Pathological Expression of CXCL12 at the Blood-Brain Barrier Correlates with Severity of Multiple Sclerosis

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Dysregulation of blood-brain barrier (BBB) function and transendothelial migration of leukocytes are essential components of the development and propagation of active lesions in multiple sclerosis (MS). Animal studies indicate that polarized expression of the chemokine CXCL12 at the BBB prevents leukocyte extravasation into the central nervous system (CNS) and that disruption of CXCL12 polarity promotes entry of autoreactive leukocytes and inflammation. In the present study, we examined expression of CXCL12 and its receptor, CXCR4, within CNS tissues from MS and non-MS patients. Immunohistochemical analysis of CXCL12 expression at the BBB revealed basolateral localization in tissues derived from non-MS patients and at uninvolved sites in tissues from MS patients. In contrast, within active MS lesions, CXCL12 expression was redistributed toward vessel lumina and was associated with CXCR4 activation in infiltrating leukocytes, as revealed by phospho-CXCR4-specific antibodies. Quantitative assessment of CXCL12 expression by the CNS microvasculature established a positive correlation between CXCL12 redistribution, leukocyte infiltration, and severity of histological disease. These results suggest that CXCL12 normally functions to localize infiltrating leukocytes to perivascular spaces, preventing CNS parenchymal infiltration. In the patient cohort studied, altered patterns of CXCL12 expression at the BBB were specifically associated with MS, possibly facilitating trafficking of CXCR4-expressing mononuclear cells into and out of the perivascular space and leading to progression of disease. (Am J Pathol 2008; 172:799–808; DOI: 10.2353/ajpath.2008.070918)

The blood-brain barrier (BBB), a specialization of capillary endothelial cells and periendothelial accessory structures, greatly restricts the movement of molecules and cells into the central nervous system (CNS), establishing immune privilege.¹ Leukocytes that traverse the microvasculature during neuroinflammatory diseases such as multiple sclerosis (MS) have been observed to congregate within a subendothelial space between the endothelial cell basement membrane and the glial limitans before entering the CNS parenchyma. Thus, one of the hallmark features of MS lesions includes an intense perivascular infiltration consisting of lymphocytes and macrophages.² Studies in MS patients suggest that myelin-reactive T lymphocytes within these lesions enter the CNS parenchyma and initiate inflammatory demyelination accompanied by phagocytosis of myelin by macrophages.^{3,4} Although several adhesion molecules essential for the migration of mononuclear cells into the CNS during MS exacerbations have been identified,^{5,6} the chemoattractants responsible for the migration of cells through the perivascular space within the human CNS microvasculature are unknown.

Recent studies using the rodent model for MS, experimental autoimmune encephalomyelitis (EAE), have demonstrated that the secondary lymphoid chemokine CXCL12 functions to localize mononuclear cells to the perivascular space, thereby limiting the parenchymal infiltration of autoreactive, effector cells.⁷ CXCL12 was originally identified as a bone marrow stromal cell-derived chemoattractant and proliferative factor for B-cell precursors.⁸ CXCL12, via its receptor CXCR4, mediates the localization of mononuclear cells into lymphoid com-

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partments, thereby promoting the development of B- and T-cell effector immune responses.^{9–11} CXCL12 is also constitutively expressed by a variety of parenchymal tissues, including the CNS, where it has been identified within neurons, endothelial cells, and glial cells.^{12,13} CXCL12 expression increases during autoimmune diseases of the thyroid, joint synovium, and salivary gland and is believed to participate in the formation of ectopic lymphoid follicles through the localization, proliferation, and activation of CXCR4-expressing effector leukocytes within inflamed tissues sites.^{14–17} In patients with a variety of neuroinflammatory diseases, including MS, CXCL12 levels are elevated in the cerebrospinal fluid (CSF).^{18,19} Several investigators have also identified CXCL12 expression within capillary endothelium and reactive astrocytes in both active and chronic inactive lesions in CNS tissues derived from MS patients.^{18,20,21} In addition, astrocyte expression of CXCL12 is increased *in vitro* in response to treatments with interleukin-1 β and myelin basic protein,²⁰ suggesting that CXCL12 plays a role in CNS autoimmunity.

