Visualization of Chemokine Binding Sites on Human Brain Microvessels

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Abstract. The chemokines monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-1α (MIP-1α) aid in directing leukocytes to specific locales within the brain and spinal cord during central nervous system inflammation. However, it remains unclear how these chemokines exert their actions across a vascular barrier, raising speculation that interaction with endothelial cells might be required. Therefore, experiments were performed to determine whether binding domains for these chemokines exist along the outer surface of brain microvessels, a feature that could potentially relay chemokine signals from brain to blood. Using a biotinylated chemokine binding assay with confocal microscopy and three-dimensional image reconstruction, spatially resolved binding sites for MCP-1 and MIP-1α around human brain microvessels were revealed for the first time. Binding of labeled MCP-1 and MIP-1α could be inhibited by unlabeled homologous but not heterologous chemokine, and was independent of the presence of heparan sulfate, laminin, or collagen in the subendothelial matrix. This is the first evidence of specific and separate binding domains for MCP-1 and MIP-1α on the parenchymal surface of microvessels, and highlights the prospect that specific interactions of chemokines with microvascular elements influence the extent and course of central nervous system inflammation.

Key words: chemokine • monocyte chemoattractant protein • macrophage inflammatory protein-1α • binding • microvessels
has not been explored in the CNS. Moreover, though adhesion of chemokines to peripheral vasculature endothelial cells in culture and tissue sections has been indicated by radiolabeled binding studies [17, 25, 29, 38, 40, 41], this has reflected attachment to the apical or luminal endothelial surfaces only. No visual depiction of perivascular chemokine binding along the parenchymal face of intact microvessels has been confirmed in any organ system.

A greater understanding of chemokine–vascular interactions should yield important clues to the pathogenesis of inflammatory disease, the objective of this study was to visualize and characterize MCP-1 and MIP-1α binding to the surface of microvessels freshly isolated from human brain. To accomplish this, high resolution confocal microscopy and three-dimensional image reconstruction were performed, for the first time, to reveal the perivascular binding of these chemokines, and to delineate the cellular and molecular binding domains. Results indicate that there are specific and separate binding sites for both MCP-1 and MIP-1α along the abluminal surface of brain microvessels. Furthermore, these sites do not appear to be coincident with perivascular macrophages or several major components of the peripheral subendothelial matrix, highlighting the prospect that may be associated with endothelial cells and/or a tightly apposed subendothelial element. The physiologic and pathophysiologic significance of these chemokine binding sites is discussed.

**Materials and Methods**

**Preparation of Microvessels**

Human brain microvessels were derived from cortical tissue acquired from patients undergoing surgery for the treatment of intractable seizures at the Yale-New Haven Hospital, and were resected from areas outside the epileptic foci. A autopsy tissue was acquired from the following sources: University of Miami and University of Maryland Brain and Tissue Banks for Developmental Disorders (through NICHD contracts NOI-HD-3-3199 and NOI-HD-1-3138), respectively. Harvard Brain Tissue Resource Center (through PHS grant MH/NS 31862) and National Disease Research Interchange (Philadelphia, PA). Final diagnoses included respiratory distress and polytrauma, with no evidence of neurologic disease. Microvessels were prepared by a modification of a previously described method for the isolation of rat brain microvessels [10]. In this case, enriched fractions of microvessels obtained by centrifugation through dextran were further purified by isopycnic sedimentation through Percoll [4]. This method yielded a population of microvascular segments that retained their basement membranes.

