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INTEGRIN/CHEMOKINE RECEPTOR INTERACTIONS IN THE PATHOGENESIS OF EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

Ghazal Banisadr^{1,*}, Samantha R Schwartz¹, Joseph R Podjol², Linda A Piccinini³, Stefan Lanker³, Stephen D Miller², and Richard J Miller¹

¹Dept of Molecular Pharmacology and Biological Chemistry, Northwestern University Medical School, 303 E Superior Ave, Chicago IL 60611

²Dept of Microbiology-Immunology, Northwestern University Medical School, 303 E Chicago Ave, Chicago IL 60611

³Biogen-Idec, Cambridge, MA 02142

Abstract

Excessive infiltration of leukocytes and the elaboration of inflammatory cytokines are believed to be responsible for the observed damage to neurons and oligodendrocytes during multiple sclerosis (MS). Blocking adhesion molecules or preventing the effects of chemotactic mediators such as chemokines can be exploited to prevent immune cell recruitment to inflamed tissues. An anti- α 4 integrin antibody (anti-VLA-4mAb/natalizumab (Tysabri®)) has been used as a treatment for MS and reduces leukocyte influx into the brain. In patients, anti-VLA-4 reduces relapses and disability progression. However, its mechanism of action in the brain is not completely understood. The anti-VLA-4mAb was demonstrated to mobilize hematopoietic progenitor cells. Interestingly, the chemokine SDF-1/CXCL12 and its receptor CXCR4 are also key factors regulating the migration of hematopoietic stem cells. Moreover, studies have revealed a crosstalk between SDF-1/CXCR4 and VLA-4 signaling in regulating cell migration. In this study, we address the effects of anti-VLA-4 on chemokine signaling in the brain during MS. We assessed the ability of anti-VLA-4 to regulate Experimental Autoimmune Encephalomyelitis (EAE) and chemokine/receptor signaling. Preclinical administration of anti-VLA-4 delayed clinical signs of EAE. We found that anti-VLA-4 treatment reduced chemokine expression. In order to further explore the interaction of anti-VLA-4 with chemokine/receptor signaling we used dual color transgenic mice. After EAE induction, the expression of both SDF-1/CXCL12 and CXCR4 receptor was upregulated, treatment with anti-VLA-4 inhibited this effect. The effects of anti-VLA-4 on chemokine signaling in the CNS may be of importance when considering its mechanism of action and understanding the pathogenesis of EAE.

*Corresponding Author: Ghazal Banisadr, Dept of Molecular Pharmacology and Biological Chemistry, Northwestern University Feinberg School of Medicine, 303 E Chicago Ave, Chicago, IL 60611, USA, Phone: (312) 503-3222, Fax: (312) 503-3202, g-banisadr@northwestern.edu.

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Keywords

Chemokine; chemokine receptor; anti-VLA-4 antibody; Experimental Autoimmune Encephalomyelitis

INTRODUCTION

Multiple sclerosis (MS) is a common neurological disorder associated with the demyelination of axons in the central nervous system (Bitsch et al. 2000; Frohman et al. 2006; Trapp et al. 1998). Although the ultimate cause of MS is unclear, it is thought to be an autoimmune disorder and is associated with neuroinflammation and the influx of populations of leukocytes into the brain. Excessive infiltration of leukocytes is a hallmark of MS and is thought to be responsible for the observed damage to neurons and oligodendrocytes (Kennedy and Karpus 1999).

Several approaches have been exploited to prevent leukocyte recruitment to inflamed tissues such as blocking adhesion molecules (selectins, integrins) e.g. an anti- $\alpha 4$ integrin antibody (anti-VLA-4 Ab or natalizumab (Tysabri®)) has been used as a treatment for MS and reduces leukocyte migration into the brain. In patients with MS, anti-VLA-4 has been shown to reduce the relapse rate and progression of disability.

