The Induction of 72-kD Gelatinase in T Cells upon Adhesion to Endothelial Cells Is VCAM-1 Dependent

Anne M. Romanic and Joseph A. Madri
Department of Pathology, Yale University School of Medicine, New Haven, Connecticut 06510

Abstract. T cell extravasation from the bloodstream into the perivascular tissue during inflammation involves transmigration through the endothelial cell layer and basement membrane into the interstitial matrix. The specific mechanisms by which T cells transmigrate, however, are poorly understood. Matrix degradation by enzymes such as 72-kD gelatinase has been implicated as an important component in tissue invasion by various types of cells. In this study, we have demonstrated that 72-kD gelatinase is induced in T cells upon adhesion to endothelial cells. We also provide evidence that the induction of 72-kD gelatinase is mediated by binding to vascular cell adhesion molecule-1 (VCAM-1). The T cells used in this study were cloned murine Th1 cells antigenic to myelin basic protein. These cells express very late antigen-4 on their cell surface and have been shown to infiltrate the brain parenchyma and cause experimental autoimmune encephalomyelitis when infused into normal mice (Baron, J. L., J. A. Madri, N. H. Ruddle, G. Hashim, and C. A. Janeway. 1993. J. Exp. Med. 177:57-68). In the experiments presented here, T cells were cocultured with VCAM-1-positive and -negative endothelial cells grown in a monolayer in order to study the expression of 72-kD gelatinase upon T cell adhesion. Additional experiments were conducted in which T cells were cocultured with VCAM-1 positive cells grown on microporous membranes suspended in transwells to study 72-kD gelatinase following T cell transmigration. T cells were also incubated with recombinant VCAM-1 in order to study the role of VCAM-1 in inducing 72-kD gelatinase. The results demonstrated that T cells adhered to both VCAM-1-positive and -negative endothelial cells. T cells that adhered to the VCAM-1-positive endothelial cells exhibited an induction in 72-kD gelatinase protein, activity, and mRNA whereas 72-kD gelatinase was not induced in the T cells that adhered to the VCAM-1-negative endothelial cells. Incubating T cells with recombinant VCAM-1 coated onto tissue culture plastic showed that T cells adhered to the molecule and that adhesion to recombinant VCAM-1 was sufficient to induce 72-kD gelatinase. Further, T cells that had transmigrated through a VCAM-1-positive endothelial cell monolayer exhibited 72-kD gelatinase activity but not mRNA expression. In addition, 72-kD gelatinase was detected on the cell surface of the transmigrated T cells by FACS analysis. In other experiments, TIMP-2 was added to the transmigration studies and was shown to reduce T cell transmigration. The results demonstrate that binding to VCAM-1 on endothelial cells induces 72-kD gelatinase in T cells which, in turn, may facilitate T cell migration into perivascular tissue.

The transmigration of T cells from the bloodstream into perivascular tissue represents a critical event in the process of inflammation. The initial step in this process is characterized by the adhesion of T cells to the endothelial cells lining the blood vessel wall via a variety of cell surface receptors (Albelda and Buck, 1990; Springer, 1990; Shimizu et al., 1991). The receptors on endothelial cells which interact with T cells can be divided into two classes. The first class consists of proteins which are members of the immunoglobulin superfamily. One member of this class is vascular cell adhesion molecule-1 (VCAM-1) which is a ligand for the α4β1 integrin also known as very late antigen-4 (VLA-4) (Osborn et al., 1989; Wayner et al., 1989; 1. Abbreviations used in this paper: EAE, experimental autoimmune encephalomyelitis; ECM, extracellular matrix; ELAM-1, endothelial leukocyte adhesion molecule-1; ICAM-1, intercellular adhesion molecule-1; IFN-γ, interferon γ; IL-2, interleukin 2; LFA-1, leukocyte function-associated antigen-1; MBP, myelin basic protein; MMP, matrix metalloproteinase; PECAM-1, platelet endothelial cell adhesion molecule-1; p-APMA, p-aminophenylmercuric acetate; rs, recombinant soluble; Th cells, helper T cells; TIMP, tissue inhibitor of metalloproteinase; tPA, urokinase plasminogen activator; VCAM-1, vascular cell adhesion molecule-1; VLA-4, very late antigen-4.)
leukocyte adhesion molecule-1 (ELAM-1), binds carbohydrate moieties called selectins (Bevilacqua and Nelson, 1990; Tanaka et al., 1992) and interacts in a homophilic manner during T cell-endothelial cell interactions (Bogen et al., 1992). The second class of receptors is a group of glycoproteins called selectins (Bevilacqua and Nelson, 1993). One member of this group, E-selectin endothelial leukocyte adhesion molecule-1 (ELAM-1), binds carbohydrate ligands on T cells. Conversely, T cells contain the receptor L-selectin (LAM-1) which interacts with carbohydrate ligands on endothelial cells.

Engagement of these and other receptor pairs triggers a number of intracellular signaling events in T cells (Shimizu and Shaw, 1991; Hynes, 1992). Binding of VLA-4 either with antibodies to the α4 subunit or with the alternatively spliced CSI domain of fibronectin stimulates tyrosine phosphorylation of a 150-kD protein in T cells (Nojima et al., 1992). Furthermore, engagement of the VLA-5 (α5β1) integrin on T cells with fibronectin has been shown to induce the expression of the AP-1 transcription factor which regulates interleukin-2 (IL-2) transcription (Yamada et al., 1991).

Engagement of cell adhesion receptors on T cells may also induce the synthesis and secretion of proteins such as matrix metalloproteinases. Matrix metalloproteinases are a family of enzymes which aid in the degradation of basement membrane and interstitial matrix proteins (Woessner, 1991). One of the proteases responsible for the degradation of the basement membrane is 72-kD gelatinase, having as substrates, collagen IV and denatured collagens (Liootta et al., 1979, 1981; Salo et al., 1983). Other enzymes in the matrix metalloproteinase family include interstitial collagenase, stromelysin, matrilysin, and 92-kD gelatinase (Woessner, 1991). Werb et al. (1989) demonstrated that engagement of the fibronectin receptor, VLA-5 or α5β1, on fibroblasts induced the expression of stromelysin and collagenase. Likewise, Seftor et al. (1992) showed that ligation of the vitronectin receptor, αvβ3, on A375M melanoma cells stimulated 72-kD gelatinase expression. These results suggest that signals transduced by the binding of integrins with their respective ligands can regulate the expression of proteases that modulate the extracellular environment.

Various studies have implicated 72-kD gelatinase as an important component in tumor migration and tissue invasion. In metastatic tumor cells which exhibit increased invasiveness into the basement membrane, the level of 72-kD gelatinase was found to be elevated (Liotta et al., 1979; Salo et al., 1983; Fessler et al., 1984; Mignatti et al., 1986; Höyhtyä et al., 1990). Further, inhibitors of 72-kD gelatinase have been shown to inhibit the invasion of metastatic cells through collagenous tissues (Mignatti et al., 1986; Schultz et al., 1988; Höyhtyä et al., 1990). These investigations suggest a role for 72-kD gelatinase in cell migration and tissue invasion. Since migrating T cells have an invasive characteristic, we propose that 72-kD gelatinase is likely an important component of T cell migration.

In inflammatory diseases, T cells invade the perivascular tissue where they cause damage to the tissue. The class of T cells which give rise to an inflammatory response are the helper T cells (Th cells), in particular the Th1 subclass. Th1 cells are involved in mediating an inflammatory response in antigen-specific delayed-type hypersensitivity reactions and autoimmune diseases (Vitetta and Paul, 1991). Experimental allergic encephalomyelitis (EAE) serves as an animal model for studying autoimmune diseases mediated by Th cells as well as for studying T cell adhesion to and transmigration through endothelial cells and invasion into perivascular tissue (Naparstek et al., 1984; Zamvil et al., 1985; Cross et al., 1990; Baron et al., 1993). Yednock et al. (1992) demonstrated that lymphocytes adhered to inflamed EAE brain blood vessels in vitro and that adhesion was inhibited by antibodies against the VLA-4 integrin, but not by antibodies against other adhesion receptors. Baron et al. (1993) demonstrated that the administration of T cells sensitized to myelin basic protein (MBP) along with antibodies against VLA-4 diminished the number of T cells that infiltrated the brain parenchyma and delayed the onset of EAE. These studies strongly suggest that the VLA-4 integrin is an important determinant for T cell entry into the central nervous system (CNS) leading to the development of EAE.