**Binding Experiments**

Purified microvessels were reacted with either biotinylated recombinant human (rh) MCP-1 or biotinylated rhMIP-1α (R&D Systems Inc.), and subsequently with fluorescein-avidin according to the manufacturer’s directions. All reactions were carried out at 4°C or room temperature to lessen possible cellular uptake of chemokines or antibodies. For negative controls, reactions were performed in the presence of anti-human MCP-1 and MIP-1α antibodies, or included the irrelevant biotinylated protein soybean trypsin inhibitor (R&D Systems Inc.). A positive control, peripheral blood monocytes (PBM), which contain receptors for both MCP-1 and MIP-1α [2, 3, 19, 35], were subjected to the identical conditions. Competition studies using a single chemokine were performed with constant concentrations of either biotinylated rhMCP-1 or rhMIP-1α in the presence of increasing concentrations of unlabeled, homologous ligand (rhMCP-1 or rhMIP-1α; both from PeproTech Inc.). Cross-competition studies between the two chemokines were conducted using a single concentration of either biotinylated rhMCP-1 or rhMIP-1α in the presence of increasing concentrations of unlabeled, heterologous ligand. Relative amounts of biotinylated MCP-1 and MIP-1α bound to microvessels were quantified using a Zeiss LSM 410 confocal microscope equipped with an argon-krypton laser (with emission at 488 and 568 nm). Microvessels were observed with an Achromat 40×/1.3 NA, oil objective, under constant conditions of aperture, pin-hole, brightness, and contrast. Images (512 × 512 pixels) were obtained and processed using A dobe Photoshop 3.0 software (A dobe Systems Inc.). To quantify the extent of labeled chemokine binding, values of mean pixel intensity were recorded from a total of 80 randomly chosen areas (192 pixels each) of microvessels from at least 10 samples. In analogous fashion, mean pixel intensities were also obtained from microvessels incubated with biotinylated soybean trypsin inhibitor. The average of the pixel values obtained from this negative control was then subtracted from each of the 80 pixel intensities obtained from all the different chemokine binding conditions so as to remove the contribution of background noise. Corrected pixel intensities were then averaged, with the resulting value representing the relative degree of specific chemokine binding along microvessels. K_d and Hill coefficient (n) values were determined using Sigma Plot software (Jandel Inc.) and using the following equation: b = (b_max * x) / (K_max + x), where b is indicated by pixel intensity values.

**Combined Chemokine Localization/Immunofluorescence**

A fter reacting isolated microvessels with biotinylated chemokines and fluorescein-avidin, as described above, or for the specific detection of chemokine receptors, microvessels were fixed in 4% (wt/vol) paraformaldehyde and seeded onto poly-L-lysine-coated glass coverslips. After blocking nonspecific binding by incubation with PBS containing 5% (vol/vol) normal goat serum (GIBCO BRL), 1% (wt/vol) BSA (Sigma Chemical Co.), and 0.5% (vol/vol) Tween 20 (Sigma Chemical Co.), microvessels were reacted with the different primary antibodies (monoclonal antiamlinin [clone LAM-89, Sigma Chemical Co.], monoclonal anti–Factor VIII [clone F8/86, DAKO], monoclonal anti–CD-68 [clone EBM11, DAKO], anti–collagen type IV collagen [Sigma Chemical Co.], and anti–heparan sulfate proteoglycan [Upstate Biotechnology]), and then exposed to rhodamine-conjugated goat anti–mouse antibody (Boehringer Mannheim Biochemicals). Monoclonal antibodies to chemokine receptors CCR1, CCR2, and CCR5 were all purchased from R&D Systems Inc., and samples reacted with these antibodies were subsequently visualized with fluorescein-conjugated goat anti–mouse antibody (Boehringer Mannheim Biochemicals). Negative control samples were processed similarly, except for the exclusion of primary antibody, while isotype control samples used similar Ig isotype, irrelevant antibody as a primary source. All samples were observed with an Olympus IX 70 inverted microscope (40x objective) to obtain simple, two-dimensional images, and a Zeiss LSM 410 confocal microscope (40×/1.3 oil objective) to acquire three-dimensional images. The latter were generated by taking a z-series of 1-μm-thick optical sections, usually 20–30, throughout the sample, and then reconstructing and editing the images using the three-dimensional reconstruction program VoxelView (Vital Images, Inc.).

**Heparinase I Digestion**

Freshly purified brain microvessels were incubated with type I heparinase (Sigma Chemical Co.; 50 U/ml in DME/F-12; GIBCO BRL) for 1 h at 37°C under constant, mild agitation. A after this time, the reaction was terminated by dilution with DME/F-12 containing 10% calf serum (GIBCO BRL), and samples of the microvessels prepared for combined immunohistochemical detection of heparan sulfate and binding with biotinylated chemokines.

**Statistical Analysis**

To determine the significance of the effect of heparinase I treatment on chemokine binding, a one-way ANOVA was performed, followed by a Bonferroni multiple comparisons test.

