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Metallothioneins Are Upregulated in Symptomatic Mice with Astrocyte-Targeted Expression of Tumor Necrosis Factor-α

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Transgenic mice expressing TNF-α under the regulatory control of the GFAP gene promoter (GFAP-TNFα mice) exhibit a unique, late-onset chronic-progressive neurological disorder with meningoencephalomyelitis, neurodegeneration, and demyelination with paralysis. Here we show that the metallothionein-I + II (MT-I + II) isoforms were dramatically upregulated in the brain of symptomatic but not presymptomatic GFAP-TNFα mice despite TNF-α expression being present in both cases. *In situ* hybridization analysis for MT-I RNA and radioimmunoassay results for MT-I + II protein revealed that the induction was observed in the cerebellum but not in other brain areas. Increased MT-I RNA levels occurred in the Purkinje and granular neuronal layers of the cerebellum but also in the molecular layer. Reactive astrocytes, activated rod-like microglia, and macrophages, but not the infiltrating lymphocytes, were identified as the cellular sources of the MT-I + II proteins. *In situ* hybridization for MT-III RNA revealed a modest increase in the white matter of the cerebellum, which was confirmed by immunocytochemistry. MT-III immunoreactivity was present in cells which were mainly round or amoeboid monocytes/macrophages. The pattern of expression of the different MT isoforms in the GFAP-TNFα mice differed substantially from that described previously in GFAP-IL6 mice, demonstrating unique effects associated with the expression of each cytokine. The results suggest that the MT expression in the CNS reflects the inflammatory response and associated damage rather than a direct role of the TNF-α in their regulation and support a major role of these proteins during CNS injury.

INTRODUCTION

Cytokines are essential mediators of cell–cell communication in the CNS, with astrocytes, microglia, and brain macrophages as major sources of cytokines as well as a number of growth factors (25, 35, 38, 45, 46, 54, 61, 65). In normal conditions, the cellular expression of cytokines in the CNS is very low or absent under tight control. Yet, in a number of neuropathological conditions the expression of some cytokines is significantly altered (see (7) for review). Such alterations could presumably make an important contribution to the clinicopathological features of many neurological disorders, but the possibility that they are simply the consequence and not the cause must be ruled out experimentally. Results obtained in transgenic mice with astrocyte-targeted expression of cytokines such as IL-6 (5, 6, 8, 12, 14, 31, 52, 60), IL-3 (13), IFN-α (1), and TNF-α (59) have unequivocally demonstrated that an abnormal cytokine production has the potential to cause devastating neurological disorders. Interestingly, each of these cytokines caused a specific repertoire of clinicopathological sequelae that presumably reflect the unique actions of the particular cytokine expressed. Therefore, these transgenic mice are useful animal models for the characterization of genes whose expression is altered by cytokine-induced pathological conditions in the CNS and that could potentially have an important role in coping with CNS damage. One such family of genes is the metallothionein (MT) family.

MTs are cysteine-rich, heavy metal binding proteins, which in rodents are composed of four isoforms (MT-I to MT-IV) (48, 53). Only three isoforms are expressed in the brain, namely MT-I + II (which are also widely expressed and regulated coordinately) and MT-III (also known as growth inhibitory factor). It is now well-established that the MT-III isoform is expressed not only in the CNS but also elsewhere (34, 41, 47). MTs bind zinc and copper and presumably function in metal ion regulation and detoxification in peripheral tissues as well as in the CNS (4, 33). Early studies suggested that MT-I + II could have significant antioxidant capacity and functions (32, 55, 63), and recent reports

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with transgenic mice overexpressing these MT isoforms and with MT-I + II KO mice fully support such roles (22, 28, 37, 39, 40, 42, 43). We have recently shown that the inflammatory response to glial cell death and the wound healing capacity of the CNS is severely impaired in MT-I + II KO mice (49, 50), suggesting a major role of these MT isoforms during CNS injury. MT-III was discovered as an inhibitory neuronal growth factor (*in vitro*) that appeared to be decreased in Alzheimer's disease brains (64); the latter, however, has not been confirmed (2, 9, 21). So far, the results obtained in MT-III KO mice suggest this MT isoform would have a rather neuroprotective role in some brain areas (20). In addition, significant alterations of the MT-III expression have been observed in several models of CNS damage, as well as that of MT-I + II in both animal models and human neuropathologies (see (3, 4, 33, 36) for review). Taken together, the results indicate that the MT family is important during CNS injury, and thus it seems important to gain insight into the factors that control the expression of the different MT isoforms in such situations.