The mechanisms by which T cells actively invade the perivascular tissue are virtually unknown. We show here that the binding of cloned MBP-specific Th1 cells to VCAM-1 on endothelial cells induces the expression of 72-kD gelatinase in T cells. In the experiments presented here, T cells were cocultured with VCAM-1-positive and -negative endothelial cells grown on collagen-coated plastic to study the induction of 72-kD gelatinase upon T cell adhesion. In other experiments, T cells were cocultured with endothelial cells grown on collagen-coated microporous membranes suspended in Transwells® to study 72-kD gelatinase expression following T cell transmigration. In the T cells that adhered to and transmigrated through VCAM-1 positive endothelial cells, 72-kD gelatinase was induced and was also detected on the cell surface by FACS analysis. Additionally, when TIMP-2 was added to the transwell system, T cell transmigration through the VCAM-1 positive endothelial cells was reduced. In further experiments, T cells were incubated with recombinant soluble VCAM-1 (rsVCAM-1) or ICAM-1 (rsICAM-1) coated onto tissue culture plastic. These experiments showed that rsVCAM-1 supported T cell adhesion and was sufficient to elicit induction of 72-kD gelatinase while adhesion to rsICAM-1 was not. Thus, we conclude that the binding to VCAM-1 on endothelial cells causes the induction of 72-kD gelatinase in T cells which, in turn, may facilitate T cell migration into perivascular tissue. The role of T cell and endothelial cell proteinase/proteinase inhibitor modulation in the process of T cell extravasation during inflammation is discussed.

Materials and Methods

Antibodies

An anti-mouse α4 integrin monoclonal antibody (Rm1-2, #HB 227; American
Type Culture Collection, Rockland, MD), and an anti-mouse LFA-1 monoclonal antibody (M17/5.2, #TIB 237, American Type Culture Collection) were provided by Dr. Charles A. Janeway, Yale University (New Haven, CT). An anti-rat VCAM-1 monoclonal antibody (SFU) and an anti-human VCAM-1 monoclonal antibody (4B9) were gifts from Dr. Roy Lobb, Biogen Inc. (Cambridge, MA). A monoclonal antibody directed against human ELAM-1 (H4/18) was a gift from Dr. Jordan S. Pober, Yale University (New Haven, CT). An anti-human ICAM-2 monoclonal antibody (CBR122/2) was a gift from Dr. Timothy Springer, Harvard University (Boston, MA). Polyclonal antibodies directed against human PECAM-1 (Houston) and bovine PECAM-1 (Elise) were provided by Dr. Steven M. Albeida, University of Pennsylvania (Philadelphia, PA). Anti-72-kD gelatinase polyclonal antibodies (Ab 31 and Ab 45) were provided by Dr. William G. Stetler-Stevenson, National Institutes of Health (Bethesda, MD). Commercial goat anti-rat IgG, goat anti-rabbit IgG, and goat anti-mouse IgG antibodies conjugated to fluorescein isothiocyanate were purchased from HyClone Laboratories (Logan, UT). A goat anti-rabbit IgG antibody conjugated to alkaline phosphatase was purchased from Promega Corp. (Madison, WI).

Materials and Reagents
Ac 1-16 peptide fragment of MBP, prepared as described (Baron et al., 1993), and recombinant IL-2 were provided by Dr. Charles A. Janeway. Lymphocyte Separation Media was purchased from Organon-Teknika Corp. (Durham, NC), Mitomycin C, p-aminophenylmercuric acetate (p-APMA), N-ethylmaleimide, PMSF, and Brij 35 were purchased from Sigma Chemical Co. (St. Louis, MO). The bicinchoninic acid protein assay kit was purchased from Pierce (Rockford, IL). Immobilon P membranes were purchased from Millipore Corp. (Bedford, MA). Recombinant soluble human VLA-4, prepared as described (Lebovitz et al., 1993), and anti-rat IgG, goat anti-rabbit IgG, and goat anti-mouse IgG antibodies were purchased from a commercial source. The anti-rabbit IgG, goat anti-rabbit IgG, and goat anti-mouse IgG antibodies were purchased from a commercial source. Immobilon P membranes were purchased from Schleicher & Schuell, Inc. (Keene, NH). X-omat AR x-ray film was purchased from Kodak (Rochester, NY). All other materials were of reagent grade.

Cells and Cell Culture
The T cells used in these studies were cloned, murine CD4⁺ Th1 cells, C19 osH, provided by Dr. Charles A. Janeway. These cells have been fully characterized and are specific for MBP antigen (Baron et al., 1993). These cells were chosen specifically for these experiments based on their invasive characteristic, as they have been shown to invade the brain parenchyma and induce EAE when injected into mice (Baron et al., 1993). T cells were maintained in culture as described by Baron et al. (1993). Briefly, 2 × 10⁶ T cells were stimulated every 14 d with 6 × 10⁵ syngeneic feeder cells, treated with mitomycin C (50 µg/ml), a peptide fragment of MBP corresponding to the first 16 amino acids of the molecule (Ac 1-16; 5 µg/ml), 10 ng/ml IL-2 (3.1), or in Dr. Karl Trygvasson’s media. Two to four days after stimulation, about 2 × 10⁷ T cells were obtained. T cells were cultured at 37°C, 5% CO₂. Before using the T cells for experiments, they were separated from the feeder cells using LCM (Organon-Teknika) according to the manufacturer’s instructions.

One of the endothelial mononuclear cell lines used in the following experiments was a microvascular endothelial cell line derived from rat epididymal fat pads, RFC cells, isolated and cultured as described by Madri and Williams (1983). Approximately 1 × 10⁶ endothelial cells were cultured in 7-T5 tissue culture flask on a substratum of collagen I (12.5 µg/ml in a 0.1 M sodium bicarbonate buffer, pH 9.4) with DMEM containing 10% FCS and 0.005 % sodium diphosphate using a cytospin (Shandon Inc., Pittsburgh, PA). These cells were detached by washing quickly one time with a warm 0.004% trypsin/0.002% EDTA solution in PBS prior to lysis. The T cell samples during the trypsin wash, aliquots of the recovered T cells were detached by washing with a warm 0.004% trypsin/0.002% EDTA solution in PBS for ~1 min. Samples of nonadherent and adherent T cells were stained with trypan blue and counted to calculate the percentage of adherent cells and to assure cell viability. To establish that endothelial cells had not contaminated the T cell samples during the trypsin wash, aliquots of the recovered T cells were viewed by differential interference contrast light microscopy. For these experiments, cells were affixed to glass slides coated with 0.1% gelatin, 0.005% sodium diphosphate using a cytospin (Shandon Inc., Pittsburgh, PA) at 1500 rpm for 5 min at room temperature. The samples of the recovered T cells were then compared to control T cell and endothelial cell samples prepared using the same technique. The size differences between the two cell types allowed identification of endothelial cells and T cells. For protein analysis, extracts of the T cells were prepared. First, however, the trypsin and EDTA might have interfered with enzyme activity or protein cell adhesion, the peptides were washed two times with cold PBS prior to lysis. The T cells were lysed with ice-cold buffer consisting of 0.05% Triton X-100, 0.01% NaN₃ in 120 mM Tris-HCl, pH 8.7. The cell extracts were then centrifuged at 14,000 rpm for 5 min at 4°C and the supernatant fractions collected and stored at -20°C. Protein concentration in the cell extracts was measured by the bicinchoninic acid protein assay (Pierce) according to the manufacturer’s instructions. The samples were then assayed for 72-kD gelatinase as described below.

Incubation of T Cells on rsVCAM-1 and rsICAM-1
To determine if engagement of the VLA-4/VCAM-1 receptor pair was sufficient for the induction of 72-kD gelatinase, T cells were incubated with a rsVCAM-1 molecule coated onto tissue culture plastic and assayed for proteinase induction as described below. As a control, T cells were incubated with a rsICAM-1 molecule coated onto tissue culture plastic. To assure for specificity of binding, T cells were also incubated in tissue culture flasks coated with 1% BSA. Tissue culture flasks were coated with rsVCAM-1 or rsICAM-1 (10 µg/ml) in a 0.1 M sodium bicarbonate buffer, pH 9.4, or with BSA (1% in PBS) overnight at 4°C. Unoccupied binding sites were blocked by incubating with 1% BSA in PBS for 1 h at 37°C. All the flasks were then washed twice with PBS warmed to 37°C. T cells (2 × 10⁷/ml) in suspension either in 50-ml polypropylene tubes or in T-75 culture flasks were added to the flasks and incubated for 5 or 20 h at 37°C. The nonadherent T cells were removed and the adherent T cells were detached by washing quickly one time with a warm 0.004% trypsin/0.002% EDTA solution in PBS. Cell extracts were prepared as described above.

