Laminin, a major glycoprotein component of vessel basement membranes, is recognized by β1- and β3-integrins expressed on endothelial cells. To determine how endothelial cell integrins might function in multiple sclerosis (MS) lesions, integrin laminin receptors and laminin were analyzed in central nervous system samples from MS patients and controls by immunohistochemistry. In active MS lesions, endothelial cell VLA-6 and β1 subunits were decreased compared to controls whereas α1 subunit and VLA-1 were increased. In chronic inactive lesions β1, VLA-6 and αv were the same as controls but VLA-1 remained increased. α3 subunit was constant in all samples. By immunoelectron microscopy VLA-1, VLA-6, β1, and laminin were distributed throughout endothelial cells; αv was adjacent to and on luminal surfaces; αv and VLA-1 were on intercellular junctions. These results indicate distinct regulation and functions of these integrins in different lesion stages. In active lesions decreased endothelial cell β1/VLA-6 could result in their detachment from laminin thereby facilitating leukocyte transvascular migration and blood-brain barrier breakdown. αv and VLA-1 on intercellular junctions may participate in re-establishing vessel integrity after leukocyte migration. Luminal surface αv also likely binds intraluminal ligands and cells. In chronic inactive plaques persistently elevated endothelial cell VLA-1 correlates with longstanding endothelial cell and blood-brain barrier dysfunction.

Laminins (Ln) are major glycoprotein components of extracellular matrix and vessel basement membranes (BM).1 Peptide sequences of Ln and of other extracellular matrix molecules are recognized by αβ1 (VLA-1), αβ1 (VLA-2), αβ1 (VLA-3), αβ4 (VLA-6), αβ4, and αβ3 integrins expressed on the surfaces of many cell types.2,3 Integrin-mediated recognition of extracellular matrix molecules results in intracellular signaling that affects a range of cell behaviors.4 In endothelial cells these signals affect focal adhesions and cytoskeletal organization, ie, actin fiber assembly. Therefore, integrin-mediated endothelial cell recognition of Ln and other BM molecules may determine cell-to-cell adhesiveness and mediate behaviors such as spreading, retraction, polarization, and migration that are essential for the maintenance and normal functioning of blood vessels.5–7

Inflammatory cytokines such as interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), interferon-γ (IFNγ), and transforming growth factor-β (TGFβ), growth factors such as fibroblast growth factor, and reactive oxygen intermediates induce changes in the levels and surface distribution of endothelial cell integrins in vitro.8–13 In immune reactions these alterations likely affect endothelial cell recognition of BM molecules and result in contraction of the endothelial cells, producing defects or denudation of the vascular lining. Cytokines may also be bound by Ln14 and influence extracellular matrix turnover.15,16 Thus, both endothelial cell integrin expression and the BM may undergo numerous modifications over the course of cellular immune reactions. Indeed, in diverse inflammatory conditions alterations of endothelial cell integrin Ln receptor expression have been documented.17–20 These studies suggest that endothelial cell integrin expression changes over time in complex patterns in vivo and that alterations may be specific for each integrin and vascular bed affected.

In central nervous system (CNS) immune reactions, particularly in acute lesions of multiple sclerosis (MS), the blood-brain barrier breaks down as the endothelial cell layer becomes porous and leukocytes migrate across blood vessel walls.21,22 Previous studies of MS have demonstrated deposition of plasma and extracellular matrix molecules on endothelial cells in acute and chronic active lesions and modulation of the expression of their integrin receptors.23,24 Increased expression of matrix metalloproteinases, enzymes that mediate vascular basement membrane and CNS extracellular matrix turnover, are also found in active MS lesions.25,26 Furthermore, in chronic MS plaques blood-brain barrier defects and endothelial cell abnormalities persist and may contribute to a parenchymal extracellular matrix that does not promote or actively impedes tissue repair.27–29 Thus, alterations of both endothelial cell integrins and vascular
Table 1. Case Material