In previous studies (11, 30), we have characterized the effect of the transgenic expression of IL-6 in astrocytes on the expression of the MT family. These studies, as well as those carried out in IL-6 KO mice (10, 51), demonstrated that IL-6 is a major cytokine regarding the control of MT isoforms in the CNS. In the present report, we have examined whether the astrocyte-targeted expression of TNF-α, which causes a chronic-progressive neurological disorder, is associated with altered expression of the MT isoforms. The results indicate that cytokine-induced brain damage, rather than the cytokine per se, is the relevant factor driving MT regulation.

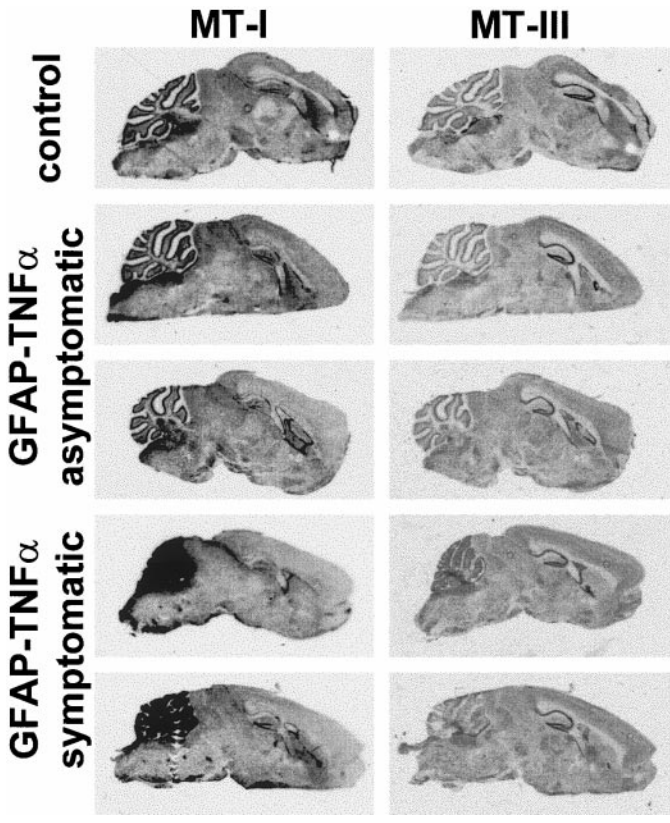

FIG. 1. *In situ* hybridization analysis of brain MT-I and MT-III RNA expression. Six 10-month-old control mice and presymptomatic and symptomatic GFAP-TNFα mice were killed, and their brains were immediately frozen in liquid nitrogen. Sagittal sections were then prepared and the *in situ* analysis was carried out in parallel for all the animals. Other animals were also evaluated, and specific quantitations were carried out in several mice per group for statistical purposes; some of the results are shown in Fig. 3.

MATERIALS AND METHODS

Production of GFAP-TNFα and GFAP-IL6 Mice

Construction and characterization of the GFAP-TNFα (GT-8 line; (59)) and GFAP-IL6 (8) transgenic mice have been described previously. In both cases, an expression vector (specific for each line) derived from the murine glial fibrillary acidic protein (GFAP) gene was used to target expression of the cytokine to astrocytes. Heterozygous offspring were studied in parallel with age- and sex-matched nontransgenic littermates. Presymptomatic as well as symptomatic (clinically affected) GFAP-TNFα mice were used in this study.

In Situ Hybridization and Radioimmunoassay

The mice were killed and their brains quickly removed, cut into two parts, frozen in liquid nitrogen, and stored at −80°C. With one of the hemispheres, serial sagittal sections (20 μm in thickness) were obtained with a cryostat and mounted on slides coated with poly(L)lysine, where an *in situ* hybridization analysis of the MT-I and MT-III isoforms was carried out as previously described (11). With the other hemisphere, the cerebellum, hippocampus, medulla + pons, and remaining brain were dissected, homogenized, and centrifuged, and MT-I + II protein levels were measured by radioimmunoassay (24, 30).