**Results**

**Expression of MCP-1 and MIP-1α Binding Sites on Brain Microvessels**

To visualize chemokine binding sites, isolated brain mi-
Chemokine binding on brain microvessels

Microvessels were reacted first with biotinylated MCP-1 or biotinylated MIP-1α, and subsequently with fluorescein-avidin (Fig. 1). The pattern of MCP-1 binding appears as a near continuous sheath encapsulating the length of the vessel fragment. In comparison, the binding of MIP-1α exhibits a discontinuous or punctate arrangement along the microvascular segment. Control experiments, using concurrent exposure of microvessels to labeled chemokines and antichemokine antibodies, revealed no such staining. PBM, which express receptors for both MCP-1 and MIP-1α (2, 3, 19, 35), also demonstrated binding of both labeled chemokines. No staining of PBM was obtained in the presence of antichemokine antibodies.

To better visualize the unique topologic distributions of MCP-1 and MIP-1α binding sites, confocal images of microvessels were reconstructed to display both endothelial cells and chemokine binding in three-dimensional space (see Figs. 2 and 4). As clearly shown in Fig. 2, the sphere of MCP-1 binding envelopes the endothelial cells in a relatively smooth and nearly continuous pattern. In striking contrast, binding of MIP-1α appears clustered in discrete patches, periodically decorating the abluminal endothelial surface in a punctate fashion.

Specificity of Chemokine Binding

As the binding patterns of biotinylated MCP-1 and biotinylated MIP-1α were clearly distinguishable, experiments were performed to confirm the presence of separate and specific binding sites for these chemokines along the microvascular surface. Specifically, studies were conducted to determine whether binding of these labeled chemokines could be antagonized by increasing concentrations of unlabeled ligand. Fig. 3 unequivocally indicates that such competition can be achieved for both MCP-1 and MIP-1α. Greater than 90% of the binding of both labeled chemokines could be inhibited by competition with 100-fold excess of the respective unlabeled chemokines. Moreover, there was no cross-inhibition between these two chemokines, i.e., unlabeled MCP-1 did not inhibit the binding of labeled MIP-1α, or vice versa. Results from these competition studies further underscore the premise of separate and specific binding sites for MCP-1 and MIP-1α along the abluminal surface of brain microvessels.

In addition to exhibiting homologous, but not heterologous, competition, the binding of both labeled chemokines to perivascular domains was shown to be saturable (Fig. 4), with Kd values of ~2 nM for MCP-1 and ~0.5 nM for MIP-1α. Corresponding Hill coefficients were n = 6 and n = 1.1 for MCP-1 and MIP-1α binding, respectively. Taken together with the qualitative depictions, these quantitative data are consistent with the presence of a limited number of spatially and biochemically distinct, high-affinity binding sites for MCP-1 and MIP-1α along the parenchymal face of microvessels from brain.

Localization of MCP-1 and MIP-1α Binding Sites

To exclude the possibility that monocyte-derived perivascular macrophages were the source of chemokine binding sites, double-label fluorescence microscopy was performed to resolve macrophage and MCP-1/MIP-1α distributions. It can be clearly seen in Fig. 5 that the binding patterns of both these chemokines and perivascular macrophages are completely distinguishable. A assessment of colocalization of chemokine binding with other perivascular cell types, such as pericytes, mast cells, plasma cells, and smooth muscle cells, which only showed very limited distribution in these microvessels preparations, additionally revealed drastically dissimilar patterns (data not shown). Lastly, as several chemokines have demonstrated affinity for proteoglycans and other extracellular matrix components (11, 45, 47, 49, 50), we examined whether...
MCP-1 and/or MIP-1α binding coincided with three major constituents of the subendothelial matrix: heparan sulfate, collagen type IV, and laminin. Fig. 5 indicates that binding sites for MCP-1 and MIP-1α lie predominantly internal to both heparan sulfate and collagen type IV domains, but appear intermixed with that of laminin. Thus, it seems unlikely that binding to brain microvessels occurs principally via attachment to either collagen type IV or heparan sulfate. However, laminin may be associated in limited context with the binding of both these chemokines. This interpretation is consistent with a recent report describing MCP-1 and MIP-1α induction of mast cell migration across microporous filters coated with laminin, but not with collagen type IV (46).

To further assure that chemokine binding to brain microvessels did not merely reflect attachment to heparan sulfate, a glycosaminoglycan (GAG) to which many chemokines, including MCP-1 and MIP-1α, have been reported to adhere (17, 25), binding experiments were also performed on microvessels stripped of heparan sulfate by treatment with heparinase I. As shown in Fig. 6, such enzymatic treatment removed nearly all perivascular heparan sulfate, as judged by immunofluorescence. Despite this, neither MCP-1 nor MIP-1α binding to microvessels was diminished. On the contrary, slightly heightened binding of both chemokines was observed (Fig. 7). It was further observed that microvessel binding of neither chemokine could be blocked by coincubation with 1,000-fold excess of...
either heparin or heparan sulfate (data not shown). Collectively, these data strongly argue against heparan sulfate, alone, being responsible for MCP-1 and MIP-1α binding to the abluminal surface of brain microvessels. Similar experiments to gauge the effects of enzymatic removal of laminin and collagen (using cathepsins B and D, and collagenase type IV, respectively) also indicated no significant diminution in the binding of either chemokine (data not shown).