<table>
<thead>
<tr>
<th>Diagnoses</th>
<th>No. of cases</th>
<th>No. of samples studied</th>
</tr>
</thead>
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<tr>
<td>Chronic MS†</td>
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<td>25</td>
</tr>
<tr>
<td>Acute disseminated leukoencephalitis‡</td>
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<td>1</td>
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<tr>
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</tr>
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</tr>
<tr>
<td>Acute herpetic encephalitis</td>
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<td>2</td>
</tr>
<tr>
<td>Cerebral infarcts†</td>
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<td>4</td>
</tr>
<tr>
<td>Hypoxic-ischemic encephalopathy</td>
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<td>4</td>
</tr>
<tr>
<td>Cervical cord compression**</td>
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<td>1</td>
</tr>
<tr>
<td>Normal CNS††</td>
<td>20</td>
<td>23</td>
</tr>
</tbody>
</table>

*One biopsy sample from a 37-year-old woman, disease duration not known; one biopsy sample from a 35-year-old woman, disease duration 6 weeks; one autopsy sample from a 30-year-old man, disease duration 11 months.
†There were 6 women and 4 men in cases for which information is available; mean age = 51.3 years (range, 33 to 78 years); clinical disease duration mean = 14.4 years (range, 3 to 37 years); postmortem intervals mean = 9.1 hours (range, 2 to 23 hours).
‡Biopsy sample from a 52-year-old man, clinical disease duration 3 to 4 weeks.
§22- and 10-year-old males. Samples contain noninflammatory chronic lesions.
¶One cerebral cortical biopsy sample and one autopsy sample.
**Secondary to vertebral metastases of adenocarcinoma.
††Neuropathologically normal autopsy samples were from cerebral hemispheres (n = 12), brain stem (n = 2), cerebellum (n = 3), optic nerve (n = 1), and spinal cord (n = 5). In cases for which information was available, there were 7 women and 11 men; mean age = 58.6 years (range, 5 days to 85 years); Mean postmortem interval = 11.7 hours (range, 3 to 22 hours).

BM components are implicated in the pathogenesis of all stages of MS lesions.

To delineate more precisely the molecular interactions between endothelial cells and Ln that occur in MS, vascular endothelial cell integrin Ln receptors and Ln in MS lesions and controls were analyzed by immunohistochemistry. Immunoelectron microscopy was used to characterize subcellular localizations of individual integrins that indicate their potential functions in situ. The results indicate complex expression patterns of endothelial cell integrin Ln receptors in different MS lesion stages and provide insight into molecular mechanisms of endothelial cell pathobiology in CNS inflammatory conditions and of blood-brain barrier dysfunction in chronic MS plaques.

Materials and Methods

Case Material (Table 1)

Samples of CNS tissues were obtained from cerebral hemisphere biopsies and from autopsies at Massachusetts General Hospital (Boston, MA), Stanford University Medical Center (Stanford, CA), and Veterans Affairs Health Care System (Palo Alto, CA). Additional autopsy samples from patients with MS were obtained from the Brigham and Women’s Hospital (Boston, MA), the National Neurological Research Bank, VA Wadsworth Medical Center (Los Angeles, CA), and the Rocky Mountain MS Tissue Bank (Englewood, CO). The samples were frozen and stored at -80°C in OCT compound (Miles Laboratories, Naperville, IL).

MS Lesion Classification

Lesions in MS tissue samples were determined to be “active” by the presence of perivascular mononuclear cell infiltrates and by the detection of numerous Oil Red O-positive myelin breakdown products in macrophages in adjacent serial sections. In autopsy samples, particularly from patients in whom the disease had been present for many years, the precise ages of specific lesions are not known and even in acute MS patients whose clinical disease courses were very brief (ie, less than one year), lesions with these features were the most prevalent. Therefore, lesions classified as “active” may have included very acute and remyelinating lesions, but the vast majority were more likely chronic active lesions or plaques. Lesions without myelin, evidence of ongoing myelin degeneration (ie, Oil Red O-positive macrophages), or recognizable mononuclear cell infiltrates were designated as inactive, demyelinated lesions (“chronic inactive”). These were the most numerous lesions in the chronic MS autopsy samples. Areas in samples from MS patients with intact white matter, as judged by tissue density on gross inspection and microscopic confirmation of the presence of intact myelinated axons, were classified as MS normal-appearing white matter (NAWM). Using these criteria, these lesions compartments were uniform in comparisons among the different MS cases.