For MT-I mRNA studies, we used the mouse cDNA kindly provided by Dr. R. D. Palmiter (University of Washington, WA). For MT-III mRNA studies, and in order to avoid cross-hybridization with MT-I and MT-II mRNAs, we have used a specific DNA fragment of 153 bp that contains the coding region for the terminal 15 amino acids and the 3′ untranslated region until the poly G stretch of MT-III mRNA, which was generously provided by Dr. G. K. Andrews (Dept. of Biochemistry, Kansas City, KS). All sections to be compared were prepared simultaneously and exposed to the same autoradiographic film. MT-I or MT-III mRNA levels were determined semiquantitatively in three sections per brain area and animal by measuring the optical densities and the number of pixels in defined areas with a

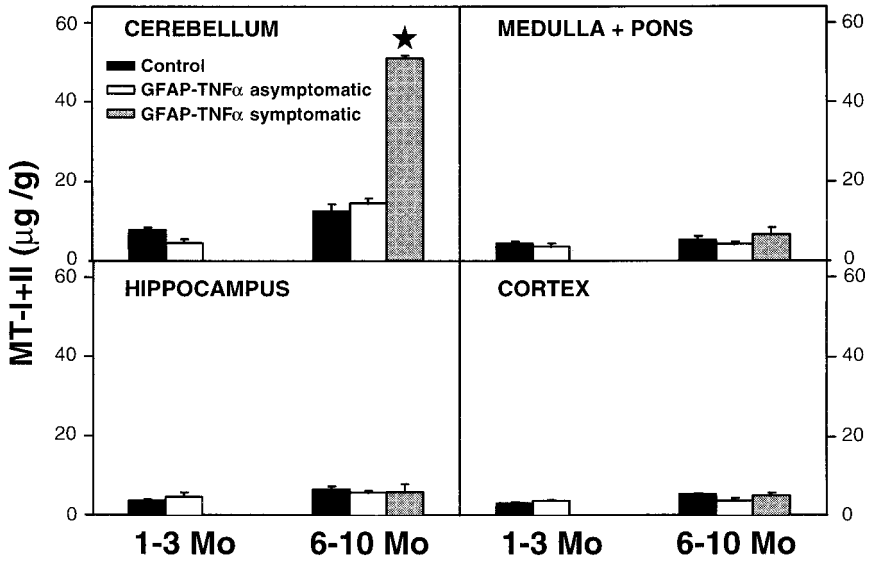

FIG. 2. Metallothionein-I + II total protein measured by radioimmunoassay. Control and presymptomatic and symptomatic GFAP-TNFα mice were killed at different ages, and their brains were frozen in liquid nitrogen and stored at −80°C. The brains were later dissected into cerebellum, medulla plus pons, hippocampus, and remaining brain (cortex) and MT-I + II were measured by radioimmunoassay. In agreement with the MT-I RNA data, the MT-I + II protein levels were dramatically increased only in the cerebellum of the symptomatic mice. Results were analyzed with one-way ANOVA. * $P < 0.001$ vs control mice of the same age.

Leica Q 500 MC system. The MT-I and MT-III mRNA values shown are expressed in arbitrary units (number of pixels × optic density).

Some slides were coated with Hypercoat LM-1 emulsion (Amersham) following the instructions of the manufacturer. The slides were exposed for 3 weeks at 4°C in a light-tight box, and then they were developed in D-19 (Kodak). For microscope observation the slides were counterstained with hematoxylin–eosin.

Histochemistry and Immunohistochemistry

For routine histology, the brains were removed, fixed in 4% buffered paraformaldehyde, and embedded in paraffin. Sections were later deparaffinized and rehydrated in graded alcohols.

