Trabajo experimental 3 — J Neuroimmunol 2001; 119: 248-260

Altered inflammatory response and increased neurodegeneration in metallothionein I+II deficient mice during experimental autoimmune encephalomyelitis

Alteración de la respuesta inflamatoria y aumento de la neurodegeneración en ratones deficientes en metalotioneína I+II durante la encefalomielitis autoinmune experimental

En este trabajo se estudia las características clínicas e histopatológicas a nivel de la respuesta inflamatoria en el SNC en ratones deficientes en MT-I y MT-II (MTKO). Para ello se inmunizaron ratones MTKO y ratones control con el péptido 40-55 de la MOG. Se observó que los ratones MTKO eran más susceptibles a desarrollar la enfermedad que los ratones de la cepa control. Además, los ratones MTKO presentaron una respuesta inmunitaria aumentada significativamente en el SNC, a nivel de producción de citocinas proinflamatorias, marcadores de estrés oxidativo y de apoptosis celular respecto a los ratones control. Estos resultados sugieren que MT-I y MT-II están implicadas en la respuesta inflamatoria que se produce en el SNC durante la EAE y donde podrían tener una función protectora.

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Altered inflammatory response and increased neurodegeneration in metallothionein I + II deficient mice during experimental autoimmune encephalomyelitis

Milena Penkowa [a,1,2], Carmen Espejo [b,1,3], Eva M. Martínez-Cáceres [b,3], Christian Bjørn Poulsen [a,2], Xavier Montalban [b,3], Juan Hidalgo [c,*]

[a] *Department of Medical Anatomy, The Panum Institute, University of Copenhagen, DK-2200, Copenhagen, Denmark*
[b] *Unitat de Neuroimmunologia Clínica, Department of Neurology, Hospital General Vall d'Hebron, Universitat Autònoma de Barcelona, Barcelona, Spain*
[c] *Departamento de Biología Celular Fisiología e Inmunología, Unidad de Fisiología Animal, Facultad de Ciencias, Universidad Autónoma de Barcelona, Bellaterra, Barcelona 08193, Spain*

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Abstract

Metallothionein-I + II (MT-I + II) are antioxidant, neuroprotective proteins, and in this report we have examined their roles during experimental autoimmune encephalomyelitis (EAE) by comparing MT-I + II-knock-out (MTKO) and wild-type mice. We herewith show that EAE susceptibility is higher in MTKO mice relatively to wild-type mice, and that the inflammatory responses elicited by EAE in the central nervous system (CNS) are significantly altered by MT-I + II deficiency. Thus, during EAE the MTKO mice showed increased macrophage and T-lymphocytes infiltration in the CNS, while their reactive astrogliosis was significantly decreased. In addition, the expression of the proinflammatory cytokines interleukin-1β, interleukin-6, and tumor necrosis factor-α elicited by EAE was further increased in the MTKO mice, and oxidative stress and apoptosis were also significantly increased in MTKO mice compared to normal mice. The present results strongly suggest that MT-I + II are major factors involved in the inflammatory response of the CNS during EAE and that they play a neuroprotective role in this scenario.

Keywords: EAE/MS; Metallothionein (MT); Cytokines; Oxidative stress; Apoptosis

1. Introduction

Experimental autoimmune encephalomyelitis (EAE) is considered to be an autoimmune disease of the CNS and is used as an animal model for multiple sclerosis (MS). Animals with EAE show paralysis and histopathological infiltrates of microglia/macrophages, T lymphocytes, and reactive astrocytes (Benveniste, 1997; Lassmann, 1999; Popovich et al., 1997; Swanborg, 1995). During EAE/MS, a number of detrimental factors are produced and these can contribute to the ongoing inflammation and brain tissue damage (Brosnan and Raine, 1996). On the one hand, the production of proinflammatory cytokines interleukin-1 (IL-1), IL-6 and tumor necrosis factor-α (TNF-α) has been correlated with enhanced EAE disease, inflammatory response, and tissue destruction (Dal Canto et al., 1999; Douni et al., 1995–1996; Eng et al., 1996; Gijbels et al., 1995; Jacobs et al., 1991; Loughlin et al., 1994; Rajan et al., 1998; Renno et al., 1995; Taupin et al., 1997; Wiemann et al., 1998). Interestingly, mice with genetical IL-6 deficiency resist induction of EAE (Mendel et al., 1998; Okuda et al., 1998, 1999; Samoilova et al., 1998). Also, transgenic mice with overexpression of TNF-α show increased severity of EAE and/or spontaneous demyelinating disease (Campbell, 1998a,b; Dal Canto et al., 1999; Douni et al., 1995–1996; Probert et al., 1995; Taupin et al., 1997).

On the other hand, reactive oxygen species (ROS) are produced during EAE (Bagasra et al., 1995; Scott et al., 1997; Van Dam et al., 1995). ROS are major mediators of

* Corresponding author. Tel.: +34-93-5812037; fax: +34-93-5812390.
E-mail address: Juan.Hidalgo@uab.es (J. Hidalgo).
[1] These authors contributed equally to this paper.
[2] Tel.: +45-35327222; fax: +45-35327217.
[3] Tel.: +34-93-2746202; fax: +34-93-2746084.

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tissue damage in the CNS (Cassarino and Bennett, 1999; Sun and Chen, 1998), and they have been implicated in EAE pathogenesis and disease (Cross et al., 1994; MacMicking et al., 1992; Ruuls et al., 1995). Moreover, a number of studies have shown that blocking inducible nitric oxide synthase (iNOS) may prevent EAE development (Brenner et al., 1997; Ding et al., 1998; Zhao et al., 1996). Thus, a concomitant inhibition of agents such as proinflammatory cytokines and ROS during EAE could have a therapeutic potential for the treatment of EAE/MS and is likely to prove superior to a single agent therapy.

During CNS inflammation including EAE, microglia/macrophages and reactive astrocytes increase their expression of metallothionein I + II (MT-I + II), which possess antioxidant and tissue protective functions (Aschner, 1996, 1998b; Espejo et al., in press; Hidalgo et al., 1997; Lazo and Pitt, 1995; Lazo et al., 1998; Molinero et al., 1998; Penkowa and Hidalgo, 2000a,b; Penkowa et al., 1999b, 2000b; Tate et al., 1995; Thornalley and Vasak, 1985). In order to examine MT functions in vivo, a genetic approach using either MT-I + II deficient (MT knock-out, MTKO) or MT-I overexpressing mice has been used (Carrasco et al., 2000; Penkowa et al., 1999a,b, 2000a; Van Lookeren Campagne et al., 1999). In MTKO mice, an altered inflammatory response, increased oxidative stress and apoptosis, and a decreased neuroregenerative capacity were observed following traumatic brain damage or kainic acid-induced seizures (Carrasco et al., 2000; Penkowa et al., 1999a, 2000a). Moreover, transgenic overexpression of MT-I reduces tissue loss, vascular edema, and improves functional outcome following focal cerebral ischemia and reperfusion (Van Lookeren Campagne et al., 1999). In addition, we recently showed that exogenous administration of metallothionein-II (MT-II) significantly decreased the clinical symptoms, mortality, and leukocyte infiltration of the CNS during EAE (Penkowa and Hidalgo, 2000b) as well as oxidative stress and apoptotic cell death (Penkowa and Hidalgo, 2000b, 2001) in Lewis rats. The antioxidant and antiapoptotic effects of MT during EAE are likely beneficial, in that oxidative stress and apoptotic cell death of both oligodendrocytes and neurons are implicated in EAE/MS pathogenesis (Aktas et al., 2000; Alcazar et al., 1998; Arbizu-Urdiain and Martinez-Yelamos, 2000; Cross et al., 1994; MacMicking et al., 1992; Ruuls et al., 1995; Sabelko-Downes et al., 1999; Hisahara et al., 2001; Honegger and Langemann, 1989; Penkowa and Hidalgo, 2000b, 2001).