Monoclonal Antibodies and Immunohistochemistry

Cryostat sections of 6-μm thickness were stained with the monoclonal antibodies (mAb) listed in Table 2 diluted in phosphate-buffered saline, pH 7.4 (PBS), using immunoperoxidase as described. In brief, air-dried sections were fixed in acetone, washed in PBS, and incubated sequentially in 10% normal horse serum, mAb, 0.03% H2O2 in PBS, biotinylated horse anti-mouse immunoglobulin (Ig) (Vector Laboratories, Burlingame, CA), and avidin-biotin-horseradish peroxidase complex (Vector), with washes in PBS between incubation steps. Immunoperoxidase reaction product was visualized with 3-amino-9-ethyl carbazole (Aldrich Chemical Co., Milwaukee, WI) and fixed in formol acetate. The sections were counterstained with hematoxylin. Samples of normal spleen were used as positive controls and for determinations of optimal staining dilutions for each mAb. Negative staining controls included substitution of irrelevant mAb and PBS for primary antibodies. Staining of normal and pathological tissues was done concurrently and there were uniform exposures to each reagent.

For semiquantitative analysis of stained cryosections, stained vessels in CNS white matter were counted by two independent observers and areas in which counts were done were calculated from ruler measurements. Vessels seen in cross-section with any endothelial cell staining were counted as positive. In samples from MS patients,
Monoclonal Antibodies

<table>
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<th>Antigen</th>
<th>Clone</th>
<th>Isotype</th>
<th>Source</th>
<th>Staining dilution*</th>
<th>Reference No.</th>
</tr>
</thead>
<tbody>
<tr>
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<td>Mouse IgG1</td>
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<tr>
<td>CD49b  (VLA-2, α2 subunit)</td>
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<td>Mouse IgG1</td>
<td>Life Technologies (Rockville, MD)</td>
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<tr>
<td>CD49c  (VLA-3, α3 subunit)</td>
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<td>Mouse IgG1</td>
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<td>Mouse IgG1</td>
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<tr>
<td>Human laminin</td>
<td>LAM-1</td>
<td>Rat IgG2b</td>
<td>BioGenex Laboratories (San Ramon, CA)</td>
<td>1:100</td>
<td>38</td>
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</table>

*Optimal staining dilution for light microscopy.

Immunoelectron Microscopy and Morphometric Analysis

Tissues from OCT blocks containing normal optic nerve, other normal CNS samples, and active MS lesions were thawed, rinsed in 10 mmol/L PBS with 2 mmol/L NaN₃ (PBSem) for 90 minutes with three changes to remove the OCT. Samples (500 μm thick) were sliced and the rinsed tissue was placed in 3.0% paraformaldehyde/0.2% glutaraldehyde in PBSem and fixed for 3 hours at room temperature (RT) with mixing. The fixed tissues were then rinsed in PBSem, cut into smaller blocks, and stored overnight at 2–8°C. The tissue blocks were then dehydrated for 10 minutes each in 50% and 70% ethanol and for 10 minutes each three times with 96% ethanol. The blocks were immediately infused with 100% LR-White (Sigma, St. Louis, MO) for 60 minutes at RT, followed by 100% LR-White overnight at 0–4°C and 100% LR-White for 60 minutes at RT. The infusion steps were all performed in the dark with rotary agitation. For polymerization the samples were transferred to size 1 gelatin capsules and incubated at 50 ± 1°C for 40 hours. Ultrathin (80- to 90-nm) sections were cut from these samples using a microtome (Reichert Om U2, Leica Inc., Deerfield, IL) and were transferred to formvar-covered carbon-coated nickel grids.