In the current study, we evaluated the expression patterns of CXCL12 and CXCR4 within CNS tissues derived from MS and non-MS patients, including non-MS patients with inflammatory and non-inflammatory neurological diseases. Using confocal microscopy, we observed that endothelial cells within arterioles and venules normally express CXCL12, exhibiting basolateral localization with respect to the CNS microvasculature. This pattern of CXCL12 expression was also observed in uninvolved areas within MS tissue specimens where there were minimal perivascular infiltrates. However, active MS lesions exhibited redistribution of CXCL12 toward the luminal sides of venules. This altered pattern of expression was specific for MS, occurring only within venules of CNS tissues derived from the MS patients in the patient cohort evaluated. The alteration in the normal pattern of CXCL12 expression correlated with increased astrocyte expression of CXCL12 within the glial limitans and with ligand activation of CXCR4 in infiltrating leukocytes. Quantitative analysis of altered CXCL12 expression within non-MS and MS tissue specimens revealed that CXCL12 redistribution is positively correlated with increased inflammation and demyelination in MS. These data suggest that loss of CXCL12 polarization at the BBB during MS enhances leukocyte adherence to vessels and promotes their migration into the CNS via activation of CXCR4.

Materials and Methods

Subjects

The study was approved by the Human Studies Committee and Institutional Review Board of the Washington University School of Medicine. Where mandated, written consent was obtained from all participants. Postmortem CNS tissue from two groups of patients was studied: 11 patients with clinically defined MS followed in the Washington University Multiple Sclerosis Center and 7 control individuals without histories of MS (Table 1). The control group consisted of three patients without any evidence of

neurological disease plus two patients with inflammatory neurological diseases (CNS lymphoma and viral encephalitis) and two patients with non-inflammatory neurological diseases, amyotrophic lateral sclerosis, and Alzheimer's disease (Table 1).

Neuropathological Classification of Multiple Sclerosis Subtypes

Patient records were reviewed by A.H.C., who was blinded to the immunohistochemical data during review. Sex, age, ethnicity, family history of MS, disease duration, MS subtype, and immunomodulatory drug exposures were recorded for each subject. The multiple sclerosis cases used in this study had a range of ages at death (35–88 years), ages at multiple sclerosis onset (20–59 years), and disease duration (18–35 years), reflecting the variability of the MS population. The cohort included one patient classified as relapsing-remitting (RR), seven as secondary progressive (SP), and three as primary progressive (PP). Table 1 summarizes the information collected on these patients.

CNS Tissues

Studies were performed on CNS tissues taken at autopsy from 11 patients with MS and 7 controls. All specimens were collected within 3 to 10 hours of death. At each autopsy, fresh CNS tissue (brain, optic nerve, brainstem, and spinal cord) was examined, and when MS lesions were identified by gross inspection, these were sampled. Areas of normal-appearing CNS, both white and gray matter, were also obtained, as in some cases, MS lesions are not visible grossly. One-half of CNS tissues were flash-frozen at the time of autopsy and stored at -80°C until use. At the time of autopsy or later, small areas of tissue were embedded in Optimal Cooling Temperature compound. The other one-half of the CNS was fixed in formalin and subsequently embedded in paraffin. For control subjects, clinical histories and cause of death were available for all cases.

For this study, one to three frozen tissue blocks (1 cm³) from various CNS regions including cerebrum, optic nerve, periventricular white matter, cerebellum, brainstem, and spinal cord were obtained from each MS case. Control subject CNS tissues were obtained from predominantly white matter areas corresponding to those areas sampled in the MS patients. Diagnosis of MS was histologically confirmed by a neuropathologist (R.E.S.).

Histological Scoring of CNS Tissues

Inflammatory cell infiltrates and demyelination were evaluated using H&E and Luxol fast blue-PAS staining, respectively, as described previously.²² Active MS lesions were identified by the presence of variable amount of perivascular and parenchymal lymphocytic infiltrates, macrophages, and demyelination.²³ Oil red O (ORO) staining was performed to demonstrate neutral lipids

Table 1. Patient Characteristics, Disease Types, CNS Regions Affected, and Histological Information

Patient no.	Age (years)	Sex	Postmortem interval (hours)	Diagnosis	CNS level	Inflammation	Demyelination	ORO
MS patients								
01	46	Female	6	SPMS	Midbrain	—	+	—
					Medulla	—	—	—
03	45	Female	3.5	PPMS	Cerebellum	—	++	—
					Medulla	+	+	+
07	87	Male	6	SPMS	Pons	—	—	—
20	45	Female	5	SPMS	Midbrain	++	+	—
21	69	Female	5	PPMS	Pons-plaque 1	++++	++++	++++
					Pons-plaque 2	—	+	+
					Cervical cord	++++	++++	++++
49	41	Female	8	SPMS	Lumbar cord	—	+	—
50	35	Male	10	SPMS	PWM	+++	++	+++
					Cervical cord	+	+++	+
59	55	Female	8	SPMS	Midbrain	+++	+++	+++
					Cervical cord	++++	++	++++
57	66	Female	9	RRMS	Thoracic cord	—	—	—
66	66	Female	6	PPMS	Medulla	+	++	+
71	69	Male	6	SPMS	Cerebellum	—	—	—
Control patients								
08	84	Female	6	Pulmonary embolus	Cerebellum	—	—	—
32	56	Female	4	Emphysema/ Atherosclerosis	PWM	—	—	—
					Cervical cord	—	—	—
38	39	Female	4	CNS lymphoma	Thoracic cord 1	—	++	++++*
					Thoracic cord 2	—	++	++++*
71	52	Male	9	Congestive heart failure	Optic nerve	—	—	—
65E	80	Male	5	WNVE	Midbrain	++++ [†]	—	—
94	73	Male	3	ALS	Cerebrum 1	—	—	—
					Cerebrum 2	—	—	—
95	34	Male	3	AML	Thoracic cord	—	—	—

