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Analyses of all matrix metalloproteinase members in leukocytes emphasize monocytes as major inflammatory mediators in multiple sclerosis

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Summary

Matrix metalloproteinases (MMPs) are implicated in multiple sclerosis where one of their roles may be to facilitate the transmigration of circulating leukocytes into the CNS. Studies have focused on only a few MMPs, and much remains unknown of which of the 23 MMP family members is/are critical to the multiple sclerosis disease process. Using quantitative real time polymerase chain reactions, we have systematically analysed the expression of all 23 MMP members in subsets of leukocytes isolated from the blood of normal individuals. We found a distinctive pattern of MMP expression in different cellular populations: MMP-11, MMP-26 and MMP-27 were enriched in B cells, while MMP-15, MMP-16, MMP-24 and MMP-28 were prominent in T lymphocytes. Of interest is the enrichment of a majority of MMP members in monocytes: MMP-1, MMP-3, MMP-9, MMP-10, MMP-14, MMP-19 and MMP-25. MMP-2 and MMP-17 were also significantly represented in monocytes, although B cells had significant amounts of these MMPs. In correspondence with their strong expression of many MMP members, monocytes migrated more rapidly across a model of the blood-brain barrier in culture than T or B lymphocytes. Finally, we found higher levels of two of the monocyte-expressed MMPs in multiple sclerosis patients compared with normal individuals: MMP-2 and MMP-14. Tissue inhibitor of metalloproteinases (TIMP)-2 was also elevated in monocytes from multiple sclerosis patients, providing a mechanism for the reported activation of MMP-2 by MMP-14 and TIMP-2. These results emphasize that monocytes are prominent contributors of the neuroinflammation in multiple sclerosis through a mechanism that involves their high MMP expression and that they identify specific MMP members as targets for novel therapeutics in the disease.

Keywords: blood-brain barrier; EAE; macrophages; metalloproteinases; multiple sclerosis

Abbreviations: C_T = cycle threshold; EAE = experimental autoimmune encephalomyelitis; HBECs = human brain-derived endothelial cells; IgG = immunoglobulin-G; MMP = matrix metalloproteinase; IFN-γ = interferon-γ; LPS = lipopolysaccharide; PBMC = peripheral blood mononuclear cell; PMA = phorbol-12,13-myristate acetate; TIMP = tissue inhibitor of metalloproteinases

Introduction

The matrix metalloproteinases (MMPs) are proteases that collectively can degrade all protein components of the extracellular matrix. They regulate many processes during development and in adulthood, particularly those that require the remodelling of the extracellular matrix such as tissue morphogenesis and wound healing. MMPs also have functions distinct from matrix remodelling, such as mediating signalling and promoting survival (Chang and Werb, 2001; McCawley and Matrisian, 2001). However, the abnormal expression of several MMP members is thought to give rise to diseases including cancers, rheumatoid arthritis, cardiac dysfunctions and lung pathology.

The mature CNS normally contains non-detectable or low levels of most MMPs, but several become upregulated in neurological diseases such as multiple sclerosis, malignant glioma and stroke (reviewed in Yong *et al*., 2001). In multiple

Table 1 *Demographic/clinical data on the nine multiple sclerosis patients and nine control subjects*

Multiple sclerosis patient	Age	Sex	Time since multiple sclerosis diagnosed	Number of relapses in last year	EDSS
1	42	M	4 months	3	3.0
2	38	F	6 years	1	2.0
3	27	M	1 month	1	1.0
4	32	F	4 years	1	2.5
5	19	M	1 month	2	1.5
6	37	M	1 month	1	2.5
7	20	M	1 month	3	1.0
8	37	F	2 years	1	2.0
9	22	F	4 months	2	1.0
Control	Age	Sex			
1	35	M			
2	28	M			
3	39	F			
4	25	F			
5	22	M			
6	27	F			
7	28	M			
8	24	M			
9	22	F			

EDSS = Expanded Disability Status Score

sclerosis brain tissues, MMP-2, MMP-7, MMP-9 and MMP-12 are reported to be elevated (Anthony *et al*., 1997; Cossins *et al*., 1997; Vos *et al*., 2003). In an animal model of multiple sclerosis, experimental autoimmune encephalomyelitis (EAE), the induction or enhanced expression of MMP family members is thought to promote the disease process (Hartung and Kieseier, 2000; Leppert *et al*., 2001; Yong *et al*., 2001). In this regard, the upregulation of some MMP members correlates with the disease course (Clements *et al*., 1997; Kieseier *et al*., 1998; Lindberg *et al*., 2001). Furthermore, synthetic inhibitors of metalloproteinase activity alleviate the severity of EAE or prevent its occurrence (Gijbels *et al*., 1994; Hewson *et al*., 1995; Norga *et al*., 1995). Young mice that are genetically deficient for MMP-9 are relatively resistant to EAE induction compared with wild-type mice (Dubois *et al*., 1999).

A key function for MMPs in multiple sclerosis and EAE is thought to be the facilitation of leukocyte entry into the CNS, since MMPs are implicated in the transmigration of leukocytes (Shipley *et al*., 1996; D'Haese *et al*., 2000; Lanone *et al*., 2002). *In vitro*, the transmigration of T cells across basement membrane matrices is inhibited by inhibitors of metalloproteinase activity (Leppert *et al*., 1996; Xia *et al*., 1996; Stuve *et al*., 1996; Brundula *et al*., 2002). Interferon-β, a drug used in the treatment for relapsing-remitting multiple sclerosis, inhibits the production of MMP-9 by T cells and their transmigration across a model of the blood-brain barrier (Leppert *et al*., 1996; Stuve *et al*., 1996). Other detrimental functions of MMPs in multiple sclerosis are thought to include the breakdown of the blood-brain barrier (Rosenberg, 2002), the promotion of inflammation within the CNS (Opdenakker and Van Damme, 1994), and direct neurotoxicity (Johnston *et al*., 2001; Newman *et al*., 2001; Gu *et al*., 2002).