FACS Analysis
T cells were stained for VLA-4, LFA-1, and 72-kD gelatinase and endothelial cells were stained for VCAM-1, ELAM-1, ICAM-2, and PECA1 cell surface expression by indirect immunofluorescence and analyzed by FACS. Endothelial cells first were trypsinized and then washed with 2% BSA in PBS to inactivate the trypsin. Approximately 1 × 10⁶ cells were incubated with their respective primary antibody for 1 h at 4°C. The cells were washed three times and then incubated with a secondary antibody conjugated to fluorescein for 30 min at 4°C. The cells were washed as above and then fixed with 1% paraformaldehyde in PBS. All antibody dilutions and washes were made with a staining buffer consisting of 5% FCS, 0.01% NaN₃ in PBS, sterile filtered. Immunofluorescent analysis was performed using a FACStar Plus® fluorescent activated cell sorter (Becton Dickinson Immunocytometry Sys., Mountain View, CA).

T Cell/Endothelial Cell Adhesion Assay
To study the effect of T cell/endothelial cell adhesion on 72-kD gelatinase induction in vitro, the cells were cocultured in the following system. Endothelial cells were cultured to form a confluent monolayer in collagen I-coated T-75 tissue culture flasks and then serum starved for 24 h. T cells (2 × 10⁶/ml), suspended in serum-free DMEM, were then added to the endothelial cells and cocultured for 5 h at 37°C, 8% CO₂, during which time T cell/endothelial cell adhesion occurred. After the coculture period, the nonadherent T cells were removed by washing with PBS warmed to 37°C. The adherent T cells were detached by washing with a warm 0.004% trypsin/0.002% EDTA solution in PBS for ~1 min. Samples of nonadherent and adherent T cells were stained with trypan blue and counted to calculate the percentage of adherent cells and to assure cell viability. To establish that endothelial cells had not contaminated the T cell samples during the trypsin wash, aliquots of the recovered T cells were viewed by differential interference contrast light microscopy. For these experiments, cells were affixed to glass slides coated with 0.1% gelatin, 0.005% sodium diphosphate using a cytospin (Shandon Inc., Pittsburgh, PA) at 1500 rpm for 5 min at room temperature. The samples of the recovered T cells were then compared to control T cell and endothelial cell samples prepared using the same technique. The size differences between the two cell types allowed identification of endothelial cells and T cells. For protein analysis, extracts of the T cells were prepared. First, however, as the trypsin and EDTA might have interfered with enzyme activity or protein cell adhesion, the peptides were washed two times with cold PBS prior to lysis. The T cells were lysed with ice-cold buffer consisting of 0.05% Triton X-100, 0.01% NaN₃ in 120 mM Tris-HCl, pH 8.7. The cell extracts were then centrifuged at 14,000 rpm for 5 min at 4°C and the supernatant fractions collected and stored at -20°C. Protein concentration in the cell extracts was measured by the bicinchoninic acid protein assay (Pierce) according to the manufacturer’s instructions. The samples were then assayed for 72-kD gelatinase as described below.
positive RFC cells; thick-lined curve represents PECAM-1 positive cells. Figure 1, A Th1 cells, C19o~4H, were stained with antibodies directed against VLA-4 integrin (R1-2) followed by fluorescein-conjugated secondary antibodies. Shaded curve represents the negative control; thin-lined curve represents VCAM-1 positive endothelial cells. (B) Rat microvascular endothelial cells, RFC cells, were stained with antibodies directed against VCAM-1 (5F10) and PECAM-1 (Elsie) followed by fluorescein-conjugated secondary antibodies. Shaded curve represents the negative control; thin-lined curve represents VCAM-1 positive RFC cells; thick-lined curve represents PECAM-1 positive RFC cells, shown here as a control.

Transmigration Assay
To determine the level of induction of 72-kD gelatinase following transmigration of the adherent T cells, a Transwell® system was employed. Before using the transwells, the bottom wells were coated with 1% BSA in PBS overnight at 4°C to prevent nonspecific attachment of transmigrated T cells to the tissue culture plastic. Endothelial cells were cultured to confluence in collagen-coated polycarbonate membrane filters (Transwell-COL® filters, 3-μm pore size, 6.5- or 24.5-mm diameter; Costar Corp., Cambridge, MA) resting in a transwell chamber and then serum starved for 24 h. T cells (2 x 10⁶/ml) were added to the transwells and cocultured with the endothelial cells for up to 24 h at 37°C. The T cells that transmigrated through the endothelial cell monolayer and the porous membrane were collected in the bottom part of the chamber, washed with PBS, lysed as described above, and then assayed for 72-kD gelatinase.

In some experiments TIMP-2 (10 μg/ml) was added to both the upper and lower chambers on transwells to investigate the effects of 72-kD gelatinase and TIMP-2 on T cell transmigration. To determine the percentage of T cells that transmigrated in the presence and absence of TIMP-2, T cells were collected from the bottom part of the chamber and counted using a Coulter counter (Hialeah, FL).

Cytokine Assays
To assay for cytokine induction upon T cell adhesion, the culture media from the coculture experiments was collected and assayed for cytokine activity by performing standard cell proliferation experiments as measured by [3H]thymidine uptake in response to a 72-h incubation. The level of cytokine was assayed by measuring the induction of proliferation of CTLL-2 and CT4S cells, respectively. IFN-γ was determined by the inhibition of proliferation of WEHI-279 cells. CTLL-2 cells, CT4S cells, and WEHI-279 cells were obtained from Dr. Kim Bottomly, Yale University (New Haven, CT). Briefly, CTLL-2 (1 x 10⁵/well), CT4S (5 x 10⁵/well), and WEHI-279 cells (1 x 10⁶/well) were incubated with varying dilutions of the culture medium obtained from the cocultured cells, with fresh DME, or with media from control endothelial cells and T cells that were cultured independently from one another. All dilutions were made with EHAA (Click's medium) plus 5% FCS. The cells were incubated for 48 h and then pulsed with [3H]thymidine for 16 h before harvesting. The relative amount of each cytokine was measured by comparing the level of cell proliferation using medium from the cocultures to the level of proliferation using fresh DMEM or media from control endothelial cells and T cells.

Western Blot Analysis
To investigate the induction of 72-kD gelatinase protein, cell extracts of the adherent and control T cells were assayed by Western analysis. Samples, normalized for protein concentration, were electrophoresed through a 10% polyacrylamide gel (Laemmli, 1970) and transferred to an Immobilon P membrane, and then processed for Western blot analysis. Unoccupied binding sites on the membrane were blocked overnight at 4°C with 5% nonfat powdered milk in a 0.1 M Tris-HCl buffer containing 1.5 M NaCl, 0.5% Triton X-100 (TBST buffer). A primary polyclonal antibody against 72-kD gelatinase, diluted in TBST buffer, was added to the membrane and allowed to incubate for 2 h at 25°C. The membrane was washed three times, 20 min each, with TBST buffer and incubated for 1 h at 25°C with a secondary antibody conjugated to alkaline phosphatase. The membrane was washed again, rinsed with water, and then incubated for 5 min with Western Blue™ (Promega, Corp.) stabilized substrate for alkaline phosphatase.

Gelatinase Zymography
72-kD gelatinase activity was assayed by zymography as described by Heussen and Dowdle (1980) and Unemori and Werb (1986) with modification. Briefly, 20 μg of cell extracts were subjected to electrophoresis, without boiling or reduction, through a 10% polyacrylamide gel impregnated with gelatin (0.2 mg/ml). Electrophoresis was conducted at 4°C to prevent gelatin degradation. After electrophoresis was complete, the gel was incubated for 1 h at 25°C in a 2.5% Triton X-100 solution, washed two times, 20 min each, with water and then incubated for 10–24 h at 37°C in a 0.05 M Tris-HCl buffer, pH 8.0, containing 0.005 M CaCl₂. As a control, duplicate samples were loaded onto another gel which was then incubated in a 0.05 M Tris-HCl buffer, pH 8.0, containing 0.001 M EDTA. The gels were fixed with 50% methanol and 10% acetic acid and stained with 0.25% Coomassie blue R250. Gelatinase activity was visualized as clear bands in a blue background.