Immunofluorescence Histochemistry

Paraffin sections were deparaffinized and, for heat-induced epitope retrieval, the sections were boiled in citrate buffer (pH 6 or 9) in a microwave oven for 2 × 10 min and/or preincubated in Digest-All-3 (Zymed Lab. Inc., code 00-3009) for 3–5 min at room temperature, followed by incubation in 10% donkey serum (Binding Site, UK, code BP 005.1) in TBS/Nonidet (TBS: 0.05 M Tris, pH 7.4, 0.15 M NaCl) with 0.01% Nonidet P-40 (TBS/Nonidet) for 30 min at room temperature. Mouse sections incubated with monoclonal mouse-derived antibodies were in addition incubated with blocking solutions A + B from the HistoMouse-SP kit to quench endogenous mouse IgG (Zymed Lab. Inc., code 95-9544). Sections were incubated overnight for triple immunofluorescence stainings with the following primary antibodies: mouse anti-horse MT-I + II (IgG) 1:50 (Dakopatts, DK, code M0639), and rabbit anti-MT-III (IgG) 1:1000 (9, 11, 51), and goat anti-human vimentin (IgG) 1:100 (Santa Cruz, code sc-7557) simultaneously. These primary antibodies were detected using donkey anti-mouse IgG conjugated with fluorescein/FITC 1:50 (Jackson ImmunoResearch Lab. Inc., code 715-095-151), donkey anti-rabbit IgG conjugated with Texas Red 1:50 (Jackson ImmunoResearch Lab. Inc., code 715-075-152), and donkey anti-goat IgG conjugated with coumarin/AMCA 1:50 (Jackson ImmunoResearch Lab. Inc., code 705-155-147) simultaneously. Other sections were preincubated in 10% goat serum (In Vitro, DK, code 04009-1B) in TBS/Nonidet with 0.01% TBS/Nonidet for 30 min at room temperature, followed by incubation with blocking solutions A + B from the HistoMouse-SP kit to quench endogenous mouse IgG (Zymed Lab. Inc., code 95-9544), followed by incubation overnight for double immunofluorescence stainings with the following primary antibodies: mouse anti-horse MT-I + II (IgG) 1:50 (Dakopatts, DK, code M0639), and rabbit anti-MT-III (IgG) 1:1000 (9, 11, 51). These primary antibodies were detected using goat anti-mouse IgG conjugated with coumarin/AMCA 1:50 (Dakopatts, DK, code W0477) and goat anti-rabbit IgG conjugated with Texas Red 1:50 (Jackson ImmunoResearch Lab. Inc., code 111-075-144) simultaneously.

In order to evaluate the extent of nonspecific binding of the antisera in the immunohistochemical experiments, 1:100–1:1000 of normal rabbit or mouse serum or just the preincubation agent was substituted for the

primary antibody step described above. Results were considered only if these controls were negative.

Statistical Analysis

When several animals were compared, statistical comparisons were carried out as follows. When two groups were compared, the Student *t* test was used; when three groups were compared, one-way ANOVA followed by post hoc comparisons of the means (SNK test) were used.

RESULTS

Presymptomatic and Symptomatic GFAP-TNFα Mice

The GT-8 transgenic mice used in this study show a characteristic physical presentation that includes symptoms of ataxia from 6 months of age or older, the severity of which increases progressively; after the onset of motor impairment, the mice display muscle spasms and become progressively cachectic (59). We have analyzed MT expression in both presymptomatic and symptomatic mice defined by these clinical criteria.

In Situ Hybridization and Radioimmunoassay

Figure 1 shows an *in situ* hybridization analysis of the MT-I and MT-III isoforms in two presymptomatic and symptomatic GFAP-TNFα mice. It is clear that the transgene expression of TNF-α was associated with a dramatic upregulation of the MT-I isoform in the cerebellum of symptomatic but not presymptomatic mice; the remaining brain areas did not show an altered MT-I expression. This contrasts with the MT-I upregulation observed in the cerebellum, brain stem, and diencephalon of GFAP-IL6 mice (11, 30). These *in situ* hybridization results were confirmed by the MT-I + II protein measurements by radioimmunoassay (Fig. 2), where again only the cerebellum of the symptomatic GFAP-TNFα mice showed a significant alteration of the MT-I + II protein levels. This was further confirmed by immunocytochemical analysis carried out in other mice (see below).