In this study, we analyzed the effect of genetical MT-I + II deficiency in 129/Sv mice (MTKO mice) during actively induced EAE. We hereby show that MT-I + II deficiency causes an increased susceptibility to the disease and an increased inflammatory response of microglia/macrophages and T lymphocytes while reactive astrogliosis is reduced. Also, the proinflammatory cytokines IL-1β, IL-6 and TNF-α and oxidative stress markers iNOS, nitrotyrosine (NITT), and malondialdehyde (MDA) were significantly increased in MTKO mice with EAE. As a likely consequence, these mice also showed an increased number of TUNEL + apoptotic cells, which were identified as neurons, oligodendrocytes, and astroglia. Therefore, we suggest that MT-I + II are potentially beneficial factors for the treatment of EAE/MS.

2. Materials and methods

2.1. Mice

Homozygous MTKO mice were generated as previously described (Masters et al., 1994). The MTKO mice were raised on the 129/Sv genetic background; therefore, mice from this strain were used as controls. Mice were 8 to 14 weeks old, and they were fed with standard chow and had access to water ad libitum. Anaesthesia was induced by intraperitoneal (i.p.) injection of 37 mg/kg of ketamine (Ketolar®, Parke and Davis, Morris Plains, NJ, USA) and 5.5 mg/kg of xylazine (Rompun®, Bayer, Leverkusen, Germany). Animal welfare was observed in compliance with the European Community regulations on this subject, and all animal experiments have adhered to the standards of the National Research Council's Guide for the care and use of laboratory animals.

2.2. Immunization

Animals were immunized by a single subcutaneous (s.c.) injection of 0.9% saline containing 100 μg of peptide 40–55 of rat myelin oligodendrocyte glycoprotein (MOG_{40-55}) emulsified in Freund's adjuvant (Sigma, St. Louis, MO, USA) (9:11 v/v) containing 4 mg/ml *Mycobacterium tuberculosis* H37RA (Difco Laboratories, Detroit, MI, USA). Rat MOG_{40-55} was synthesised by Dr. D. Andreu (Departament de Síntesi de Pèptids, Facultat de Química, Universitat de Barcelona, Spain). Mice received 0.05 ml of emulsion s.c. in the four limb flanks. At days 0 and 2 post-immunization, each mouse received 0.2 ml (2 IU/ml) of inactivated *Bordetella pertussis* (Vaxicoq®, Pasteur Merieux, Lyon, France) intravenously.

2.3. Clinical evaluation

All animals were weighed and examined daily, from day 7 to the end of the experiment (day 37 post-immunization), for neurological signs according to the following criteria: 0, no clinical signs; 0.5, partial loss of tail tonus for two consecutive days; 1, paralysis of whole tail; 2, mild paraparesis of one or both hind limbs; 3, paraplegia; 4, tetraparesis; 5, tetraplegia; 6, death. All experiments were performed in a blinded manner. The blind was established and preserved throughout the entire experimental process in such a way that the investigator examining and

clinically evaluating the animal was kept unaware of the strain of the mice.

2.4. Tissue processing

Animals were sacrificed with CO_2 at day 37 post-immunization and the CNS was resected. Tissues were fixed overnight in 4% paraformaldehyde/PBS. Following fixation, the samples were cryoprotected in 30% sucrose/PBS, frozen in 2-methylbutane cooled in liquid nitrogen and then stored at −80 °C until use.

Brain and spinal cord were cut into 30 μm thick serial, coronal sections on a cryostat and used for histochemistry and immunohistochemistry. The sections were incubated in 3.0% H_2O_2 in TBS/Nonidet for 15 min at room temperature to quench endogenous peroxidase. After washing in running tapwater, sections were washed in TBS/Nonidet three times for 5 min. The sections were preincubated with pronase E (protease type XIV) (Sigma, code P5147) (0.025 g dissolved in 50 ml TBS for 10 min, pH 7.4, at 37 °C). All the sections were incubated in 10% normal goat serum in TBS/Nonidet for 30 min at room temperature to block nonspecific binding. Moreover, sections prepared for incubation with monoclonal mouse-derived antibodies were in addition incubated with Blocking Solutions A + B from HistoMouse-SP Kit to quench endogenous mouse IgG (Zymed Lab., South San Francisco, CA, USA, code 95-9544).

2.5. Histochemistry

Standard HE stainings and stainings for biotinylated tomato lectin from *Lycopersicon esculentum* (Sigma, code L9389) were performed as previously described (Penkowa and Hidalgo, 2000b).

2.6. Immunohistochemistry

Sections were incubated overnight at 4 °C with one of the following primary antibodies: rabbit anti-rat MT-I + II 1:500 (Gasull et al., 1993; Penkowa et al., 1997, 1999a,b, 2000b); mouse anti-human CD14 (as a marker for monocytes-macrophages) 1:20 (Zymed, code 180121); mouse anti-rat CD3 (as a marker for T lymphocytes) 1:50 (Serotec, Oxford, UK, code KD MCA 772); rabbit anti-cow GFAP (as a marker for astrocytes) 1:250 (Dakopatts, Glostrup, DK, code Z 334); mouse anti-human IL-1β1.50 (Biogenesis, Kingston, NH, USA, code 5375-4329); rat anti-mouse IL-6 1:10 (Harlan Sera Lab., Sussex, UK, code MAS 584); rabbit anti-mouse TNF-α 1:100 (Biosource Int., Camarillo, CA, USA, code AMC 3012); rabbit anti-mouse inducible nitric-oxide synthase (iNOS) (as a marker for oxidative stress) 1:100 (Alexis Biochemicals, San Diego, CA, USA, code 210-503-R050); rabbit anti-nitrotyrosine (NITT) (as a marker for oxidative stress) 1:100 (Alpha Diagnostic Int., San Antonio, TX, USA, code NITT 12-A); rabbit anti-malondialdehyde (MDA) (as a marker for oxidative stress) 1:100 (Alpha Diagnostic, code MDA 11-S); rabbit anti-mouse ICE/Caspase-1 1:100 (Santa Cruz Biotech., Santa Cruz, USA, code sc-1218R); and rabbit anti-human caspase-3 1:50 (Santa Cruz Biotech., code sc-7148). The primary antibodies were detected by using biotinylated mouse anti-rabbit IgG 1:400 (Sigma, code B3275), or biotinylated goat anti-mouse IgG 1:200 (Sigma, code B8774), or biotinylated goat anti-mouse IgM 1:10 (Jackson ImmunoResearch Lab., West Baltimore Pike, West Grove, PA, USA, code 115-065-020), or biotinylated goat anti-rat IgG 1:1000 (Amersham Pharmacia Biotech., Buckinghamshire, UK, code RPN 1005) for 30 min at room temperature. These secondary antibodies were detected by StreptABComplex/HRP (StreptABComplex/HRP, Dakopatts, code K377) prepared at manufacturer's recommended dilution and incubated for 30 min at room temperature. The immunoreaction was visualized using 0.015% H_2O_2 in DAB/TBS (TBS: 0.05 M TRIS, pH 7.4, 0.15 M NaCl) for 10 min at room temperature.