Despite the accumulating evidence of a pathogenic role for MMPs in multiple sclerosis, much remains unknown of which of the 23 MMP family members is/are critical to the disease process and which immune cell subset produces them. Most studies have focused on the gelatinase subfamily of MMPs (MMP-2 and MMP-9) or on MMP-7, in part because of their relative ease of measurements. There is virtually no information on MMP members beyond MMP-14 (i.e. MMP-15 to MMP-28). In addition, while multiple sclerosis is thought to be initiated through the activation of autoreactive T cells in the periphery and their subsequent migration into the CNS, there are important roles for infiltrating B cells and monocytes (which become macrophages in tissue) in the pathology of the disease (Martin *et al*., 2001; O'Connor *et al*., 2001; Hemmer *et al*., 2002). Indeed, macrophages and CNS-intrinsic microglia, which have many similar characteristics and can be difficult to differentiate from one another, are the predominant group of inflammatory cells in active demyelinating plaques (Prineas *et al*., 1978). In detailed neuropathological studies of various types of demyelinating lesions in multiple sclerosis, macrophages/microglia numbers exceed those of lymphocytes by ~10-fold (Lucchinetti *et al*., 2000). Little is known of the role of MMPs in the trafficking of monocytes into the CNS.

In this study, we have systematically analysed the expression of all 23 MMP members in subsets of leukocytes isolated

from the blood of normal individuals. We found several MMPs to be abundant in monocytes compared with B or T lymphocytes. Correspondingly, monocytes migrated more rapidly across a model of the blood-brain barrier than lymphocytes, a process that was antagonized by an MMP inhibitor, tissue inhibitor of metalloproteinases (TIMP)-1. Finally, we found higher expression of two of the MMPs that are enriched in monocytes in multiple sclerosis patients compared with normal individuals. These results emphasize that monocytes are prominent contributors of the neuroinflammation in multiple sclerosis through a mechanism that involves their high MMP expression.

Material and methods

Cell isolation

Venous blood from normal adult volunteers and from consecutively evaluated active patients with relapsing-remitting multiple sclerosis (Table 1) was collected into tubes containing EDTA in accordance with the guidelines of the McGill Ethical Review Board. Relapsing-remitting multiple sclerosis was defined by the McDonald criteria (McDonald *et al*., 2001). Patients were considered active since all were experiencing a clinical relapse (defined as the onset of new and objective neurological findings, lasting at least 48 h, in the absence of fever) and/or the presence of gadolinium enhancing lesions on brain MRI at the time of phlebotomy. All multiple sclerosis patients were untreated and all had relatively early multiple sclerosis with average time from multiple sclerosis diagnosis of 17 months (range: 1 month to 6 years) and Expanded Disability Status Score (EDSS) range of 1.0–3.0 (Table 1). Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density centrifugation (Pharmacia Biotech, Uppsala, Sweden). $CD14^+$ monocytes, $CD19^+$ B cells and $CD3^+$ T cells were purified using the MACS system (Miltenyi Biotech, Auburn, CA, USA) following the manufacturer's protocol without modification. Isolated cells were washed twice in medium to remove residual EDTA. The medium used was RPMI 1640 supplemented with 10% foetal calf serum (FCS), penicillin (100 U/ml), streptomycin (100 μg/ml) and 2 mM L-glutamate (all from Sigma, St. Louis, MO, USA). Cell viability was assessed by Trypan blue exclusion and was invariably >98%. The efficacy of MACS purification was assessed by flow cytometry using triple staining (anti-CD3-FITC (fluoroisothiocyanate) and anti-CD19-Cy3 (BD PharMingen, Mississauga, ON, Canada), and anti-CD14-PE from DAKO, Glostrup, Denmark). The purity of monocytes, B cell and T cell preparations was routinely ⩾97%.

Cell stimulation

Purified cell populations were harvested into Trizol (Gibco-BRL, Burlington, Ontario, Canada) either immediately following isolation ('immediately *ex vivo*') or following 8 h of cell culture with or without activation. When activated, B cells were stimulated with anti-IgG (immunoglobulin-G)/IgM (2 μg/ml, Jackson ImmunoResearch, West Grove, PA, USA); monocytes were stimulated for 2 h with 10 U/ml interferon-γ (IFNγ) (1 ng/ml, Biosource, Missisauga, Ontario, Canada) followed by the addition of 100 ng/ml lipopolysaccharide (LPS) (Sigma) for another 6 h; T cells were stimulated with 5 ng/ml phorbol-12,13-myristate acetate (PMA) and 2 μM ionomycin (both from Sigma). Trizoled samples were stored at –70°C for subsequent RNA extraction.

RNA isolation and reverse transcription

Total RNA was isolated from cell lysates according to the instructions provided with the Trizol. RNA was resuspended in diethyl pyrocarbonate-treated water (diethyl pyrocarbonate from Sigma Aldrich, Poole, UK) and concentrations were determined by spectrophotometry using a GeneQuant pro RNA–DNA calculator (Amersham Pharmacia Biotech, Little Chalfont, UK). One microgram of total RNA was reverse transcribed using 2 μg random hexamers (Amersham Pharmacia Biotech) and Superscript II reverse transcriptase (Invitrogen Life Technologies, Paisley, UK) according to the supplier's instructions. cDNA was stored at –20°C until used in the polymerase chain reaction (PCR).