Detection of MCP-1 and MIP-1α Receptors

Insofar as both kinetic and cytological data indicated that both MCP-1 and MIP-1α were binding to high-affinity sites that were closely associated with the endothelium, we further assessed whether brain microvessels express receptors for these chemokines. These receptors, designated by the abbreviation CCR, have been well documented in leukocytes, and their sequences cloned (35). CCR 2 is considered the lone, specific receptor for MCP-1, while both CCR 1 and CCR 5 are the recognized receptors for MIP-1α. As indicated in Fig. 8, brain microvessels display immunoreactivity with specific antibodies to each of these receptors. All three antibodies were similarly reactive with PBM (data not shown), consistent with these cells’ ability to bind both chemokines (as visualized above). However, it is important to note that microvascular staining with the anti-receptor antibodies was not similar to that obtained with anti-CD68 staining of perivascular macrophages (Fig. 5), the latter clearly exhibiting immunoreactivity in discrete cellular domains that lie outside the immediate vascular wall. In a contrary manner, antireceptor antibodies decorate the contour of microvessels in a more continuous manner, and apparently bind to sites in close apposition to the abluminal endothelial surface. Thus, antichemokine receptor staining of microvessels may include, but is not restricted to, perivascular macrophages.

Discussion

Specific binding of MCP-1 and MIP-1α to separate do-
Figure 5. Codistribution of MCP-1 and MIP-1α binding with perivascular markers along brain microvessels. Microvessels were incubated with biotinylated chemokines (either MCP-1, left column, or MCP-1α, right column) and the indicated antibodies (top to bottom). Chemokine binding is displayed in green, and antibody reactions are in red. Insets show three-dimensional reconstructions, obtained as described in Materials and Methods. For orientation purposes, asterisks indicate the direction looking into the lumen of each microvessel fragment. Anti-CD68 specifically stains monocyte-derived macrophages. Arrowheads denote staining of the individual matrix components, which appear outside the domains of chemokine binding. Bars, 40 μm.
mains along the microvessel outer surface was determined according to the following criteria. First, the distribution patterns of MCP-1 and MIP-1α binding were remarkably different from each other. Second, binding of labeled MCP-1 and MIP-1α could each be inhibited nearly 95% by 100-fold excess of their unlabeled homologues, but not by unlabeled derivatives of the other chemokine. Third, binding of both chemokines was saturable. Finally, neither MCP-1 nor MIP-1α binding was observed to colocalize with three major components of the basement membrane or to be quantitatively dependent upon the presence of these constituents, implying a lack of any significant chemokine trapping within the subendothelial matrix.

It is important to reemphasize that the chemokine binding sites visualized and characterized here reside strictly on the abluminal surface of the microvessels. These results are thus to be distinguished from descriptions of radiolabeled chemokine binding to the apical surface of cultured endothelial cells, which represents the luminal microvascular surface in situ. In the latter case, chemokine binding has been demonstrated to be largely dependent upon the presence of GAGs (17, 40, 41). Attachment of chemokines to such GAGs is thought to serve the passive role of sequestering chemokines in the luminal space, raising their effective concentration and, thus, their probability of encountering a chemokine receptor on a loosely tethered leukocyte (17). In contrast to that observed in these previous reports, MCP-1 and MIP-1α binding to isolated brain microvessels was not diminished as a result of enzymatic removal of perivascular heparan sulfate, a prominent chemokine-binding GAG. As similar heparinase treatment to that performed here has been shown to completely antagonize the binding of numerous chemokines to both cultured cells and isolated subendothelial matrix (11, 25), our findings infer that, at the very least, attachment to heparan sulfate is not the sole mechanism underlying MCP-1 and MIP-1α binding to human brain microvessels. Of course, these chemokines might interact with GAGs other than heparan sulfate present along the abluminal surface of brain microvessels, and do so with varying affinities (49). However, the Kd values obtained here (≤2.0 nM) are significantly lower than those generally reported for chemokine attachment to various GAGs, e.g., mid-nanomolar to low millimolar range (27). That enzymatic removal of both laminin and collagen was additionally unable to lessen the degree of MCP-1 and MIP-1α binding sustains the concept that these perivascular chemokine binding domains are more closely associated with integral elements of the brain microvasculature than with components of the subendothelial matrix. The additional lack of coincidence between chemokine binding and the presence of perivascular cells also eliminates these cells as being the major target of chemokine binding.