[3H]Collagen IV Digestion Assay
To measure the specific digestion of collagen IV in solution, the cell extracts, normalized for protein concentration, were incubated with [3H]collagen IV (10 μg/ml) as described previously (Liotta et al., 1981) with modification. In summary, 30 μg of the cell extract were activated with 1 mM p-APMA for 1 h at 37°C (Stetler-Stevenson et al., 1989). Following activation, N-ethylmaleimide and PMSF were added to the reaction mixtures to final concentrations of 65 μg/ml which were then incubated with [3H]collagen IV (7,000 cpm) in a 0.05 M Tris–HCl buffer, pH 7.6, containing 0.005 M CaCl₂, 0.2 M NaCl, and 0.002% Brij 35 for 18 h at 35°C. The final volume of the reaction mixture was 380 μl. The reaction was stopped by the addition of 10 mM EDTA and placed on ice for 30 min. The samples were centrifuged at 14,000 rpm for 10 min at 4°C using NanoSpin filtration units with a cut-off range of 100,000 mol wt (Gelman Sciences Inc.). Digested [3H]collagen IV was collected in the filtrate and measured for radioactivity by liquid scintillation using an LKB liquid scintillation counter. As controls, some of the samples were not activated with p-APMA in order to examine the precursor–product ratio and to show that p-APMA was indeed able to activate the samples. In other samples 10 mM EDTA was added to the initial reaction mixtures to show that the collagen IV degradation could be inhibited.
Figure 2. Differential interference contrast light microscopy of T cells adherent to VCAM-1-positive endothelial cells. T cells were cocultured with VCAM-1-positive RFC endothelial cells, grown in monolayer, for 5 h at 37°C. (A) adherent and nonadherent T cells cocultured for 5 h. (B) T cells adherent to endothelial cells after nonadherent T cells were removed. 88 ± 8% of the T cells adhered to the VCAM-1-positive endothelial cells. (C) About 90% of the adherent T cells were effectively removed without disturbing the endothelial cell monolayer. Arrowheads denote the few T cells that were not removed. Arrows indicate T cells that had transmigrated through the endothelial cells and were now beneath the endothelial cell monolayer. (D) Control VCAM-1-positive RFC endothelial cells. Bar, 100 μm.

Northern Blot Analysis

The expression of 72-kD message was analyzed by Northern blot analysis as follows. Total cellular RNA was extracted from adherent and control T cells using standard protocols as described by Sambrook et al. (1989). Approximately 5 μg of total RNA was denatured with 20 mM MOPS, 0.5 mM EDTA in 5 mM sodium acetate, electrophoresed through a 1% agarose gel, and then transferred to a Nytran membrane (Schleicher & Schuell, Inc.). To prepare for hybridization, the blot was incubated with Rapid-hyb buffer (Amersham Corp.) for 30 min at 65°C. A 32P-cDNA probe encoding 72-kD gelatinase (prepared from the cDNA clone MC-1 as described by Reponen et al., 1992) was radiolabeled using the Prime-It II Random Primer Labeling Kit (Amersham Corp.) then added directly to the Rapid-hyb buffer and incubated for 2.5 h at 65°C. The blot was washed at 67°C two times, 30 min each, with 2× SSC (300 mM NaCl, 30 mM trisodium citrate, pH 70) containing 0.1% SDS, dried, and exposed to X-omat AR film (Kodak). To standardize the relative amount of 72-kD mRNA per lane, the blot was also hybridized with 32P-cDNA probe for γ-actin.

Results

T Cells Adhere to VCAM-1-positive Endothelial Cells

Th1 cells sensitized to MBP and capable of infiltrating the brain parenchyma and inducing EAE when injected into syngeneic mice were immunolabeled with an antibody to VLA-4 and shown to be positive for the receptor (Fig. 1; Baron et al., 1993). These cells were cocultured with microvascular endothelial cells that express constitutive levels of VCAM-1, as demonstrated by FACS analysis (Fig. 1). Within a 5-h coculture period, 88 ± 8% of the T cells adhered to the endothelial cells with some cells observed to transmigrate through the monolayer (Fig. 2 and Table I). For analysis of 72-kD gelatinase induction, it was necessary to remove the T cells from the endothelial cell monolayer without damaging the T cells or removing the endothelial cells. A gentle washing procedure using trypsin and EDTA was utilized. As demonstrated in Fig. 2, ~90% of the T cells were successfully removed leaving the endothelial cell monolayer intact. Also, staining with trypan blue indicated no loss of viability in the T cells collected (not shown). To establish that endothelial cells were not removed during the washing procedure, aliquots of the recovered cells were affixed to glass slides using a cytospin and analyzed by DIC light microscopy. Of the cells collected from the cocultures, less than 1% were endothelial cells (not shown).

As shown in fibroblasts and synovial cells, cytokines in-
The presence and absence of TIMP-2 was significant (P = 0.033). Also, transmigrated T cells for 20 h in the presence or absence of TIMP-2 (10 μg/ml) were subjected to electrophoresis through a 10% polyacrylamide gel impregnated with gelatin (0.2 mg/ml) as described in Materials and Methods. Lane 1, control T cells; lane 2, adherent T cells. 72-kD gelatinase protein was observed only in the adherent T cells. The small arrow indicates 7-actin. Lane 1, control T cells; lane 2, adherent T cells. Expression of 72-kD gelatinase protein was observed only in the adherent T cells. (C) T cells were assayed for expression of 72-kD gelatinase mRNA by Northern blot analysis. For each sample, 5 μg of total RNA was resolved by electrophoresis in a 1% agarose gel. The samples were transferred to a Nytran membrane and hybridized with a 2.8-kb 32P-labeled cDNA probe encoding 72-kD gelatinase as described in Materials and Methods. To standardize the relative amount of mRNA per lane, the blot was also hybridized with a 1.4-kb 32P-labeled cDNA probe encoding γ-actin. Lane 1, control T cells; lane 2, adherent T cells. Expression of 72-kD gelatinase mRNA was detected only in the adherent T cells, as denoted by the large arrow. The small arrow indicates γ-actin mRNA.

**Table 1. Percentage of T Cells That Adhere to and Transmigrate through VCAM-1-Positive and -Negative Endothelial Cell Monolayers**

|                      | % Adhesion          | % Transmigration |
|----------------------|---------------------|-----------------|
| VCAM-1-positive cells| 88 ± 8% n = 7       |                 |
| VCAM-1-negative cells| 60 ± 3% n = 6       |                 |

VCAM-1-positive endothelial cells - TIMP-2 84 ± 11% n = 4
VCAM-1-positive endothelial cells + TIMP-2 38 ± 16% n = 4
VCAM-1-negative endothelial cells - TIMP-2 45 ± 10% n = 4
VCAM-1-negative endothelial cells + TIMP-2 39 ± 8% n = 4

T cells (2 × 10^6/ml) were allowed to adhere to VCAM-1-positive or -negative endothelial cells for 5 h, removed, and then counted by trypan blue exclusion as described in Materials and Methods. Each experiment consisted of three cocultures. For transmigration studies, T cells (2 × 10^6/ml) were added to transwells containing VCAM-1-positive or -negative endothelial cells for 20 h in the presence or absence of TIMP-2 (10 μg/ml). Transmigrated T cells were collected in the bottom chambers and counted using a Coulter counter as described in Materials and Methods. Each experiment consisted of four transwells. Results are presented as mean and SD. The difference in transmigration of T cells through VCAM-1-positive endothelial cells in the presence and absence of TIMP-2 was significant (P = 0.033). Also, transmigration of T cells through VCAM-1-positive and -negative endothelial cells in the absence of TIMP-2 was significantly different (P = 0.024). Transmigration of T cells through VCAM-1-negative endothelial cells in the presence and absence of TIMP-2 was not significantly different (P = 0.432).

**Adhesion of T Cells to VCAM-1-Positive Endothelial Cells Induces 72-kD Gelatinase Expression and Activity**

Samples of adherent T cells collected from coculture were analyzed for 72-kD gelatinase by Western blot. Results showed that adherent T cells expressed 72-kD gelatinase protein while control T cells did not (Fig. 3 B). 72-kD gelatinase activity was also assayed by zymography. As shown in Fig. 3 A, 72-kD gelatinase activity was induced in adherent T cells whereas it was absent in control T cells. To investigate the specific degradation of collagen IV, cell extracts were incubated with [3H]collagen IV in solution. Collagen IV digestion was measured by counting the amount of degraded radiolabeled substrate collected in the filtrate of the reaction mixture. Fig. 4 A indicates that the adherent T cells digested collagen IV 1.7-fold more compared to the control which had no activity. The results also showed that colla-