It has been recently shown that the anti-VLA-4 mAb natalizumab mobilizes hematopoietic progenitor cells in humans (Zohren et al. 2008). Interestingly, the chemokine Stromal cell-Derived Factor-1 (SDF-1)/CXCL12 and its receptor CXC chemokine receptor 4 (CXCR4) have been demonstrated to be principal factors regulating the migration and mobilization of hematopoietic stem cells (Nagasawa et al. 1996). Furthermore, recent studies have revealed “crosstalk” between the CXCR4/SDF-1 chemokine system and VLA-4 in regulating neutrophil retention in the bone marrow (Petty et al. 2009; Ngo et al. 2008). Indeed, synergistic mobilization of these stem cells by anti-VLA-4 and the CXCR4 antagonist, AMD3100, has been described. Although interactions between VLA-4 and CXCR4 signaling have now been described (Bonig et al. 2009; Petty et al. 2009), the molecular mechanisms underlying these interactions are not known and the implications of this interaction for effects in MS are unclear.

In addition to disrupting leukocyte trafficking to inflamed brain tissue, promoting remyelination has also been suggested as a therapeutic strategy in MS. Two different approaches have been advanced: either by blocking inhibitory signals that prevent resident oligodendrocytes progenitor cells (OPs) from maturing into myelin-producing oligodendrocytes, or by inducing the migration of endogenous or transplanted OPs. We have recently shown that chemokines, and SDF-1 in particular, are upregulated in the brain during neuroinflammation and are key factors in regulating the migration of OPs to demyelinated regions in the brains of mice with EAE (Banisadr et al. 2011) where OPs can potentially induce remyelination.

In the present series of experiments we wished to address the question of how anti-VLA-4 may affect chemokine signaling in the brain. Because VLA-4 and chemokine receptor

signaling has been shown to cooperate in regulating the mobilization of hematopoietic stem cells and neutrophil retention in the bone marrow (Bonig et al. 2009; Petty et al. 2009), it is possible that interference with VLA-4 signaling might also impact chemokine mediated processes within the brain. Chemokine expression in the brain is upregulated by several means one of which is probably the effect of inflammatory cytokines produced by infiltrating leukocytes (Glabinski et al. 1997). Hence, we hypothesized that inhibition of inflammatory cell influx into the brain during EAE may also inhibit upregulated chemokine expression in this situation. In addition, since neural progenitor cells have been shown to express both VLA-4 and chemokine receptors (e.g. CXCR4) (Pluchino et al. 2005), interactions between integrin cell adhesion molecules and chemokine signaling pathways might be expected to regulate neural progenitor function, as in the case of hematopoietic stem cells. The overall consequences of blocking VLA-4 in the brain of MS patients remain to be fully elucidated. In the present series of experiments we have demonstrated that blocking VLA-4 does inhibit the expression of SDF-1/CXCL12 in the brain, an effect that may have multiple consequences.

Materials and methods

Animals

SJL mice were used for EAE induction. SDF-1-RFP/CXCR4-EGFP transgenic mice were also used in this study. SDF-1-RFP/CXCR4-EGFP bi-transgenic mice were made in our laboratory by generating SDF-1-RFP mice and crossing them with CXCR4-EGFP mice (Bhattacharyya et al. 2008). All of the procedures performed on animals within this study were conducted in accordance with the guidelines of Northwestern University Animal Care and Use Committee.

Induction and clinical evaluation of peptide-induced EAE

Six to seven week old female SJL mice were immunized s.c. with 100 μ l of an emulsion containing 200 μ g of Mycobacterium tuberculosis H37Ra (Difco) and 50 μ g proteolipid protein (residues 139-151, HSLGKWLGHDPKF; PLP₁₃₉₋₁₅₁) distributed over three spots on the flank (Fuller et al. 2004). Clinical signs of EAE appeared typically after 10-14 days. Naïve mice were used as controls for EAE.

Clinical evaluation

The animals were observed daily and clinical severity assessed in a blinded fashion on a 0-5 scale as follows: 0, asymptomatic; 1, loss of tail tonicity; 2, atonic tail and hind leg weakness; 3, hind limb paralysis; 4, hind limb paralysis and forelimb weakness; 5, moribund (Fuller et al. 2004). A relapse was defined as a sustained increase (more than 2 days) in at least one full grade in clinical score after the animal had improved previously at least a full clinical score and had stabilized for at least 2 days. The data are plotted as the mean clinical score for all animals in a particular treatment group.

