Differential transcription of matrix-metalloproteinase genes in primary mouse astrocytes and microglia infected with Theiler’s murine encephalomyelitis virus

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The BeAn strain of Theiler’s murine encephalomyelitis virus (TMEV) induces demyelinating disease in susceptible mice comparable to human multiple sclerosis. Recent in vivo studies showed that matrix metalloproteinases (MMPs) and their inhibitors (tissue inhibitors of MMPs, TIMPs) are associated with demyelination in Theiler’s murine encephalomyelitis. The present study was performed to evaluate the in vitro MMP and TIMP expression in astrocytes and microglia following TMEV infection. Brain cell cultures from SJL/J mice were infected with the BeAn strain of TMEV and the expressions of 11 MMPs and 4 TIMPs were evaluated by reverse-transcription quantitative polymerase chain reaction (RT-qPCR) at different time points post infection (p.i.). In control astrocytes and microglia, a constitutive expression of MMP-2, -3, -9, -10, -12, -13, -14, -15, -24 and TIMP-2 to -4 was detected. In addition, TIMP-1 and MMP-11 was found in astrocytes only, and MMP-7 was absent in both cells cultures. RT-qPCR demonstrated high virus RNA copy numbers in astrocytes and a low amount in microglia. In accordance, TMEV antigen was detected in astrocytes, whereas it was below the limit of detection in microglia. MMP-3, -9, -10, -12, and -13 as well as TIMP-1 were the enzymes most prominently up-regulated in TMEV-infected astrocytes. In contrast, TMEV infection was associated with a down-regulation of MMPs and TIMPs in microglia. Conclusively, in addition to inflammatory infiltrates, TMEV-induced astrocytic MMPs might trigger a proteolysis cascade leading to an opening of the blood-brain barrier and demyelination in vivo. Journal of NeuroVirology (2008) 14, 205–217.

Keywords: brain cell culture; mouse; MMPs; TIMPs; TMEV

Introduction

Theiler’s murine encephalomyelitis virus (TMEV), a cardiovirus of the Picornaviridae family (Theiler, 1934), induces a demyelinating encephalomyelitis (Theiler’s murine encephalomyelitis, TME) depending on the virus strain used and the mouse strain infected (Lipton, 1975). TME is a relevant model for the chronic progressive form of human multiple sclerosis (MS; Stohlman and Hinton, 2001; Welsh et al, 1990).
In vivo and in vitro studies revealed that TMEV can be found in astrocytes, macrophages, microglia, and oligodendrocytes. However, the cellular tropism of TMEV seems to differ depending on the virus strain and the detection system used (Aubert et al., 1987; Lipton et al., 1995; Oleszak et al., 2004; Tsunoda et al., 1996; Zheng et al., 2001; Zoécklein et al., 2003). In BeAn-infected SJL/J mice, TMEV antigen was mainly detected in macrophages, and to a lesser extend in oligodendrocytes and astrocytes (Dal Canto and Lipton, 1982; Lipton et al., 1995). In contrast, in SJL/J mice infected with the DA strain, viral RNA was more often found in oligodendrocytes, astrocytes, and to a lesser extend in microglia/macrophages (Aubert et al., 1995; Oleszak et al., 2004; Tsunoda et al., 1996; Zheng et al., 2001; Zoécklein et al., 2003). In BeAn-infected SJL/J mice, TMEV antigen was mainly detected in macrophages, and to a lesser extend, in oligodendrocytes and astrocytes (Dal Canto and Lipton, 1982; Lipton et al., 1995). In contrast, in SJL/J mice infected with the DA strain, viral RNA was more often found in oligodendrocytes, astrocytes, and to a lesser extend in microglia/macrophages (Aubert et al., 1987). Furthermore, 100 times more viral RNA and 4 times more viral antigen-positive cells were noticed in DA-infected mice compared to BeAn-infected mice (Zoécklein et al., 2003). The induction of the innate immune response by TMEV and other Picornaviruses seems to be mediated by multiple cytoplasmic double-stranded RNA (dsRNA)-sensor proteins such as melanoma differentiation-associated gene-5 (Gitlin et al., 2006), protein kinase R (Carpentier and Williams, 2007), as well as endosomal Toll-like receptor 3 (So et al., 2006). TMEV infection of primary astrocytes leads to the induction of type I interferons and other proinflammatory chemokine and cytokine genes (So et al., 2006; Carpentier and Williams, 2007), most likely via the nuclear factor (NF)-κB pathway (Palma et al., 2003; Palma and Kim, 2004).

Rubio and Martin-Clemente (1999) found that TMEV was quickly internalized in mouse brain astrocytes and actively replicated in the cytoplasm. c-fos expression peaked 30 min post infection (p.i.) and disappeared 2 h.p.i. and was directly virus induced. Studies with astrocytes originating from mice susceptible and resistant to demyelination showed that following TMEV infection, macrophage inflammatory protein (MIP)-2 chemokine was only induced in astrocytes from genetically susceptible mice (Rubio et al., 2006). Interleukin (IL)-1 gene expression is restricted to astrocytes originating from susceptible mouse strains after TMEV infection (Rubio and Capa, 1993).