Quantitative real-time PCR

For PCR reactions, specific primers and fluorogenic probes for all human MMP and TIMP genes were designed using Primer Express 1.0 software (PE Applied Biosystems, Warrington, UK) and synthesized by PE Applied Biosystems; sequences for primers and probes are as previously described (Nuttall *et al*., 2003). The 18S ribosomal RNA gene was used as an endogenous control to normalize for differences in the amount of total RNA in each sample. 18S rRNA primers and probe were purchased from PE Applied Biosystems. PCR reactions were performed using the ABI Prism 7700 Sequence Detection System (PE Applied

Fig. 1 Expression of MMPs in mononuclear cells subsets obtained from normal volunteers. The *y*-axis for each MMP is relative RNA levels expressed as a ratio, and thus normalized, to 18S RNA. Each bar represents the mean ±SD of data from six to nine subjects, with the majority of the results from nine normal individuals. For each graph, the histograms from the extreme left are: immediately *ex vivo* peripheral blood mononuclear cells (black bar), unstimulated and stimulated (with anti-IgG/IgM) B cells (open bars), unstimulated and stimulated (with IFN-γ and LPS) monocytes (striped bars), and unstimulated and stimulated (with PMA and ionomycin) T cells (grey bars). MMP-4, MMP-5 and MMP-6 do not exist, while there is no human homolog for MMP-18 and MMP-22. Levels for MMP-12, MMP-13 and MMP-20 transcripts were below detection levels in all subsets.

Biosystems), following the manufacturer's protocol. The cycle number (termed cycle threshold, or C_T) at which amplification entered the exponential phase was determined and this number was used as an indicator of the amount of target RNA in each tissue, i.e. a lower C_T indicated a higher quantity of starting RNA.

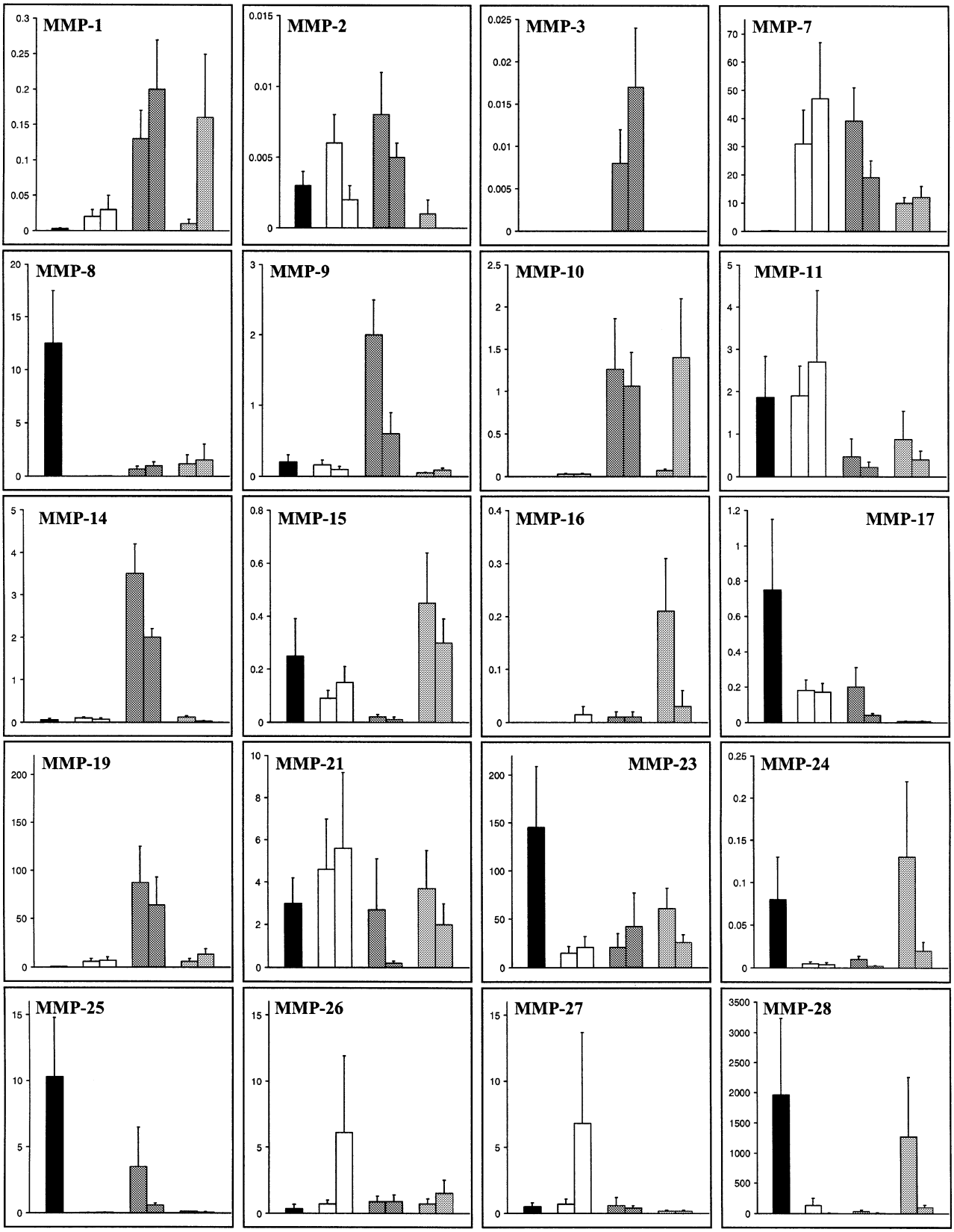

Table 2 *Summary of MMPs preferentially expressed by various mononuclear cell subsets*

Leukocyte subset	MMP member preferentially expressed
Monocytes	MMP-1, MMP-2*, MMP-3, MMP-9, MMP-10, MMP-14, MMP-17*, MMP-19, MMP-25
B lymphocytes	MMP-2*, MMP-11, MMP-17*, MMP-26, MMP-27
T lymphocytes	MMP-15, MMP-16, MMP-24, MMP-28

*Expressed by two of three cell subsets. MMPs expressed by all three subsets in roughly equal amounts: MMP-7, MMP-8, MMP-21 and MMP-23.