In order to evaluate the extent of non-specific binding in the immunohistochemical experiments, control sections were incubated in the absence of primary antibody. Results were considered only if these controls were negative. A further control for the cytokines was performed by preabsorption of the primary antibodies with their corresponding antigenic proteins. For this purpose we used: mouse IL-1β (RD Systems, Minneapolis, MN, USA, code 401-ML-005); mouse IL-6 (RD Systems, code 406-ML-005); mouse TNF-α (RD Systems, code 410-MT-010). Results were considered only if these controls were negative. Comparisons of sections, which were either incubated or not incubated with Blocking Solutions A + B from Histo-Mouse-SP Kit, were also done.

2.7. In situ detection of DNA fragmentation by terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP)-digoxigenin nick end labeling (TUNEL)

TUNEL staining was performed as previously described (Penkowa et al., 1999a).

2.8. Immunofluorescence histochemistry and TUNEL

In order to examine which cells are expressing IL-1β, IL-6, and TNF-α during EAE, triple immunofluorescence was performed after tissue processing as mentioned above. Sections were incubated overnight at 4 °C with TexasRed (TXRD)-linked tomato lectin from *L. esculentum* 1:50 (Sigma, code L9139), polyclonal rabbit anti-cow GFAP (as mentioned above), and one of the following: mouse anti-human IL-1β (as mentioned above); rat anti-mouse IL-6 (as mentioned above); or mouse anti-human TNF-α 1:50 (Santa Cruz Biotech., code sc-7317). The anti-GFAP antibodies were detected by using goat anti-rabbit IgG linked with aminomethylcoumarin (AMCA) 1:40 (Jackson Im-

munoRes. Lab., code 111-155-144). The anti-IL-1β and anti-TNF-α antibodies were detected by using goat anti-mouse IgG linked with fluorescein (FITC) 1:30 (Jackson ImmunoRes. Lab., code 115-095-146). The anti-IL-6 antibodies were detected by using FITC-linked goat anti-rat IgG 1:30 (Jackson ImmunoRes. Lab., code 112-095-143).

In order to examine which cells are suffering from oxidative stress, triple immunofluorescence stainings were performed by incubating sections overnight at 4 °C with TXRD-linked tomato lectin (as mentioned above), and mouse anti-human NF 1:10 (Biogenex, USA, code AM073-10M), and either rabbit anti-mouse iNOS, or rabbit anti-NITT, or rabbit anti-MDA (all as mentioned above). The anti-NF antibodies were detected by using FITC-linked goat anti-mouse (as above). The anti-iNOS, anti-NITT, and anti-MDA antibodies were detected by using AMCA-linked goat anti-rabbit IgG (as above).

In order to examine which cells were undergoing apoptotic cell death, other triple fluorescence stainings were performed by incubating sections with FITC-linked TUNEL (Oncor, USA, code S7111-KIT) performed according to manufacturer's protocol, and afterwards these sections were incubated overnight at 4 °C with mouse anti-porcine vimentin (as a marker for macrophages and reactive astrocytes) 1:50 (Dakopatts, code M0725), and rabbit anti-human NSE 1:1000 (Calbiochem, USA, code D05059). The anti-vimentin antibodies were detected by using AMCA-linked goat anti-mouse IgG 1:30 (Jackson ImmunoRes. Lab., code 115-155-146). The anti-NSE antibodies were detected by using TXRD-goat anti-rabbit IgG 1:50 (Jackson ImmunoRes. Lab., code 111-075-144). Also, other sections were incubated with FITC-linked TUNEL (as mentioned above) and mouse anti-human CNPase (as a marker for oligodendrocytes) 1:50 (Biogenesis, code 2406-3006). The anti-CNPase antibodies were detected by using AMCA-linked goat anti-mouse IgG 1:30 (as mentioned above).

In order to confirm the notion of apoptotic cell death as judged by TUNEL, neighbouring sections were incubated with rabbit anti-mouse caspase-1 1:100 (Santa Cruz Biotech., code sc-1281R), and mouse anti-human caspase-3 1:50 (Santa Cruz Biotech., code sc-7272). These primary antibodies were detected as mentioned above for anti-NSE and anti-vimentin antibodies. The sections were embedded in 20 μl fluorescent mounting (Dakopatts, code S3023) and kept in darkness at 4 °C. Control sections were processed in parallel, in which the primary antibody was omitted. Results were considered only if these controls were unstained. For the simultaneous examination and recording of the three stains, a Zeiss Axioplan2 light microscope equipped with a triple band (DAPI/FITC/Texas Red) filter was used.

2.9. Cell counts of stained cells

For most of the variables analyzed, cell counts were carried out in 1 mm^2 matched areas of brain stem, spinal cord, and cerebellum of all mice for statistical evaluation of the results. To this end, positively stained cells, defined as cells with staining of the soma (cytoplasm), or in the case of TUNEL cells with nuclear staining, were counted. Cell counts were performed in at least three sections of brain stem, spinal cord, and cerebellum of each mouse, and a mean value was calculated for each animal. Cell counts were performed in a blinded manner in at least three mice per group.

2.10. Statistical analysis

Clinical results were analyzed with the Student *t*-test or Chi-square test depending on the variables analyzed (see Table 1). For cell counts, the results were evaluated by two-way analysis of variance (ANOVA), with strain and EAE as main factors. When the interaction was significant, it was interpreted to be the consequence of the effect of MT-I + II deficiency on EAE effects. A significant difference was considered when $p < 0.05$.

3. Results

3.1. General

In three independent experiments, 32 wild-type mice ($n_1 = 12$, $n_2 = 11$ and $n_3 = 9$) and 30 MTKO mice ($n_1 = 11$, $n_2 = 10$ and $n_3 = 9$) were immunized with $MOG_{40\text{-}55}$ as described in Materials and methods. Pooled results of the clinical data are shown in Table 1. Daily score was

Table 1
Clinical evaluation of the mice during EAE
Clinical course of normal and MTKO mice after EAE induction with MOG_{40-55} peptide.