Mun-Bryce et al. (2002) found that matrix metalloproteinases (MMPs) are up-regulated prior to the induction of cytokines such as tumor necrosis factor (TNF)-α after lipopolysaccharide (LPS)-induced neuroinflammation. MMPs are enzymes degrading extracellular matrix (ECM) molecules (Matrisian, 1990; Stamenkovic, 2003; Woessner, 1991). According to their substrate specificity, they are categorized into collagenses (MMP-1, -8, -13), gelatinases (MMP-2, -9), stromelysins (MMP-3, -10, -11) including matrilysin (MMP-7), and membrane-type MMPs (MMP-14, -15, -16, -17, -24). MMP-12 or metalloelastase does not belong to a specific group (Massova et al., 1998; McCawley and Matrisian, 2001; Yong et al., 1998). Activity of MMPs is under control of tissue inhibitors of MMPs (TIMPs; Brew et al., 2000). MMPs are of importance for the development of demyelinating diseases as they open the blood-brain barrier, favor invasion and migration of inflammatory cells, trigger the release of TNF-α, and decompose myelin proteins (Baker et al., 2002; Ries and Petrides, 1995; Rosenberg et al., 1995). Association of MMPs with demyelinating diseases has been shown for MS (Cuzner and Opdenakker, 1999; Hartung and Kieseier, 2000; Leppert et al., 2001; Lindberg et al., 2001; Rosenberg, 2001, 2002, 2005). TMEV (Ulrich et al., 2006), experimental allergic encephalomyelitis (EAE; Toft-Hansen et al., 2004), canine distemper encephalomyelitis (Alldinger et al., 2006; Gröters et al., 2005; Miao et al., 2003), and mouse hepatitis virus (MHV) JHM strain (JHMV) infection of mice (Zhou et al., 2002, 2005). Recently, it was shown, that MMP-3 was significantly up-regulated 1 day p.i. (d.p.i.) and in the demyelinating phase of TMEV infection (28 to 196 d.p.i.). MMP-12 mRNA was prominently up-regulated in the demyelinating phase of infection and MMP-12 protein was present in intralesional microglia/macrophages and astrocytes. TIMP-1 mRNA was significantly elevated throughout the observation period (1 to 196 d.p.i.; Ulrich et al., 2006). Based on these findings the aim of this study was to determine the role of astrocytes and microglia as a source of MMPs and TIMPs following TMEV infection.

Results

TMEV infection of astrocytes and microglia
TMEV-infected astrocytes formed a confluent monolayer of viable cells throughout the studied time period. Control astrocytes displayed a flat polygonal shape, whereas in infected cultures astrocytes were enlarged consisting of prominent somata and processes. In addition, a mild to moderate cytopathic effect (CPE) consisting of round and detached cells (Figure 1) was noticed. TMEV protein was detected as early as 6 h.p.i. in the cytoplasm of glial fibrillary acidic protein (GFAP)-positive astrocytes. The percentage of TMEV antigen-positive cells increased from 2.0% ± 2.4% (6 h.p.i.) to 13.1% ± 6.5% and 13.1% ± 10.4% (48 and 72 h.p.i.) and decreased to 2.1% ± 3.7% 240 h.p.i. (Figure 2). Infectious virus was present throughout the observation period and dramatically increased from 8 × 10^2 plaque-forming units (PFU)/ml at 6 h.p.i. to 3.3 × 10^5 PFU/ml at 48 h.p.i. (Figure 2). TMEV RNA was first detected 6 h.p.i. and peaked 48 h.p.i. and was present until 240 h.p.i. (Figure 3A).

Control microglia cultures showed a mixed population of ameboid and resting microglia, whereas infected cultures mainly consisted of activated (phagocytic) microglia and single resting forms. Following infection, only a minimal brief transient CPE characterized by increased necrotic detached cells was observed; however, the total number of cells prominently declined towards 240 h.p.i. Virus protein...
MMPs in TMEV-infected astrocytes

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Figure 1 Primary mouse astrocytes infected with the BeAn strain of TMEV at 48 hours post infection (h.p.i.). Infected astrocytes are hypertrophic with an enlarged soma and an increased length of processes and with minimal cytopathic effect consisting of round and detached cells. (A) Phase-contrast microscopy; (B) green fluorescing TMEV antigen–positive cells (Cy2); (C) Red fluorescing GFAP-positive cells (Cy3); (D) Overlay of A and B (Cy2, Cy3, and blue fluorescing nuclei stained with bisbenzimide 0.01%). Scale bar = 6 μm; immunocytochemistry.

could not be detected in the infected microglia cell cultures by immunofluorescence at any time point. In contrast, TMEV RNA was firstly detected and peaking at 6 h.p.i. followed by a prominent decline at 240 h.p.i. (Figure 3B). In comparison, astrocytes displayed approximately 1000 to 10000 higher absolute copy numbers of TMEV RNA than microglia (Figure 3A and B).