LFB, luxol fast blue; AML, acute myelogenous leukemia; ALS, amyotrophic lateral sclerosis; PWM, periventricular white matter; AD, Alzheimer's disease; WNVE, West Nile virus encephalitis.

*Oil red O-positive macrophages associated with tract degeneration.

[†]All perivascular cuff, no inflammatory cell in the parenchyma.

within macrophages (indicative of myelin phagocytosis), also known as lipid-laden or foamy macrophages. Extent of inflammation and demyelination within each section were graded using a five-point scale (negative, +, ++, +++, and +++++), as previously described.²⁴ Inflammation was scored based on the number of perivascular or parenchymal mononuclear infiltrating cells accordingly to the following scale: 0, no infiltrating cells; +, fewer than 10 infiltrating cells/×40 magnified microscopic field; ++, 10 to 20 infiltrating cells/×40 magnified microscopic field; +++, 20 to 40 infiltrating cells/×40 magnified microscopic field; and +++++, >50 infiltrating cells/×40 magnified microscopic field. Demyelination was scored based on the percentage of the section that was demyelinated: negative, no demyelination; +, 10 to 20%; ++, 20 to 40%; +++, 40 to 70%; and +++++, 70 to 100%. The amount of lipid-laden macrophages infiltrating the tissue was also evaluated using a five-point scale: 0, no ORO⁺ cells; +, rare, fewer than 10 ORO⁺ cells/×40 magnified microscopic field; ++, 10 to 20 ORO⁺ cells/×40 magnified microscopic field; +++, 20 to 40 ORO⁺ cells/×40 magnified microscopic field; and +++++, >50 ORO⁺ cells/×40 magnified microscopic field. All scores were determined by an observer blinded to the CXCL12/CXCR4 results (A.H.C.).

Antibodies

The following antibodies were used for immunohistochemistry: CXCL12 rabbit polyclonal antibody (Peprotech, Rocky Hills, NJ), polyclonal CXCR4 antibodies (panCXCR4; Leinco, St. Louis, MO) and polyclonal rabbit anti-mouse phosphoserine 339-CXCR4-specific antibodies (pS³³⁹-CXCR4)²⁵ monoclonal mouse anti-human-CD31 (hCD31) (a generous gift from Dr. P.J. Newman, Blood Center of Wisconsin), and hamster anti-CD45 antibodies were all purchased from BD Pharmingen (San Diego, CA). Mouse anti-human glial fibrillary acidic protein (GFAP) antibody was purchased from Dako (Glostrup, Denmark). Normal goat and rabbit sera and IgG isotype control antibodies were from Jackson ImmunoResearch (West Grove, PA).

Immunohistochemistry and Confocal Microscopy

Frozen sections were permeabilized, blocked, and stained as previously described.²⁶ Additional blocking with image-iT Fx signal enhancer (Molecular Probes, Eugene, OR) solution was used according to the manufacturer's instructions. Detection of CXCR4 with panCXCR4

and pS³³⁹-CXCR4 antibodies was performed as previously described.²⁵ Primary antibodies (see above) were used at the following dilutions: anti-CXCL12 (1:20), pS³³⁹-CXCR4 (1:150), anti-hCD31 (1 μ g/ml), panCXCR4 (1 μ g/ml), GFAP, or CD45 (1 to 10 μ g/ml). Primary antibodies were detected with secondary goat or donkey anti-rabbit or mouse IgG conjugated to Alexa 555 or Alexa 488 (Molecular Probes). For immunofluorescence staining, nuclei were counterstained with ToPro3. Control sections were incubated with antisera in the presence of a 100 μ mol/L excess of peptide or with isotype-matched IgG. Sections were analyzed using a Zeiss LSM 510 laser scanning confocal microscope and accompanying software (Zeiss, Welwyn Garden City, UK). Velocity image analysis software (Improvion) was used to generate and analyze three-dimensional renderings of confocal images. Stained regions were identified by applying a classifier to exclude objects smaller than 0.1 μ m³ and pixels of intensity less than 45 (scale 0 to 225).