	Incidence (%)	Mortality (%)	Day of onset[a]	Maximum score[a]	Daily score[a]
$MT^{+/+}$ ($n = 32$)	43.75	9.37	23.93 ± 4.25	1.66 ± 2.15	1.43 ± 1.88
$MT^{-/-}$ ($n = 30$)	70.00 *	16.67	24.75 ± 5.07	2.52 ± 2.09	2.03 ± 1.71

[a] Results are mean ± SD.
* $p < 0.05$ vs. wild-type mice.

performed from day 7 to day 37 post-immunization. The day of onset of EAE was similar in both strains, but an increased susceptibility to EAE induction in the MTKO mice compared to normal mice was evident. Thus, 70% of the immunized MTKO mice developed EAE, while only 43.75% of the wild-type mice did ($p < 0.05$), while mortality, mean daily score and maximum score also tended to be higher in the MTKO mice (Table 1). These results were consistent in the three separate experiments carried out. A detailed histopathological analysis of the effects of EAE in the CNS of normal and MTKO mice was carried out in one experiment and is described below.

3.2. Inflammatory response

Examination of HE stained sections showed pronounced cell infiltrates in all EAE-sensitized mice relatively to mice without EAE (Fig. 1). However, in MTKO mice with EAE, the number of cells infiltrating the CNS tissue was significantly increased when compared to that of normal mice (Fig. 1C,D).

As expected, the number of lectin + microglial/macrophage cells and of CD14 + macrophages was increased throughout the CNS in all mice with EAE relatively to mice without EAE, and these cells were further increased in MTKO mice (Fig. 2A–F; Table 2). In EAE-sensitized wild-type mice, microglia/macrophages had a bushy appearance and both lectin + and CD14 + cells frequently displayed cell processes (Fig. 2B,E). In MTKO mice, the activated microglial/macrophages were amoeboid or round with short, retracted processes (Fig. 2C,F).

The number of CD3 + T lymphocytes in the CNS was also increased in all mice with EAE relatively to mice without EAE. Hence, the latter hardly showed any T cells, while in EAE-sensitized mice, several CD3 + cells were observed throughout the CNS (Fig. 2G–I; Table 2). The number of CD3 + T cells was significantly increased in MTKO mice relatively to normal mice (Fig. 2H,I; Table 2).

The expected EAE-induced astrogliosis was observed, and both the number and size of GFAP + astrocytes were increased in EAE-sensitized mice relatively to mice without EAE (Fig. 2J–L; Table 2). However, the EAE-induced reactive astrogliosis was significantly decreased in MTKO mice when compared to that of normal mice (Fig. 2K,L; Table 2).

3.3. Proinflammatory cytokines

As expected, the cytokines IL-1β, IL-6, and TNF-α were not significantly expressed in mice without EAE (Fig. 3A,D,G). However, following EAE induction their expression was increased throughout the CNS of all mice, although this upregulation was higher in MTKO mice (Fig.

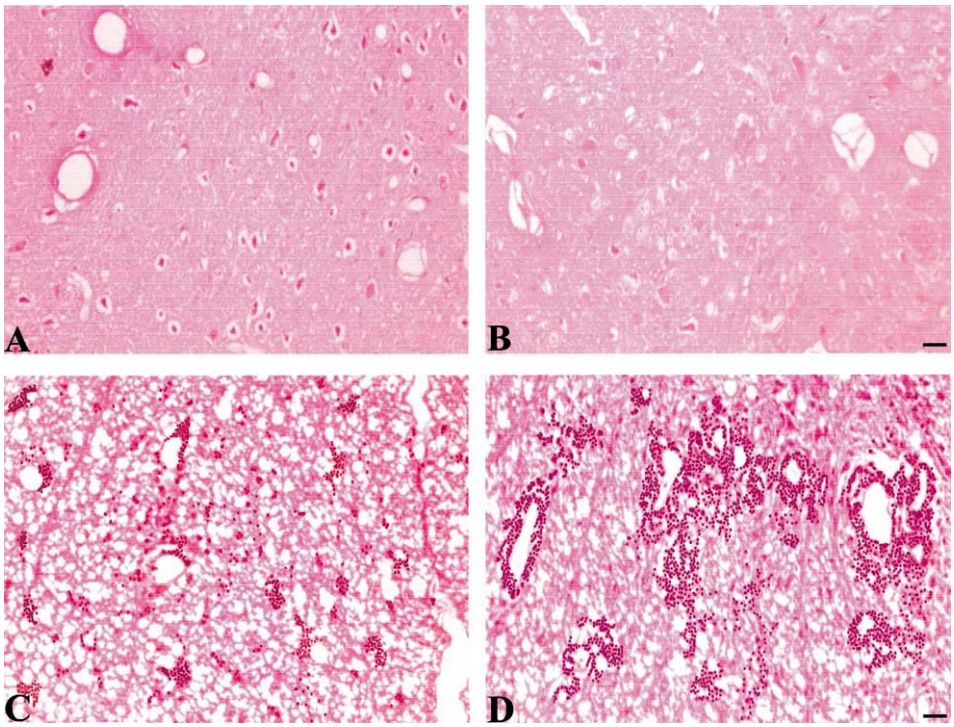

Fig. 1. HE stainings of mice without EAE and EAE-sensitized mice. (A,B) HE stained tissue sections of normal (A) and MTKO (B) mice without EAE. (C,D) HE stained tissue sections of EAE-sensitized normal (C) and MTKO (D) mice. As seen in (C,D), the number of infiltrating cells during EAE is significantly increased in MTKO mice relatively to normal mice. Scale bars: 57 μm.