What then might be the nature of these abluminal chemokine binding sites? MCP-1 and MIP-1α binding may reflect attachment to specific chemokine receptors on the surface of endothelial cells. In this regard, the fluorescence...
competition assays described here mirror those reported for chemokine binding to cloned CCR1 and CCR2 receptors (31, 32), in that a 100-fold excess of unlabeled ligand abrogated nearly 95% of labeled chemokine binding. That we were further able to detect immunostaining with antibodies to CCR1, CCR2, and CCR5 is yet additional support. Lastly, the estimated $K_d$ values for both MCP-1 and MIP-1α binding to brain microvessels are within the low nanomolar range generally reported for these chemokines’ binding to their respective receptors (reviewed in 27, 43). The relatively high degree of apparent cooperativity in MCP-1 binding, as indicated by a Hill coefficient of $n = 6$, may reflect cooperative interactions between high-affinity chemokine receptors and low-affinity extracellular matrix component(s) (17). Thus, our observations could be the first evidence of expression of receptors for MCP-1 and MIP-1α on, or around, the abluminal surface of microvascular endothelium. In agreement with this contention, recent studies have indicated both expression and activity of chemokine receptors on cultured endothelial cells (7, 8, 15), although no cytological distributions of these receptors were described.

A side from binding to specific receptors on brain microvessels, MCP-1 and MIP-1α may also be engaging the Duffy antigen receptor for chemokines (DARC), a promiscuous receptor that binds several chemokines (24) and is expressed in endothelial cells (33). Consistent with this possibility, Horuk et al. (18) demonstrated immunocytochemical staining of capillaries and postcapillary venules of human brain sections with an antibody to DARC. However, considering the different binding patterns for MCP-1 and MIP-1α reported here, and the noted lack of affinity of MIP-1α for DARC (24), binding to DARC cannot be the exclusive means by which both these chemokines associate with brain microvessels.

What could be the functional significance of these perivascular chemokine binding domains? Middleton et al. (29) presented evidence that another chemokine, interleukin 8, is transcytosed from the abluminal to the luminal microvascular surface in skin, where it appears to reside on the tips of endothelial microvilli. Such luminal “presentation” of bound chemokines is thought to provide the proper context for efficient activation of leukocyte $\beta$-integrins (2, 6, 44, 45). The binding sites described here might represent domains to which the MCP-1 and MIP-1α initially dock, before transcytosis across the microvascular endothelium of brain. MCP-1 and MIP-1α binding to brain microvessels may additionally signal vascular permeability changes through cytoskeletal reorganization, as has been suggested most recently for interleukin 8 (9). Furthermore, binding to these abluminal sites may function to prevent these chemokines from being diluted within the perivascular space and, thus, augment the intensity, prolong the duration, and/or influence the site of inflammatory reactions (11). While all these possibilities remain a priori, the pleiotropic nature of chemokines (30, 43) is consistent with these binding sites subserving several functions.

Are these perivascular chemokine binding sites restricted to, or enriched in, microvessels from brain? While this remains to be explored, the stringent requirements for maintaining an effective blood-brain barrier dictates that specialized mechanisms, possibly receptors, exist within brain microvessels for the purpose of efficiently communicating chemokine signals. In contrast, less restrictive vascular beds may simply allow tissue-derived chemokines to percolate through leaky endothelial junctions into the vascular lumen. That Randolph and Furie were not able to demonstrate significant binding of MCP-1, when this chemokine was applied to the basolateral (abluminal) surface of cultured monolayers of human umbilical vein endothelial cells (36), may, in part, reflect this phenotypic diversity. It may also be that specific endothelial chemokine binding sites are rapidly lost, or their membrane polarity altered, upon adaptation to culture conditions (39).
so will their pathogenic role(s) in disease become more appreciated. In turn, this will lead to greater efforts to specifically antagonize chemokine action at sites of inflammation and infection. Heightened understanding of the interactions of chemokines with their binding sites on the vascular surface will be a significant step in this direction.

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