Figure 3 shows the quantification of MT-I RNA levels carried out in the cerebellum of mice of different ages (1–3 and 6–10 months old). Other brain areas were also analyzed, but since no effects of TNF-α were observed, these results are not shown. A dramatic upregulation of MT-I was observed in symptomatic GFAP-TNFα mice in the Purkinje, granular, and molecular layers. This again contrasts with the GFAP-IL6 mice, which do not show significant MT-I upregulation in the molecular layer (11, 30). For further analysis, microautoradiographies were carried out (Fig. 4). Note that a slight upregulation of MT-I was observed in the Purkinje cell layer of presymptomatic mice, apparently in the Bergmann glia (Fig. 4B), while a dramatic upregulation was observed in all cerebellar areas of the

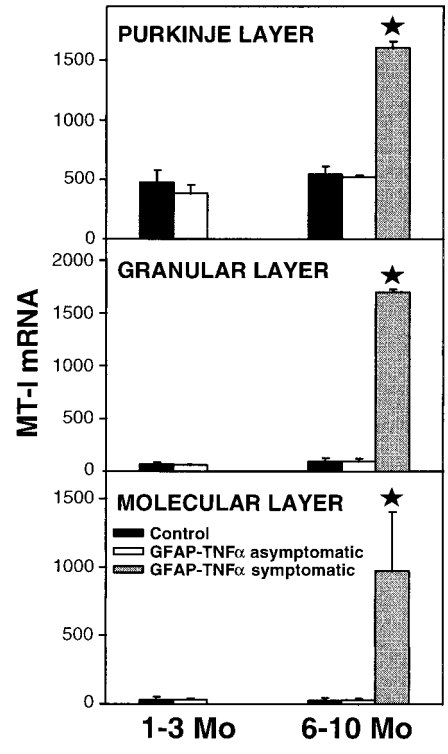

FIG. 3. Effect of the transgenic expression of TNF-α on cerebellar MT-I RNA levels. Levels were measured in defined areas of the Purkinje, granular, and molecular layers. A moderate (Purkinje) and dramatic (granular and molecular) upregulation of MT-I were observed in the symptomatic mice (see also Fig. 4). Results were analyzed with one-way ANOVA. * *P* at least <0.05 vs the control mice of the same age.

symptomatic mice (Fig. 4C). As expected (59), the transgenic expression of TNF-α caused a severe lymphocyte infiltration of the CNS, particularly in the white matter of the cerebellum (see for instance Figs. 4C and 4D). These infiltrates were completely devoid of MT-I RNA signal (Figs. 4C and 4D).

In contrast to MT-I, the MT-III expression was not altered significantly in either presymptomatic or symptomatic GFAP-TNF mice (Fig. 1). A slight upregulation in MTIII expression, however, was noticed in the cerebellum (especially in the white matter) of some GFAP-TNFα mice (Figs. 4E and 4F). This was also observed by immunocytochemistry for the MT-III protein. Specific quantification of the MT-III RNA levels in selected areas, such as the CA1–CA3 neuronal layers of the hippocampus, did not reveal significant changes (data not shown). The perivascular lymphocyte infiltrates of the CNS were devoid of MT-III RNA signal, similar to the MT-I isoform (Fig. 4F).

Histochemistry and Immunocytochemistry

As expected (59), the transgenic expression of TNF-α caused a severe lymphocyte infiltration of the CNS, particularly in the white matter of the cerebellum (see for instance Figs. 4C and 4D). These infiltrates caused