MMP and TIMP transcripts

Uninfected astrocytes (day 0, controls) showed a high expression of MMP-2, -11, -14, -15, TIMP-2, -3, and -4. MMP-3, -9, -10, -12, and -24 were moderately expressed and MMP-13 and TIMP-1 showed a low expression. MMP-7 was absent (Figure 4A). Noninfected microglia (day 0, controls) showed a high expression of MMP-9, -12, -14, and TIMP-2. MMP-2, -3, -10, -13, -15, -24, and TIMP-4 were moderately expressed and TIMP-3 transcripts were only found in low amounts. MMP-7, -11, and TIMP-1 transcripts were not detected (Figure 4B).

In infected astrocyte cultures, MMP and TIMP mRNAs varied for individual proteins and time points. The MMPs/TIMPs most prominently and

Figure 2 Percentage of GFAP-positive cells (dot bar) and infected cells (grey bar) on the left y-axis (mean ± standard deviation) as determined by immunocytochemistry. The black line displays infectious virus on the right y-axis as determined by plaque assays.

Figure 3 Theiler's murine encephalomyelitis virus (TMEV) RNA transcripts measured by reverse transcriptase–quantitative polymerase chain reaction (RT-qPCR) in astrocytes and microglia. Box and whisker plots exhibit the median and quartiles of normalized copy numbers per 10 ng RNA. Extreme values are shown as circles. (A) In astrocytes, TMEV RNA was first detected at 6 h post infection (h.p.i.), peaked at 48 h.p.i. and declined at 240 h.p.i. (B) In microglia, TMEV RNA was first detected and peaked at 6 h.p.i. and prominently declined until 240 h.p.i. A significant difference between groups as detected by two-way ANOVA with post hoc independent t tests is marked as follows: *** p ≤ .001.
significantly affected by TMEV infection were MMP-3, -9, -10, -12, -13, and TIMP-1. Minor significant up- and down-regulations below a 2.0-fold change in infected compared to controls were observed for MMP-11, -14, -15, -24 and TIMP-3 and -4. Enzymes not significantly influenced by TMEV infection at the time points investigated included MMP-2 and TIMP-2. Similar to controls, MMP-7 was not detected. The relative expression of MMPs and TIMPs is summarized as the ratio of the geometric means of TMEV infected versus noninfected astrocytes in Table 1. MMP-3 was highly significantly up-regulated 48 h.p.i., followed by a peak at 72 h.p.i. and a decline at 240 h.p.i. (Figure 5A). MMP-9 was significantly down-regulated at 12 h.p.i. and significantly up-regulated at 48 h.p.i. and peaked at 72 h.p.i. (Figure 5B). The course of MMP-10 regulation was similar to that of MMP-3, showing a highly significant up-regulation 48 and 72 h.p.i. and a moderate up-regulation at 240 h.p.i. (Figure 5C). The significant up-regulation of MMP-12 was present at 48, 72, and 240 h.p.i. (Figure 5D). MMP-13 was the MMP most prominently up-regulated following TMEV infection and was significantly up-regulated as early as 12 h.p.i. Expression peaked at 72 h.p.i. and declined at 240 h.p.i. (Figure 5E). TIMP-1 was moderately significantly up-regulated beginning at 6 h.p.i., with a peak at 72 h.p.i. and a slight decline at 240 h.p.i. (Figure 5F).

In infected microglia cultures, an overall down-regulation of MMP and TIMP transcripts was observed. The relative expression of MMPs and TIMPs is summarized as the ratio of the geometric means of TMEV infected versus noninfected microglia in Table 2. MMPs/TIMPs displaying moderate (0.5- to 0.1-fold) down-regulations following TMEV infection were MMP-3, -9, -10, -11, -12, -24 and TIMP-2, and -3.

Though the recombinant murine MMP-3, -9, and -12 positive controls were readily detected in the Western blots, no MMP proteins were found in the concentrated supernatants of both astrocyte and microglia cell cultures.

**Correlation between TMEV, MMP, and TIMP expressions in astrocyte cultures**

The amount of viral RNA as measured by reverse-transcription quantitative polymerase chain reaction (RT-qPCR) and infectious virus expressed as PFU/ml showed a marked significant positive linear correlation ($r = .865, p < .001$). TMEV RNA expression was most prominently positively correlated to MMP-13.
(r = .673; p < .001) and TIMP-1 (r = .662, p < .001). Moderate positive correlations were calculated for TMEV RNA and MMP-3 (0.522, p < .001), MMP-10 (0.582, p < .001), and TIMP-3 (r = 0.568, p < .001). Further minor significant positive correlations were found for TMEV RNA and MMP-12 (r = 0.377, p < .001) and MMP-14 (r = 0.389, p < .001). MMP-11, -15, and TIMP-4 showed minor significant negative correlations to TMEV RNA (r = −.398, p < .001; r = −.291, p = .010; r = −.327, p = .003). MMP-13 was the only enzyme showing a moderate significant positive correlation to the amount of infectious virus (r = 0.552; p < .05).