Statistical Analysis

Percentages of CXCL12 redistribution are expressed as means $\pm$ SEM. Comparison of median values for each group using the Mann-Whitney test was used to determine the statistical significance of vessel CXCL12 redistribution within CNS tissues derived from MS versus non-MS patients. Linear regression analyses were performed using Prism Statistical Analysis software.

Results

Multiple Sclerosis Cohort

Tissue specimens from 18 subjects (11 with and 7 without MS) were included in the study (Table 1). For the MS patients, median disease duration was 19.5 years (range, 18 to 35 years). The MS cohort included 1 patient with RRMS, 7 patients with SPMS, and 3 patients with PPMS. Histological characterization of 17 MS and 12 non-MS tissue blocks revealed that the majority of MS specimens contained active lesions, which were graded for inflammation, demyelination, and infiltration by lipid-laden macrophages as described (Table 1). In contrast, the blocks from non-MS patients did not show any pathological abnormalities, with the exception of CNS lymphoma case, in which there were extensive areas infiltrated with lipid-laden macrophages consequent to tract degeneration, and the West Nile virus encephalitis case, in which there were intense perivascular infiltrates. Blocks from MS patients also contained areas with normal appearing white matter, whose staining patterns were used as additional controls for CXCL12 and CXCR4 immunohistochemical analyses (see below). Demyelination in active MS lesions was associated with intense perivascular infiltrates comprising mononuclear cells (Figure 1, a–f). Areas of extensive demyelination partially overlapped with plaque areas containing large numbers of foamy macrophages, as evidenced by staining with ORO (Figure 1, d and f). Infiltrates within MS lesions consisted mainly of perivas-

Figure 1. Active multiple sclerosis lesion within medulla. **a:** A section from the medulla of a postmortem specimen from a patient with MS stained with Luxol fast blue reveals multiple, irregularly bordered areas of demyelination. The boxed area indicates the region depicted at higher magnification after staining with H&E (**b**), Luxol fast blue (**c**), and ORO (**d**). Note the focus of demyelination with an irregular border (**c**, arrowheads), which includes a defined region bordered abruptly by ORO⁺ macrophages (**d**, arrowheads). The demyelinated region contains a venule with perivascular infiltration of small lymphocytes (**e**) that are adjacent to foamy ORO⁺ macrophages (**f**). Magnification: $\times 8$ (**a**), $\times 40$ (**b–d**), and $\times 100$ (**e** and **f**).

cular ORO-negative lymphocytes and parenchymal ORO⁺ macrophages (Figure 1, e and f). CNS tissues derived from non-MS patients exhibited normal microvasculature without infiltrating mononuclear cells and normal myelination with the exception of the two patients who had CNS lymphoma and West Nile virus encephalitis (data not shown).

Redistribution of CXCL12 at the BBB Occurs During MS

In previous studies, CXCL12 has been localized to the BBB in MS patients and in patients without neurological disease.^{18,21,27} In these limited analyses, CXCL12 expression patterns were not extensively analyzed nor were the cellular sources of CXCL12 in normal and diseased tissues definitively determined. In animal studies, CXCL12 expression at the BBB displayed a basolateral polarity that was lost during induction of EAE.⁷ To determine whether similar dynamic expression of CXCL12 occurs at the human BBB, we evaluated CXCL12 expression in all tissue blocks from MS and non-MS patients via double-label, immunofluorescent confocal microscopy. In all tissue and all CNS regions examined, CXCL12 protein was detected adjacent to CD31⁺ endothelial cells, within both gray (not shown) and white matter along both arterioles and venules (Figures 2, a–c, and 3, a and

Figure 2. CXCL12 redistribution occurs in venules within CNS tissues derived from MS patients. Endothelial cell localization (CD31, Alexa-488, green) of CXCL12 (Alexa-555, red) in arterioles and venules within postmortem CNS tissues derived from non-MS (**a** and **b**) and MS (**c** and **d**) patients. Note lack of CD31 staining within elastin layer of arteriole wall (**large arrows**). CXCL12 expression is detected along the basolateral (**small arrow**) and luminal (**arrowhead**) surfaces of venules only in MS specimens (**d**). Nuclei are counterstained with ToPro3 (blue). Scale bar = 8 μ m.