**Figure 3. Gelatinase zymography, Western blot analysis, and Northern blot analysis for 72-kD gelatinase of T cells adherent to VCAM-1-positive endothelial cells.** T cells were cocultured with VCAM-1-positive RFC endothelial cells for 5 h at 37°C. Then the adherent T cells were collected and assayed for 72-kD gelatinase activity by zymography, for protein by Western blot, and for mRNA expression by Northern blot. (A) To assay for 72-kD gelatinase by zymography, cell extracts (20 μg each) of control and adherent T cells were subjected to electrophoresis through a 10% polyacrylamide gel impregnated with gelatin (0.2 mg/ml) as described in Materials and Methods. Lane 1, control T cells; lane 2, adherent T cells. Lane 3, control T cells; lane 4, adherent T cells. Gel containing lanes 1 and 2 was incubated with CaCl2 and gel containing lanes 3 and 4 was incubated with EDTA. Arrows pointing to clear bands denote the 72-kD proenzyme form and the 67-kD active form of 72-kD gelatinase present in the adherent T cell samples. 72-kD gelatinase activity was not detected in control T cells or in the samples incubated with EDTA. Molecular mass markers are as indicated. (B) Cell extracts (20 μg each) of control and adherent T cells were subjected to electrophoresis through a 10% polyacrylamide gel and transferred to an Immobilon P membrane for Western blot analysis as described in Materials and Methods using a polyclonal antibody directed against 72-kD gelatinase (Ab 31). Lane 1, control T cells; lane 2, adherent T cells. 72-kD gelatinase protein was observed only in the adherent T cells. (C) T cells were assayed for expression of 72-kD gelatinase mRNA by Northern blot analysis. For each sample, 5 μg of total RNA was resolved by electrophoresis in a 1% agarose gel. The samples were transferred to a Nytran membrane and hybridized with a 2.8-kb 32P-labeled cDNA probe encoding 72-kD gelatinase as described in Materials and Methods. To standardize the relative amount of mRNA per lane, the blot was also hybridized with a 1.4-kb 32P-labeled cDNA probe encoding γ-actin. Lane 1, control T cells; lane 2, adherent T cells. Expression of 72-kD gelatinase mRNA was detected only in the adherent T cells, as denoted by the large arrow. The small arrow indicates γ-actin mRNA.
HEG.

Figure 4. [3H] Collagen IV digestion graph of T cells cultured under various experimental conditions. T cells were assayed for the specific digestion of collagen IV in solution. Results are presented as fold increase in [3H]collagen IV digestion relative to the control. Each experiment was done in duplicate and the number of experiments done are shown in parentheses. Values represent the mean and standard error of 3-5 experiments. The average cpm collected in the filtrate ranged from ~700 cpm in the control to ~2500 cpm in the adherent and transmigrated samples. (A) Cell extracts (30 µg each) were activated for 1 h at 37°C with 1 mM p-APMA and then incubated for 18 h at 35°C with [3H]collagen IV (7,000 cpm) as described in Materials and Methods. Samples assayed are as follows: Control T cells; T cells that adhered to VCAM-1-positive RFC endothelial cells for 5 h; T cells that adhered to VCAM-1-negative ECV304 endothelial cells for 5 h; T cells that adhered to rsVCAM-1 for 5 h; T cells that transmigrated through a VCAM-1-positive endothelial cell monolayer and collected 20 h after the beginning of the experiment; T cells that transmigrated through a VCAM-1-negative endothelial cell monolayer and collected 20 h after the beginning of the experiment. (B) Cell extracts (30 µg each) were activated for 1 h at 37°C with 1 mM p-APMA and then incubated for 18 h at 35°C with [3H]collagen IV (7,000 cpm) in the presence or absence of 10 mM EDTA. (Open bars) EDTA-treated samples; (shaded bars) untreated samples. Samples assayed are as follows: Control T cells; T cells that adhered to VCAM-1-positive RFC endothelial cells for 5 h; T cells that adhered to rsVCAM-1 for 5 h; T cells that transmigrated through a VCAM-1-positive endothelial cell monolayer and collected 20 h after the beginning of the experiment.

Figure 5. FACS analysis of VCAM-1-negative endothelial cells. ECV304 human endothelial cells were immunolabeled with antibodies directed against VCAM-1 (4B9) and PECAM-1 (Houston) followed by fluorescein-conjugated secondary antibodies as described in Materials and Methods. Shaded curve represents the negative control; thin-lined curve represents ECV304 cells that are negative for VCAM-1; thick-lined curve represents ECV304 cells that are positive for PECAM-1.

T Cells Adhere to VCAM-1-negative Endothelial Cells but Do Not Exhibit Increased 72-kD Gelatinase Expression and Activity

To determine if the induction of 72-kD gelatinase was due to binding to VCAM-1, T cells were cocultured with endothelial cells that did not express VCAM-1 on the cell surface. First, however, to establish that ECV304 endothelial cells did not express VCAM-1 in the culture conditions used, cells were immunolabeled with an antibody directed against VCAM-1 and analyzed by FACS. ECV304 cells were also im-
on VCAM-1 expression differ from those reported by Saunders et al. (1992).

PECAM-1 antibodies. Results demonstrated that VCAM-1, ELAM-1, and ICAM-2 were not expressed on the cell surface nor were their expressions induced with TNFα or IL-1β (Fig. 5 and data not shown). PECAM-1, on the other hand, was expressed on these cells (Fig. 5). Notably, our results on VCAM-1 expression differ from those reported by Saikusa et al. (1992).

When cocultured with ECV304 endothelial cells, 60 ± 3% of the T cells adhered (Table I). Analysis of the adherent T cells by zymography (Fig. 6 A) and the [3H]collagen IV digestion assay (Fig. 4 A) revealed that 72-kD gelatinase activity was not induced. 72-kD gelatinase mRNA expression also was not induced (Fig. 6 B).

**Adhesion of T Cells to Recombinant VCAM-1 is Sufficient for the Induction of 72-kD Gelatinase Expression and Activity**

To examine further the role of VCAM-1 in inducing 72-kD gelatinase, T cells were cultured for 5 h with recombinant soluble human VCAM-1 that had been coated onto tissue culture plastic. Fig. 7 A shows that T cells bound to the rsVCAM-1. Specificity for binding was determined by culturing T cells on tissue culture plastic coated with 1% BSA. Virtually no T cells adhered under these conditions (Fig. 7 C).

Analysis by zymography demonstrated that adhesion of T cells to rsVCAM-1 induced 72-kD gelatinase activity (Fig. 8 A). Also, the adherent T cells degraded [3H]collagen IV 2.7-fold more than the control (Fig. 4 A) and the reaction was inhibited by EDTA (Fig. 4 B). Treatment of cell extracts with and without p-APMA indicated that 70% of the enzyme in the cell extract was of the precursor form (Fig. 4 C). Additionally, Northern blot analysis indicated that 72-kD gelatinase mRNA expression was induced (Fig. 8 B). To investigate if induction of 72-kD gelatinase activity was cumulative over time, cells were allowed to adhere to rsVCAM-1 for 20 h. While more T cells adhered to rsVCAM-1 during this period (not shown), the level of induction, i.e., the specific activity, was about the same as in the T cells adherent for only 5 h (Fig. 4 A). As a control, T cells were also incubated with human rsICAM-1. The results showed that T cells adhered to rsICAM-1 (Fig. 7 B) but that 72-kD gelatinase activity was not induced as measured by zymography (Fig. 8 A). Also, mRNA expression of 72-kD gelatinase was not induced in T cells adherent to rsICAM-1 (Fig. 8 B). A minimal amount of [3H]collagen IV digestion, however, was detected (Fig. 4 A).

**72-kD Gelatinase Activity Persists in T Cells That Have Transmigrated through a VCAM-1-positive Endothelial Cell Monolayer**

The induction of 72-kD gelatinase was investigated following T cell transmigration through a VCAM-1-positive endothelial cell monolayer using a Transwell® system. During a period of up to 20 h, T cells adhered to and transmigrated through the endothelial cell monolayer (Table I). Transmigrated T cells collected at 8 and 20 h exhibited comparable levels of 72-kD gelatinase induction as measured by zymography (Fig. 9 A) and [3H]collagen IV digestion was 2.5-fold greater than control (Fig. 4 A). Collagen IV digestion also was inhibited by EDTA (Fig. 4 B) and treatment with and without p-APMA indicated that 48% of the enzyme in the cell extract was of the precursor form (Fig. 4 C). Expression of 72-kD gelatinase message in the transmigrated T cells, however, was not detected by Northern blot analysis (Fig. 9 B). Similar experiments were also done using the VCAM-1-negative endothelial cells. Modest amounts of T cells transmigrated through the VCAM-1-negative endothelial cell monolayer (Table I) but 72-kD gelatinase expression was not induced (Figs. 4 A, 9 A and B).
Figure 7. Differential interference contrast light microscopy of T cells adherent to VCAM-1, ICAM-1, and BSA coated onto plastic. Tissue culture flasks were coated with rsVCAM-1 (10 µg/ml), rsICAM-1 (10 µg/ml), or BSA (1%) overnight at 4°C as described in Materials and Methods. T cells were then added to the flasks and incubated for 5 h at 37°C. (A) T cells adherent to rsVCAM-1-coated plastic. (B) T cells adherent to rsICAM-1-coated plastic. (C) T cells incubated in BSA-coated flasks. T cells adhered to rsVCAM-1 and rsICAM-1 but not to BSA-coated plastic. Bar, 100 µm.