Tissue preparation

Mice were treated every other day with 125 μ g control rat IgG or anti-VLA4 Ab administered intraperitoneally in a total volume of 100 μ l. At day 14 and day 26 after

priming (3 and 9 treatments respectively; for mice treated preclinically) and day 20 and 33 after priming (3 and 9 treatments respectively; for mice treated at peak of acute disease), 4 mice per time point (per experiment) were anesthetized and perfused transcardially with cold PBS, followed by a freshly prepared solution of 4% paraformaldehyde (PFA) in PBS, pH 7.4. The brains were rapidly removed and post-fixed overnight in 4% PFA at 4°C. Forty-micrometer thick coronal sections were cut with a vibratome (Leica VT 1000S; Leica Biosystems, Nussloch, Germany) and collected in cold PBS. Sections were then processed for histology or immunohistochemistry.

Histology and immunohistochemistry

In order to assess the expression of chemokines and their receptors in EAE mice, sections of SDF-1-RFP/CXCR4-EGFP mice (SJL background) induced with EAE as described above, and treated with anti-VLA-4mAb (rat anti-mouse VLA-4, kindly provided by Biogen Idec, Cambridge, MA, USA) were analyzed by a confocal microscope and compared to control EAE mice injected with purified rat IgG control antibody (Millipore, Billerica, MA, USA).

In order to characterize the cell types expressing chemokines and their receptors, sections were processed for immunohistochemistry. Immunohistochemistry was performed on free-floating sections using the following primary antibodies: anti-GFAP (1:2000, Sigma, St. Louis, MO, USA) to characterize astrocytes, anti-IBA1 (1:1000, Wako, Richmond, VA, USA) to label microglia, Olig1 (1:500, Millipore, Billerica, MA, USA) oligodendrocyte lineage marker and CD68 to detect macrophages (1:300, Millipore, Billerica, MA, USA). The appropriate isotype-specific secondary antibodies consisted of AlexaFluor 633 or 647-conjugated preparations (1:300; Molecular Probes, Grand Island, NY, USA).

Sections were incubated in PBS/4% goat serum/0.1% triton for 90 minutes. They were then incubated with primary antibodies diluted in PBS/2% normal serum/0.1% triton overnight at 4°C. The sections were then washed with PBS and incubated with secondary antibodies (Molecular Probes, Grand Island, NY, USA) for 1 hour. Sections were washed with PBS, mounted on slides, and analyzed by confocal microscopy. Image acquisition software (Fluoview) was used. All histology studies presented in this paper are shown after 3 treatments of anti-VLA4.

Protein extraction

Mice were perfused intracardially with PBS, forebrain, cerebellum and spinal cords were obtained from animals treated preclinically and at the peak of the disease. Samples were homogenized in ice-cold lysis buffer (Cell Lysis Buffer, Cell Signaling Technology, Beverly, MA) containing protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN). Tissue lysates were sonicated and centrifuged for 10 minutes at 14,000g, 4°C, and supernatants were collected and stored at -80°C until used. Protein concentration was determined using a BCA Protein Assay (Pierce Biotechnology, Rockford, IL) with bovine serum albumin as standard.

Enzyme-Linked Immunosorbent Assays (ELISA) for SDF-1/CXCL12

Quantitative determination of SDF-1/CXCL12 was performed using Quantikine mouse SDF-1/CXCL12 ELISA kit (R&D systems, Minneapolis, MN, USA), according to manufacturer's protocol. Briefly, all reagents, standard dilutions, and samples were prepared as directed in the product insert. 50 μ L of Assay Diluent was added to each well. 50 μ L of standard, control, or sample was then added, and incubated at room temperature for 2 hours on a horizontal orbital microplate shaker followed by 4 washes. 100 μ L of Conjugate was added to each well and incubated at room temperature for 2 hours on the shaker. After washing, 200 μ L of Substrate Solution followed by 50 μ L of Stop Solution were added. Optical densities were read at 450nm (correction wavelength set at 540nm) by using an automated plate reader, and chemokine levels were calculated by interpolation from the standard curve. Values were corrected for protein concentration.