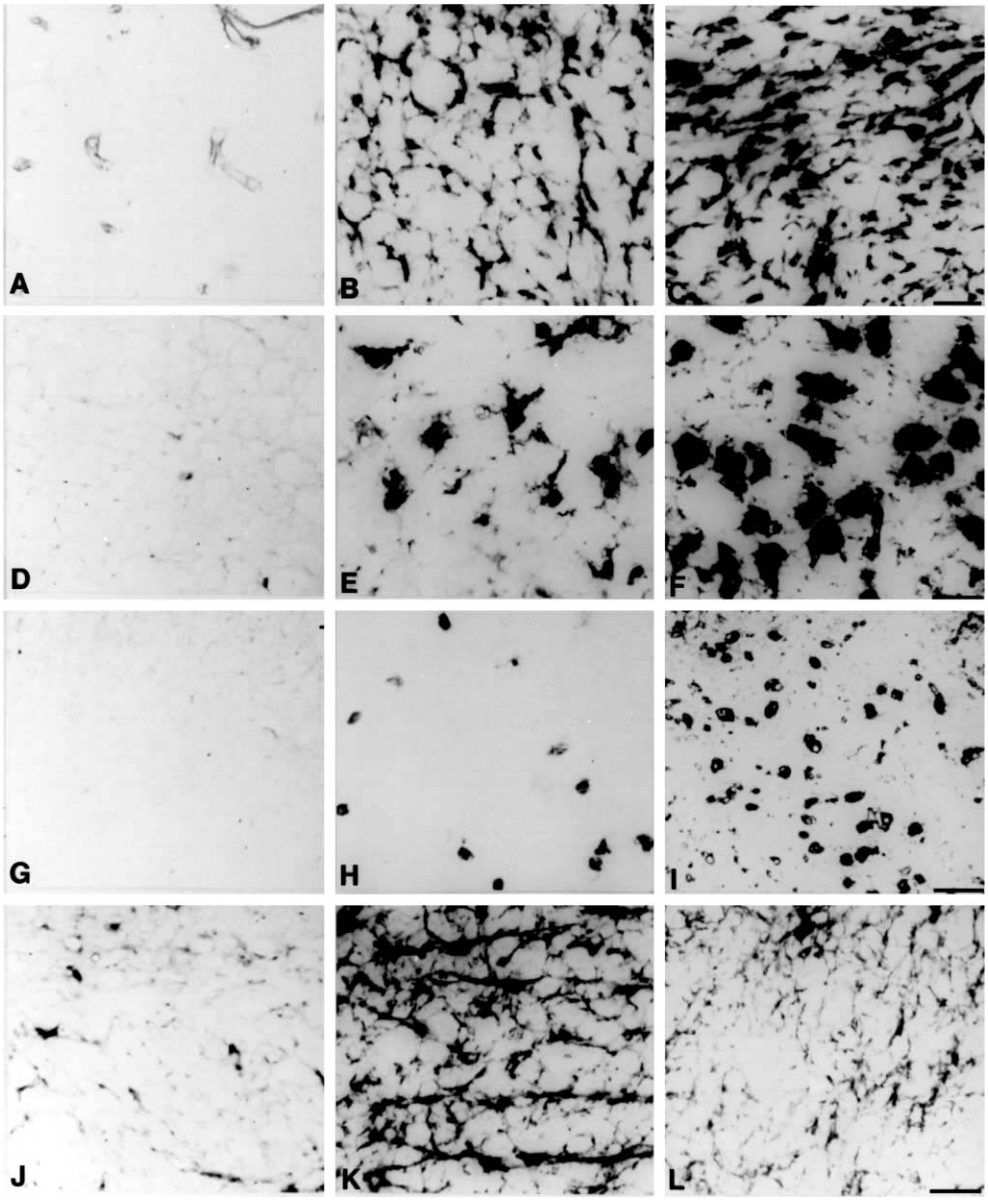

Fig. 2. Lectin + and CD14 + macrophages/microglia, CD3 + T lymphocytes, and GFAP + astrocytes of the brain stem of mice with and without EAE. (A) In normal mice without EAE, lectin staining is mainly seen in vessels. (B) During EAE, normal mice show several lectin + activated microglia/macrophages in both white and grey matter areas. (C) In MTKO mice with EAE, numerous lectin + microglia/macrophages are seen in white and grey matter areas throughout the CNS. (D) CD14 + macrophages are hardly seen in normal mice without EAE. (E) During EAE, normal mice display many CD14 + macrophages. (F) In MTKO mice with EAE, the number of CD14 + macrophages is significantly increased relatively to normal mice. (G) CD3 + T lymphocytes are hardly seen in normal mice without EAE. (H,I) The number of CD3 + T lymphocytes is clearly increased during EAE. However, normal mice (H) display fewer T cells relatively to MTKO mice (I). (J) Showing GFAP + astrocytes in normal mice without EAE. (K) During EAE, a significant GFAP + reactive astrogliosis is observed in normal mice. (L) In MTKO mice with EAE, the GFAP + reactive astrogliosis is reduced compared to that of normal mice. Scale bars: (A–C) 22 μm. (D–I) 18 μm. (J–L) 21 μm.

3; Table 2). Expression of IL-1β, IL-6, and TNF-α was increased in some of the recruited microglia/macrophages and reactive astrocytes during EAE, as judged by using triple immunofluorescence (data not shown).

3.4. Oxidative stress

Oxidative stress was examined by using immunoreactivity for the oxidative stress markers iNOS, NITT, and MDA. In mice without EAE, immunoreactivity for iNOS, NITT, and MDA was basically undetectable (Fig. 4A,D,G). During EAE, all the mice showed increased immunostaining throughout the CNS grey and white matter for these three markers, and the MTKO mice showed increased levels compared to wild-type mice (Fig. 4; Table 2). As determined by using triple immunofluorescence, mainly microglia/macrophages but also neurons and astrocytes showed iNOS immunoreactivity during EAE (data

Table 2
Immunohistochemical cell countings
Cell counts of positively stained cells in 1 mm^2 matched areas of 3-μm-thick sections of brain stem, spinal cord, and cerebellum of all mice. Results are mean ± SD. Results were evaluated by two-way analysis of variance (ANOVA), with strain and EAE as main factors. EAE increased all variables (p at least < 0.05); while MT-I + II deficiency affected all variables ($p < 0.001$) except CD8 levels.

	Normal mice without EAE	MTKO mice without EAE	Normal mice with EAE	MTKO mice with EAE
Lectin + cells	86.3 ± 2.08	85.7 ± 0.58	181 ± 4.6	278 ± 1
CD14 + cells	2 ± 1	2 ± 1.73	100 ± 2.6	194 ± 0.58
CD3 + cells	0.67 ± 0.55	2 ± 0	36.3 ± 1.5	71.7 ± 2.5
CD4 + cells	0.67 ± 0.58	1.67 ± 0.55	34.7 ± 1.5	68.7 ± 1.5
CD8 + cells	0.0 ± 0.0	0.67 ± 0.58	3.33 ± 1.53	6.67 ± 2.89
GFAP + cells	97.3 ± 4.04	91 ± 1.0	195 ± 2.6	105 ± 2.5
IL-1β + cells	0.67 ± 0.58	1.33 ± 0.58	100 ± 3.6	155 ± 3.8
IL-6 + cells	0.33 ± 0.55	1.33 ± 0.55	60.3 ± 1.53	107.7 ± 2.3
TNF-α + cells	1.0 ± 0.0	1.33 ± 0.58	104 ± 5.6	155 ± 3.6
iNOS + cells	0.0 ± 0.0	0.67 ± 0.58	130 ± 1.7	262 ± 5.7
NITT + cells	0.33 ± 0.58	0.37 ± 0.55	128.7 ± 3.5	260 ± 7.1
MDA + cells	0.67 ± 0.55	0.67 ± 0.58	112.3 ± 1.5	281 ± 5.3
TUNEL + cells	0.67 ± 0.58	0.67 ± 0.58	125.7 ± 4.7	276.3 ± 3.8
Caspase-1	0.33 ± 0.58	0.37 ± 0.55	122.5 ± 5.8	273.1 ± 4.9
Caspase-3	0.0 ± 0.0	0.0 ± 0.0	123.8 ± 3.4	275.0 ± 3.2

not shown). NITT immunostaining was mainly present in neurons, but also many microglia/macrophages showed NITT labeling during EAE (Fig. 5A,B). NITT immunoreactivity in astrocytes was observed only in MTKO mice (Fig. 5B). Immunostaining for MDA was observed in neurons, microglia/macrophages, and astrocytes as judged by using triple immunofluorescence (data not shown).

3.5. Apoptotic cell death

Apoptotic cell death was determined by using TUNEL technique and immunoreactivity for caspase-1 and -3 as well as by evaluating apoptotic morphology.

