Key Metalloproteinases Are Expressed by Specific Cell Types in Experimental Autoimmune Encephalomyelitis

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Key Metalloproteinases Are Expressed by Specific Cell Types in Experimental Autoimmune Encephalomyelitis

Henrik Toft-Hansen, Robert K. Nuttall, Dylan R. Edwards, and Trevor Owens

Metalloproteinases (MPs) include matrix metalloproteinases (MMPs) and metalloproteinase-disintegrins (ADAMs). Their physiological inhibitors are tissue inhibitor of metalloproteinases (TIMPs). MPs are thought to be mediators of cellular infiltration in the pathogenesis of multiple sclerosis and its animal model, experimental autoimmune encephalomyelitis (EAE). We used real-time RT-PCR to profile the expression of all 22 known mouse MMPs, seven ADAMs, and all four known TIMPs in spinal cord from SJL/J mice and mice with adoptively transferred myelin basic protein (MBP)-specific EAE. A significant and >3-fold alteration in expression was observed for MMP-8, MMP-10, MMP-12, ADAM-12, and TIMP-1, which were up-regulated, and for MMP-15, which was down-regulated. Expression levels correlated with disease course, with all but ADAM-12 returning toward control levels in remission. To examine potential cellular sources of these strongly affected proteins in the inflamed CNS, we isolated macrophages, granulocytes, microglia, and T cells by cell sorting from the CNS of mice with EAE and analyzed their expression by real-time RT-PCR. This identified macrophages as a major source of MMP-12 and TIMP-1. Granulocytes were a major source of MMP-8. ADAM-12 was expressed primarily by T cells. Cellular localization of MMP-10, TIMP-1, and ADAM-12 in perivascular infiltrates was confirmed by immunostaining or in situ hybridization. Microglia from control mice expressed strong signal for MMP-15. Strikingly, the expression of MMP-15 by microglia was significantly down-regulated in EAE, which was confirmed by immunostaining. Our study identifies the cellular sources of key MPs in CNS inflammation. The Journal of Immunology, 2004, 173: 5209–5218.

L eukocyte infiltration of the CNS is a hallmark of multiple sclerosis (MS) and experimental autoimmune encephalomyelitis (EAE), a widely used animal model for MS. To infiltrate the CNS parenchyma, leukocytes must migrate across the blood-brain barrier (BBB), which, under normal circumstances, restricts the entrance of cells and large molecules into the CNS. Disruption of the integrity of the BBB accompanies the extensive cellular infiltration and pathology seen in MS and EAE and is assumed to be a prerequisite for disease.

The functional BBB includes endothelial cells joined by tight junctions and a basement membrane made up of extracellular matrix (ECM) proteins, such as laminin, collagen, and fibronectin. Leukocyte extravasation to CNS parenchyma is facilitated by proteolysis of ECM proteins conducted by a family of Zn²⁺- and Ca²⁺-dependent proteinases called matrix metalloproteinases (MMPs; also designated matrixins). There are at least 25 MMPs (22 identified in mice), which, among them, can degrade virtually all components of the ECM (1). The MMPs have therefore attracted attention as potential critical mediators of diseases involving inflammation of the CNS, including MS (2, 3), as well as in other diseases where degradation of ECM is part of the pathogenesis, such as in cancer metastasis and rheumatoid arthritis (4). Besides their role in ECM proteolysis, MMPs can also act as signaling molecules and regulators of cell fate by shedding or cleaving adhesion molecules, growth factors, chemokines, and cytokines (5, 6).

Metalloproteinase-disintegrins (ADAMs; also called adamalysins) are another family of metalloproteinases, which potentially can act as proteases, signaling molecules, and regulators of cell fate. They comprise >30 members with diverse actions (7–9). There are indications that ADAMs are involved in regulating inflammatory processes, as exemplified by ADAM-17, which can cleave membrane-bound TNF-α, leading to shedding of the more proinflammatory soluble TNF-α (10); hence, the synonym for ADAM-17 is TNF-α-converting enzyme (TACE).

Tissue inhibitors of metalloproteinases (TIMPs) are physiological inhibitors of metalloproteinases. There are four known TIMPs, and they inhibit metalloproteinases by binding to the catalytic site in a 1:1 stoichiometry. No comprehensive study of the binding of all four TIMPs to all MMPs and ADAMs has been undertaken, but some generalizations can be made. TIMPs generally inhibit all MMPs to various degrees, with the exception that TIMP-1 is unable to inhibit MMP-14, -15, -16, and -24 (11). These are four of the six membrane-bound MMPs, the remaining two being MMP-17 and MMP-25. The ADAMs show a more restricted pattern of inhibition by TIMPs, with only a few ADAMs being inhibited by TIMPs (12).