To determine the relative RNA levels within the samples, standard curves for the PCR reaction were prepared by using the cDNA from one sample and making two-fold serial dilutions covering the range equivalent to 20 ng to 0.625 ng of RNA (for 18S analyses the range was from 4 ng to 0.125 ng). These dilutions were subject to real-time PCR as described above. Relative standard curves for C_T versus input RNA were prepared and relative levels of starting RNA in each sample were determined.

The results of each MMP member were normalized to those from 18S ribosomal RNA from the same sample.

Migration assays

Migration assays were conducted in Boyden® chambers, as previously described (Biernacki *et al*., 2001). Briefly, each chamber (3 μm pore size membrane), pre-coated with fibronectin (BD PharMingen), was inserted into a well of a 24 well plate to create a two-compartment migration system. The membranes were overlaid with 2.5×10^4 human brain-derived endothelial cells (HBECs) in a total volume of 500 μl endothelial cell media (Biernacki *et al*., 2001). Migration assays were carried out 3 days later, at which time the HBECs formed a confluent layer over the fibronectin. The HBECs were derived from temporal lobe specimens resected from young adults undergoing surgical treatment for intractable epilepsy. On the day of migration, the endothelial cell medium was aspirated and the upper chambers, containing the HBEC/fibronectin layer, were transferred to new wells containing 500 μl of fresh medium. 5×10^5 *ex vivo* monocytes, B cells or T cells were added in parallel to the top compartment of different wells, in a total volume of 500 μl of medium, and incubated at 37°C/5% carbon dioxide. At this point, recombinant TIMP-1 was added where indicated. After 24 h, 50 μl of 0.5 mol/l EDTA was added to the bottom compartment to mobilize cells and the plates were placed on a flatbed shaker for 15 min. Cells were collected from the bottom compartment, centrifuged, resuspended and counted with a haemocytometer.

Results

Expression of MMPs and TIMPs in subsets of mononuclear cells from normal subjects

We analysed the expression of all known MMPs in purified B cells, monocytes and T cells from nine normal individuals using real time PCR. These cell populations were either left untreated for 8 h in culture ('unstimulated') or they were stimulated as described above during the 8 h *in vitro*. Fig. 1 shows a distinctive pattern of MMP expression in the different cellular populations, in that some MMPs were preferentially expressed in particular cell subsets. Thus, MMP-11, MMP-26 and MMP-27 were prominent in B cells, while MMP-15, MMP-16, MMP-24 and MMP-28 were enriched in T lymphocytes. Of interest is the enrichment of a majority of MMP members in monocytes: MMP-1, MMP-3, MMP-9, MMP-10, MMP-14, MMP-19 and MMP-25. We note that MMP-1 and MMP-10 could also be found in T cells, but after stimulation. MMP-2 and MMP-17 expression were also significant in monocytes, although B cells also contained these members. Some MMPs were expressed fairly uniformly by all cell subsets; these were MMP-7, MMP-8, MMP-21 and MMP-23. The differential expression of MMP members is presented in Table 2.

We investigated whether stimulation *in vitro* with selected stimuli would alter MMP expression by each cell subset. Stimulation increased the expression of MMP-3 in monocytes, MMP-1 and MMP-10 in T cells, and MMP-26 and MMP-27 in B cells (Fig. 1). Some MMP members were decreased upon cell stimulation such as: MMP-7, MMP-9, MMP-14, MMP-21 and MMP-25 in monocytes; MMP-16, MMP-24 and MMP-28 in T cells; and MMP-2 in B cells.

While the data in Fig. 1 provide a quantitative comparison between samples for each MMP member normalized to 18S RNA levels, the analyses did not indicate the expression levels of each gene. To resolve this, C_T results and their ranges are presented in Fig. 2. Such analyses further emphasize the high expression of many MMP members in monocytes compared with T and B cells, since a lower range of C_T is evident for many MMP members (e.g. MMP-1, MMP-2, MMP-3, MMP-9, MMP-10, MMP-14, MMP-19 and MMP-25) in this cell subset.

The presence of proteases in many physiological systems is accompanied by the simultaneous expression of their inhibitors. The endogenous antagonists of MMP activity are the four TIMPs. As with the levels of numerous MMPs, transcript levels for TIMP-1, TIMP-2 and TIMP-4 are enriched in monocytes compared with T and B lymphocytes (Fig. 3).

In summary, the comprehensive analyses of all existent human MMPs in the various mononuclear cell subsets emphasize a higher expression of several MMP members in