72-kD Gelatinase Is Present on the Surface of T cells That Have Transmigrated through a VCAM-1-positive Endothelial Cell Monolayer

To determine if the 72-kD gelatinase activity detected in the T cells that transmigrated through the VCAM-1–positive endothelial cells was cell surface associated, the transmigrated T cells were immunolabeled with an anti-72-kD gelatinase antibody and assayed for surface expression by FACS analysis. The results demonstrated that the transmigrated T cells were positive for 72-kD gelatinase on the cell surface (Fig. 10A). Further, we demonstrated that the surface-associated 72-kD gelatinase was T cell derived and that the T cells were not merely collecting an endothelial cell–derived enzyme as they transmigrated. Gelatinase zymography of the culture media and cell extracts of control VCAM-1–positive and negative endothelial cells indicated that both cell types made and secreted 72-kD gelatinase (Fig. 10, C and D).

Figure 8. Gelatinase zymography and Northern blot analysis of T cells adherent to rsVCAM-1 and rsICAM-1. T cells were incubated in rsVCAM-1-coated and rsICAM-1-coated tissue culture flasks for 5 h at 37°C. (A) Adherent T cells were collected, lysed, and assayed for 72-kD gelatinase activity by zymography. Cell extracts (20 µg each) of control and adherent T cells were subjected to electrophoresis through a 10% polyacrylamide gel impregnated with gelatin (0.2 mg/ml) as described in Materials and Methods. Lane 1, control T cells; lane 2, T cells adherent to rsVCAM-1 for 5 h; lane 3, control T cells; lane 4, T cells adherent to rsICAM-1 for 5 h. Arrows pointing to clear bands denote the 72-kD proenzyme form and the 67-kD active form of 72-kD gelatinase present in the T cells adherent to rsVCAM-1. 72-kD gelatinase activity was not detected in the T cells that adhered to rsICAM-1. A separate gel was incubated with EDTA and demonstrated that gelatinolytic activity was inhibited (not shown). Molecular mass markers are as indicated. (B) T cells were assayed for the expression of 72-kD gelatinase mRNA by Northern blot analysis. For each sample, 5 µg of total RNA was resolved by electrophoresis in a 1% agarose gel. The samples were transferred to a Nytran membrane and hybridized with a 2.8-kb 32P-labeled cDNA probe encoding 72-kD gelatinase as described in Materials and Methods. To standardize the relative amount of mRNA per lane, the blot was also hybridized with a 1.4-kb 32P-labeled cDNA probe encoding γ-actin. Lane 1, control T cells; lane 2, T cells adherent to rsVCAM-1 for 5 h; lane 3, T cells adherent to rsICAM-1 for 5 h. Expression of 72-kD gelatinase mRNA was detected only in the T cells that adhered to rsVCAM-1, as denoted by the large arrow. The small arrow indicates γ-actin mRNA.
Endothelial Cell Monolayer

To examine the role of 72-kD gelatinase in T cell transmigration, recombinant TIMP-2, an inhibitor of 72-kD gelatinase, was added to the transwell system. The results demonstrated that T cell transmigration was reduced from 84 ± 11% in the control to 38 ± 16% in the presence of TIMP-2 (Table I). Also, 45 ± 10% of the T cells transmigrated through the VCAM-1-negative endothelial cells in the absence of TIMP-2 while 39 ± 8% transmigrated in the presence of TIMP-2 (Table I).

**Discussion**

An initial event in the process of transmigration is characterized by the adhesion of T cells to endothelial cells lining the blood vessel wall. Adhesion occurs via a variety of cell surface receptor pairs including VLA-4 and VCAM-1 (Albelda and Buck, 1990; Springer, 1990; Shimizu et al., 1991). Following adhesion, T cells transmigrate through the endothelium and the subendothelial basement membrane into the interstitial matrix (Albelda and Buck, 1990; Springer, 1990; Shimizu and Shaw, 1991). The mechanisms by which T cells actively modulate their interactions with endothelial cells or with the ECM during the process of transmigration, however, are essentially unknown. In this study we have focused on the concept that degradation of the extracellular matrix (ECM) is one means by which T cells modulate their surrounding environment, particularly in the process of transmigration. We have demonstrated that 72-kD gelatinase is induced in T cells upon adhesion to endothelial cells. We also provide evidence that the induction of 72-kD gelatinase is mediated by binding to VCAM-1.

Our experiments demonstrated that during a 5-h coculture period T cells adhered to both VCAM-1-positive and -negative endothelial cells, although fewer T cells bound to the VCAM-1-negative cells. Of particular importance, however, was the observation that only T cells which adhered to the VCAM-1-positive endothelial cells exhibited an induction in 72-kD gelatinase protein, activity, and mRNA. Neither 72-kD gelatinase activity nor message was induced in the T cells that adhered to the VCAM-1-negative endothelial cells. Notably, we assayed for the secretion of the cytokines IL-2, IL-4, and IFN-γ and found that they were not induced during the coculture period. This is an important observation since cytokines have been shown to induce metalloproteinase expression in other cells such as fibroblasts and synovial cells (Dayer et al., 1985, 1986; Murphy et al., 1985). Further, incubating fresh T cells with conditioned medium obtained from the cocultures demonstrated that the induction of 72-kD gelatinase was not due to a soluble factor secreted into the medium. Indirectly, we also demonstrated that cell surface-associated cytokines were not a significant con-

**Figure 9.** Gelatinase zymography and Northern blot analysis of T cells that have transmigrated through VCAM-1-positive and -negative endothelial cells. T cells were cocultured for up to 20 h at 37°C with endothelial cells grown on microporous membranes suspended in transwells. Transmigrated T cells were collected in the bottom of the transwell chamber and then prepared either for gelatinase zymography or Northern blot analysis. (A) For zymography, cell extracts (20 μg each) of control and transmigrated T cells were subjected to electrophoresis through a 10% polyacrylamide gel impregnated with gelatin (0.2 mg/ml) as described in Materials and Methods. Lane 1, control T cells; lane 2, T cells that transmigrated through a VCAM-1-positive endothelial cell monolayer and collected 8 h after the beginning of the experiment; lane 3, T cells that transmigrated through a VCAM-1-positive endothelial cell monolayer and collected 20 h after the beginning of the experiment; lane 4, control T cells; lane 5, T cells that transmigrated through a VCAM-1-negative endothelial cell monolayer and collected 8 h after the beginning of the experiment; lane 6, T cells that transmigrated through a VCAM-1-negative endothelial cell monolayer and collected 20 h after the beginning of the experiment. Molecular mass markers are as indicated. Arrows pointing to clear bands denote the 72-kD proenzyme form and the 62-kD active form of 72-kD gelatinase present in the T cells that transmigrated through the VCAM-1-positive endothelial cells and collected at 8 and 20 h. A band correlating in size to ~50 kD was also detected in these T cells and is possibly interstitial collagenase. A duplicate gel was incubated with EDTA and demonstrated that all gelatinolytic activity was inhibited (not shown). 72-kD gelatinase activity was not detected in control T cells or in T cells that transmigrated through the VCAM-1-negative endothelial cells. (B) T cells were assayed for the expression of 72-kD gelatinase mRNA by Northern blot analysis. For each sample, 5 μg of total RNA was resolved by electrophoresis in a 1% agarose gel. The samples were transferred to a Nytran membrane and hybridized with a 2.8-kb 32P-labeled cDNA probe encoding 72-kD gelatinase as described in Materials and Methods. To standardize the relative amount of mRNA per lane, the blot was also hybridized with a 1.4-kb 32P-labeled cDNA probe encoding γ-actin. Lane 1, control T cells; lane 2, T cells that transmigrated through a VCAM-1-positive RFC endothelial cell monolayer and collected 20 h after the beginning of the experiment; lane 3, T cells that transmigrated through a VCAM-1-negative ECV304 endothelial cell monolayer and collected 20 h after the beginning of the experiment. Expression of 72-kD gelatinase mRNA was not detected in any of the T cell samples, as denoted by the large arrow. The small arrow indicates γ-actin mRNA.
Figure 10. FACS analysis of T cells that transmigrated through VCAM-1-positive and -negative endothelial cells and gelatinase zymography of VCAM-1-positive and -negative endothelial cells. VCAM-1-positive and -negative endothelial cells were grown in monolayer on microporous membranes suspended in transwells as described in Materials and Methods. T cells were added to the top of the transwell chamber and cocultured for 20 h at 37°C. Transmigrated T cells were collected in the bottom chamber and immunolabeled with an antibody directed against 72-kD gelatinase (Ab 45) followed by a fluorescein-conjugated secondary antibody. (A) T cells that transmigrated through VCAM-1-positive endothelial cells. (B) T cells that transmigrated through VCAM-1-negative endothelial cells. Shaded curves represent the negative control; thin-lined curves represent control T cells; thick-lined curves represent transmigrated T cells. Surface-associated 72-kD gelatinase was detected only in the T cells that transmigrated through the VCAM-1-positive endothelial cells. (C) VCAM-1-positive RFC endothelial cells and (D) VCAM-1-negative ECV304 endothelial cells were assayed for 72-kD gelatinase activity by gelatinase zymography. Culture media (40 µl) and cell extracts (20 µg) obtained from serum-starved endothelial cells were subjected to electrophoresis through a 10% polyacrylamide gel impregnated with gelatin (0.2 mg/ml) as described in Materials and Methods. Lane 1, culture media; lane 2, cell extract. Molecular mass markers are as indicated. Arrows pointing to clear bands denote the 72-kD proenzyme form and the 62-kD active form of 72-kD gelatinase present in both endothelial cell types. Top arrow points to an additional band of ~92 kD and is identified as 92-kD gelatinase.