b). In all venules examined within non-MS CNS tissues and in normal-appearing white matter regions of those derived from MS patients, CXCL12 expression was localized to the parenchymal side of the endothelium (Figure 3, a and b). Thus, patients with histories and CNS post-mortem exams consistent with CNS lymphoma, amyotro-

phic lateral sclerosis, Alzheimer's disease, or viral encephalitis did not exhibit CXCL12 redistribution to the luminal side of the endothelium in any areas of the CNS. Interestingly, CXCL12 immunoreactivity in venules within MS specimens often displayed punctate staining, suggesting astrocyte end-feet may be a source of the chemokine (Figure 3b). Analysis of venules within active MS lesions displayed a redistribution of CXCL12 with chemokine detected on both luminal and parenchymal sides of CD31-expressing endothelial cells (Figures 2c and 3d). In CNS tissues derived from non-MS patients and in normal-appearing white matter regions of MS patients, quantification of fluorescence intensity revealed a polarity in peak CXCL12 expression localizing it to the basolateral surface of endothelial cells of both arterioles (data not shown) and venules (Figure 3, d and e). Quantitative confocal microscopy of inflamed venules detected a shift in the distribution of CXCL12 with respect to CD31, with peak levels of intensity of CXCL12 colocalizing with peak levels of CD31 (Figure 3f). Three-dimensional reconstructions of venules stained with CXCL12 (red) and CD31 (green) best demonstrate the redistribution of CXCL12 observed in venules within active lesions of MS tissues with noninflamed venules exhibiting red exteriors and green interiors (Figure 3, g and h) and inflamed venules exhibiting green exteriors and red interiors (Figure 3i). Experiments using control IgG antibodies did not demonstrate any specific staining (data not shown).

Quantitative analyses of CXCL12 expression patterns revealed that average percentages of venules displaying loss of CXCL12 polarity ranged from 10 to 100% in CNS sections from MS patients, whereas CXCL12 remained polarized within all venules in CNS sections of non-MS patients (Figure 3j). In general, the maximum number of

Figure 3. CXCL12 redistribution is specifically altered during MS. Endothelial cell localization (CD31, Alexa-488, green) of CXCL12 (Alexa-555, red) in cerebellar tissue obtained from non-MS (amyotrophic lateral sclerosis) (**a**) and MS patients (**b** and **c**). Nuclei are counterstained with ToPro3 (blue). Scale bar = 10 μ m. Quantification of fluorescence intensity acquired by confocal microscopy for CXCL12 (red stain and line) and CD31 (green stain and line) are shown for venules within non-MS (**d**) and MS (**e** and **f**) tissues. **Double-headed arrows** indicate location of line graph analysis. Three-dimensional reconstructions of microvessels stained with anti-CXCL12 (red) and anti-CD31 (green) antibodies are shown for venules depicted in **a-c**: non-MS (**g**), MS (**h** and **i**). The percentage of venules with loss of CXCL12 polarity in each patient in our non-MS and MS cohorts (**j**) were determined by examining CD31 and CXCL12 staining patterns in approximately 5 to 72 venules per patient ($n = 6$ control, 11 MS patients).

Figure 4. CXCL12 redistribution occurs in venules within active MS lesions regardless of extent of perivascular infiltrates. **a:** Combined analysis of all venules with ≥ 10 perivascular leukocytes within MS specimens were examined for redistributed CXCL12 expression (light gray bar) versus polarized expression (dark gray bar). **b:** Combined analysis of venules with CXCL12 redistribution for venules with ≥ 10 (light gray bar) versus ≤ 10 (dark gray bar) leukocytes adjacent to CD31⁺ endothelium. **c:** Endothelial cell localization (CD31, Alexa-488, green) of CXCL12 (Alexa-555, red) reveals loss of CXCL12 polarity in a venule without perivascular infiltrates (<10 associated leukocytes). **d:** Combined analysis of all venules with >15 versus ≤ 15 perivascular leukocytes for loss of perivascular CXCL12 expression. **e:** A CD31⁺ venule (Alexa-488, green) with intense perivascular infiltrates displays weak intraluminal (arrowhead) and no perivascular (arrow) expression of CXCL12. Scale bar = 8 μ m.

venules analyzed per patient varied with the quality of the tissue available and ranged from 5 to 72 venules per patient. Importantly, blinded, independent scoring for CXCL12 redistribution identified all MS specimens without false positives. To determine whether CXCL12 redistribution correlates with the presence of perivascular infiltrates within MS lesions, we evaluated CXCL12 expression patterns in venules in which there were ≥ 10 leukocytes in contact with the endothelial cells. This criterion was based on assessment of un-inflamed, control specimens in which all venules had <10 leukocytes in perivascular locations (data not shown). Approximately 97% of venules meeting this criterion exhibited redistribution of CXCL12 (Figure 4a). However, analyses of all venules with redistributed CXCL12 for perivascular infiltrates demonstrated that 72.2% had <10 perivascular leukocytes while 27.8% had ≥ 10 perivascular leukocytes (Figure 4b), suggesting that CXCL12 redistribution may be detected in venules with minimal perivascular infil-

trates (Figure 4c). Examination of CXCL12 expression within venules with extensive perivascular infiltrates (>15 adjacent cells, Figure 4e) exhibited complete loss of CXCL12 on the parenchymal side of 43.8% of venules compared with 8.7% of vessels with fewer perivascularly located cells (<15 adjacent cells) (Figure 4d). These results suggest that CXCL12 is normally expressed within the perivascular spaces of arterioles and venules and disruption in this pattern of expression occurs exclusively within active lesions of MS patients. In addition, the data suggest that CXCL12 normally functions to localize leukocytes to the perivascular spaces of the CNS microvasculature and that redistribution of CXCL12 to the luminal side of venules during MS may facilitate increased parenchymal entry of infiltrating cells.