Immunostaining was carried out at RT unless otherwise noted. The grids were incubated in 20 mmol/L glycine in 10 mmol/L PBS for 10 minutes at 37°C, blocked with 5% normal goat serum in 10 mmol/L PBS with 0.8% BSA and 0.1% IGSS gelatin (PBSAG) for 30 minutes, mAb diluted 1:50 in PBSAG with 1% normal goat serum for 2 hours at RT and then overnight at 2–4°C. The optimal dilution for each mAb was determined in preliminary studies to maximize labeling and eliminate nonspecific staining of cells, nuclei, and BMs. The grids were then incubated with the appropriate 15-nm gold particle-conjugated goat anti-mouse or anti-rat IgG reagent (E-Y Laboratories, Inc., San Mateo, CA) diluted 1:25 in PBSAG with 1% normal goat serum for 2 hours at RT. PBS washes were performed between each step and after the gold-labeled antibody incubation. The grids were then postfixed in 2% glutaraldehyde in PBSem for 10 minutes, washed in PBS followed by deionized water (DIH₂O). They were then stained with 4% uranyl acetate oxalate, washed with boiled DIH₂O, washed with 20 mmol/L NaOH, and further stained with 0.2% lead citrate in a Petri dish with NaOH granules. The grids were then washed once in NaOH and in boiled DIH₂O four times, air-dried, and examined in a Zeiss electron microscope. Fields for photographs were selected on the basis of tissue preservation and the presence of multiple gold particles. Immunoelectron microscopy was performed on samples for VLA-1, VLA-6, αv, and β1 subunits, and LN. A spleen sample was used as a positive control and normal mouse serum substituted for mAb as a negative control.

Localization of the gold particles within endothelial cell cytoplasm were assessed in electron photomicrographs of preparations of preparations stained for VLA-6 and αv and β1 subunits. Distances of each particle in the cytoplasm from the luminal surface (Dlum) and from the abluminal surface (Dablium) were measured and a Dlum/Dablium ratio was determined for each particle. A ratio of <1 indicates that the particle is localized closer to the luminal surface and >1 indicates that the particle is closer to the abluminal surface.

Results

VLA-6 and β1 Subunit

VLA-6 was abundantly expressed in a uniform pattern on capillaries and small venules in normal white matter (Figure 1A). Endothelial cells were also stained in gray matter, leptomeninges, and spinal nerve roots. Vascular smooth muscle, neurons, and glia were VLA-6-negative.
In MS NAWM, compared to normal white matter, VLA-6 vessel staining was slightly decreased. Many vessels with perivascular inflammatory cells in active lesions were either completely negative or had incomplete staining, i.e., only a few stained endothelial cells (Figure 1B). In chronic inactive MS plaques the extent of vessel staining was similar to that in normal white matter (Figure 1C). In other inflammatory and noninflammatory conditions VLA-6 expression was also observed but no consistent patterns of immunostaining were identified and counts of stained vessels were not done in those samples.

As previously reported and illustrated, expression of the β1-integrin subunit was prominent on CNS microvessels in control white matter and was reduced in active MS lesions compared to control and MS NAWM. Staining patterns were similar to those of VLA-6 but in active lesions additional staining of macrophages and other cells was also noted.

Results of semiquantitative analysis of vascular staining for VLA-6 and β1 subunit are shown in Figure 2. Numbers of VLA-6-positive vessels/mm² were lower in MS NAWM compared to control white matter (P < 0.01) and in active MS lesions (P < 0.02 compared to MS NAWM, P < 0.001 compared to control white matter). Similarly, active MS plaques had fewer β1 subunit-positive vessels than were found in MS NAWM (P < 0.02) or controls (P < 0.01). Numbers of VLA-6- and β1 subunit-positive vessels/mm² were the same in chronic inactive MS lesions as in controls.

By immunoelectron microscopy in normal CNS samples immunogold labeling for VLA-6 subunit was found to be dispersed throughout endothelial cell cytoplasm. Although no striking polarization of labeling was identified many gold particles were found on or near abluminal cell surfaces (Figure 3). In active lesions there were fewer gold particles on endothelial cells and other cells were negative. Immunogold labeling for β1 subunit was also dispersed through endothelial cell cytoplasm in normal samples. As expected from light microscopic analyses and previous studies, cells in perivascular and parenchymal areas in active MS lesions were also labeled for the β1 subunit (not shown).