In mice without EAE, TUNEL + cells were basically undetectable, and immunoreactivity for caspase-1 and -3

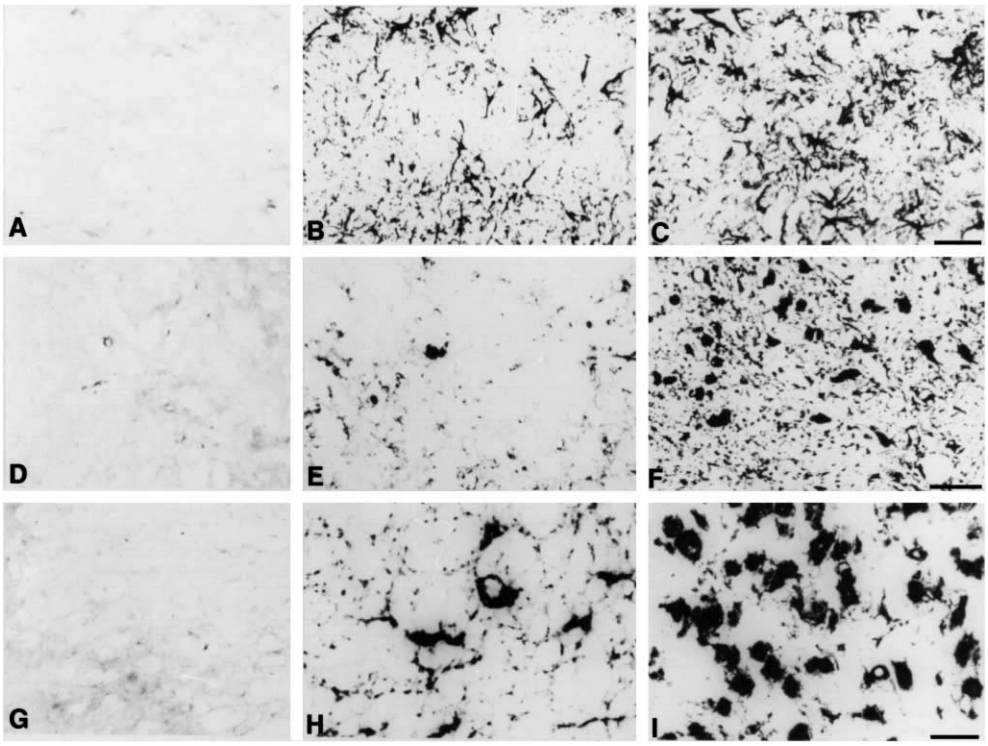

Fig. 3. Cytokines IL-1β, IL-6, and TNF-α in the brain stem of mice with and without EAE. In normal mice without EAE, expression of IL-1β (A), and IL-6 (D), and TNF-α (G) was hardly seen. During EAE, the normal mice increased their expression of cytokines IL-1β (B), and IL-6 (E), and TNF-α (H). In the MTKO mice with EAE, an increased expression of IL-1β (C), and IL-6 (F), and TNF-α (I) was seen throughout the CNS relatively to normal mice. Scale bars: (A–C) 44 μm. (D–F) 33 μm. (G–I) 29 μm.