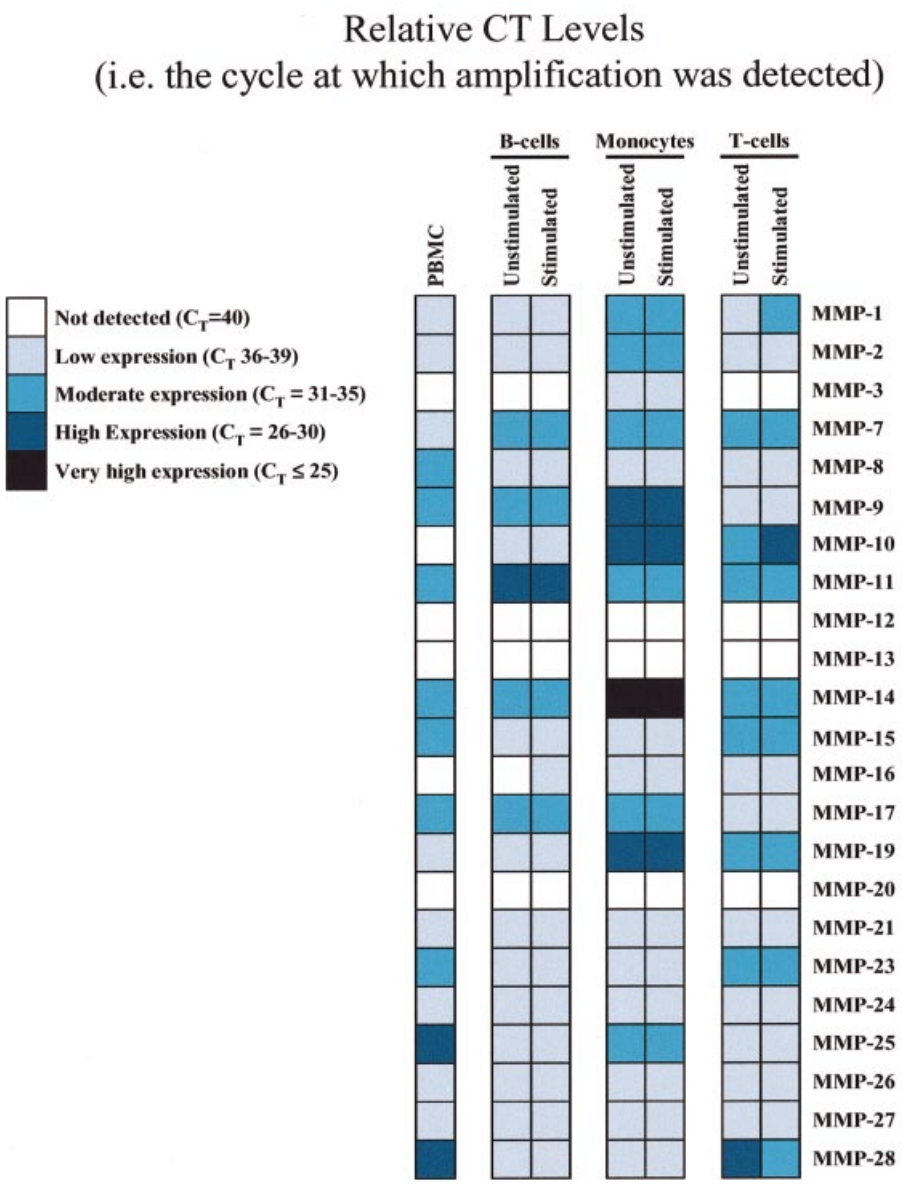

Fig. 2 Expression levels of the MMPs determined by comparing cycle threshold (C_T) levels. Boxes represent the mean of six to nine subjects, with the majority of the results from nine normal individuals.

the monocyte population, even though B and T cells also have their own distinctive profile of MMP expression.

Monocytes migrate more efficiently across a model of the blood-brain barrier than B and T cells

We investigated whether the prominent expression of many MMP members by monocytes has functional consequences. Since MMPs are thought to be important in the transmigration of various leukocytes subsets into tissues (Leppert *et al*., 1996; Xia *et al*., 1996; Shipley *et al*., 1996; Stuve *et al*., 1996; D'Haese *et al*, 2000; Lanone *et al*., 2002), we investigated the relative capacity of each mononuclear cell subset to transmigrate across a model of the blood-brain barrier that is composed of HBECs and a fibronectin matrix (simulating the basement membrane). Figure 4A demonstrates that, when seeded at the same density, significantly more monocytes transmigrated compared with T cells; the transmigration of B cells was intermediate in level. In correspondence with the hypothesis that MMPs mediate transmigration, the proportion of monocytes that crossed the endothelial/fibronectin barrier was reduced by TIMP-1 (Fig. 4B).

Overall, the higher expression of many MMP members in monocytes is reflected in their significantly higher capacity to transmigrate a model of the blood-brain barrier compared with B and T cells. Indeed, the transmigration of T cells,