**VLA-1 and αν Subunit**

VLA-1 expression was observed on small numbers of microvessels in control and MS NAWM (Figure 4A). Vessel expression was increased in active MS lesions and
appeared to be localized on endothelial cells. No VLA-1 expression on mononuclear or CNS resident cells was observed (Figure 4B). Vessel expression of VLA-1 was as prominent in chronic inactive MS lesions as in active MS lesions (Figure 4C). Small numbers of VLA-1-positive vessels were also observed in other neurological disease controls with no clear trends identified.

In sections stained for the αv subunit, endothelial cell localization, vessel staining, and an increase in expression in active MS lesions similar to that of VLA-1 were observed. The αv subunit was additionally expressed on macrophages and astrocytes in active lesions (Figure 4D).

Results of semiquantitative analysis of VLA-1 and αv subunit are shown in Figure 5. In active MS lesions numbers of VLA-1-positive vessels/mm² were higher than in controls and MS NAWM (P < 0.01 for both). In chronic inactive MS lesions numbers of VLA-positive vessels/mm² remained significantly greater than in control and MS NAWM (P < 0.03 for combined data). Similar numbers of stained vessels/mm² for αv were seen in active MS lesions, but in contrast to VLA-1, αv-positive vessels/mm² were the same as in controls in chronic inactive lesions.24

By immunoelectron microscopy detection of VLA-1 was generally low but when gold particles were found in endothelial cell cytoplasm, they appeared to be randomly distributed. Specific labeling for VLA-1 was present, however, on interendothelial cell junctions and adjacent areas (Figure 6). No VLA-1 was observed on parenchymal or other cells or on the BM.

In control samples, αv subunit immunogold labeling was extremely sparse, but in samples with active MS lesions endothelial cell labeling was more abundant. Individual and clustered gold particles were localized on or near endothelial cell membrane luminal surfaces. Within the cytoplasm they were found more frequently in the luminal than abluminal portions (Figure 7). Labeling was also specifically localized on or near intercellular tight junctions (Figures 7 and 8). Some labeled cell fragments within the vessel lumens may represent platelets. Scattered particles were also seen on perivascular and parenchymal cells.

Morphometric analyses demonstrated that in each of seven endothelial cells labeled for αv subunit, more gold particles were localized within the cytoplasm closer to the luminal than the abluminal surface (Figure 9A). By contrast, in a similar analysis of endothelial cells immunolabeled for VLA-6, no consistent intracytoplasmic polarization of gold particle localization was evident, indicating a random distribution (Figure 9B).

αv and α2 Subunits

In control samples meningeal artery smooth muscle and rare parenchymal venules were α3 subunit-positive whereas gray and white matter were otherwise negative. In active MS lesions small numbers of white matter microvessels were stained but there was no clear relationship between the presence of perivascular inflammatory cells and α3 vascular expression (Figure 10A). In some active plaques more diffuse staining of macrophages and glia, including Creutzfeldt astrocytes,22 were observed (not shown). Similar patterns of staining of αv-positive vessels were seen in inactive MS lesions and no differences in the numbers of vessels stained in the different sample groups were identified (Figure 5).

The anti-αv subunit mAb immunostained neuron cell bodies in gray matter but no consistent vessel staining in normal or pathological tissues was observed. Some active MS samples showed prominent staining of macrophages and astrocytes and old plaques showed high diffuse background staining (not shown).

Lm

By light microscopy Lm was detected on large meningeal vessels and on arteries, venules, and microvessels in...
Figure 4. VLA-1 and α₅ subunit immunostaining. A to C, mAb TS2/7 with hematoxylin. A: Two capillaries (arrow) express VLA-1 whereas a larger vessel (right center) is mostly VLA-negative in normal control white matter. Magnification, ×114. B: Prominent staining of a venule in an active MS lesion. Perivascular mononuclear cells, astrocytes (arrowheads) and other cells are unstained. Magnification, ×171. C: Numerous capillaries retain immunoreactivity in a chronic inactive MS plaque. Magnification, ×114. D: α₅ subunit is expressed on a venule and on parenchymal macrophages and astrocytes in an active MS lesion. mAb VNR 147. Magnification, ×171.