Administration of synthetic broad-spectrum MP inhibitors alleviated symptoms of EAE, presumably by curbing leukocyte infiltration (13–15). However, in addition to their detrimental roles,
MPs might also have beneficial effects in MS. For example, MMPs are suggested to be involved in repair processes such as remyelination, as indicated by the finding that MMP-9 is necessary for outgrowth of oligodendrocyte processes (16). The use of current generation, broad-spectrum MP inhibitors would inhibit such beneficial actions of MPs as well as their detrimental actions. It is likely that inhibition of specific MPs or MP products by specific cell types at specific times in the pathogenesis of MS would be of greater benefit than using broad-spectrum inhibitors indiscriminately. Detailed knowledge of the MP and TIMP expression profiles by the cell types involved in CNS infiltration is required to enable such approaches. Until now, only a limited number MMPs, ADAMs, and TIMPs have been studied in MS or EAE.

In this study we profiled the expression of all 22 known mouse MMPs, all four known TIMPs, as well as seven ADAMs in spinal cord (SC) from unmanipulated control mice and from mice with EAE at peak disease using quantitative real-time RT-PCR. The seven ADAMs chosen all have a putative functional protease domain and are not restricted to expression in the testis, unlike other members of this family (8, 9). We found a significant and >3-fold up-regulation of the expression of MMP-8, MMP-10, MMP-12, ADAM-12, and TIMP-1 and a significant and >3-fold down-regulation of MMP-15. We have identified macrophages as a major source of MMP-12 and TIMP-1, and granulocytes as a major source of MMP-8. None of the cell types we studied by PCR emerged as a single major source of MMP-10, but low level expression by multiple infiltrating cell types was observed by in situ hybridization (ISH). Unmanipulated microglia expressed MMP-15, and this MMP-15 expression by microglia was significantly down-regulated in EAE. ADAM-12 was found to be expressed almost exclusively by T cells.

Materials and Methods

Adoptive transfer EAE
Female SJL/J mice (6–8 wk old) were purchased from The Jackson Laboratory (Bar Harbor, ME). EAE was induced by passive transfer of MBP-reactive T cells. Donor mice were immunized s.c. at the base of the tail with 100 µl of an emulsion containing 400 µg of MBP (Sigma-Aldrich, Oakville, Canada) and 100 µg of Mycobacterium tuberculosis H37 RA (Difco, Detroit, MI) in IFA (Difco) and were boosted in the flanks 7 days later with the same amount. A single-cell suspension was prepared from the draining lymph nodes 14 days after the first immunization by passing lymph nodes through a 70-µm pore size cell strainer (BD Biosciences, Franklin Lakes, NJ), and cells (4 × 10⁶/ml) were cultured in the presence of 50 µg/ml MBP in RPMI 1640 (Invitrogen Life Technologies, Burlington, Canada) supplemented with 10% FCS (Sigma-Aldrich), 50 µM µ-ME (Sigma-Aldrich), 2 mM L-glutamine (Invitrogen Life Technologies), and 100 U/ml penicillin-streptomycin (Invitrogen Life Technologies). After 4 days in culture, cells were collected by centrifugation on Ficoll-Paque (Amersham Biosciences, Baie d’Urfe, Canada), and 4 × 10⁶ lymphoblasts were injected into the tail vein of naive mice. The MBP reactivity of the lymph node cells was measured by [³H]thymidine (ICN Biomedicals, Irvine, CA) incorporation assay. After transfer of MBP-reactive cells, mice were weighed and monitored daily for clinical signs of EAE, which were scored as: 1, flaccid tail; 2, hind limb weakness and poor righting ability; 3, one hind limb paralyzed; 4, both hind limbs paralyzed with or without forelimb paralysis and incontinence; and 5, moribund. All mice with EAE included in this study were killed at peak disease (grade 4). Mice were kept in a specific pathogen-free environment. Animal maintenance and all experimental protocols were performed in accordance with Canadian Council for Animal Care guidelines and approved by McGill University animal care committee.

Isolation of RNA

For SC analysis, mice were anesthetized with a lethal dose of Somnotol (MTC Pharmaceuticals, Cambridge, Canada) and intracardially perfused with ice-cold PBS, followed by removal of the SC. Total RNA was purified using TRIzol RNA isolation reagent (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer’s protocol for whole tissue RNA extraction. For sorted cells, TRIsol was used according to the protocol for RNA extraction for low amounts of RNA.