Discussion

In this study the presence of transcripts of 11 MMPs and 4 TIMPs was evaluated in primary mouse astrocytes and microglia at different time points following infection with the BeAn strain of TMEV. Infected astrocytes showed an increased transcription of MMP and TIMP genes including most prominently MMP-3, -9, -10, -12, -13, and TIMP-1. Additionally, infected astrocytes displayed a mild to moderate cytotoxic effect characterized by single-cell necrosis and infectious virus was retrieved throughout the observation period. The reduced detection of infectious virus at 6 h.p.i. by using the plaque assay compared to the detection of viral RNA by RT-qPCR is most likely related to the early stage of the virus replication cycle, which lasts for 5 to 10 h for Picornaviridae (Racaniello, 2001). During this stage, viral RNA but not progeny virus can be detected easily. In addition, TMEV antigen was readily detected in infected cultures, indicating viral replication and viral protein synthesis. Astrocytes allowed TMEV BeAn to replicate at a higher rate compared to microglia, as reported previously in in vitro studies (Zheng et al., 2001). Microglia exhibited a restricted TMEV infection and allowed virus replication on a low level only. According to Oleszak et al. (2004), the susceptibility of microglia to TMEV infection is different from macrophages, and blood-borne activated macrophages represent the main cell type responsible for viral persistence within the lesions. Accordingly, in vitro studies showed that TMEV preferentially infects activated macrophages (Jelachich et al., 1999). Interestingly, delayed apoptosis of macrophages is a well-known feature of such an infection (Lipton et al., 2016).
Figure 5  Matrix metalloproteinase (MMP)-3, -9, -10, -12, -13, and tissue inhibitor of metalloproteinase (TIMP)-1 transcripts in cultured astrocytes as measured by reverse transcriptase–quantitative polymerase chain reaction (RT-qPCR). Box and whisker plots show the median and quartiles of normalized copies/10 ng RNA. Extreme values are shown as circles. (A) Moderate up-regulation of MMP-3 mRNA at 48 h post infection (h.p.i.), peaking at 72 h.p.i. and declining at 240 h.p.i. in infected cultures compared to controls. (B) Moderate down-regulation of MMP-9 mRNA at 12 h.p.i. and moderate up-regulation at 48 and 72 h.p.i. (C) A severe up-regulation of MMP-10 at 48 and 72 h.p.i. (D) Marked up-regulation of MMP-12 at 48, 72, and 240 h.p.i. (E) Significant up-regulation of MMP-13 from 24 to 240 h.p.i., peaking at 72 h.p.i. (F) Moderate up-regulation of TIMP-1 beginning from 48 h.p.i., peaking at 72 h.p.i. and slightly declining at 240 h.p.i. in infected cultures compared to controls. A significant difference between the groups as detected by two-way ANOVA with post hoc independent t tests is marked as follows: * $p \leq .05$; ** $p \leq .01$; *** $p \leq .001$. 
Table 2 Relative MMP and TIMP expressions during the time course of TMEV infection of microglia.

| Gene    | 6     | 48    | 72    | 240   |
|---------|-------|-------|-------|-------|
| **MMP-2** | 1.53  | 1.49* | 1.30  | 0.70* |
|         | (0.91–2.55) | (1.10–2.00) | (0.97–1.75) | (0.53–0.93) |
| **MMP-3** | 0.93  | 0.20* | 0.38  | 0.357* |
|         | (0.56–1.57) | (0.10–0.40) | (0.23–0.64) | (0.19–0.67) |
| **MMP7** | Not detected | Not detected | Not detected | Not detected |
| **MMP-9** | 0.50*** | 0.31*** | 0.33*** | 0.56*** |
|         | (0.39–0.65) | (0.23–0.43) | (0.24–0.45) | (0.43–0.72) |
| **MMP-10** | 1.29  | 0.40** | 0.73  | 0.80  |
|         | (0.72–2.31) | (0.29–0.56) | (0.49–1.07) | (0.55–1.15) |
| **MMP-11** | Not detected | 1.00  | 2.03  | 0.38*  |
|         | (0.37–2.69) | (0.68–6.01) | (0.16–0.92) | |
| **MMP-12** | 1.05  | 0.51*** | 0.76* | 0.20*** |
|         | (0.77–1.41) | (0.42–0.61) | (0.60–0.95) | (0.16–0.26) |
| **MMP-13** | 4.62*** | 0.61* | 0.91  | 0.41  |
|         | (3.14–6.78) | (0.39–0.94) | (0.60–1.39) | (0.12–1.37) |
| **MMP-14** | 0.81  | 1.63*** | 1.10  | 0.68*** |
|         | (0.61–1.07) | (1.31–2.03) | (0.81–1.51) | (0.57–0.81) |
| **MMP-15** | 0.62** | 0.94  | 1.52* | 0.61  |
|         | (0.47–0.82) | (0.67–1.34) | (1.03–2.25) | (0.33–1.15) |
| **MMP-24** | 0.67* | 1.02  | 0.82  | 0.18*** |
|         | (0.49–0.93) | (0.74–1.39) | (0.53–1.28) | (0.09–0.39) |
| **TIMP-1** | Not detected | Not detected | 1.33  | Not detected |
|         | (0.64–2.77) | |
| **TIMP-2** | 0.79* | 0.85  | 0.53*** | 0.11*** |
|         | (0.64–0.98) | (0.65–1.10) | (0.40–0.71) | (0.09–0.14) |
| **TIMP-3** | 0.94  | 1.46* | 1.21  | 0.15*** |
|         | (0.21–4.29) | (1.05–2.04) | (0.86–1.66) | (0.11–0.22) |
| **TIMP-4** | 0.83  | 1.03  | 0.85  | 0.18*** |
|         | (0.59–1.18) | (0.69–1.53) | (0.58–1.24) | (0.10–0.31) |