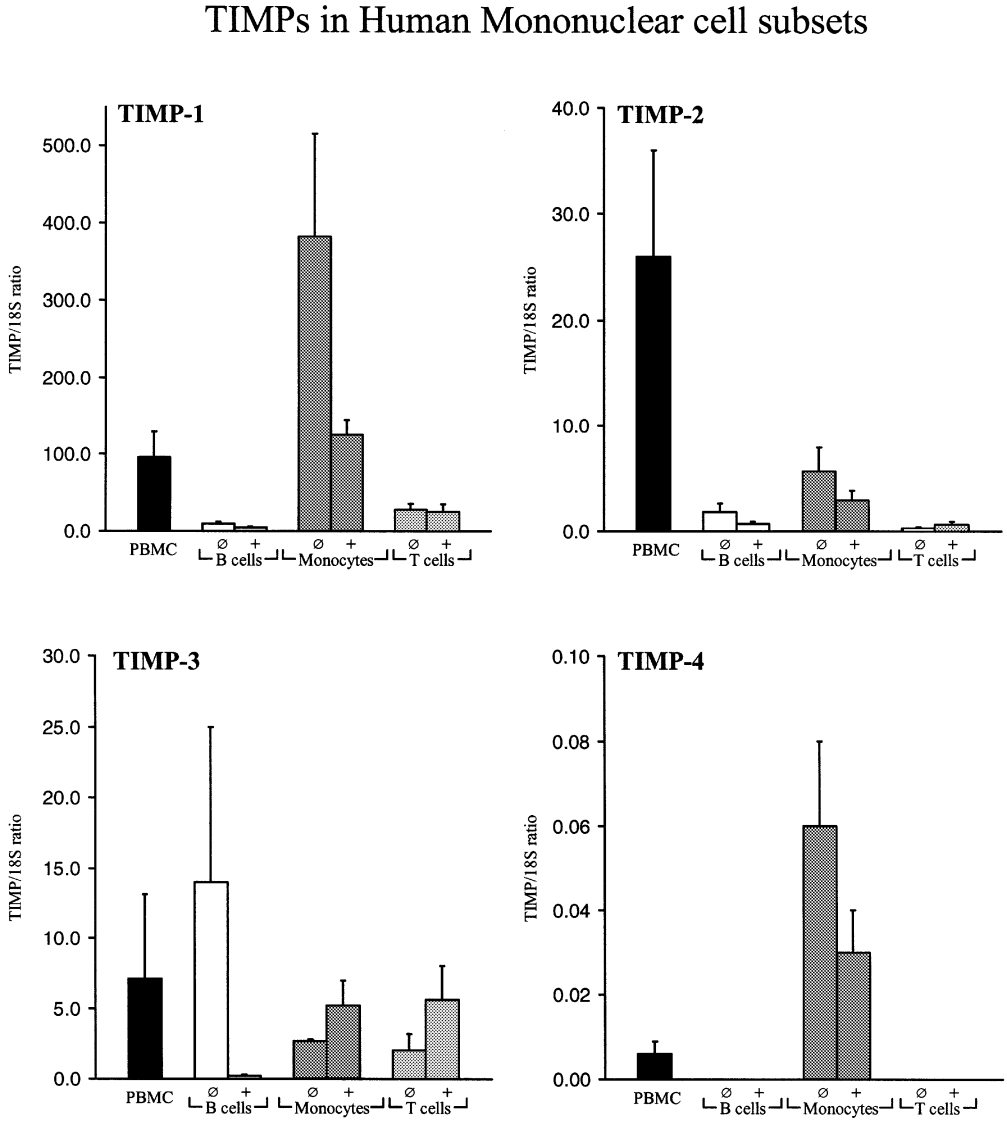

Fig. 3 Levels of TIMP transcripts in mononuclear cell subsets obtained from six to nine normal volunteers. Bars are mean ±SD. PBMC represents the immediately *ex vivo* peripheral blood mononuclear cells. For the B cells, monocytes and T cells, conditions are either unstimulated in culture (ø), or stimulated (+) with the appropriate stimulus.

researched in several studies of relevance to multiple sclerosis (Martin *et al.*, 2001; O'Connor *et al.*, 2001; Hemmer *et al.*, 2002), is at least an order of magnitude lower than that of monocytes.

Comparison of MMP and TIMP profiles in monocytes from multiple sclerosis patients and normal individuals

Since monocytes have a higher representation of several MMP members than T and B lymphocytes and since they have a greater capacity to transmigrate a model of the blood-brain barrier, we compared the capacity of monocytes from multiple sclerosis patients to express particular MMPs relative to controls. Cell subsets were isolated from nine consecutive multiple sclerosis patients diagnosed as having active relapse-remitting multiple sclerosis based on objective evidence for an ongoing clinical relapse and/or the presence of gadolinium-enhancing MRI scans at the time of phlebotomy. We focused on the MMPs with high expression in monocytes. Figure 5 shows a higher expression in monocytes from multiple sclerosis individuals of MMP-2 and MMP-14 compared with levels in control subjects. Other monocyte-enriched MMPs (MMP-1, MMP-3, MMP-9, MMP-10, MMP-17, MMP-19 and MMP-25) were distinctly not different between multiple sclerosis patients and controls, whether the monocytes were stimulated in culture or not ($P > 0.05$, data

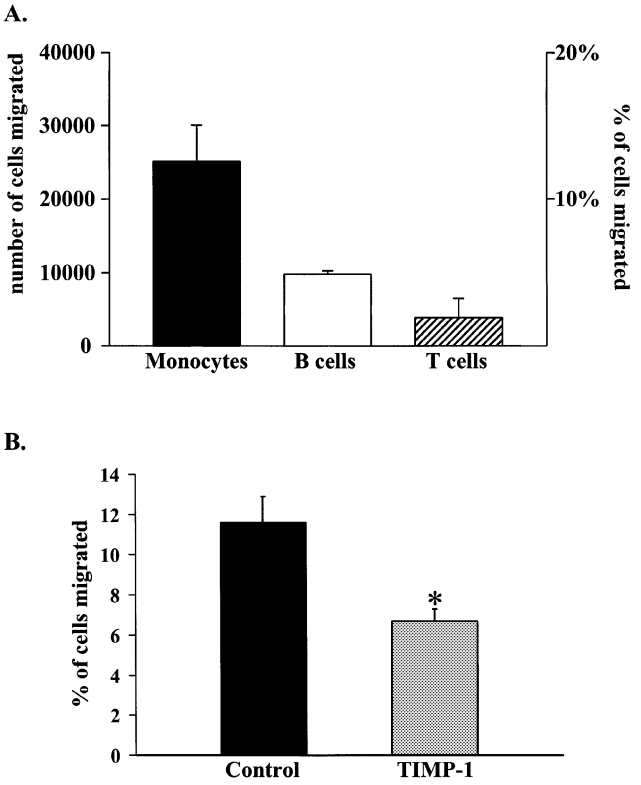

Fig. 4 Comparative migration of leukocyte subsets. (**A**) Representative experiment (one of six) shows that monocytes migrate more efficiently than B cells ($P = 0.03$) and T cells ($P = 0.02$). As all data sets passed tests for normality, the paired *t*-test for parametric data was used to compare groups using GraphPad Prism® software. (**B**) Addition of TIMP-1 at 160 ng/ml ($n = 4$ migration wells) reduced the migration of monocytes compared with controls ($n = 6$ wells); $^*P < 0.05$ (Student's *t*-test).

not shown). Altogether, these results indicate a higher expression of specific MMP members, MMP-2 and MMP-14, in monocytes from individuals with multiple sclerosis compared with controls.

Finally, we determined the levels of the four TIMPs in monocytes from multiple sclerosis patients. Figure 5 documents a higher expression of TIMP-2 in multiple sclerosis monocytes compared with controls. TIMP-1, TIMP-3 and TIMP-4 were not different in monocytes between multiple sclerosis and controls (data not shown).