Table I. Primer and probe sequences for mouse ADAMs

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
<th>Probe</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>ADAM-10</td>
<td>GTGCCGATACGGGCTCTTGCC</td>
<td>CACAGGCTCTTGAGCACATTACATG</td>
<td>ACTATCACCTGCAAGGGCGCTCTCC</td>
<td></td>
</tr>
<tr>
<td>ADAM-12</td>
<td>ATCCAGTCTTCTGCAGCGTCA</td>
<td>GCGATCTCTTGCTGTGTTACATCC</td>
<td>CCATGCAGTGCCACGGC</td>
<td></td>
</tr>
<tr>
<td>ADAM-15</td>
<td>GAGCACTTCCCAACAGCATCTTAGG</td>
<td>GGGAGATCATGGTCCAAACCC</td>
<td>CCATGCCTCTCATGGAATTTG</td>
<td></td>
</tr>
<tr>
<td>ADAM-17</td>
<td>AAGTGGCAAGGCTGGGAATG</td>
<td>CACACGGGCCAGAAAGT</td>
<td>CCTGCCCTCCTGATCCAGGACAAC</td>
<td></td>
</tr>
<tr>
<td>ADAM-19</td>
<td>CGGGCCCACCTCGAA</td>
<td>CGGTTCATCTGCAAGGTT</td>
<td>TGGGCCCTCTGATTTACACTGACAAC</td>
<td></td>
</tr>
<tr>
<td>ADAM-28</td>
<td>TACTGGTGAAGGGCGAATG</td>
<td>TGCCCCACCTTTCCAGGCTT</td>
<td>TCCAGGAACCAAGGTTGCAATACTCATGTTGAC</td>
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</tr>
<tr>
<td>ADAM-33</td>
<td>CAGGCACCTGCAAGATCTACCT</td>
<td>CCTATTGGCAACCCCAAGCTTTA</td>
<td>TGGGAAGTTTGTGGCACTGGC</td>
<td></td>
</tr>
</tbody>
</table>

All probes are in the 5′–3′ orientation. For the probes, a FAM fluorescent reporter is coupled to the 5′ end, and a TAMRA quencher is coupled to the 3′ end.
Cell sorting

Mice were anesthetized with a lethal dose of Somnotol (MTC Pharmaceutical) and were intracardially perfused with 5 ml of ice-cold PBS, followed by 20 ml of 4% PFA (Fisher Scientific, Fairlawn, NJ). Brain and SC were dissected, postperfusion fixed 1 h in 4% PFA, and incubated overnight in PBS with 20% sucrose (EMD Chemicals, Gibbstown, NJ). Tissues were embedded in OCT (EMS, Hatfield, PA) and frozen in a 2-methylbutane (EMD Chemicals) bath immersed in liquid nitrogen. Immunohistochemical staining was performed on 10-μm cryostat sections. Frozen sections were blocked in 10% normal goat serum (Vector Laboratories, Burlingame, CA) in PBS for 1 h at room temperature, followed by incubation with primary rabbit Abs: MMP-15 (Chemicon International, Temecula, CA) and ADAM-12 (Chemicon International) overnight at 4°C, then with biotinylated goat anti-rabbit Ig (Vector Laboratories) for 1 h at room temperature. Sections were treated with 3% H₂O₂ to quench endogenous peroxidase activity, then were incubated with an avidin-HRP complex (Vector Laboratories) following the manufacturer’s instructions. HRP activity was detected by the use of 3,3′-diaminobenzidine (Lab Vision, Fremont, CA) as chromagen according to the manufacturer’s instructions. Control sections were incubated with equal concentration of rabbit Ig (DAKO, Mississauga, Canada) purified from serum of immununized rabbits. For H&E staining, hybridized in 50% (v/v) formamide, 4× SSC, 10% (w/v) dextran sulfate, 1× Denhardt’s solution, with salmon sperm DNA (1 mg/ml) and appropriate riboprobe (100 ng/section) for 20 h at 50°C in a sealed humidified chamber. Sections were washed in 2× SSC, treated with RNase A (20 μg/ml), and processed for immunological detection of the DIG-labeled cRNA using anti-DIG Abs at a dilution of 1/500 (Roche). Color development was allowed to continue until a blue-purple precipitate was detected with the antisense probe; the color reaction for slides treated with the sense probe was terminated at the same time as that for the antisense slides.

Immunostaining

Mice were anesthetized with a lethal dose of Somnotol (MTC Pharmaceutical) and were intracardially perfused with 5 ml of ice-cold PBS, followed by 20 ml of 4% PFA (Fisher Scientific, Fairlawn, NJ). Brain and SC were dissected, postperfusion fixed 1 h in 4% PFA, and incubated overnight in PBS with 20% sucrose (EMD Chemicals, Gibbstown, NJ). Tissues were embedded in OCT (EMS, Hatfield, PA) and frozen in a 2-methylbutane (EMD Chemicals) bath immersed in liquid nitrogen. Immunohistochemical staining was performed on 10-μm cryostat sections. Frozen sections were blocked in 10% normal goat serum (Vector Laboratories, Burlingame, CA) in PBS for 1 h at room temperature, followed by incubation with primary rabbit Abs: MMP-15 (Chemicon International, Temecula, CA) and ADAM-12 (Chemicon International) overnight at 4°C, then with biotinylated goat anti-rabbit Ig (Vector Laboratories) for 1 h at room temperature. Sections were treated with 3% H₂O₂ to quench endogenous peroxidase activity, then were incubated with an avidin-HRP complex (Vector Laboratories) following the manufacturer’s instructions. HRP activity was detected by the use of 3,3′-diaminobenzidine (Lab Vision, Fremont, CA) as chromagen according to the manufacturer’s instructions. Control sections were incubated with equal concentration of rabbit Ig (DAKO, Mississauga, Canada) purified from serum of immununized rabbits. For H&E staining, hybridized in 50% (v/v) formamide, 4× SSC, 10% (w/v) dextran sulfate, 1× Denhardt’s solution, with salmon sperm DNA (1 mg/ml) and appropriate riboprobe (100 ng/section) for 20 h at 50°C in a sealed humidified chamber. Sections were washed in 2× SSC, treated with RNase A (20 μg/ml), and processed for immunological detection of the DIG-labeled cRNA using anti-DIG Abs at a dilution of 1/500 (Roche). Color development was allowed to continue until a blue-purple precipitate was detected with the antisense probe; the color reaction for slides treated with the sense probe was terminated at the same time as that for the antisense slides.