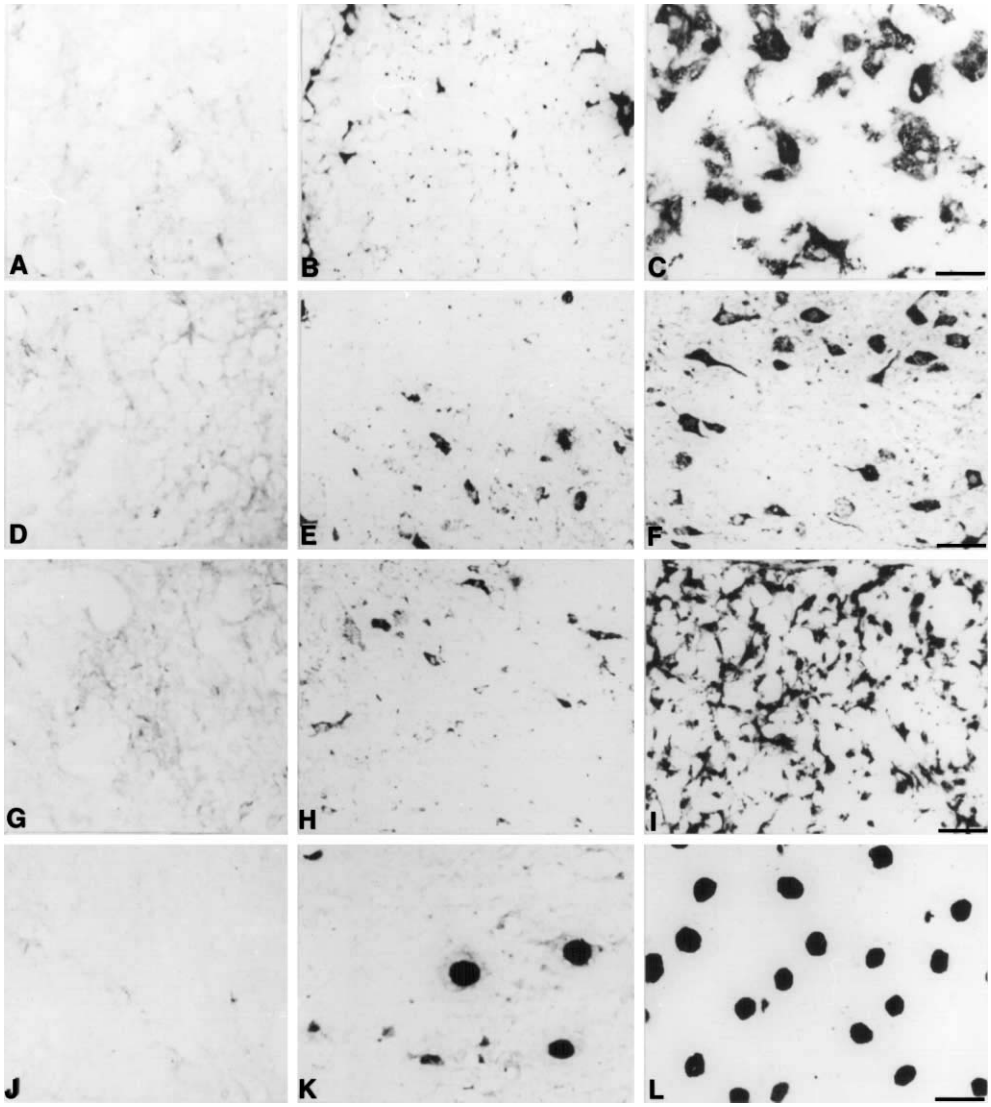

Fig. 4. Oxidative stress markers iNOS (A–C), NITT (D–F), and MDA (G–I) and TUNEL labeling of apoptotic cells in the brain stem of mice with and without EAE. (A) Immunoreactivity for iNOS was hardly detected in normal mice without EAE. (B) In normal mice with EAE, iNOS immunostaining was increased compared to that of mice without EAE. (C) In MTKO mice with EAE, iNOS immunoreactivity was significantly increased in both white and grey matter relatively to normal mice. (D) In normal mice without EAE, NITT immunoreactivity was hardly detected. (E) In EAE-sensitized normal mice, NITT immunostaining was increased in brain stem white and grey matter relatively to mice without EAE. (F) In EAE-sensitized MTKO mice, NITT immunoreactivity was significantly increased in both white and grey matter relatively to normal mice. (G) In normal mice without EAE, immunoreactivity for MDA was barely seen. (H) In normal mice with EAE, increased MDA immunoreactivity was detected compared to that of mice without EAE. (I) In MTKO mice with EAE, MDA immunoreactivity was significantly increased relatively to normal mice with EAE. (J) TUNEL + apoptotic cells were hardly detected in normal mice without EAE. (K) In EAE-sensitized normal mice, many cells of the brain stem were TUNEL + . (L) In EAE-sensitized MTKO mice, an increased number of TUNEL + cells were observed throughout the CNS relatively to normal mice with EAE. Scale bars: (A–C) 22 μm. (D,G) 30 μm. (E,F,H,I) 40 μm. (J–L) 16 μm.

was barely observed (Fig. 4J; Table 2). During EAE, the number of apoptotic cells increased throughout the CNS grey and white matter as judged by TUNEL labeling and caspase-1 and -3 immunoreactivity, and the MTKO mice displayed further increases of these apoptotic markers (Fig. 4K,L; Table 2). In all mice with EAE, the TUNEL + cells displayed apoptotic morphology such as compaction of chromatin, cell shrinkage, and/or membrane blebbing.

By using triple fluorescence for NSE (a neuronal marker), vimentin (a marker for macrophages and reactive astrocytes), and TUNEL, it was observed that mainly NSE + neurons were undergoing apoptotic cell death (Fig. 5C,D). Only in MTKO mice, a few vimentin + astrocytes were TUNEL + during EAE (Fig. 5D). In normal mice with EAE, astrocytes were not labeled by TUNEL (Fig. 5C). Some other cells were in addition TUNEL + (as seen

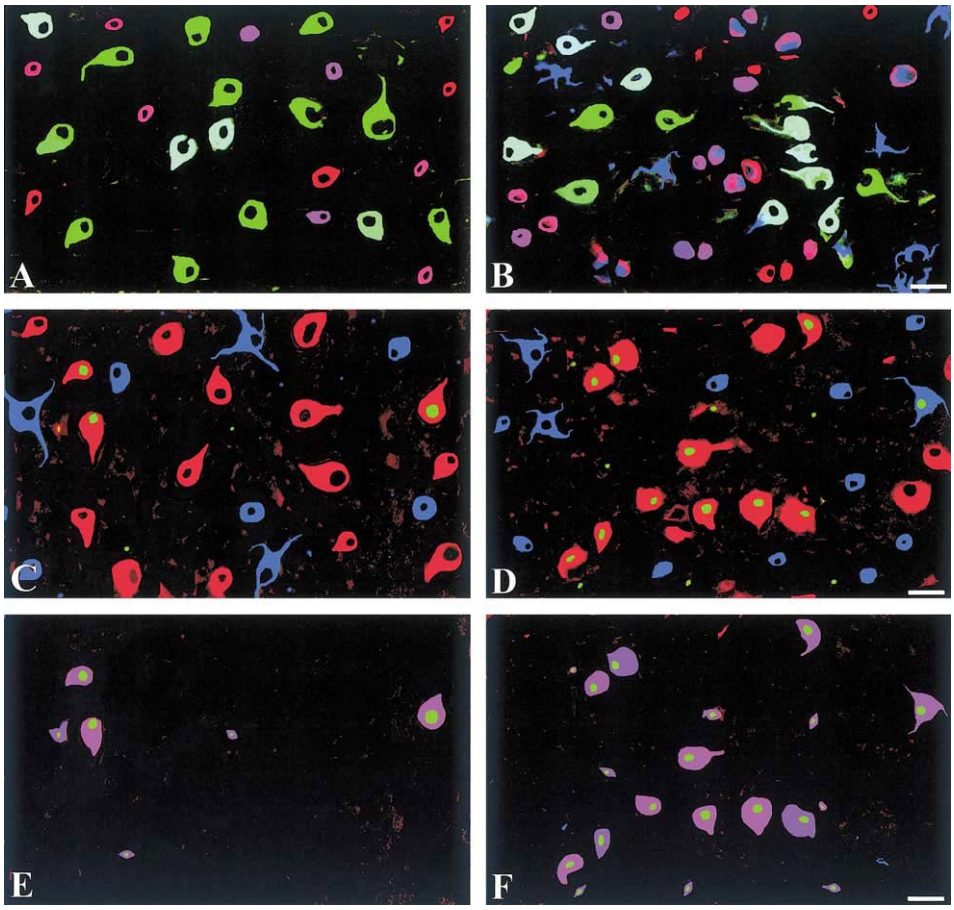

Fig. 5. Triple fluorescence stainings of brain stem sections of normal and MTKO mice with EAE. (A,B) Stainings of NF + neurons (green), lectin + microglia/macrophages (red), and NITT (blue) in normal (A) and MTKO (B) mice. NITT immunoreactivity is seen in both neurons (turquoise) and in microglia/macrophages (pink) of all mice. However, the numbers of NITT + neurons and microglia/macrophages of MTKO mice are increased relatively to normal mice. Hence, most of the neurons and microglia/macrophages of MTKO mice are suffering from oxidative stress. Moreover, in MTKO mice only, NITT + astrocytes are seen (blue stellate cells). In normal mice, most of the NF + neurons are devoid of NITT. (C,D) Stainings of NSE + neurons (red), vimentin + macrophages and astrocytes (blue), and TUNEL (green) in normal (C) and MTKO (D) mice. The number of TUNEL + neurons in MTKO mice is significantly increased when compared to that of normal mice. Also, astrocytic labeling with TUNEL is seen in MTKO mice only. (E,F) Neighboring sections to (C,D), which shows triple fluorescence stainings of TUNEL (green), caspase-1 (red), and caspase-3 (blue). The TUNEL + cells seen in (C,D) are showing coexpression of caspase-1 and -3 (pink), which supports the notion of apoptotic cell death. Scale bars: (A,B) 22 μm. (C–F) 18 μm.

in Fig. 5C,D), which were oligodendrocytes as judged by using double immunofluorescence for the oligodendrocytic marker CNPase and TUNEL (data not shown).

Confirming the notion of apoptotic cell death are immunostainings for caspase-1 and -3, which were co-expressed in TUNEL + cells (Fig. 5E,F) of both normal and MTKO mice with EAE.

4. Discussion

The present report demonstrated that genetical deficiency of MT-I + II is accompanied by increased disease susceptibility to actively induced EAE in 129/Sv mice. This clinical effect of MT-I + II deficiency was associated with an increased inflammatory response of microglia/macrophages and T lymphocytes including expression of proinflammatory cytokines IL-1β, IL-6, and TNF-α, and with a diminished reactive astrogliosis. Furthermore, during EAE the levels of oxidative stress, as judged by immunoreactivity for iNOS, NITT, and MDA, and the number of apoptotic cells, as judged by using TUNEL technique and immunoreactivity for caspase-1 and -3, were increased in MTKO mice relatively to normal mice.