Figure 5. VLA-1- and α₅ subunit positive microvessels/mm² are more numerous in active MS lesions (samples from 4 cases) than in NAWM (samples from 6 cases) and controls (samples from 14 cases). Numbers of VLA-1-positive microvessels in chronic inactive lesions (samples from 6 cases) are also higher than in control samples whereas numbers of α₅ subunit-positive microvessels are the same as controls. α₅ integrin subunit microvessel staining is essentially the same in all samples and conditions. See also legend to Figure 2.

Figure 6. Immunoelectron microscopic localization of VLA-1 on interendothelial cell junction (arrow) in a normal optic nerve microvascular endothelial cell. Three gold particles are on and two additional particles are in the general vicinity of the junction. Background labeling of cytoplasm, lumen and BM are minimal. L, lumen. mAb TS2/7. Magnification, ×20,200.
normal CNS parenchyma (Figure 10B). In active lesions vessel staining was sometimes disrupted or not apparent (Figure 10C). These alterations were focal, however, and no significant differences in numbers of stained vessels among the sample groups were observed (data not shown). By immunoelectron microscopy Ln labeling was observed in endothelial cells and predominantly on the BM but not in pericytes or other cells (Figure 11).

**Discussion**

This study was undertaken to understand how the expression of integrin Ln receptors and their recognition of BM Ln may contribute to the physiological alterations and functions of CNS microvascular endothelial cells over the course of evolution of MS lesions. The necessary use of autopsy tissue samples from MS patients for such analyses is complicated by the fact that specific lesions’ ages are unknown and the majority of lesions likely were present for many years. Although the use of samples from clinically acute MS cases improves the likelihood that specific lesions are pathologically early, the precise stages of endothelial cell activation and injury in the samples are not known. Thus, some of the present observations, even in active MS lesions, may well relate to more chronically altered or regenerating rather than acutely injured endothelial cells or they may reflect the simultaneous occurrence of both types of processes. Furthermore, cellular expression of \(\beta_1\) integrins does not necessarily completely correlate with affinity for specific extracellular matrix molecule ligands and integrins clearly have complex binding capacities and multiple biological roles. Therefore, inferences of specific functions based solely on integrin and integrin subunit expression need to be made with caution.

On the other hand, the findings on immunostaining of specific integrins in control tissue samples were essentially as reported in other studies of human CNS tissues and the alterations identified in MS lesions are similar to those reported in non-CNS inflammatory conditions. Furthermore, the immunostaining pattern and subcellular localizations of specific integrins identified are also similar to those in human endothelial cells in vitro and many of the alterations identified in the present study can also be induced in these cells by specific inflammatory mediators. Thus, the complex patterns of integrin Ln receptor expression identified are likely relevant to acute as well as chronic MS lesions in vivo.

Vascular expression of VLA-6 (\(\alpha_6\) subunit) was less in MS NAWM and in active MS lesions than in normal controls. Down-regulation of the \(\beta_1\) subunit expression paralleled that of VLA-6, but in view of the multiple potential pairings of the \(\beta_1\) subunit with other \(\alpha\) chains, it is not certain whether these patterns are directly related. In-
deed, decreased $\beta_1$ expression has been reported in a model of vascular injury and may therefore be a more general indicator of endothelial cell injury. Down-regulation of $\alpha_v$ subunit expression has also previously been documented in inflamed proliferative synovia from patients with rheumatoid arthritis and this effect can be mimicked in synovial cells using a combination of TNF-$\alpha$ and IFN-$\gamma$ in vitro. Because these cytokines are present in inflammatory MS lesions, it is likely that the effects on VLA-6 expression in inflammatory MS lesions are similarly mediated at least in part by these cytokines. In addition, certain chemokines may also modulate $\beta_1$ integrin-mediated T cell affinity for Ln and other extracellular matrix proteins without altering $\beta_1$ expression levels. These chemokines are also likely present in inflammatory MS lesions and they may modify endothelial cell proliferation. It is not known at present, however, if they affect human CNS endothelial cell-extracellular matrix molecule interactions.