Quantitative real-time PCR (qPCR)

For SC analysis and for analysis of T cells and myeloid cells, qPCR was performed using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) according to previously described methods (17). The qPCR was performed for all mouse MMP and TIMP genes (primer and probe sequences in Ref. 18) and several mouse ADAMs (Table I). Conditions for the PCR were 2 min at 50°C, 10 min at 95°C, and then 40 cycles, each consisting of 15 s at 95°C, and 1 min at 60°C. To account for differences in the extraction and RT of total RNA.

Table II. Analysis of MP and TIMP expression profiles in CNS of mice with EAE at peak disease compared to unmanipulated controls

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold Increase in EAE</th>
<th>Fold Decrease in EAE</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-1a</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>MMP-1b</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>MMP-2</td>
<td>1.6</td>
<td></td>
<td>0.0642</td>
</tr>
<tr>
<td>MMP-3</td>
<td>2.2</td>
<td></td>
<td>0.1248</td>
</tr>
<tr>
<td>MMP-7</td>
<td>1.5</td>
<td></td>
<td>0.3662</td>
</tr>
<tr>
<td>MMP-8</td>
<td>90.1</td>
<td></td>
<td>&lt;0.0001</td>
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<td>MMP-9</td>
<td>2.6</td>
<td></td>
<td>0.0941</td>
</tr>
<tr>
<td>MMP-10</td>
<td>70</td>
<td></td>
<td>0.0183</td>
</tr>
<tr>
<td>MMP-11</td>
<td>1.3</td>
<td></td>
<td>0.3477</td>
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<tr>
<td>MMP-12</td>
<td>132.9</td>
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<td>0.0134</td>
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<td>MMP-13</td>
<td>1.2</td>
<td></td>
<td>0.6135</td>
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<td>MMP-14</td>
<td>2.2</td>
<td></td>
<td>0.0322</td>
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<tr>
<td>MMP-15</td>
<td>5.5</td>
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<td>0.0026</td>
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<tr>
<td>MMP-16</td>
<td>2.2</td>
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<td>MMP-17</td>
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<td>0.0606</td>
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<td>0.901</td>
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<td>MMP-21</td>
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<td>MMP-23</td>
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<td>MMP-24</td>
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<td>MMP-25</td>
<td>1.9</td>
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<td>ADAM-15</td>
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<td>ADAM-28</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>ADAM-33</td>
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<td>0.0023</td>
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<td>TIM-3</td>
<td>1.9</td>
<td></td>
<td>0.0344</td>
</tr>
<tr>
<td>TIM-4</td>
<td>1.2</td>
<td></td>
<td>0.1007</td>
</tr>
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</table>

n = 4 for each group. Genes showing a >3-fold alteration with P < 0.05 in an unpaired t test were chosen for detailed analysis. ND, Not detected.
sections were incubated for 5 min in Harris hematoxylin (Surgipath, Winnipeg, Canada), followed by 1 min in eosin (Surgipath).

**Results**

*MMP-8, -10, -12, and -15; ADAM-12; and TIMP-1 are the most strongly affected MP genes in severe EAE*

The aim of our study was to identify significant differences in expression levels of MMP, TIMP, and ADAM genes as a consequence of inflammation in EAE SC, then to localize the expression of these genes to individual cell populations. Our criteria for genes to be studied in more detail were that they showed a statistically significant and >3-fold difference in expression levels. The analysis was performed using quantitative real-time RT-PCR, and the results for SC are shown as fold alteration in expression level between mice with EAE and control mice (Table II). Six genes fulfilled our criteria: MMP-8, MMP-10, MMP-12, MMP-15, ADAM-12, and TIMP-1. These genes are marked with arrows in Fig. 1a, which is a graphical representation of the data in Table II. MMP-15 was the only gene of the six that showed decreased expression. MMP-15 is one of the six membrane-bound MMPs (MMP-14, -15, -16, -17, -24, and -25), of

**FIGURE 1.**  
*a,* Graphical representation of the data in Table II. , Down-regulated expression. The vertical lines represent specified fold alterations in expression.  
*b,* The ΔCT values for each of the genes analyzed are shown as ranges, indicated by shading of the boxes. ΔCT is the difference in CT between the gene of interest and the internal reference control gene, 18S rRNA. Arrows indicate the genes chosen for detailed analysis. ND, not detected.
which four (MMP-15, -16, -17, and -24) were down-regulated in EAE (Table II and Fig. 1a).