Astrocyte Up-Regulation of CXCL12 Occurs at the BBB within Active MS Lesions

The alteration in the pattern of CXCL12 expression within inflammatory MS lesions suggested that the cellular sources of CXCL12 might differ between normal and inflamed BBB. Given that CXCL12 has been detected within astrocytes both *in vitro* and *in vivo*,^{20,21,27} we examined CXCL12 expression via confocal microscopy using antibodies to GFAP, a marker for activated astrocytes. In CNS sections from non-MS patients, low levels of CXCL12 were observed in microvasculature-associated astrocytes compared with endothelial cells, which expressed higher levels of CXCL12 (Figure 5, a–c). In contrast, in CNS sections from MS patients, CXCL12 expression by endothelial cells and astrocytes was comparable, and the redistributed CXCL12 remained distinct from GFAP immunoreactivity (Figure 5, d–f). Within the MS specimens, non-redistributed CXCL12 and GFAP immunoreactivity were extensively colocalized within the glial limitans (Figure 5, d–f). These data suggest that astrocytes contribute to the increased levels of CXCL12 observed at the BBB within the CNS of patients with MS.

CXCR4 Activation within Active MS Lesions

Given that the expression pattern of CXCL12 at the BBB was altered within the CNS of MS patients, we evaluated the expression patterns of its receptor, CXCR4, within these tissues using both panCXCR4 and pS³³⁹-CXCR4 antibodies. The latter antibody recognizes a ligand-induced phosphorylation of CXCR4 serine339.²⁵ Pan-CXCR4 antibodies detected CXCR4 in a majority of cells within perivascular infiltrates of active MS lesions and in scattered cells within the parenchyma (Figure 6a). In five of the MS specimens, subsets of cells within these infiltrates exhibited ligand-activated CXCR4 (Figure 6, a and b), as did the adjacent endothelial cells (Figure 6, b and c). In addition, luminal CD45-expressing leukocytes within venules exhibiting CXCL12 redistribution also contained activated CXCR4 (Figure 6, d–g). The MS specimens in which ligand-activated CXCR4 could be detected were characteristically from those with the shortest postmortem interval versus those in which no staining

Figure 5. Astrocytes are a source of CXCL12. Activated astrocyte localization (GFAP, Alexa-488, green) (**a**) of CXCL12 (Alexa-555, red) (**b**, **arrow**) in the glial limitans adjacent to CXCL12-expressing endothelial cells (**b**, **arrow-heads**) within a section from the optic nerve from a non-MS patient. **c**: Merged image. Scale bar = 8 μ m. End-feet of activated astrocytes (GFAP, Alexa-488, green) (**d**) express increased staining for CXCL12 (Alexa-555, red) (**e**, **arrowhead**) within a section from the spinal cord from an MS patient. **f**: Merged image depicts colocalization of GFAP and CXCL12 (**arrow**).

with pS³³⁹-CXCR4 antibodies was apparent (Table 1). Decreased staining with phospho-specific antibodies due to postmortem delays has been previously observed.²⁸ In contrast, only one non-MS patient specimen, obtained from a patient who succumbed to CNS lymphoma, contained leukocytes with activated CXCR4, which were located in the perivascular space (Figure 6h). In all control specimens, CD45-expressing cells detected within the vessel lumina did not exhibit activated CXCR4 (Figure 6, h and i; data not shown), although some of these specimens had short postmortem intervals in which pS³³⁹-CXCR4 should be detectable (Table 1). These data suggest that CXCR4 activation occurs on luminal mononuclear cells in MS, perhaps induced by the redistribution of CXCL12 in active MS lesions. This could lead to inappropriate trafficking of these cells into the CNS and the development of inflammatory lesions.

Loss of CXCL12 Polarity at the BBB Correlates with Severity of MS

Given the association between CXCL12 redistribution and CXCR4 activation, we hypothesized that altered CXCL12 expression within MS lesions might correlate with severity of disease. Thus, we performed correlation analyses comparing the percentages of vessels with

CXCL12 redistribution with extents of inflammation, demyelination, and macrophage infiltration observed within tissues derived from MS patients (Table 1) (Figure 7). While CXCL12 redistribution was significantly correlated with all measures of histological severity, the highest correlation coefficients were seen with demyelination and macrophage infiltration, suggesting that CXCL12 redistribution is a sensitive marker of disease severity. These data, together with the previously published data in the EAE model, also suggest that the redistribution of CXCL12 is pathological and may play a role in the ongoing capture of CXCR4-expressing leukocytes at the BBB, leading to the development of active MS lesions and associated demyelination.

