 31-9790, does not inhibit the MMP-related astacin/tolloid metalloproteinase family suggesting that Ro 31-9790 could exhibit selectivity within the metzincin superfamily of metalloproteinases. Ro 31-9790 did not simply function as a nonselective inhibitor of metalloproteinases due to its zinc chelating properties because it was ineffective against ACE, a zinc-dependent endopeptidase belonging to the thermolysin family, which is distinct from MMPs. To explore the potential role of MMPs further we tested a naturally occurring MMP inhibitor, TIMP1, which is structurally unrelated to the hydroxamate inhibitors. We had previously shown recombinant hTIMP1 does not inhibit phorbol ester–induced L-selectin shedding from mouse lymphocytes. Interestingly, TIMP1 had no affect on lymphocyte transendothelial migration across HEVs in mice. Human TIMP1 inhibits murine MMP9 and the invasion of amnion membranes by B16-F10 murine melanoma cells in vitro. In addition administration of hTIMP1 to mice reduces the colonization of lungs by B16-F10 melanoma cells indicating cross-species inhibition, although the full spectrum of murine MMPs that are inhibited by human TIMP1 is not known. The lack of effect of hTIMP1 is unlikely to be due to insufficient inhibitor because the circulating plasma level achieved in this study (~15 μM/350 μg/mL) was 50- to 150-fold higher than that shown to completely inhibit murine MMP9 and matrix degradation by mouse blastocysts. The dose of hTIMP1 used was similar to that used to block experimental metastasis in mice and melanoma cell invasion of basement membranes in vitro.

The requirement for metalloproteinase activity during transendothelial migration across HEVs has not been shown before and

Table 3. Effect of Ro 31-9790 on lymphocyte migration into the spleen

<table>
<thead>
<tr>
<th>MMP inhibitor</th>
<th>CFSE-labeled cells/mm² *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>697 ± 42</td>
</tr>
<tr>
<td>Ro 31-9790</td>
<td>601 ± 26</td>
</tr>
</tbody>
</table>

*Results are mean number of CFSE-labeled cells/mm² ± SEM (n = 6).

Table 4. Effect of matrix metalloproteinases inhibitors on lymphocyte localization inside high endothelial venules

<table>
<thead>
<tr>
<th>MMP inhibitor</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ro 32-1541</td>
<td>1810 ± 110*</td>
</tr>
<tr>
<td>TIMP1</td>
<td>1150 ± 80†</td>
</tr>
</tbody>
</table>

*Results are mean number of CFSE-labeled cells/mm² ± SEM inside HEVs and are pooled from brachial and inguinal LNs of 3 mice.
†Lower value compared to control mice was also seen in the number of lymphocytes outside HEVs (data not shown), paralleling a lower number of CFSE-labeled cells injected into mice treated with Ro 32-1541.
‡Results are mean number of CFSE-labeled cells/mm² ± SEM inside HEVs and are pooled from brachial and inguinal LNs of 4 mice.

Figure 4. Ro 31-9790 arrests lymphocytes in the endothelial lining and not in the basement membrane or the lumen of HEVs. (A) Laminin in the HEV basement membrane was stained and the number of lymphocytes adjacent to or within the basement membrane counted and expressed per unit length of laminin. Although the number of CFSE-labeled cells inside HEVs was visibly increased in Ro 31-9790–treated mice, lymphocyte numbers in the basement membrane were not significantly different (ns). Bar indicates 20 μm. (B) Ultrastructural analysis of laminin in the HEV endothelial lining and not in the basement membrane or the lumen of HEVs.
metalloproteinase-dependent down-regulation of L-selectin expression during migration into LN s provides independent evidence for metalloproteinase activation, but further experiments will be required to identify the enzyme activities responsible. It is possible that L-selectin shedding and transmigration across HEVs are regulated by different metalloproteinases that are both inhibited by Ro 31-9790; identification of all murine metalloproteinases that are inhibitable by Ro 31-9790, but not by Ro 32-1541 or TIMP1, will be useful in this regard. MMPs have been implicated in T-lymphocyte transmigration across the blood-brain barrier of mice from studies using the encephalitogenic mouse CD4+ T-cell clone C19. MMP2 is induced in C19 cells following binding to VCAM-1 and overexpression of MMP2 by C19 cells facilitates their migration across the blood–brain barrier in vivo. Transmigration across VCAM-1-expressing microvascular endothelial cells grown on collagen-coated filters is inhibitable by TIMP2 and the synthetic hydroxamate MMP inhibitor GM6001 prevented C19 cells from penetrating the collagen-coated Transwells, supporting a role for MMPs in basement membrane penetration. However, the metalloproteinase-dependent transendothelial migration of lymphocytes across HEVs that we report here is distinguishable in that it is a rapid event (half-life 30 minutes), whereas VCAM-1-induced MMP expression is maximal after 5 hours. In addition, MMP2 and MMP9 do not cleave cell surface L-selectin and binding to VCAM-1 does not stimulate L-selectin shedding within the time frame of transmigration studied here (A.A. and G.P., unpublished observations, 2000). Studies so far have shown clearly that L-selectin sheddase cleaves L-selectin in the same but not an adjacent membrane suggesting that the metalloproteinase responsible for L-selectin shedding is expressed by lymphocytes. However, we cannot rule out the possibility that the metalloproteinase responsible is expressed on the HEV membrane and cleaves L-selectin from the lymphocyte surface at sites of close membrane apposition (4 nM), which occur during transmigration.