The analysis of alteration in expression levels of genes clearly shows up- and down-regulation of gene expression, but is not informative about the absolute levels of expression of the individual genes. A good estimation of this can be obtained from the maximum cycle threshold (ΔCT) values obtained from the quantitative real-time PCR analysis (Fig. 1b). A small ΔCT value corresponds to a high level of expression, and vice versa. It is evident from Fig. 1b that the large fold increase in expression of MMP-8, -10, and -12 in EAE SC reflected a very low level of expression in the unmanipulated SC, rather than a high level of expression in EAE. By contrast, TIMP-1 was expressed at a fairly low level in unmanipulated SC, but, in EAE, became the most highly expressed of the genes studied. MMP-15 fell from an intermediate to a low level of expression. ADAM-12 falls in the low range in both cases.

Expression of MP genes correlates with disease course

Our screen of a wide range of MMPs, ADAMs, and TIMPs showed that the expression of six genes was altered >3-fold in SC of mice with severe (grade 4) EAE (Table II and Fig. 1a). To further characterize the expression of these six genes in EAE, we studied the kinetics of their expression at the onset of disease, in severe disease, and after the first episode of symptoms. Fig. 2 shows the clinical course of EAE in individual mice after transfer of MBP-reactive T cells. As evident from Fig. 2, mice progressed rapidly from onset to grade 2, most often overnight. Mice at grade 2 EAE were therefore used to represent the onset of disease. Mice in each group in the kinetic study of expression were sacrificed at equivalent stage of disease. Fig. 3 reveals a clear correlation between the increased expression levels and the increased severity of symptoms for MMP-8, MMP-10, MMP-12, and TIMP-1 (Fig. 3, a, b, c, and f). Notably, the expression levels of these genes were strongly reduced after remission from symptoms, probably due to the loss of a significant number of the relevant infiltrating cell types (macrophages and granulocytes) expressing these genes after remission. The results for MMP-15 indicate an inverse relationship between expression levels and disease severity (Fig. 3d), with expression in remitted mice returning to levels comparable to those in controls. There was a clear up-regulation of ADAM-12 in mice with severe (grade 4) EAE (Fig. 3e), but in contrast to the other five genes analyzed, the expression of ADAM-12 persisted after remission at a level comparable to grade 4 disease.

Myeloid cells are the major producer of MP

We first screened the expression of all 22 MMPs, four TIMPs, and seven ADAMs in two cell populations (T cells and myeloid cells) sorted from EAE CNS using flow cytometric sorting. The cells were sorted from brain and SC of individual mice; we included brain to retrieve a greater number of cells. The trends in the expression levels of the analyzed genes were similar between SC and brain samples (data not shown). We sorted T cells as CD45<sup>high</sup>CD3<sup>+</sup> cells and a population of CD45<sup>low</sup>Mac1/CD11b<sup>+</sup> cells, which includes macrophages and granulocytes (21) (Fig. 4, b and c). This allows us to compare expression by infiltrating T cells and myeloid cells. The results of this analysis for genes showing an up-regulation in SC EAE of 1.2-fold or more (see Table II) are shown in Fig. 5. Of the 19 genes analyzed in this way, most (10) were expressed at significantly higher levels by myeloid cells than by T cells. Five genes (MMP-3, MMP-10, MMP-13, MMP-25, MMP-28, and ADAM-33) were expressed at equivalent levels (no statistically significant difference). Three genes (MMP-11, ADAM-12, and ADAM-19) were expressed at significantly higher levels by T cells than by myeloid cells.

MP genes are expressed by specific cell types

To assess the expression of the six most strongly affected genes (MMP-8, MMP-10, MMP-12, MMP-15, ADAM-12, and TIMP-1) in more detail by individual cell types, we isolated infiltrating macrophages, granulocytes, and T cells as well as resident microglia from the CNS of mice with EAE using flow cytometric sorting. As mentioned above, the population isolated as CD45<sup>high</sup>Mac1/CD11b<sup>+</sup> cells included both macrophages and granulocytes (Fig. 4c). To assess expression from granulocytes alone, we isolated CD45<sup>high</sup>Gr-1<sup>high</sup> cells, a population we have shown to consist of polymorphonuclear leukocytes (S. P. Zehntner and T. Owens, unpublished observations; Fig. 4d). Subtraction of the granulocyte contribution from the total allowed estimation of expression by macrophages alone. T cells were identified as CD45<sup>high</sup>Gr-1<sup>high</sup> cells and microglia as CD45<sup>4</sup>Mac1/CD11b<sup>+</sup> cells (22, 23). Unlike infiltrating leukocytes, microglia can be isolated in significant numbers both from unmanipulated and inflamed SC (Fig. 4, a and c, respectively).

Fig. 4, e and f, shows the average proportions of the cell types isolated from CNS that were used in the real-time PCR analysis. The total number of cells in CNS increased by 56% in EAE, mainly due to infiltration of macrophages, granulocytes, and T cells. The proportion of microglia remained at 23%, which, given the increase in total cells, actually reflects an increase in the number of microglia in EAE compared with unmanipulated mice.