Statistical analysis

Data were reported as mean $\pm$ standard error of the mean and statistically evaluated for difference by Student t test using SigmaStat 3.1 software. A *P* value less than 0.05 was considered significant.

RESULTS

Preclinical administration of anti-VLA-4mAb delays the signs of EAE

Treatment with anti-VLA-4mAb initiated 7 days after priming effectively delayed the clinical signs of EAE. As shown in figure 1A, by day 11 after priming, mice treated with the control antibody exhibited clear clinical signs of EAE ($n=12$, mean clinical score of 1.2) whereas none of the anti-VLA-4-treated mice displayed any signs of the disease ($n=12$). Control mice were at the peak of the disease at day 14 post-priming with a mean clinical score of 4.1.

By day 18 after priming mice treated with the anti-VLA-4mAb started to exhibit clinical signs of EAE (mean clinical score 1.1) and the peak of the disease was observed at day 24 with a mean clinical score of 4 (Fig 1A, Table 1). Interestingly, mice in the anti-VLA-4 mAb-treated group clearly entered remission faster, displaying a shorter acute phase stage.

Treatment with anti-VLA-4 mAb at the peak of the acute phase does not interfere with clinical signs of EAE

Anti-VLA-4 mAb was also administrated at the peak of the acute phase of the disease and the subsequent effects on EAE were monitored. It is important to note that animals in the control antibody-treated group had similar mean day of onset of EAE (11) and severity (1.3) compared with the anti-VLA-4-treated group prior to the start of the treatment (Fig. 1B, Table 1). In both groups, the mean day of the peak of the disease was 14.1 ($n=12$ per group) with a mean clinical score of 4.1. Both groups followed a similar pattern of disease severity.

Effect of anti-VLA-4 mAb treatment on SDF-1/CXCR4 expression in EAE mice

In order to examine the expression of SDF-1/CXCL12 and CXCR4 after anti-VLA-4 mAb treatment in EAE, SDF-1-RFP/CXCR4-EGFP dual transgenic mice were used. Brain and

spinal cord tissue sections from representative animals of the different treatment groups described in figure 1 were examined using confocal microscopy. Anti-VLA-4 treatment did not affect the chemokine/receptor expression in naïve mice. As we have previously reported, both SDF-1/CXCL12 and CXCR4 were upregulated in EAE mice compared to naïve animals. Upregulation was mainly observed in the corpus callosum (cc), subventricular zone (SVZ), cortical area, cerebellum and blood vessels. As shown in figure 2, preclinical treatment with anti-VLA-4 mAb reduced the expression of CXCR4 in the posterior part of the SVZ, cc and cerebellum. SDF-1 expression was also downregulated in the SVZ and cc (Figs. 2B and 2D respectively). On the other hand expression patterns of SDF-1/CXCL12 and CXCR4 appeared similar in control and anti-VLA-4 treated mice in the spinal cord (not shown).

Similarly, reduced expression in the SVZ, cc and cerebellum was observed when anti-VLA-4 was administrated at the peak of the acute phase (day 14 after EAE induction, not shown).

Characterization of SDF-1/CXCL12 and CXCR4 expressing cells in SDF-1RFP/CXCR4-EGFP EAE mouse brain

We performed immunohistochemistry on SDF-1-RFP/CXCR4-EGFP transgenic mice to characterize the cells expressing SDF-1/CXCL12 and CXCR4 in EAE mice prior to or after treatment with anti-VLA-4 mAb. We noted that CXCR4 was expressed by some cells exhibiting the morphology of migrating OPs in the posterior part of the SVZ (Fig. 3). Immunostaining using an anti-Olig1 antibody demonstrated that these CXCR4-expressing cells also expressed Olig1 a transcription factor expressed by OPs (Fig. 3). CXCR4 was also upregulated in CD68 expressing macrophages in EAE mouse brain. SDF-1/CXCL12 expression was mainly confined to GFAP positive astrocytes and IBA1 positive microglia (Fig. 3). Similar results in the pattern of OPs were observed when anti-VLA-4 was injected preclinically or at the peak of the acute phase.