Discussion

We examined the expression patterns of CXCL12 and its receptor CXCR4 within CNS tissues derived from non-MS patients and from those with various forms of MS. Results of this study indicate that CXCL12 is normally sequestered within the perivascular spaces of the human CNS microvasculature, suggesting that this chemokine acts to localize infiltrating, CXCR4-expressing leukocytes to this site. In both normal CNS tissues and in nonlesioned areas of CNS tissues derived from MS patients, CXCL12 was detected along the basolateral surfaces of endothelial cells and along GFAP-expressing, astrocyte end-feet, providing an adherent barrier to leukocyte entry whereas the redistribution of CXCL12 from the parenchymal to the luminal side of venules was observed only in tissues with active MS lesions. Analysis of CXCR4 expression revealed that luminal mononuclear cells exhibiting CXCR4 activation were also only detected within active MS lesions, suggesting that redistribution of CXCL12 may lead to inappropriate, CXCR4-mediated trafficking of immune cells into the CNS during MS. Given that perivascular infiltrates develop during the evolution of all neuroinflammatory diseases, regardless of etiology,²⁹ the polarized expression of CXCL12 at the BBB is likely to be of fundamental significance in the formation of these infiltrates during a wide variety of autoimmune and infectious diseases of the CNS. Consistent with this, the redistribution of CXCL12 in MS tissues was significantly correlated with extensive macrophage infiltration and demyelination, suggesting that dysregulation of CXCL12 expression disrupts its normal localizing, homeostatic function and contributes to the neuropathogenesis of MS.

The altered pattern of CXCL12 expression observed in MS lesions is consistent with studies evaluating CXCL12 expression at the BBB in mice with EAE, a mouse model for MS.⁷ CXCL12, which was detected along the basolateral surfaces of endothelial cells within the non-inflamed murine CNS microvasculature, exhibited redistribution during the peak of EAE when there is extensive leukocyte trafficking. Furthermore, CXCR4 antagonism with a small molecule inhibitor led to loss of perivascular cuffs, widening of infiltrates, and worsened disease. Although the murine EAE model shares many characteristics with MS, it is not MS. Thus, the observed alteration in

Figure 6. CXCR4 activation occurs within infiltrating leukocytes in active MS lesions. Cellular localization with panCXCR4 (Alexa-488, green) (**a**) and pS³³⁹-CXCR4 (Alexa-555, red) (**b**) antibodies in a section of the medulla with an active MS lesion. **c**: Note a subset of perivascular CXCR4-expressing cells (**arrowheads**) and endothelial cells (**white arrow**) contain activated CXCR4. Analyses of an inflamed CD31⁺ (Alexa-488, green) venule within sister-sections derived from an active MS lesion in the midbrain reveals redistribution of CXCL12 (Alexa-555, red) with decreased perivascular CXCL12 staining (**d**) is associated with activation of CXCR4 (Alexa-555, red) (**f**, **arrowheads**) within CD45⁺ leukocytes (Alexa-488, green) (**e**). The white line delineates the CD31⁺ venule perimeter (**d**, **arrow**) detected in the merged image (**g**). Analysis of CXCR4 activation in control CNS specimens reveals CD45⁺ (Alexa-488, green), pS³³⁹-CXCR4⁺ (Alexa-555, red) cells within the perivascular space in a thoracic cord section from a patient with CNS lymphoma (**h**) but no CXCR4 activation in CD45⁺ cells within the lumen of a vessel in the cerebrum of an amyotrophic lateral sclerosis patient (**i**). Scale bar = 20 μ m.

perivascular CXCL12 expression in our MS specimens demonstrates a role for redistributed CXCL12 in leukocyte exit from this site in humans. Almost all venules with perivascular infiltrates exhibited redistribution of CXCL12. In addition, we were unable to detect perivascular CXCL12 in venules with the highest numbers of endothelium-associated cells, suggesting that redistributed CXCL12 leads to extensive parenchymal infiltration. However, the majority of redistributed venules did not exhibit perivascular infiltrates, and the correlation between histopathological inflammation scores and CXCL12 redistribution was the weakest. These apparent discrepancies may result from a combination of acute and chronic activity within our tissue specimens. Thus, CXCL12 redistribution might be an early phenomenon associated with the initial trafficking of autoreactive

mononuclear cells that persists in chronic active lesions, in which perivascular infiltration is variable,² and in chronic silent lesions, which do not exhibit inflammation. The highly significant correlations between macrophage infiltration and demyelination strongly suggest, however, that altered CXCL12 expression contributes to ongoing disease, worsening MS severity.