Fig. 6 shows the gene expression levels of the six most strongly affected genes, in the four isolated cell types. It is clear that macrophages were the major sources of MMP-12 (Fig. 6c) and TIMP-1 (Fig. 6f), whereas granulocytes were the major source of MMP-8 (Fig. 6a). For MMP-10, expression was seen by microglia and macrophages (and in T cells in only one of four samples; Fig. 6b). None of the cell types investigated stood out as a major producer of MMP-10. ADAM-12 was expressed almost exclusively by T cells (Fig. 6e). Uniquely among the genes we analyzed, MMP-15 was strongly expressed by resting microglia, and this was reduced 15-fold in EAE (Fig. 6d).

When expression data from cell subpopulations from EAE SC were normalized per relative cell proportions (results not shown), the overall conclusions from Fig. 6 were unchanged. That is, TIMP-1 was expressed mainly by macrophages, MMP-8 mainly by granulocytes with some expression by macrophages and T cells, MMP-10 by microglia and macrophages, MMP-12 mainly by macrophages with some expression by microglia and T cells, MMP-15 by microglia with a small contribution from macrophages, and ADAM-12 by T cells.
ADAM-12 protein is expressed by infiltrating T cells, whereas parenchymal MMP-15 levels decrease in EAE

To verify that the expression of ADAM-12 message localized by flow cytometry and PCR to T cells (Fig. 6e) was reflected by the production of protein, we used immunohistochemistry to analyze the production of ADAM-12 in SC white matter of mice with EAE (Fig. 7, a and b). By comparison with staining of the same infiltrate in a separate section with control rabbit IgG (Fig. 7c), we found that ~50% of cells in perivascular infiltrates were producing ADAM-12. Analysis of the cell proportions in EAE CNS (Fig. 4f) showed that infiltrating T cells account for ~50% of the infiltrating cells. The fact that about half the infiltrating cells stained positively for ADAM-12 and had a round morphology suggests that these cells are T cells.

In whole SC, we found MMP-15 message to be down-regulated 5.5-fold in EAE (Table II and Fig. 1a). In contrast to ADAM-12, anti-MMP-15 Ab did not stain cells within infiltrates in EAE SC more intensely than control serum (Fig. 7c, d and e). This is consistent with cell sorting data showing that infiltrating cells in EAE express virtually no MMP-15 (Fig. 6d). The analysis of MMP-15 expression by sorted cells pointed to a down-regulation of MMP-15 expression by microglia in EAE. Immunostaining showed a general reduction of MMP-15 staining intensity between SC white matter in unmanipulated control mice (Fig. 7d) and a comparable uninfiltrated area in mice with EAE (Fig. 7f). This reduction of staining intensity was evident both on cell bodies and in the stroma, probably corresponding to cell processes, thereby indicating that the level of MMP-15 protein is indeed reduced in the SC of mice with EAE.

MMP-10 and TIMP-1 expression localizes to perivascular infiltrates

Expression analysis from sorted cells did not provide clear information about whether the increase in MMP-10 expression in the CNS was due to infiltrating cells, or whether another cell type was responsible (Fig. 6b). To address this question, we used ISH to analyze MMP-10 expression (Fig. 8). We also analyzed TIMP-1 expression, which had been shown to be expressed by cell-sorted macrophages (Fig. 6f). Brain sections from the same mice whose SCs were used for the initial screening of gene expression (Fig. 1) were analyzed by ISH. These mice had severe EAE (grade 4), and numerous cerebellar infiltrates could be identified by H&E staining (Fig. 8a). Sections were hybridized with antisense and control (sense) probes for TIMP-1 or MMP-10. A uniformly strong signal for TIMP-1 was detected in about half the infiltrating cells (Fig. 8b). By contrast, MMP-10 message was expressed by a majority of infiltrating cells, but at variable levels of intensity (Fig. 8d).

For both TIMP-1 and MMP-10, other cellular sources of message, most notably neurons in the granular cell layer and Purkinje cells, were observed in cerebellum (not shown). Cells associated with blood vessels, with morphology similar to endothelial cells, were also positive for MMP-10 (not shown). There was no difference in ISH signal intensity for neuronal or endothelial expression between EAE samples and controls. We conclude that the increase in message levels for MMP-10 in EAE was due to combined expression by multiple types of infiltrating cells.

Discussion

To our knowledge, this is the first and most comprehensive study of the expression of all known mouse MMPs and TIMPs as well as seven ADAMs in CNS tissue from animals with EAE. This is also the first analysis of the expression of MMPs, ADAMs, and TIMPs in cell populations sorted from the CNS of mice with EAE. Our findings show distinct patterns of expression, which probably reflect functional differences in these genes in CNS inflammation.