Discussion

The inflammatory process involves the recruitment of systemic leukocytes into tissues. Many factors and molecules regulate the entry of leukocytes, and the MMPs are considered one of the major requirements in the process of transmigration of leukocytes into tissues. In mice deficient for MMP-9, the chemotaxis of neutrophils to intradermally injected granulocyte chemotactic protein-2 was decreased compared with controls (D'Haese *et al*., 2000). In MMP-12 null mice, there was impaired lung recruitment of macrophages in response to cigarette smoke, which protected against smoke-induced emphysema (Hautamaki *et al*., 1997). During interleukin 13-induced inflammation in the lungs of mice, MMP-12 contributed to the accumulation of eosinophils and macrophages (Lanone *et al*., 2002). A requirement for metalloproteinases can also be demonstrated *in vitro*, where the capacity of various leukocyte subsets to transmigrate models of blood-tissue barriers is impaired by synthetic metalloproteinase inhibitors (Leppert *et al*., 1996; Xia *et al*., 1996; Graesser *et al*., 2000; Brundula *et al*., 2002) or by TIMP-1 (Delclaux *et al*., 1996; Uhm *et al*., 1999). In a very elegant experiment, Graesser *et al*. (1998) found that T lymphocytes could crawl across an endothelial cell barrier despite the presence of a metalloproteinase inhibitor, but they were hampered from penetrating the underlying basement membrane layer. Clearly, the role of MMPs in various inflammatory conditions deserves attention.

The infiltration of leukocytes into the CNS is generally agreed to be an important event in the evolution of the multiple sclerosis disease process (Martin *et al*., 2001; O'Connor *et al*., 2001; Hemmer *et al*., 2002). With respect to the leukocyte subsets, a critical role for T lymphocytes in initiating the pathology of multiple sclerosis has been inferred from animal experiments where the adoptive transfer of myelin reactive $CD4^+$ or $CD8^+$ T cells (Pettinelli and McFarlin, 1981; Zamvil *et al*., 1985; Huseby *et al*., 2001) into recipient animals led to a multiple sclerosis-like disease, EAE. Nonetheless, monocytes (which become macrophages when these infiltrate into tissues) may be the final mediators of the disease since the *in vivo* depletion of these cells (by the use of mannosylated liposomes containing dichloromethylene diphosphonate) led to a marked suppression of disease (Huitinga *et al*., 1990). In animals depleted of monocytes, lymphocytes tended to accumulate in the subarachnoid spaces of the leptomeninges around spinal cord and did not infiltrate the CNS parenchyma (Tran *et al*., 1998). Other potential roles of monocytes or microglia in the neuroinflammatory process may be the damage to the CNS through several mechanisms including the production of proinflammatory cytokines and free radicals (Benveniste, 1997). Axonal injury observed in the brains of patients with multiple sclerosis is correlated not only with the presence of $CD8^+$ T lymphocytes, but also with the presence of macrophages and microglia (Bitsch *et al*., 2000; Kuhlmann *et al*., 2002).

Since leukocyte infiltration is critical to disease evolution in multiple sclerosis and as MMPs are important to the transmigration of cells into tissues, the expression of MMPs by particular leukocyte subsets deserves attention. While there are a number of publications on mixed peripheral blood leukocyte populations, there are only sparse reports of MMP expression in distinct leukocyte subsets in normal conditions, let alone in multiple sclerosis cases. Kouwenhoven *et al*. (2001) used *in situ* hybridization protocols and determined that there was a larger number of monocytes from multiple

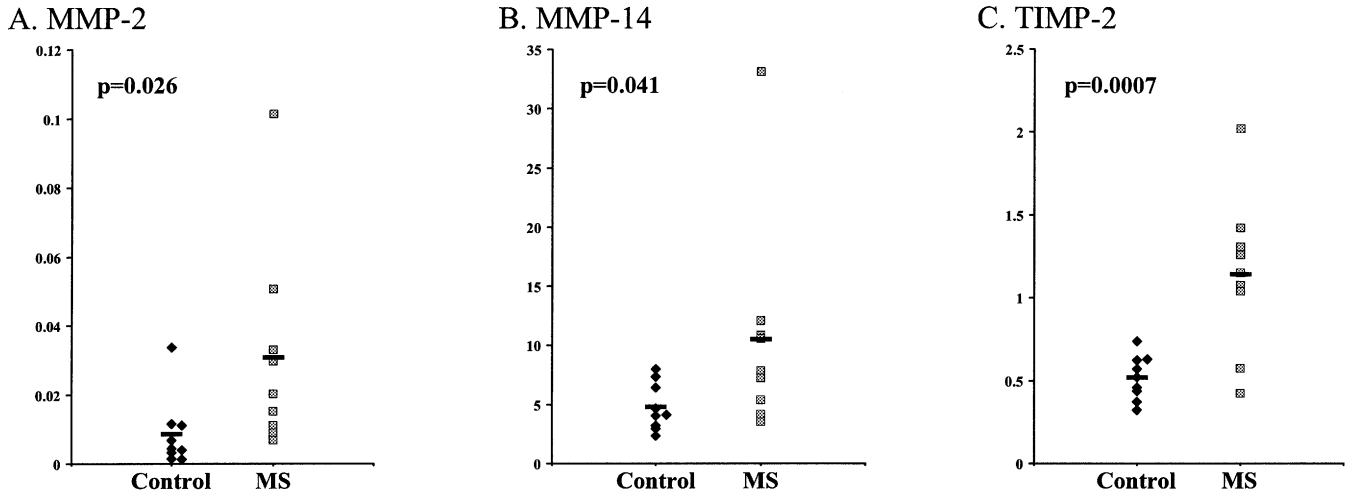

Fig. 5 Monocytes from multiple sclerosis patients with active disease have higher levels of certain MMPs compared with controls. MMP-2 and MMP-14 in unstimulated monocytes from multiple sclerosis patients are statistically different from those of controls, as is that for TIMP-2. Each point is of a different individual and the bar represents the mean. Unpaired Student's *t*-test was used for statistical comparisons. MS = multiple sclerosis.