The detection of activated CXCR4 on CD45-expressing luminal leukocytes within our MS specimens is also consistent with a possible role for CXCL12 redistribution in promoting CNS leukocyte entry. Control specimens obtained from patients with various neurological diseases, including two neuroinflammatory conditions, CNS lymphoma and viral encephalitis, exhibited neither loss of CXCL12 polarity nor activation of CXCR4 within luminal

Figure 7. Redistribution of CXCL12 significantly correlates with histological markers of MS disease severity. Extents of inflammation (a), demyelination (b), and presence of macrophages (c) within CNS sections derived from MS patients were determined as described in the Materials and Methods, and severity scores versus percentage of CXCL12 redistribution for each block analyzed in the MS patient group are depicted. Correlation coefficient of best-fit line (r^2) and P values are shown for each graph.

leukocytes. Thus the increased activation of CXCR4 on leukocytes within active MS lesions may indicate that the shift in CXCL12 expression during autoimmunity is part of a pathological process that leads to leukocyte capture on the luminal surface of the microvasculature.

Previous studies have identified secondary lymphoid chemokines, including CXCL12, and CXCR4-expressing leukocytes within the CSF of patients with neuroinflammatory diseases, including MS.^{19,27,30} In these studies, levels of CSF CXCL12 were positively correlated with evidence of BBB disruption, suggesting that CXCL12 plays a role in the permeability of the CNS microvasculature. Analyses of CNS tissues similarly detected CXCL12 within endothelial cells and astrocytes in both active and inactive lesions.²⁷ These studies, however, did not detect alterations in the pattern of CXCL12 expression at the BBB and did not evaluate the role of CXCR4 on infiltrating leukocytes. Based on the endothelial cell expression of CXCL12, most authors have suggested that CXCL12 acts to promote extravasation of leukocytes into the CNS. Given the ubiquitous presence of this molecule at a site that restricts leukocyte entry, we would argue that the normal basolateral expression of CXCL12 instead prevents leukocyte trafficking out of the perivascular space and into the CNS parenchyma. During autoimmune disease, interactions between immune, endothelial, and/or glial cells may alter CXCL12 expression patterns and contribute to the release of leukocytes from their perivascular localization and promote leukocyte entry into the CNS proper. The appearance of CXCL12 in the CSF may therefore be a marker of leukocyte extravasation.

We observed CXCL12 expression within microvasculature-associated astrocytes in both non-MS and MS patients. However, CXCL12 immunoreactivity was considerably increased in activated astrocytes with elevated GFAP expression in specimens derived from MS patients. Increased expression of CXCL12 could contribute to its redistribution across venules via enhanced binding

to endothelial cell CXCR4, which was also detected in this study. This is consistent with recent data describing endothelial cell-mediated translocation of CXCL12 via trafficking of CXCR4 within bone marrow cells.³¹ Alternatively, alterations in the display of CXCL12 during MS may occur through changes in its binding to cell surface heparan sulfate proteoglycans on endothelial cells.³² In recent studies using endothelial cells from rheumatoid synovial tissues, CXCL12 attachment to heparan sulfate proteoglycans was enhanced by inflammatory cytokines such as tumor necrosis factor- α .³³ In addition, prior studies have observed that microvasculature-associated astrocytes express CXCL12 within active MS lesions^{20,27} and that astrocyte CXCL12 expression may be up-regulated by treatment with interleukin-1 β *in vitro*.^{20,34} Thus, alterations in the pattern of CXCL12 expression might be a general mechanism to alter leukocyte migration during inflammation.

The specificity of CXCL12 redistribution and increase in CXCR4 activation in MS tissues suggest that CXCR4 activation within CNS leukocytes may, in fact, be a marker for CXCL12 redistribution. If so, there exists a potential therapeutic role for CXCR4 blockade to prevent MS exacerbations. Potential CXCR4 blockers would have to prevent interactions between CXCR4-expressing leukocytes and CXCL12-expressing endothelium without entering the CNS, because CXCR4 inactivation within the CNS has been observed to promote leukocyte exit from the perivascular space. Various studies have attempted to link chemokine receptor expression and MS through analysis of peripheral blood mononuclear cells derived from MS patients versus those without the disease. Increased surface expression of CXCR3 has been observed on T cells, CCR5 on both T and B cells, and CXCR3 and CXCR4 on macrophages/microglia.^{29,35,36} Current studies are underway to evaluate CXCR4 activation via phospho-specific CXCR4 antibody staining in CSF mononuclear cells from patients with MS and other neurological diseases. In conclusion, loss of CXCL12 polarity at the BBB was specifically associated with MS in the present study, suggesting that it may provide a novel diagnostic and a therapeutic target to prevent leukocyte entry and decrease inflammation in MS.

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