Clearly, macrophages are an important source of MP and TIMP expression in EAE. Macrophage infiltration is required for EAE and, if blocked, prevents disease (24, 25). Our study shows fairly high expression by macrophages of all but two (MMP-10 and ADAM-12) of the six most strongly affected genes. The finding that TIMP-1 was expressed by macrophages suggests a potential regulatory role for macrophages in EAE and MS by controlling the

![Graphs showing kinetics of MP gene expression during EAE.](Image)

**FIGURE 3.** Kinetics of MP gene expression during EAE. Expression levels of MMP-8, -10, -12, and -15; ADAM-12; and TIMP-1 in SC from unmanipulated mice, mice with early onset (grade 2) and severe, established (grade 4) EAE, and mice in remission were measured by real-time PCR. The significance of comparisons to unmanipulated mice was determined using an unpaired t test. An unpaired t test was also used to compare grade 4 mice with the remitted group. n = 4 for all groups except remitted mice (n = 3). *p < 0.05; **p < 0.01; ***p < 0.001. Error bars represent SEM.
MP/TIMP ratio and thereby controlling the level of MP activity. Also, astrocytes would be suspected to play a role in controlling the MP/TIMP ratio, as activated astrocytes have been demonstrated to express TIMP-1 in EAE (26).

The MMP-9/TIMP-1 ratio has received much attention since the finding that the serum MMP-9/TIMP-1 ratio is increased in patients with active MS, mainly due to increased MMP-9 levels (27, 28). Hence, MMP-9 has become a candidate in the pathogenesis of MS and EAE. It has been shown that MMP-9-deficient mice

**FIGURE 4.** Flow cytometric analysis of cell populations in unmanipulated (a) and EAE CNS (b–d). a and c, Staining for PE-CD45 vs FITC-Mac1/CD11b. b, PE-CD45 vs CD3-biotin coupled to streptavidin-Cy-Chrome. d, PE-CD45 vs FITC-Gr-1. Boxes are drawn to identify microglia (a and c), T cells (b), macrophages and granulocytes (c), and granulocytes (d). Fluorescence levels are matched between panels a and b and between panels c and d. e–f. Average numbers and proportions of cells sorted from CNS preparations: e, unmanipulated controls (n = 8); f, EAE (n = 16 for microglia and macrophages, n = 6 for neutrophils and T cells). Mac., macrophages; Gran., granulocytes.

**FIGURE 5.** Relative expression of selected genes by T cells (T) and myeloid cells (M; macrophages and granulocytes) from EAE CNS at peak disease (n = 6 for each group). Values on the ordinate are relative levels of gene expression compared with the expression of 18S rRNA. The values are arbitrary and cannot be compared between different genes. Error bars represent SEM.
younger than 4 wk were less susceptible to EAE, but adult mice were unaffected (29). MMP-9 has previously been reported to be up-regulated in both mouse and rat EAE (15, 26). In a kinetic study (15), the up-regulation of MMP-9 was seen at the onset of disease and had virtually disappeared when the rats reached maximum disease. In our study we found a 2.6-fold up-regulation of MMP-9 (Table II), but it was not significant and was not chosen for further study. This relatively small increase in MMP-9 expression is probably due to the fact that the mice analyzed in our study were at an advanced stage in the disease, when an early large rise in MMP-9 expression will have subsided.

MMP-7 has previously been found to be up-regulated in rat models of EAE (15, 30). We found a nonsignificant 1.5-fold decrease in MMP-7 expression in grade 4 disease. However, our finding of no up-regulation of MMP-7 is consistent with an earlier observation that MMP-7 was not up-regulated in mouse EAE (26). This points to a possible species difference between mice and rats with respect to MMP-7 expression in EAE.

We found that whereas T cells are a minor source of MMP-8 and MMP-12, they are virtually the only source of ADAM-12 among the infiltrating cells (Fig. 6e). The cellular sources of ADAM-12 as well as its functions have not been well studied. A study of ADAM-12-deficient mice suggested that ADAM-12 plays a role in adipogenesis and myogenesis (31). To our knowledge, the only previous demonstration of ADAM-12 expression in the CNS identified oligodendrocytes as sources of ADAM-12 (32). These could be the cells responsible for the expression of ADAM-12 we found in the unmanipulated CNS (Fig. 1b). ADAM-12 was the only gene of the six analyzed that was expressed at levels comparable to severe disease after remission from disease (Fig. 3e). One may speculate that this is due to persisting T cells in the CNS after remission. Our demonstration of the expression of this gene by T cells could indicate a novel role for ADAM-12 in T cell function.

The differential expression of MPs by infiltrating T cells and myeloid cells is of interest. In a study of MMP expression by subsets of human leukocytes isolated from blood, monocytes were found to express more MMP members than T cells or B cells and to have greater migratory potential (33). In our study we found myeloid cells to be the major source of MP message compared with T cells, with MMP-11, ADAM-12, and ADAM-19 being the exceptions.

FIGURE 6. Relative expression of selected genes in cell populations sorted from CNS. n = 7 for unmanipulated microglia, and n = 10 for EAE microglia. The n = 8, 6, and 4 for macrophages and granulocytes, granulocytes alone, and T cells, respectively. For MMP-10 (b), only one of four samples showed detectable expression in T cells. Where possible, expression level differences between control microglia and EAE microglia were analyzed using an unpaired t test. Mac., macrophages; Gran., granulocytes; ND, not detected. **, p < 0.01.