The observed patterns of VLA-6 expression generally parallel changes in vessel wall integrity and blood-brain barrier function, ie, both may be severely compromised in acute lesions. Furthermore, the significant although lesser difference in VLA-6-positive vessel staining in the NAWM samples compared to the controls might correlate with abnormalities in blood-brain barrier function, ie, increased water content, that are detected in MS NAWM in patients in areas where there is little or no histological evidence of injury. Because VLA-6 may be involved in matrix guidance pathways that permit endothelial cells to locate each other in angiogenesis and other pathological processes, VLA-6 down-regulation might be related to disengagement of endothelial cells from BM Ln and from each other. Indeed, a recent study suggests that in the absence of engagement with an extracellular matrix molecule substrate VLA-6 may contribute to cell motility. Thus, in active MS lesions VLA-6 down-regulation in endothelial cells would result in their detachment from Ln and this might both facilitate leukocyte passage through the vessel wall and contribute to the characteristic breaches of the blood-brain barrier. The apparent return of VLA-6 and $\beta_1$ subunit staining patterns to normal in chronic lesions implies that these aspects of endothelial cell dysfunction may be reversible.

In contrast to VLA-6 and $\beta_1$ subunit, vascular $\alpha_v$ subunit was increased in active MS lesions. An increase in $\alpha_v$ expression is consistent with the finding of enhanced $\alpha_v$ integrin-mediated leukocyte adhesion.

Figure 8. Interendothelial cell junction in a microvessel adjacent to the vessel in Figure 7 shows gold particle labeling on the junctional complex (arrow) and on the luminal surface (arrowheads) but not in the abluminal cytoplasm or on the BM. L, Lumen. Magnification, $\times20,300$.

Figure 9. Morphometric analysis of subcellular localizations of $\alpha_v$-integrin and VLA-6. Data are expressed as numbers of gold particles with $D_{Lum}/D_{Ablum}$ ratios $<1$ or $>1$ in each endothelial cell analyzed. A: In photomicrographs of samples immunolabeled for $\alpha_v$ integrin subunit, there were more intracytoplasmic gold particles with $D_{Lum}/D_{Ablum}$ ratios $<1$ in each of seven cells, indicating that in each cell more particles were closer to or on the luminal surface than were close to the abluminal surface. $P = 0.0006$ by Fisher test. B: In contrast to data in A, in ten endothelial cells immunolabeled for VLA-6 the $D_{Lum}/D_{Ablum}$ ratios did not show a consistent pattern of localization, ie, in seven cells there were more particles closer to abluminal surfaces and in three there were more particles closer to luminal surfaces, indicating random distribution of particles within the cytoplasm.
and αβ₃ on microvessels in experimental cerebral ischemic lesions by Okada et al.⁵² although in that study up-regulation was found in smooth muscle rather than endothelial cells. On the other hand, Defilippi et al.⁵³ showed that αβ₃ on cultured human umbilical vein endothelial cells (HUVECs) decreases when they are treated with TNF-α and IFN-γ and that this is due to a selective effect on the β₃ rather than the α₃ subunit. Because these cytokines are present in active MS lesions, the explanation for this possible discrepancy with the present data are unclear but differences between the in vitro and in vivo conditions, the type of cells studied, and the timing and levels of cytokines are likely significant.