Note. Values show the ratio of the geometric means of normalized copy numbers/10 ng RNA from infected versus control microglia, with the upper and the lower limits of the 95% confidence interval. Moderate (0.5- to 0.1-fold) down-regulations are marked in light gray. Significant difference of the ratio from one as revealed by two-way ANOVA with post hoc independent t tests is marked as follows: *p ≤ .05; **p ≤ .01; ***p ≤ .001.

2005). This delayed apoptosis might also occur in virus-infected microglia and is accompanied by interrupted host protein synthesis and thus results in a down-regulation of MMPs and TIMPs in microglia as seen in the present study.

Constitutive MMP and TIMP expression in noninfected astrocyte and microglia cell cultures

MMP-2, -3, -9, -10, -11, -12, -13, -14, -15, and -24 mRNAs were constitutively expressed in control astrocyte cultures, whereas MMP-7 was absent. In addition, all four known TIMPs were present in control astrocytes. The observed expression profile of astrocytes and microglia is in accordance with previous findings (Crocker et al., 2006; Gottschall et al., 1995; Liuzzi et al., 2004). Similarly, MMP-12 is among the most highly expressed genes in microglia and the total copy number is approximately 100-fold higher than in astrocytes.

MMPs in TMEV-infected astrocytes

In the present study, infection of primary astrocytes from SJL/J mice with the BeAn strain of TMEV induced increased transcription of MMP and TIMP genes, including most prominently MMP-3, -9, -10, -12, -13, and TIMP-1. Minor significant changes were found for MMP-11, -14, -15, -24 and TIMP-3 and -4. Enzymes not significantly influenced by TMEV infection included MMP-2, -7, and TIMP-2. Though a high degree of purity of enriched astrocyte cultures was obtained, a contributing role of few contaminating other glial cells, especially microglia, as a potential source of the observed MMP and TIMP up-regulation cannot be ruled out. Subsequently, isolated and infected microglia were investigated for MMP and TIMP expression. However, in contrast to astrocytes, TMEV infection of microglia resulted in an overall down-regulation of MMP and TIMP transcripts.

MMP-2 transcripts were not significantly changed after TMEV infection of astrocytes. This finding parallels the results of an in vivo study (Ulrich et al., 2006). In MS, however, MMP-2 up-regulation seems to be associated with the early events in plaque formation (Lindberg et al., 2001).

MMP-3 gene expression was significantly up-regulated in infected astrocytes as early as 48 h.p.i. and until the end of the observation period at 240 h.p.i. Similarly, in an in vivo study evaluating MMP and TIMP gene expression following TMEV BeAn infection of SJL/J mice, MMP-3 was found to be significantly up-regulated on 1 d.p.i. and from 28 to 196 d.p.i. (Ulrich et al., 2006), although the transferability of the in vivo situation to the more complex in vivo situation remains unknown. Interestingly, in coronavirus infection of mice, another viral model for MS, increased MMP-3 expression seemed to be restricted to astrocytes (Zhou et al., 2005). In acute and subacute demyelinating canine distemper encephalitis, MMP-3 proteins, together with MMP-1, -7, -9, -12, -13, and -14, were present in astrocytes and microglia/macrophages (Miao et al., 2003). In MS, MMP-3 mRNA transcripts were up-regulated in early lesions characterized by an inflammatory edema of myelin sheaths; however, overt demyelination was not observed (Lindberg et al., 2001).

In infected astrocytes, MMP-9 displayed an increased expression pattern similar to MMP-3 at 48 and 72 h.p.i.; however, there was no significant difference compared to controls at 240 h.p.i. In contrast, MMP-9 belonged to the genes significantly down-regulated in infected microglial cultures and seemed not to be affected by TMEV BeAn infection in vivo (Ulrich et al., 2006). This difference might be explained by the absence of immune cell-mediated signals following virus-infection in vitro. MMP-9 activity and protein expression is inhibited by interferon-β (IFN-β) and -γ (Ma et al., 2001). Decreased MMP-9 mRNA levels and suppressed MMP-9
promoter activity are dependent on the transcription factor STAT-1α (Ma et al., 2001). Interestingly, in in vivo studies, IFN-γ gene expression is significantly up-regulated following TMEV BeAn infection (Gerhauser et al., 2007b) and, as expected, it is absent in TMEV BeAn–infected astrocytes (Gerhauser et al., unpublished observation). In MS, MMP-9 is up-regulated (Cossins et al., 1997; Lindberg et al., 2001) and is believed to be a key element in the blood-brain barrier breakdown (Rosenberg et al., 1995; Rosenberg, 2002). In mice with EAE, contradictory results have been obtained concerning MMP-9 expression (Clements et al., 1997; Pagenstecher et al., 1998; Teesalu et al., 2001; Toft-Hansen et al., 2004).