Following transmigration through a VCAM-1-positive endothelial cell monolayer, 72-kD gelatinase activity remained detectable. Transmigrated T cells collected 8 and 20 h after coculture showed that the level of induction of activity was relatively the same at both time points. Expression of 72-kD gelatinase mRNA, on the other hand, was not detected in the transmigrated T cells collected at 20 h. These results indicated that the expression of 72-kD gelatinase activity persisted after the T cells detached from the endothelial cells. Recently, 72-kD gelatinase has been shown to be cell surface associated (Emonard et al., 1992; Monsky et al., 1993; Kleiner and Stefler-Stevenson, 1993). These investigators suggest that 72-kD gelatinase is bound to a receptor as has been demonstrated for urokinase plasminogen activator (uPA) (Blasi et al., 1987; Estreicher et al., 1989). To determine if the 72-kD gelatinase activity we detected in the transmigrated T cells was cell surface associated, transmigrated T cells were collected and assayed for 72-kD gelatinase surface expression by FACS analysis. The results...
demonstrated that the T cells did express 72-kD gelatinase on the cell surface. As described for uPA, it is possible that cell surface-associated 72-kD gelatinase is more stable and has an increased half-life compared to free enzyme (Blasi et al., 1987). This would explain why 72-kD gelatinase activity was present in the transmigrated T cells while mRNA expression was absent. Thus we conclude that the binding of T cells to VCAM-1, in the context of cell adhesion, leads to an induction of 72-kD gelatinase activity and that the enzyme is cell surface associated. We hypothesize that, in the process of T cell transmigration, a prolonged expression of 72-kD gelatinase activity localized to the pericellular region may be necessary for T cells to degrade the ECM and invade the perivascular tissue.

We also showed that inhibition of 72-kD gelatinase with TIMP-2 reduced T cell transmigration. These results indicated that 72-kD gelatinase facilitated T cell transmigration. It should be noted, however, that the process of T cell transmigration is complex and utilizes many mechanisms in addition to 72-kD gelatinase induction. This was made evident with the observations that modest amounts of T cells transmigrated through the VCAM-1-negative endothelial cells in which 72-kD gelatinase was not induced and that small amounts of T cells still managed to transmigrate through the VCAM-1-positive endothelial cells when TIMP-2 was added to the transwell system. It cannot be ruled out, though, that a higher concentration of TIMP-2 might have inhibited transmigration completely. However, since we have shown previously that uPA is also induced in T cells upon adhesion to endothelial cells (Romanic and Madri, 1994), we hypothesize that proteinases such as uPA may also be involved in transmigration.

Due to the difficulties in obtaining endothelial cells that constitutively express VCAM-1 as well as endothelial cells that lack VCAM-1 expression, endothelial cells from different species were used. This was of concern to us since species' differences might have affected T cell adhesion and 72-kD gelatinase induction. The results, however, demonstrated that species differences did not interfere with these experiments. The murine T cells used in these experiments adhered to and transmigrated through both the rat VCAM-1-positive RFC endothelial cells and the human VCAM-1-negative ECV304 endothelial cells quite well. Also, the rςVCAM-1 and rςCAM-1 molecules effectively mediated T cell adhesion. Furthermore, adhesion to the rςVCAM-1 elicited an induction in 72-kD gelatinase in the T cells. In support of these observations, others have demonstrated that recognition between T cells and endothelial cells is functional across species due to the high degree of evolutionary conservation between cell adhesion molecules, particularly between VLA-4 and VCAM-1 (Wu et al., 1988; Miyake et al., 1991; Brady et al., 1992).

The question remains as to the nature of the signaling cascades initiated upon binding to VCAM-1. The ligand for VCAM-1 is the VLA-4 integrin (Elices et al., 1990; Shimizu et al., 1991). Integrins have been demonstrated to mediate signals between the intracellular and extracellular compartments (Hynes, 1992; Juliano and Haskill, 1993). Intracellular signals modulating the expression of matrix-degrading proteinases or their inhibitors upon cell–cell or cell–ECM contact provide a mechanism by which cells influence their surroundings. Since migrating T cells undergo a series of transient attachments to matrix proteins, proteolysis of these potential ligands could facilitate the process of T cell migration. Werb et al. (1989) have shown that engagement of the fibronectin receptor, α5β1, on fibroblasts signals the induction of stromelysin and collagenase and Seftor et al. (1992) have demonstrated that binding of the vitronectin receptor, αvβ3, induces the expression of 72-kD gelatinase in A375M melanoma cells. Although speculative, induction of matrix-degrading proteinases via integrin binding may occur through some common mechanisms. Several studies have demonstrated that ligation of integrins affects tyrosine phosphorylation of intracellular proteins (Hynes, 1992; Juliano and Haskill, 1993). Ligation of VLA-4 has been shown to induce the tyrosine phosphorylation of a 150-kD protein in T cells (Nojima et al., 1992). Others have suggested that intracellular signaling upon engagement of integrins can be due to calcium flux (Ng-Sikorski et al., 1991), changes in intracellular pH (Schwartz et al., 1991), and changes in cell shape and cytoskeletal architecture (Aggeler et al., 1984; Unemori and Werb, 1986). Further experiments will reveal if any of these intracellular changes contribute to the induction of 72-kD gelatinase expression in T cells.

T cell–endothelial cell interactions and T cell–ECM interactions during migration may also be mediated either by regulating the expression of specific ligands and their receptors or by altering the affinity of a ligand for its receptor (Shimizu et al., 1990, 1991; Shimizu and Shaw, 1991). We have demonstrated that T cells which have transmigrated through endothelial cells in vitro and in vivo exhibit a specific decrease in α4 expression at the cell surface (Baron, 1994; Romanic, A. M., I. Visintin, J. L. Baron, C. A. Janeway, and J. A. Madri, manuscript in preparation). This observation supports the concept that adhesion via the VLA-4 integrin acts as a signal to elicit an intracellular response. In particular, this suggests that the α4 subunit confers a signaling response to regulate its own expression as well as the expression of 72-kD gelatinase.

Matrix-degrading proteinases including interstitial collagenase, stromelysin, matrilysin, and 72- and 92-kD gelatinases as well as the tissue inhibitors of metalloproteinases, TIMP-1 and TIMP-2, have been implicated in many biological scenarios such as inflammation, wound healing, metastasis, and development (Woessner, 1991). In T cell-mediated autoimmune diseases such as rheumatoid arthritis, multiple sclerosis, and EAE, T cells invade tissues and cause damage (Zamvil et al., 1985; Boyle and McGeer, 1990; Cross et al., 1990; Laffon et al., 1991; Yednock et al., 1992; Baron et al., 1993). The results presented here provide the first direct evidence that 72-kD gelatinase is induced in T cells upon endothelial cell adhesion.

It appears, then, that T cell interactions with both endothelial cells and the ECM are tightly coordinated and that T cells actively respond to and modulate their environment through a number of mechanisms including proteolysis of matrix proteins. Further elucidation of these mechanisms will likely provide insight into the processes of metastasis, inflammation, and lymphocyte homing and may lead to new therapies and new ideas for drug design for treatment of these disease-related processes.

We would like to thank Dr. Jody L. Baron for her help with this project, and Drs. Kim Bottomly and Charles A. Janeway for their interest and helpful suggestions and for the use of their facilities. We would also like to...
References

Aagger, J., S. M. Frisch, and Z. Werb. 1984. Changes in cell shape correlate with collagenase gene expression in rabbit synovial fibroblasts. J. Cell Biol. 99:1662-1671.

Albelda, S. M., and C. A. Buck. 1990. EndoCAM: a novel endothelial-cell cell adhesion molecule. J. Cell Biol. 110:1227-1237.

Albelda, S. M., A. A. Muller, C. A. Buck, and P. J. Newman. 1991. Molecular and cellular properties of PECA-1 (endoCAM/CD31): a novel vascular cell-cell adhesion molecule. J. Cell Biol. 114:1039-1068.