In contrast, our findings of luminal surface expression and intracytoplasmic localization of α₃ in CNS endothelial cells are consistent with the polarization of αβ₃ demonstrated in HUVECs by Conforti et al.⁵⁴ Luminal surface expression implies the potential for α₃-integrins to bind to plasma molecules, including fibronectin, fibrinogen and vitronectin, and to intraluminal leukocytes that have these RGD-containing proteins bound on their surfaces.⁵⁵ The membrane localization also implies that endothelial cell α₃ or portions of it may be shed into the circulation to a greater degree than would molecules bound to the BM. Therefore, their detection in MS patients might indicate disease activity. Up-regulation of α₃ expression also correlates with other evidence of endothelial cell activation in MS lesions (Van der Maesen et al, manuscript in preparation).⁵⁶–⁵⁸ As in HUVECs,⁵⁹ the α₃ subunit was also found on interendothelial cell junctions. This localization correlates with the dynamic redistribution of these molecules and cytoskeletal reorganization induced in HUVECs by H₂O₂ and TNF in vitro¹¹,¹² and likely also relates to their loss of intercellular adhesiveness.

In active lesions microvascular endothelial cell immunostaining for VLA-1 was also greater than that in normal samples. Unlike α₃ subunit, however, VLA-1 was not preferentially localized to luminal membranes and the amount of immunostaining was also greater in chronic lesions than in controls. Therefore, VLA-1 is differently regulated and likely has functions distinct from α₃-integrins. Indeed, VLA-1 expression is increased in HUVECs by TNF-α and other inflammatory mediators and this enhanced expression is associated with increased adhesiveness to Ln as...
well as to other BM components. Increased endothelial cell adhesion to the BM would presumably be more important as the endothelial cell lining becomes re-established after leukocytes have migrated through the vessel wall than during active inflammation. The localization of VLA-1 on interendothelial junctions supports this view because it implies involvement in maintaining tight intercellular contacts. The absence of a detectable increase in the amount of VLA-1 in NAWM (Figure 5) is also consistent with a role for VLA-1 in later stages because endothelial cells in areas where the white matter is intact, even if they are functionally abnormal, would not have undergone the same degree of pathological alteration as those in acute and chronic lesions. Although endothelial cells in chronic MS plaques show evidence of impaired barrier function, specific morphological abnormalities of interendothelial cell tight junctions have not been identified to date. Nevertheless, the failure of VLA-1 to return to normal patterns of expression in chronic lesions might indicate that at the molecular level the junctions continue to be abnormal, and this could be related to persistent blood-brain barrier dysfunction of chronic plaques.

As documented in previous studies of MS lesions, Ln immunoreactivity was primarily localized around blood vessels and there was slight BM Ln thickening in chronic plaques. Ln immunoreactivity may be fixation-dependent, and there are also newly discovered Ln variants in the CNS. Therefore, a more comprehensive survey of the various Ln chains and molecular isoforms might demonstrate additional alterations in Ln and other BM component immunoreactivity in MS lesions. In some active lesions vascular Ln did appear to be disrupted and even to disappear from vessels (Figure 10C), suggesting that there may be active breakdown of BM Ln in conjunction with the inflammation. Indeed, a cytokine-dependent endothelial cell-derived sulfatase that can degrade subendothelial BM has recently been described and proteolytic digestion of Ln may release peptides with immunological functions not present in intact Ln. Thus, endothelial cells actively modify as well as respond to their extracellular matrix environment as part of the immunopathological response.

In conclusion, we have identified alterations in the expression and subcellular localization of endothelial cell integrin Ln receptors in MS lesions. In active lesions decreased β1/VLA-6 may result in endothelial cell detachment from BM, thereby facilitating leukocyte emigration and blood-brain barrier breakdown. Enhanced αv subunit could promote endothelial cell binding to other ligands and cells as well as to Ln and, because of its luminal surface localization, may be shed into the circulation. The αv subunit and VLA-1 may be particularly important in re-establishing vessel wall integrity and the blood-brain barrier through intercellular junctions. These data support many in vitro observations on endothelial cell pathophysiology in immune reactions and provide new insights into mechanisms of endothelial cell dysfunction over the course of evolution of MS lesions. The findings may also have implications for the diagnosis of active disease and for therapeutic targeting of specific endothelial cell molecules in MS patients.

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References


47. Woldemar M, Alon R, Springer TA: The C-C chemokine MCP-1 differentially modulates the avidity of b1, and b2 integrins on T lymphocytes. Immunity 1996, 1:479–1661