Treatment of anti-VLA-4 Ab reduces the expression of SDF-1/CXCL12 in EAE brain

In order to quantitate the expression of SDF-1/CXCL12 after treatment with anti-VLA-4 mAb, ELISA was performed. We found that SDF-1/CXCL12 expression was lower in EAE brains treated with anti-VLA-4 preclinically (Fig. 4A) or at the peak of the acute phase (Fig. 4B).

DISCUSSION

In these experiments we observed that anti-VLA-4 mAb reduced the expression of the chemokine SDF-1/CXCL12 as well as its receptor CXCR4 in the brain during EAE suggesting that anti-VLA-4 treatment can affect chemokine signaling in the brain associated with this syndrome. Previous reports in the literature have indicated that chemokine receptors are expressed by populations of circulating immune cells and also by neural stem cells including OPs within the brain. In addition to regulating the movement of leukocytes, chemokines also control the migration of neural stem cells both during development and in the context of brain disease, where they help to target the migration of neural stem cells to

damaged areas of the brain so that they can participate in repair related functions. Hence, interference with chemokine signaling in the brain may have important consequences for both the influx of leukocytes (McCandless et al. 2008) and for the migration of OPs during EAE (Banisadr et al. 2011).

In addition to disruption of leukocyte trafficking to inflamed brain tissue, promoting remyelination by inducing the migration of endogenous or transplanted OPs, or by inducing maturation of pre-existing OPs at the site of lesions, has also been suggested as a therapeutic strategy in MS. We have recently shown that the expression of chemokines including SDF-1/CXCL12 is upregulated in the brain during neuroinflammation and these are key factors in regulating the migration of OPs to demyelinated regions in EAE brain (Banisadr et al. 2011) where OPs can potentially induce remyelination. Because VLA-4 and chemokine receptor signaling have been shown to cooperate in regulating the mobilization of hematopoietic stem cells and neutrophil retention in the bone marrow (Bonig et al. 2009; Petty et al. 2009), it is possible that interference with VLA-4 signaling may also impact chemokine mediated processes within the brain. This is particularly so because, in addition to immune cells, neural progenitor cells have been shown to strongly express both VLA-4 and chemokine receptors (e.g. CXCR4) (Pluchino et al. 2005; Tran et al 2007). Hence, interactions between these signaling pathways may be expected in the regulation of neural progenitor function, as reported for hematopoietic stem cells. Indeed, Pluchino et al reported that treatment of OPs *in vitro* with anti-VLA-4 reduced their recruitment to the brains of EAE mice following their intravenous injection. Moreover, we have now demonstrated that the expression of CXCR4 in OPs is reduced by treatment with anti-VLA-4 which would also be expected to produce the same effect.

Generally speaking OPs in the adult brain are found distributed in the parenchyma. However, migration of additional OPs from the SVZ has been reported to occur under some circumstances including in the context of syndromes such as EAE (Menn et al. 2006; Nait-Oumesmar et al. 2007). Since anti-VLA-4 has been shown to decrease the severity of EAE, we may hypothesize that during MS the potential effects of the antibody on OP migration is not as important as its overall inhibitory effect on the influx of leukocytes. While it is possible that the potential inhibitory effects of anti-VLA-4 on OP migration may have some detrimental effects on the course of the disease in EAE, it is assumed that local OPs are the primary mediators of remyelination in MS. Thus, a potential reduction in migration of OPs from the SVZ to sites of demyelination might be negligible compared to the beneficial effect of preventing inflammatory leucocyte migration across the blood brain barrier (BBB) into the brain parenchyma.

Interestingly we also observed that anti-VLA-4 treatment reduced the expression of SDF-1/CXCL12 in EAE mice. In EAE the expression of chemokines such as SDF-1/CXCL12 and MCP-1/CCL2 is greatly upregulated in astrocytes and microglia. It is possible that much of this expression is the result of the influence of inflammatory cytokines secreted by an initial wave of infiltrating leukocytes. Upregulated chemokine expression may then encourage further leukocyte influx. Hence, if the initial migration of these cells is inhibited, the subsequent upregulation of chemokine signaling by astrocytes and microglia might also be modified. It is therefore apparent that the beneficial effects of anti-VLA-4 treatment may be