Baron, J. L., J. A. Madri, N. H. Ruddle, G. Hashim, and A. A. Janeway. 1993. Association of murine CD31 with transmigrating lymphocytes following antigenic stimulation. Am. J. Pathol. 141:843-854.

Boyle, E. A., and P. L. McGee. 1990. Cellular immune response in multiple sclerosis plaques. Am. J. Pathol. 137:575-584.

Buehler, L. M., F. S. C. Watkins, J. L. Madri, N. H. Ruddle, P. A. Marsden, and T. F. Tedder. 1992. Neutrophils, monocytes, and lymphocytes bind to cytokine-activated glomerular endothelial cells through L-selectin (LAM-1) in vitro. J. Immunol. 149:2437-2444.

Carmel, A. B., D. L. Quick, D. M. Rothstein, K. Sugita, S. F. Schlossman, and C. Morimoto. 1990. Activation of peripheral blood T cells by signalling through both VLA-4 and VLA-5 integrin molecules. J. Immunol. 145:785-793.

Dayer, J. M., B. Testard, and A. Cerami. 1985. Cancer/tumor necrosis factor stimulates collagenase and prosta glandin E2 production by human synovial and dermal fibroblasts. J. Exp. Med. 162:2163-2168.

Dayer, J.-M., B. de Roochemonteix, B. Burrus, S. Desmazac, and C. A. Dummer. 1990. Human immunoblastic sarcoma invades human skin via a 150 kDa protein. J. Immunol. 144:645-648.

Elices, M. J., L. Osborn, Y. Takada, C. Crouse, S. Luohsky, M. E. Hemler, and R. L. Oettgen 1990. VLA-4 on an activated endothelium interacts with the leukocyte integrin VLA-4 at a site distinct from the VLA-4/fibronectin binding site. J. Cell Biol. 100:577-584.

Emonard, H. P., A. G. Remacle, A. C. No61, J.-A. Grimand, W. G. Steiler, and M. Na-7. 1991. Characterization of a soluble form of vascular cell adhesion molecule-1, a cytokine-induced endothelial protein that binds to lymphocytes. Cell. 59:1203-1211.

Reponen, P. C., Sahlberg, P. Hulttia, T. Hursttianen, I. Thiesf, and K. Tryggvason. 1992. Molecular cloning of murine 72-kDa type IV collagenase and its expression during mouse development. J. Biol. Chem. 267:7856-7862.

Romancic, A. M., and J. A. Madri. 1994. Extracellular matrix-degrading proteases in the nervous system. Brain Pathol. In press.

Salo, T. L., L. Osborn, and K. Tryggvason. 1983. Purification and characterization of a murine basement membrane collagen-degrading enzyme secreted by metastatic tumor cells. J. Biol. Chem. 258:3058-3063.

Sambrook, J. E., F. Frisch, and T. Maniatis. 1989. In Molecular Cloning: A Laboratory Manual. 2nd ed. C. Nolan, editor. Cold Spring Harbor Labora-

tory Press, Cold Spring Harbor, N.Y. 7.37-7.87.

Sawaski, Y. 1992. Rare spontaneous transformed human endothelial cell line provides useful research tool. In Vitro Cell. & Dev. Biol. 28A:380-382.

Schultz, R. M., S. Silberman, B. Pesky, A. S. Bajkowski, and D. F. Car-

michael. 1988. Inhibition by human recombinant tissue inhibitor of metalloproteinases of human aminion invasion and lung colonization by murine B16-F10 melanoma cells. Cancer Res. 48:5539-5545.

Schwartz, M. A., C. Lenchene, and D. E. Ingber. 1991. Insoluble fibroblast activates the Na+H+ antiporter by clustering and immobilizing integrin α5β1, independent of cell shape. Proc. Natl. Acad. Sci. USA. 88:7849-7853.

Seligman, E. B., E. A. Seftor, G. Chi-Rosso, W. G. Steiler-Stevenson, D. Brown, K. E. Rosenthal, and M. J. Hendrix. 1992. Role of the αvβ3 integrin in human melanoma cell invasion. Proc. Natl. Acad. Sci. USA. 89:1557-1561.

Shibata, Y., G. A. van Seventer, K. J. Horgan, and S. Shaw. 1990. Regulated expression and binding of three VLA (β1) integrin receptors on T cells. Nature (Lond.). 354:250-253.
Shimizu, Y., W. Newman, T. V. Gopal, K. J. Horgan, N. Graber, L. D. Beall, G. A. van Seventer, and S. Shaw. 1991. Four molecular pathways of T cell adhesion to endothelial cells: roles of LFA-1, VCAM-1, and ELAM-1 and changes in pathway hierarchy under different activation conditions. J. Cell Biol. 113:1203-1212.

Shimizu, Y., and S. Shaw. 1991. Lymphocyte interactions with extracellular matrix. FASEB (Fed. Am. Soc. Exp. Biol.) J. 5:2292-2299.

Springer, T. A. 1990. Adhesion receptors of the immune system. Nature (Lond.). 346:425-434.

Staunton, D. E., M. L. Dustin, and T. A. Springer. 1989. Functional cloning of ICAM-2, a cell adhesion ligand for LFA-1 homologous to ICAM-1. Nature (Lond.). 339:61-64.

Sternler-Stevenson, W. G., H. C. Krutzsch, M. P. Wacher, I. M. K. Marguiles, and L. A. Liotta. 1989. The activation of human type IV collagenase proenzyme. J. Biol. Chem. 264:1353-1356.

Takahashi, K., Y. Sawaki, J.-I. Hata, K. Mukai, and T. Goto. 1990. Spontaneous transformation and immortalization of human endothelial cells. In Vitro Cell. & Dev. Biol. 25:265-274.

Tanaka, Y., S. M. Albelda, K. J. Horgan, G. A. van Seventer, Y. Shimizu, W. Newman, J. Hallam, P. J. Newman, C. A. Buck, and S. Shaw. 1992. CD31 expressed on distinctive T cell subsets is a preferential amplifier of $\beta_1$ integrin-mediated adhesion. J. Exp. Med. 176:245-253.

Utsumori, E. N., and Z. Werb. 1986. Reorganization of polymerized actin: a possible trigger for induction of procollagenase in fibroblasts cultured in and on collagen gels. J. Cell Biol. 103:1021-1031.

Vennegoor, C. J. G. M., E. van de Wiel-van Kemenade, R. J. F. Huijbers, F. Sanchez-Madrid, C. J. M. Metief, and C. G. Figdor. 1992. Role of LFA-1 and VLA-4 in the adhesion of cloned normal and LFA-1 (CD11/CD18)-deficient T cells to cultured endothelial cells. J. Immunol. 148:1093-1101.

Vitetta, E. S., and W. E. Paul. 1991. In Peptide Growth Factors and Their Receptors II. M. B. Sporn and A. B. Roberts, editors. Springer-Verlag, New York. 401-426.

Wayner, E. A., A. Garcia-Pardo, M. J. Humphries, J. A. McDonald, and W. G. Carter. 1989. Identification and characterization of the T lymphocyte adhesion receptor for an alternative cell attachment domain (CS-1) in plasma fibronectin. J. Cell Biol. 109:1321-1330.

Werb, Z., P. M. Tremble, O. Behrendtse, E. Crowle, and C. H. Damsky. 1989. Signal transduction through the fibronectin receptor induces collagenase and stromelysin gene expression. J. Cell Biol. 109:877-889.

Woessner, J. F. 1991. Matrix metalloproteinases and their inhibitors in connective tissue remodeling. FASEB (Fed. Am. Soc. Exp. Biol.) J. 5:2145-2154.

Wu, N. W., S. Jalkanen, P. R. Streeter, and E. C. Butcher. 1988. Evolutionary conservation of tissue-specific lymphocyte-endothelial cell recognition mechanisms involved in lymphocyte homing. J. Cell Biol. 107:1845-1851.

Zamvil, S., P. Nelson, J. Trotter, D. Mitchell, R. Knobler, R. Fritz, and L. Steinman. 1985. T-cell clones specific for myelin basic protein induce chronic relapsing paralysis and demyelination. Nature (Lond.). 317:355-358.

Yamada, A., T. Nikaido, Y. Nojima, S. F. Schlossman, and C. Morimoto. 1991. Activation of human CD4 T lymphocytes: Interaction of fibronectin with VLA-5 receptor on CD4 cells induces the AP-1 transcription factor. J. Immunol. 146:53-56.

Yednock, T. A., C. Cannon, L. C. Fritz, F. Sanchez-Madrid, L. Steinman, and N. Kain. 1992. Prevention of experimental autoimmune encephalomyelitis by antibodies against $\alpha_4\beta_1$ integrin. Nature (Lond.). 356:63-66.