Another significantly up-regulated MMP after TMEV BeAn infection of astrocytes was MMP-10. This enzyme is among the most prominently up-regulated MMPs and TIMPs in EAE, another model for MS (Toft-Hansen et al., 2004). In this study, MMP-10 expression was associated with cells forming perivascular cuffs. A strong MMP-10 expression has further been observed in neoplastic gemistocytic astrocytes and seems to be related to a worse prognosis of astrocytic tumours compared to oligodendrogliomas (Thorns et al., 2003). MMP-10 up-regulation might thus be a stereotypical sign of astrocytic activation following various insults.

In infected astrocytes, MMP-12 expression was significantly up-regulated 24 h later than the other MMPs and TIMP-1. In vivo, MMP-12 was the enzyme most prominently up-regulated in the demyelinating phase of TME (Ulrich et al., 2006). Although MMP-12 expression is up-regulated in astrocytes following TMEV infection, it remains unaltered or moderately down-regulated in microglia. Still, the absolute amount of MMP-12 mRNA in microglia cultures was higher than in astrocyte cultures. Because it is unclear whether the in vitro situation is directly transferable to the more complex in vivo situation, the previously reported up-regulation of MMP-12 in the spinal cord in the late phase of TME can be the result of (a) an up-regulation of MMP-12 transcription in reactive astrocytes and (b) an increase in the number of microglia and macrophages or (c) a combination of both. In addition, it still remains a possibility that microglia cocultured with other brain cells including astrocytes might respond differently to TMEV infection compared to single-cell cultures. A stimulation of microglial/macrophase MMP-12 expression by other indirectly virus-induced pathways might therefore play an essential role during the in vivo pathogenesis of TMEV-induced myelin loss. This is supported by the immunohistochemical demonstration of MMP-12 in astrocytes as well as microglia/macrophages within lesions in vivo (Ulrich et al., 2006). Furthermore, there is a severely increased number of intraleisonal microglia/macrophages following TMEV infection of SJL/mice (Gerhauser et al., 2007a). MMP-12 or macrophase metalloelastase is able to cleave various molecules including myelin basic protein (MBP; Chandler et al., 1996; Gronski et al., 1997). In MS and EAE, MMP-12 was present in demyelinating lesions and is suspected to be involved in myelin destruction (Pagenstecher et al., 1998; Vos et al., 2003). In coronavirus infection of mice, MMP-12 was expressed by central nervous system (CNS) resident cells and inflammatory cells (Zhou et al., 2005). In chronic demyelinating distemper encephalitis, the protein of MMP-12 was detected in macrophages, microglia, astrocytes, and perivascular mononuclear infiltrates (Miao et al., 2003).

Surprisingly, in infected astrocytes, MMP-13 showed the fastest and most prominent up-regulation post infection. In addition, MMP-13 mRNA was most strikingly positively correlated to TMEV RNA and infectious virus expression. MMP-13 up-regulation is described in demyelinating canine distemper encephalitis, a disease with prominent infection of astrocytes (Miao et al., 2003) and in bacterial meningitis (Kieseier et al., 1999). Although a robust mesenchymal expression and collagenolytic activity of MMP-13 is known to be a key feature in pathological conditions such as osteoarthritis and rheumatoid arthritis (Im et al., 2007; Neuhold et al., 2001), the function and contributing role of MMP-13 during neuroinflammatory and neurodegenerative disease remains undetermined and warrants further studies.

The TIMP most significantly affected by TMEV BeAn infection in astrocytes was TIMP-1, whereas its expression was below the detection limit in microglia and remained unchanged after infection. In vivo, TIMP-1 mRNA was significantly up-regulated in the acute and chronic disease (Ulrich et al., 2006). Although the transferability of the in vitro situation to the more complex in vivo situation is unclear, up-regulation of TIMP-1 represents a well-known response of astrocytes to injury (Jaworski, 2000). However, TIMP-1 protein was mainly found in CD4+ lymphocytes and not in astrocytes and CD8+ cells in coronavirus infection of mice (Zhou et al., 2005).

In summary, the present study shows that TMEV BeAn infection of astrocytes induced increased transcription of MMP-3, -9, -10, -12, -13, and TIMP-1. In contrast, TMEV infection of microglia resulted in an overall down-regulation of MMP and TIMP transcripts. Thus, enhanced transcription of proteolytic enzymes following TMEV infection of astrocytes might be the initial event in proteolysis observed in demyelinating diseases and astrocytes might be the key cells triggering processes leading to demyelination. However, the complex interactions between astrocytes, microglia and TMEV need to be investigated in detail in further studies.