exerted both by a direct influence on leukocyte influx and a subsequent reduction in chemokine expression. In our experiments SDF-1/CXCL12 expression was reduced when anti-VLA-4 treatment was received preclinically or at the peak of the acute phase of EAE. In consequence, decreased leukocyte migration and disease amelioration are anticipated. However, our results show that preclinical treatment has beneficial effects where as a disease progression similar to control mice was observed when anti-VLA-4 was administrated at the peak of the disease. These findings indicate that once the BBB is sufficiently compromised, the migration of activated cells into the central nervous system (CNS) might be independent of anti-VLA-4/ vascular cell adhesion molecule-1 (VCAM-1) interactions. Furthermore, it may also indicate that early effects of leukocyte influx and chemokine production produce negative effects that are difficult to reverse even if integrin and chemokine signaling are subsequently inhibited.

Because chemokine receptors are known to be expressed by both populations of leukocytes and by neural stem cells it is clear that a complete understanding of the mechanism of action of therapeutic agents such as anti-VLA-4 that affect chemokine signaling may be of importance when considering its mechanism of action in the CNS.

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Figure 1.

A: Preclinical administration of anti-VLA-4 Ab effectively delays the clinical signs of EAE. By day 11 after priming, mice treated with the control antibody showed clinical signs of EAE (n=12, mean clinical score of 1.2) whereas none of the anti-VLA-4-treated mice were showing any signs of the disease (n=12). B: Treatment with anti-VLA-4 Ab at the peak of acute phase does not interfere with clinical signs of EAE. (Arrows show the beginning of the treatment). Both groups followed a similar pattern of disease severity. Arrows show the start of the treatment.

Figure 2.

Effect of anti-VLA-4 Ab treatment on SDF-1/CXCR4 expression in EAE mice. In order to examine the expression of SDF-1/CXCL12 and CXCR4 after anti-VLA-4 Ab treatment in EAE, SDF-1-RFP/CXCR4-EGFP dual transgenic mice were used. Preclinical treatment with anti-VLA-4 Ab reduces the expression of CXCR4 (green) in the posterior part of the subventricular zone (SVZ) (B), corpus callosum (cc) (D) and cerebellum (F). SDF-1/CXCL12 (red) is downregulated in the SVZ and cc (B and F respectively). Panels A, C, and E show the expression of CXCR4 and SDF-1/CXCL12 in EAE mice injected with the control antibody.

Figure 3.

Characterization of SDF-1/CXCL12 and CXCR4 expressing cells in SDF-1RFP/CXCR4-EGFP EAE mouse brain. CXCR4 is expressed by cells exhibiting the morphology of migrating progenitors in the posterior part of the SVZ (A). Immunostaining using an anti-Olig1 antibody shows that these CXCR4-expressing cells (panel A, green) colocalize with Olig1 (panel B, red). Arrowheads in panel C show the colocalization of CXCR4 and Olig1. CXCR4 is also upregulated in CD68 expressing macrophages in EAE mouse brain (BV: blood vessel). Arrowheads show the colocalization of CXCR4 and CD68 expression (D). SDF-1/CXCL12 expression is mainly confined to GFAP positive astrocytes (arrowheads in E) and IBA1 positive microglia (arrowheads in F).

Figure 4.

Treatment of anti-VLA-4 Ab reduces the expression of SDF-1/CXCL12 in EAE brain. In order to quantify the expression of SDF-1/CXCL12 after treatment with anti-VLA-4 Ab, ELISA was performed. SDF-1/CXCL12 expression is lower in EAE brains treated with anti-VLA-4 preclinically (A) or at the peak of the acute phase (B).

Table 1

Effect of anti- VLA-4 antibody on relapsing-remitting EAE when administered during the preclinical and peak acute phase of disease.

Disease stage at treatment initiation	Number of mice	Mean day of onset	Mean score at onset	Mean day of peak	Mean score at peak
Preclinical					
Control	12	11	1.2	14	4.1
Anti- VLA-4 Antibody	12	18	1.1	24	4
Acute phase					
Control	12	11	1.3	14.1	4.1
Anti- VLA-4 Antibody	12	11	1.3	14.1	4.1

* $p < 0.05$