sclerosis patients expressing MMP-1, MMP-3, MMP-7, MMP-9 and of TIMP-1 mRNA compared with normal subjects. Using immunohistochemistry analyses of active multiple sclerosis lesions, macrophages were found to be positive for MMP-2, MMP-7, MMP-9 and MMP-12 (Anthony *et al*., 1997; Vos *et al*., 2003). Nonetheless, these few existing studies have focused on a small group of MMPs. Thus, in this study, we have undertaken the analyses of all known human MMPs and have performed these in distinct leukocyte subsets under unstimulated or stimulated conditions. We have found a distinctive pattern of expression of certain MMPs in specific subsets and the enrichment of many MMPs in monocytes. When evaluated under identical conditions, monocytes were more migratory than B lymphocytes, which were correspondingly more transitory than T cells. We propose that the high migratory capacity of monocytes and their significant expression of MMP members is causally related, since transmigration across an endothelial barrier is reduced by an inhibitor of MMP activity, TIMP-1 (Fig. 4B); the inhibition of monocyte migration by TIMP-1 has also been reported recently by others (Séguin *et al*., 2003).

It is emphasized that our current analyses of MMP expression is at the transcriptional level, as this allows the simultaneous investigation of all known mammalian MMP members; protein reagents to examine many MMP members are still lacking or not optimized. MMPs are regulated at multiple points, one of which is at the level of mRNA expression. Other levels include pro-enzyme activation, post-translational modification, and regulation of activity by endogenous inhibitors (Yong *et al*., 2001; Chang and Werb, 2001). Future experiments will address whether the analyses of MMP transcripts here are reflected in changes of ultimate enzyme activity; in most systems, however, there is good correspondence between transcript and expression of MMP enzyme activity.

When comparing monocytes from multiple sclerosis and normal individuals, we found that MMP-2, MMP-14 and TIMP-2 were concordantly elevated in the multiple sclerosis samples. Although TIMP-2 is an inhibitor of activated MMPs, it serves an additional and contrasting role in the case of pro-MMP-2, which it activates. It had been noted that the cell surface activation of pro-MMP-2 by MMP-14 required the interaction with TIMP-2 (Emmert-Buck *et al*., 1995; Strongin *et al*., 1995). Subsequently, it was shown that MMP-14 functions as a receptor for TIMP-2 (but not TIMP-1) and/or the pro-MMP-2/TIMP-2 complex, and that this facilitates the activation of pro-MMP-2 by an adjacent MMP-14 (reviewed by Murphy and Knauper, 1997). Our results thus indicate that monocytes from multiple sclerosis patients not only express specific MMPs and TIMPs in higher amounts than controls, but that they are also endowed with the mechanisms to activate pro-MMP-2 to achieve various functions. The tripartite of MMP-2, MMP-14 and TIMP-2 could be targets for therapeutic intervention in multiple sclerosis to alleviate the entry of monocytes into the CNS.

In the current study, MMP-12, MMP-13 and MMP-20 were below detection limits either when leukocytes were in unstimulated or stimulated conditions. That the primers and probes for these MMPs are functional is indicated by analyses of other cell types. MMP-12 and MMP-13 were detected in various cell lines in our recent study of MMPs in gliomas (Nuttall *et al*., 2003), while we have found MMP-20 in tooth pulp tissue (data not shown). The lack of detection for MMP-12, MMP-13 and MMP-20 does not necessarily mean that these MMPs are not important in leukocyte biology or in multiple sclerosis; rather, it is possible that optimal stimulation to upregulate these MMPs was not used in this study. For

example, MMP-12 can be upregulated in monocytoid cells by granulocyte-macrophage colony stimulating factor (GM-CSF) (Feinberg *et al*., 2000; Wu *et al*., 2000), and it would be of interest to use GM-CSF activated monocytes for future studies to evaluate a role for MMP-12 in human leukocyte biology. MMP-13 has been reported in macrophages in horses with chronic obstructive pulmonary diseases (Raulo *et al*., 2001) and in rat alveolar macrophages when stimulated with LPS, PMA or IgA immune complexes (Gibbs *et al*., 1999). Besides stimulation conditions, our inability to detect MMP-13 in this study could be related to species differences.

As noted earlier, other groups have found the upregulation of MMP-2, MMP-7, MMP-9 and MMP-12 in the brain of patients with multiple sclerosis (Anthony *et al*., 1997; Cossins *et al*., 1997; Lindberg *et al*., 2001; Vos *et al*., 2003). We did not find an elevation of MMP-7 and MMP-9 in the monocytes taken from multiple sclerosis patients in this study, indicating that other cell types may be contributing primarily to these elevated MMPs in the lesions of patients with multiple sclerosis; alternatively, these genes are turned on only when monocytes/macrophages are within the CNS. Indeed, the results of our study have revealed new areas for further studies involving other leukocyte subsets, such as the role of MMP-11 and MMP-28 in B and T cells, respectively, in multiple sclerosis.

In summary, our investigation sheds light on the increasing roles that MMPs are thought to play in the multiple sclerosis disease process (Hartung and Kieseier, 2000; Leppert *et al*., 2001; Yong *et al*., 2001). We have conducted the most comprehensive profiling of all MMPs to date in leukocytes and have further presented the data with respect to distinct leukocyte subsets. Our results reveal the abundance of MMP expression in monocytes, which correlates with their high migratory capacity, and emphasizes the role that monocytes play in the neuroinflammatory process in multiple sclerosis. Finally, we specify MMPs for inhibition in order to alleviate monocyte entry into the brain of patients with multiple sclerosis: MMP-2 and MMP-14. The elevation of TIMP-2 in monocytes in multiple sclerosis provides a mechanism for the activation of MMP-2 by MMP-14 in the disease process.

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