Materials and methods

Animals

Adult SJL/J mice were purchased from Harlan Winkelmann (Borchen, Germany) and maintained...
in a microisolator cage system (Tecniplast, Hohenpeissenberg, Germany) for breeding. One- to 3-day-old pups were used for cell isolation. Breeding and killing of animals was authorized by the ethical commission of the Landeshauptstadt Hannover (permission number 42500/1H).

**Astrocyte and microglia cultures**

Mixed glial cell cultures were established from cerebral hemispheres of 1- to 3-day-old mice according to McCarthy and deVellis (1989) and Giulian and Baker (1986). Briefly, cerebral cortices were isolated under a dissecting microscope and cleaned of meninges followed by mechanical dissociation in calcium-free phosphate-buffered (PBS), pH 7.4, containing 0.8 g/L Na-EDTA, 2 mg/ml trypsin, and 0.2 mg/ml DNase I (Roche Diagnostics, Mannheim, Germany) and incubation at 37°C for 20 min. After centrifugation at 250 × g at 4°C for 10 min, cell pellets were resuspended in defined Sato’s medium (Bottenstein and Sato, 1979), supplemented with 10% fetal calf serum (FCS), and cells were singulated using a fire-polished Pasteur pipette. Cells (5 × 10⁶) were seeded on poly-l-lysine (100 μg/ml)–coated 75-ml flasks (Nunc, Wiesbaden, Germany). Cultures were maintained in Sato’s medium–10% FCS under standard conditions (5% CO₂, 37°C) and medium was changed 24 h, 72 h, and 6 days after seeding.

Seven days after seeding, the cells reached confluence. Flasks were sealed with Parafilm and microglia were separated by shaking at 37°C, 150 rpm, on a rotary platform shaker (Innova 2000; New Brunswick Scientific, New Jersey, USA) for 45 min. The flasks were then washed twice and the remaining cells were cultured in Sato’s medium–10% FCS for at least 4 h. Then the flasks were agitated twice overnight to remove oligodendrocyte precursor cells. The flasks were sealed with Parafilm and shaken at 200 rpm, 37°C, on a rotary platform shaker. The remaining astrocyte cultures reproducibly displayed a purity of 90% to 95%. The remaining cells included few fibroblasts and other glial cells like microglia. Microglia cultures were 97% pure. The enriched astrocytes and microglia were seeded onto 24-well plates (Costar; Corning, Germany; 1 × 10⁵ cells/well) and cultured in Sato’s medium–10% FCS. For microglia, the medium was supplemented with macrophage colony-stimulating factor (M-CSF; 40 ng/ml). The medium was changed every second day. The experiment started 24 h after seeding. Quantification of cells was performed by counting five different high-power fields in one coverslip preparation.

**Immunofluorescence and controls**

TMEV was visualized by using a mouse monoclonal antibody directed against VP1 of the DA strain of TMEV diluted 1:100 (DAmAb2, kindly provided by Prof. Raymond P. Roos, Department of Neurology, University of Chicago Medical Center, Chicago Illinois). Astrocytes were identified using a polyclonal rabbit anti-glial fibrillary acidic protein (GFAP) antibody (Dako Cytomation, Hamburg, Germany) diluted 1:400. Microglia were identified using a polyclonal rabbit anti-Iba-1 antibody (Wako Chemicals, Neuss, Germany) diluted 1:250 and 1’,1’-dioctadecyl-3,3’,3’-tetramethylindocarbocyanine perchlorate (Dil-acLDL; Paesel+Lorey, Hanau, Germany). Primary mouse fibroblasts served as negative controls for immunocytochemistry. For double immunostaining, cultures grown on coverslips were washed twice with Sato’s medium, followed by PBS and fixed with 4% paraformaldehyde, pH 7.4, for 15 min at room temperature. Primary antibodies were added to PBS containing 5% horse and goat normal serum and 0.25% Triton X-100. Cells were incubated with this solution overnight at 4°C. Secondary antibodies included Cy2-conjugated AffiniPure goat anti-mouse immunoglobulin G (IgG) (H+L) and Cy3-conjugated AffiniPure goat anti-rabbit IgG (H+L; Jackson ImmunoResearch Laboratories, Dianova, Hamburg, Germany) diluted 1:200. Cultures were finally subjected to a nuclear stain with 0.01% bis-benzimide (Sigma-Aldrich, Munich, Germany) in distilled water 10 min at room temperature. Coverslips were mounted with fluorescent mounting medium (Dako Cytomation) and observed under an inverted microscope (Olympus IX-70; Olympus Optical, Hamburg, Germany). Microphotographs were taken using the PM-30 photo system (Olympus Optical) and a color reversal film ISO400 (Ektachrome 400X EPL135-36; Kodak, Rochester, New York).