Although we cannot draw any conclusions about the role of L-selectin shedding in lymphocyte transmigration across HEVs from this study, the correlation between inhibition of shedding and transendothelial migration is interesting and warrants some discussion. Studies of human neutrophils rolling on L-selectin ligands (MECA 79 antigen) in vitro and of mouse neutrophils rolling in vivo in exteriorized postcapillary venules on unidentified L-selectin ligands have shown that the rolling velocity is reduced in the presence of the hydroxamate-based MMP inhibitor KD-IX-73-4. Although L-selectin expression on rolling neutrophils was not measured, these authors concluded that rolling was regulated by limited proteolysis of L-selectin induced by ligand engagement. An increase in the number of neutrophils that bound from flow was also noted, but the effect of KD-IX-73-4 on the diapedesis of neutrophils in vivo was not determined. Whether L-selectin shedding regulates lymphocyte rolling in HEVs and the relationship between rolling velocity and the subsequent extravasation of leukocytes remains to be determined. We found that systemic treatment with Ro 31-9790 did not increase the binding of lymphocytes or other leukocytes in the lumen of HEVs, where L-selectin mediates adhesion and shedding is thought to occur, nor did it increase the overall recruitment of lymphocytes by HEVs; however, it did inhibit L-selectin down-regulation on lymphocytes entering LNs. Because lymphocytes were able to initiate transendothelial migration, we conclude that the effect of Ro 31-9790 is downstream of events in the lumen of HEVs and, therefore, may be completely independent of L-selectin shedding. It is unlikely that the metalloproteinases responsible regulate lymphocyte chemotaxis because Ro 31-9790 did not affect chemokine-dependent migration of lymphocytes from the MZ into the splenic white pulp, which, like LN entry, is dependent on CCR7. However, the generation and mainte-

nance of a gradient of chemokine across the endothelial layer lining HEVs could be dependent on metalloproteinases, as shown for dorsoventral patterning in the embryo, which is regulated by another MMP-related family of metalloproteinases, the tissuetype metalloproteinases.

Metalloproteinase activation could be just one step in a cascade of events required for lymphocytes to find and penetrate interendothelial cell adhesions and the underlying basement membrane to complete diapedesis and enter the LN parenchyma. Recent studies of monocyte transmigration across human umbilical vein endothelial cells have shown that staining for the VE-cadherin/β-catenin/β-catenin/plakoglobin complex is lost from adherens junctions at sites of monocyte penetration of the endothelial layer and rapidly regained after transmigration, but the mechanism was not worked out. It is possible that metalloproteinases regulate the degradation of adhesion complexes between adjacent HECs at the basolateral, rather than the apical, side and the effect of Ro 31-9790 is to trap lymphocytes within the endothelial lining of HEVs.

An interesting observation was that none of the MMP inhibitors tested blocked lymphocyte transmigration across the basement membranes of HEVs in mice. Hydroxamate inhibitors and TIMP1 have been used repeatedly to block tumor cell and lymphocyte migration across basement membranes in vitro and these observations formed the basis for the in vivo study reported here. However, the role of MMPs in regulating the extravasation of tumor cells has been called into question recently. Studies of TIMP1-expressing B16 melanoma cells have shown reduced colonization of mouse lungs and chick embryos following intravenous injection in comparison with wild-type B16 cells. However, intravital microscopy of blood vessels in the chicken chorioallantoic membrane showed that the expression of TIMP1 have no effect on the extravasation of injected B16 melanoma cells but reduced the growth and number of tumors after extravasation. Systemic treatment of mice with batimastat, another hydroxamate-based MMP inhibitor, reduced B16 melanoma cell growth in the liver, but intravital microscopy showed that extravasation of tumor cells was normal in inhibitor-treated mice. These studies do not eliminate a role for MMPs in tumor cell extravasation because, as discussed above, TIMP1 does not inhibit all MMPs and the efficacy of these inhibitors against murine MMPs is not worked out. However, they do highlight the difficulties in reproducing the complex interactions that regulate diapedesis in in vitro assays. It would be interesting to test Ro 31-9790 in models of B16 melanoma cell extravasation because we have shown that, in contrast to TIMP1, it is an effective inhibitor of transendothelial migration.

In conclusion, the use of MMP inhibitors with differing selectivities for MMP-related enzymes has identified a novel role for metalloproteinases in controlling lymphocyte transendothelial migration. Identification of the metalloproteinase, the development of selective and long-lived synthetic inhibitors, and the effective delivery of inhibitors to the blood vessel wall will all be required for further analysis of this step in extravasation. It will be interesting to determine whether transendothelial migration of leukocytes at inflammatory sites or of metastasizing tumor cells is also metalloproteinase dependent. Identification of the metalloproteinases that affect lymphocyte transendothelial migration is therefore a major goal for the future.

Acknowledgments

Thanks go to Liz Hirst for help with confocal microscopy, Stamatis Pagakis for image analysis, and Joe Brock for the figures. We gratefully acknowledge Bill Luscinskas, Dylan Edwards, Gillian Murphy, and Ian Frayling for helpful comments on the manuscript and John Nixon and Kevin Bottomley for advice about inhibitors.
Transendothelial migration of lymphocytes across high endothelial venules into lymph nodes is affected by metalloproteinases

Christelle Faveeuw, Graham Preece and Ann Ager