EAE/MS is considered an autoimmune disease mediated by macrophages and T lymphocytes secreting proinflammatory cytokines, which play a crucial role in the induction of the disease (Brosnan and Raine, 1996; Eng et al., 1996; Gijbels et al., 1995; Jacobs et al., 1991; Lassmann, 1999; Mendel et al., 1998; Okuda et al., 1998, 1999; Rajan et al., 1998; Samoilova et al., 1998; Tanuma et al.,

1999; Wiemann et al., 1998; Zhu et al., 1998). In vivo, MT-I + II significantly decrease the CNS inflammatory response of macrophages including expression of IL-1β, IL-6, and TNF-α (Carrasco et al., 2000; Penkowa and Hidalgo, 2000b, 2001; Penkowa et al., 1999a, 2000a). Daily treatment of EAE with MT-II in Lewis rats significantly reduced activation and recruitment of macrophages and T cells. This could explain in part the amelioration of clinical signs observed in these animals (Penkowa and Hidalgo, 2000b). Interestingly, inhibitory effects of MT-I + II on macrophages were also observed in vitro, in that induction of MT-II mRNA and total MT-I + II protein in human monocytes was followed by suppression of monocyte activation including inhibition of the oxidative burst, adherence to plastic, and IL-1β levels following LPS treatment (Koropatnick and Zalups, 1997; Leibbrandt and Koropatnick, 1994). Moreover, MT-I + II may ameliorate EAE by reducing IL-1, IL-6 and TNF-α production, in that activation of these cytokines results in recruitment to the CNS and activation in situ of large numbers of lymphocytes and macrophages (Brosnan and Raine, 1996; Eng et al., 1996; Woodroofe, 1995). Accordingly, an inhibition of these cytokines can suppress EAE (Gijbels et al., 1995; Klinkert et al., 1997; Jacobs et al., 1991; Rajan et al., 1998; Wiemann et al., 1998).

Furthermore, MT-I + II may have a regenerative effect during EAE, in that MT-I + II increase astrogliosis and expression of growth factors such as transforming growth factor-β (TGF-β), which both may participate in the healing phase following CNS damage (Aschner, 1998a; Benveniste, 1997; Carrasco et al., 2000; Eddleston and Mucke, 1993; Eng et al., 1996; Penkowa et al., 1999a, 2000a). In agreement with this, MT-induced amelioration of EAE in rats was associated with increased astrogliosis (Penkowa and Hidalgo, 2000b), and it was reported that EAE can be prevented and treated by TGF-β (Racke et al., 1991; Santambrogio et al., 1993). Moreover, in MTKO mice subjected to a focal brain injury, reactive astrogliosis, growth factor production, and the following wound healing were dramatically impaired (Penkowa et al., 1999a, 2000a). Hence, accumulating data suggest that MT-I + II can ameliorate pathological conditions of the CNS, and this is associated with downregulation of macrophage and T cell responses including production of proinflammatory cytokines, as well as upregulation of astrogliosis and TGF-β in the CNS.

MT-I + II are also antioxidant factors protecting against ROS (reactive oxygen species) and oxidative damage (Aschner, 1996, 1998b; Lazo et al., 1995, 1998; Molinero et al., 1998; Pitt et al., 1997; Schwarz et al., 1995; Tamai et al., 1993; Tate et al., 1995; Thornalley and Vasak, 1985,). ROS are involved in EAE/MS pathogenesis (Bagasra et al., 1995; Benveniste, 1997; Cross et al., 1994; MacMicking et al., 1992; Ruuls et al., 1995; Scott et al., 1997; Van Dam et al., 1995), and in vitro, ROS can easily damage myelin (Konat and Wiggins, 1985) and induce degeneration of oligodendrocytes (Griot et al., 1990; Kim and Kim, 1991). In addition, ROS are key mediators of apoptotic cell death (Cassarino and Bennett, 1999; Sun and Chen, 1998). Apoptotic cell death of both oligodendrocytes and neurons is certainly implicated in EAE/MS pathogenesis (Aktas et al., 2000; Alcazar et al., 1998; Arbizu-Urdiain and Martinez-Yelamos, 2000; Sabelko-Downes et al., 1999; Hisahara et al., 2001; Honegger and Langemann, 1989; MacMicking et al., 1992; Ruuls et al., 1995; Penkowa and Hidalgo, 2001). In addition, MT-II treatment during EAE in rats could significantly inhibit neuronal and oligodendrocyte apoptosis, while elimination of autoreactive T cells by apoptosis was unaffected (Penkowa and Hidalgo, 2001). The latter phenomenon is likely very important, since apoptosis during EAE/MS has a dual role: apoptosis is detrimental and pathological when it occurs in neurons and oligodendrocytes, but on the other hand, apoptosis is beneficial and a physiological control mechanism inducing disease remission when it occurs in inflammatory cells such as T cells (Zipp, 2000).

Whether or not this antiapoptotic effect of MT-II treatment is due to antioxidant functions of MT or yet unknown mechanisms of actions is currently not elucidated, but indeed, treatment of EAE-sensitized rats or mice with antioxidant factors or inhibitors of ROS can ameliorate EAE (Brenner et al., 1997; Cross et al., 1994; Malfroy et al., 1997; Ruuls et al., 1995; Zhao et al., 1996; Zielasek et al., 1995). Thus, antioxidant scavengers are needed during EAE in order to neutralize ROS. MT-I + II function as thiol donors, scavenge free radicals and reduce NO-induced cyto- and nuclear toxicity (Lazo et al., 1995, 1998; Radi et al., 1991; Schwarz et al., 1995). Moreover, NO synthesis inhibition during immobilization stress decreased MT-I + II (Molinero et al., 2000a), which supports the role for MT-I + II in NO detoxification. Hence, MT may significantly affect the levels of oxidative stress during EAE by neutralization of ROS. Accordingly, treatment with MT-II during EAE in rats leads to a significant decrease of oxidative stress (Penkowa and Hidalgo, 2000b) and apoptotic cell death (Penkowa and Hidalgo, 2001). In agreement with this, MTKO mice are also suffering from severely increased oxidative stress and neuronal apoptosis in other models of brain injury (Carrasco et al., 2000; Penkowa et al., 1999a, 2000a). Indeed, in the present report, oxidative stress markers (iNOS, NITT, and MDA) and apoptotic cell death were significantly increased in MTKO mice relatively to normal mice during EAE. These effects of MT during EAE could indeed have a therapeutical potential, in that oxidative stress and apoptosis of both oligodendrocytes and neurons are implicated in EAE/MS.

In summary, we have demonstrated here that MT are involved at different levels in the pathogenesis of EAE, playing a protective role. Our data suggest that MT-I + II, acting alone or in concert with other anti-inflammatory or antioxidant mediators, may be potential candidates in the treatment of CNS disorders such as EAE/MS.

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