FIGURE 7. Immunostaining for ADAM-12 and MMP-15 in EAE. Sequential sections from a perivascular infiltrate in SC white matter of a mouse with EAE were stained with H&E (a), anti-ADAM-12 Ab (b), anti-MMP-15 Ab (c), and control rabbit Ig (e). An equivalent area of SC white matter from an unmanipulated mouse was stained with anti-MMP-15 Ab (d). f, MMP-15 staining for an uninfiltrated area adjacent to the infiltrate shown in a–c. Results shown are representative of four mice with EAE and four unmanipulated mice. Original magnification, ×63.
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FIGURE 8. ISH for TIMP-1 and MMP-10 expression in EAE. Perivascular
filtrates in cerebellar white matter of mice with EAE were identified
using H&E staining (a). Infiltrates were hybridized on separate sections
with TIMP-1 antisense probe (b) and MMP-10 antisense probe (d), c and
e. Lack of hybridization with TIMP-1 and MMP-10 sense probes, re-
respectively. Results shown are representative of three mice with EAE. Original magnification, ×63.

(Fig. 5). However, both cell populations were isolated from the CNS
and have therefore demonstrated capacity to infiltrate, and both T cells
and myeloid cells colocalize within CNS infiltrates. One could spec-
ulate that the differences in MP expression reflect different timings for
entry of T cells and myeloid cells into the CNS. If a particular cell
type were responsible for extensive breakdown of the BBB at initia-
tion of inflammation, then other cells, with a smaller or different ar-
senal of MPs, would have an easier route into the CNS. The sequence
of cellular infiltration in EAE could be orchestrated by changing che-
mokine profiles over the course of disease (34).

From Fig. 6a, it is evident that granulocytes were a major source of
MMP-8 (also named neutrophil collagenase), consistent with
previous findings (35). In addition to the mRNA messages detected
in our study, granulocytes may contribute to the MP load by re-
leasing MPs from preformed vesicles upon infiltration, without the
need for de novo MP synthesis.

In our analysis we also included microglia, the resident macro-
phage-like cells in the CNS. Microglia have been implicated as
critical cells in MS/EAE pathogenesis (36, 37), perhaps through
production of MMPs (38). We found that microglia expressed
MMP-10, -12, and -15 in EAE. These cells share bone marrow
origin and many functional properties with macrophages, so shared
expression of MMPs would be expected. The down-regulation of
membrane-associated MMP-15 by microglia was a striking demon-
stration of a specific down-regulation of an MMP by a specific
cell type in EAE. This finding raises questions regarding the func-
tion of this membrane-associated MMP. Because it is down-regu-
lated in EAE, it would not appear to play a role in facilitating
infiltration. Indeed, four of the six membrane-associated MMPs
were down-regulated in EAE (Table II and Fig. 1a). It will be
interesting to determine how many are expressed by microglia and
whether membrane-associated MMPs play a special role in micro-
glial biology. A study of membrane-associated MMPs in gliomas
showed them to generally be elevated (17). The functional conse-
quences of this contrasting response of membrane-associated
MMPs in the context of different CNS diseases remain to be
elicited.

We also note an increase in absolute numbers of microglia in the
CNS in EAE (Fig. 4, e and f), which is consistent with an earlier
study (39). This increase is probably due to the proliferation of
resident microglia as a consequence of activation in the inflam-
atory milieu, although a contribution from immigrating, blood-
derived cells cannot be excluded (40, 41). One can speculate
whether the change from resting to activated microglia is reflected in
their expression profiles of membrane-associated MMPs, in-
cluding the down-regulation of MMP-15. Fig. 3d shows that
MMP-15 levels after remission from EAE return to a level compar-
able to that in unmanipulated controls. This may indicate a
change in the activation status of the microglia that produce MMP-
15, returning to a more resting state with higher MMP-15 expres-
sion after remission.

The MS therapeutic, IFN-β, is proposed to work in part by in-
hibiting MMPs, as indicated by a decrease in the transcription of
MMP-7 and -9 in PBLs from patients with relapsing-remitting MS
receiving IFN-β treatment (42). Likewise, the attenuation of EAE
symptoms in mice treated with the antibiotic minocycline may be
mediated in part by the MMP inhibitory effect of this drug, because
minocycline decreases transcription levels of MMPs and inhibits T
cell migration across a fibronectin membrane (43). The use of syn-
thetic broad-spectrum MP inhibitors in MS might be problematic
given the current lack of knowledge about potential beneficial and
detrimental roles of specific MPs in CNS inflammation. The use of
such drugs in clinical trials involving cancer patients was termin-
dated due to low efficacy and serious side effects (44). Our study
provides comprehensive information about MP and TIMP expres-
sion in EAE as well as information about cell type-specific ex-
pression of MPs and TIMPs. We anticipate that our findings will
aid in the development of rational MS therapies based on manipu-
ulating specific MP and TIMP actions.

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