**Virus and experimental design**

The TMEV BeAn 8386 strain was originally isolated from a feral mouse in Belem, Brazil, in 1957 (Rozhon et al, 1983; a gift from Dr. H. L. Lipton, Department of Neurology, Northwestern University Medical School, Chicago, Illinois). It was propagated in baby hamster kidney (BHK)-21 cells (a gift from Prof. Raymond P. Roos) cultured in Dulbecco’s modified Eagle’s medium (DMEM; PAA Laboratories, Pasching, Germany) supplemented with 10% FCS and 100 U/ml penicillin/100 μg/ml streptomycin. Virus with a titer of 5.5 × 10⁶ PFU/ml was used for the experiments. Cells were infected with TMEV BeAn at a multiplicity of infection (MOI) of 1.0 at 24 h post seeding using 2 × 10⁵ PFU per well. Mock-infected controls received Sato’s medium–10% FCS only. Supernatants and total cellular RNA of TMEV-infected astrocyte cultures and controls were harvested 0, 6, 12, 24, 48, 72, and 240 h.p.i. Supernatants and total cellular RNA of TMEV-infected microglia cultures and controls were harvested 0, 6, 48, 72, and 240 h.p.i. Plaque assays were done in duplicates. Briefly, supernatants serially diluted from 10⁻² to 10⁻⁶ were used for inoculation of confluent monolayer cultures of murine lung tumor cells (L2 cells; a gift from Prof. C. Jane Welsh, Department of Veterinary Integrative Biosciences, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, Texas) with TMEV. Plaques were counted after 4 days of incubation.
Station, Texas) in 6-well plates (Nunc, Wiesbaden, Germany), including 1 well for negative controls. L2 cells were incubated with the serially diluted supernatants for 1 h at room temperature, followed by the application of 0.4% methylcellulose in DMEM with 2% FCS. Plaques were stained with 1% crystal violet after 72 h and the number of plaques was evaluated and expressed as PFU/ml.

RT-qPCR
RNA was isolated from 6-cell culture wells/time point using the RNasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions as described (Gerhauser et al., 2005; Markus et al., 2002). RNA concentration was measured at 260 nm employing a spectral photometer (GeneQuant Pro; Amersham Biosciences Europe, Freiburg, Germany) and final RNA concentration was set to 10 ng/μl. RNA was reversely transcribed using the Omniscript Reverse Transcriptase Kit (Qiagen), 10 μM random hexamers (Random Primers; Promega, Mannheim, Germany), and 0.5 U/μl Rnase inhibitor (RNase OUT; Invitrogen, Karlsruhe, Germany). Quantitative polymerase chain reaction (qPCR) was performed with 1 μl cDNA/25 μl reaction with the Brilliant SYBR Green qPCR Core Reagent Kit (Stratagene Europe, Amsterdam, The Netherlands) using the MX3005P™ Multiplex Quantitative PCR System (Stratagene) with optimized reaction conditions. Primer pairs specific for TMEV, MMP (-2, -3, -7, -9, -10, -11, -12, -13, -14, -15, -24), TIMP (-1 to -4), and housekeeping gene GAPDH, HPRT, SDHA, β-actin) RNAs were used as positive controls. The separated proteins were transferred to Trans-Blot Transfer Medium (Bio-Rad Laboratories, Munich, Germany) by a tank blotting system (Carl Roth, Karlsruhe, Germany) at 250 V and 400 mA for 1.5 h. After blocking of nonspecific binding by 5% skim milk powder (Merck, Darmstadt, Germany), the membranes were incubated with either a monoclonal rabbit anti-MMP-3 antibody diluted 1:1000 (Epitomics, Burlingame, USA), a polyclonal rabbit anti-MMP-9 antibody diluted 1:2000 (Abcam, Cambridge, UK), or a polyclonal goat anti-MMP-12 antibody diluted 1:250 (Santa Cruz Biotechnology, Santa Cruz, USA), respectively. After incubation with a horseradish peroxidase–conjugated secondary antibody, protein was visualized using Super Signal West Pico Chemiluminescent Substrate ( Pierce, Rockford, USA).

Statistical analysis
Statistical analysis was accomplished using the SPSS for Windows Version 13.0 software package (SPSS). The numbers of immunofluorescence-positive cells are presented as mean and standard deviation. The normalized copy numbers/10 ng RNA obtained from the RT-qPCR were log-transformed prior to statistical analysis. RT-qPCR samples without measurable mRNA (no C_T) were set to detection limit divided by 2. Hereby, detection limit was either the external standard, the positive control, or the sample with the lowest amount of detectable mRNA and a correct melting point. Box and whisker plots were drawn to display the median and the quartiles. Statistical differences were evaluated by using two-way analysis of variance (ANOVA) for the factors group (control or TMEV infected) and time (time post infection) simultaneously with post hoc independent t tests for the different time points. Pearson’s product moment correlation coefficient was calculated to demonstrate the relationship between TMEV RNA/PFU, MMP, and TIMP mRNA expression. Statistical significance was designated as p ≤ .05.
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