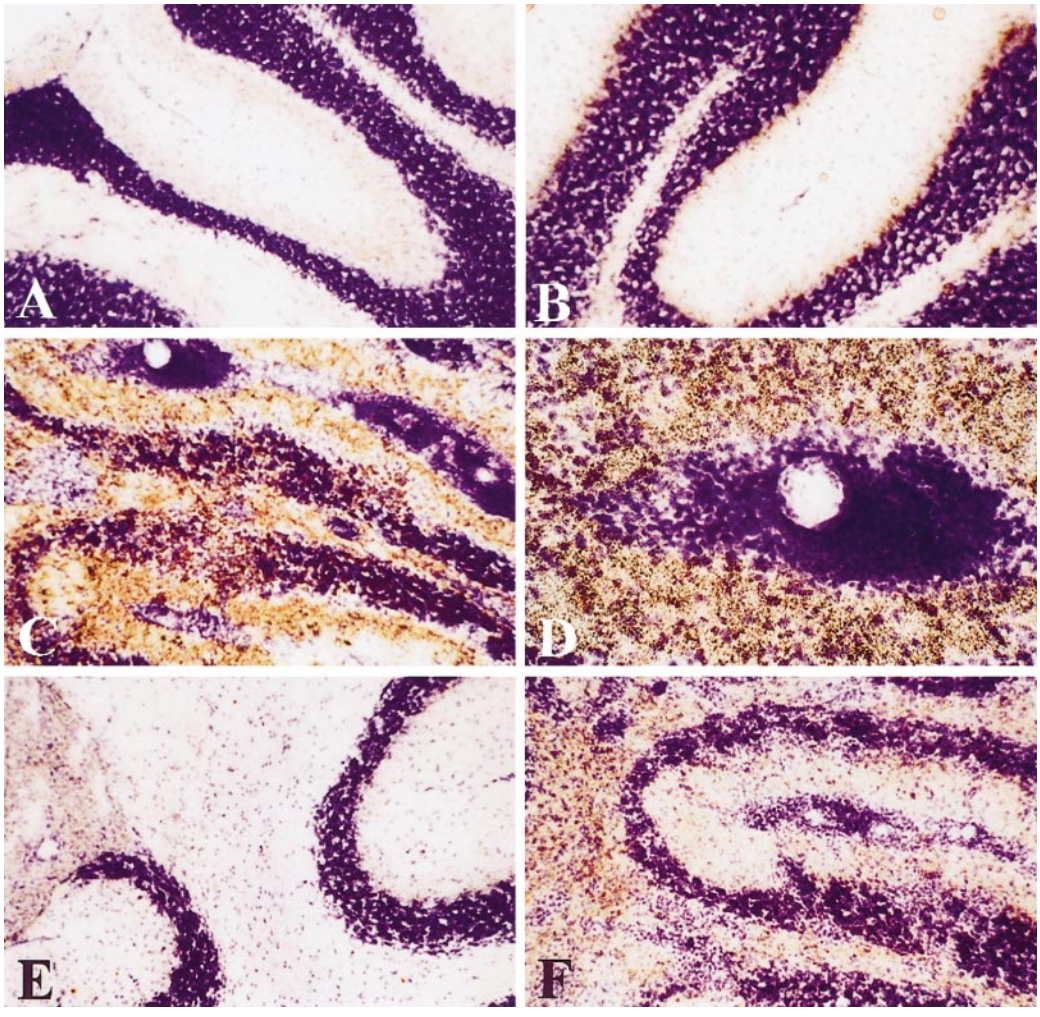

FIG. 4. Representative microautoradiographies of the cerebellum of normal and GFAP-TNFα mice for MT-I (A–D) and MT-III (E, F) RNAs. Presymptomatic GFAP-TNFα mice show a slight upregulation of the MT-I isoform (B) compared to control mice (A) in the Purkinje cell layer, while a strong upregulation is observed in symptomatic mice in all cerebellar areas (C, D). The infiltrates are devoid of MT-I signal (D). In control mice, a clear MT-III RNA signal is observed in the plexus choroid cells, while the signal is weak in all cerebellar areas (E). In symptomatic GFAP-TNFα mice (F), MT-III RNA levels are increased in the white matter and slightly in the molecular layer. The infiltrates are also devoid of MT-III signal (F). Original magnifications: A–C, E, F: 133×; D: 333×.

significant activation of microglia, recruitment of macrophages, and the appearance of GFAP-positive reactive astrocytes (see (59) for a detailed analysis). In agreement with the MT-I expression, MT-I + II immunoreactivity was increased in the GFAP-TNFα mice especially in the cerebellum (Fig. 5). These MT-I + II-positive cells were mainly reactive astrocytes, round or amoeboid mononuclear macrophages, and rod-like or bushy microglial cells. In normal mice MT-III expression was seen in choroid plexus cells, ependymal cells, and meningeal cells. In the white matter of the cerebellum, brain stem, and corpus callosum a very few astrocytes and microglial cells expressed MT-III. In other brain areas MT-III expression was hardly detected. In GFAP-TNFα mice an increased number of MT-III-expressing cells was seen when compared to normal mice. The MT-III-positive cells were situated in the white matter of the cerebellum, brain stem, medulla spinalis, and forebrain. These cells were mainly round or amoeboid monocytic/macrophage cells seen around vessels in the mentioned white matter areas (Fig. 5). Gray matter areas of GFAP-TNFα mice were devoid of increased MT-III expression.

DISCUSSION

TNF-α is a major inflammatory cytokine in the CNS and it is therefore important to gain knowledge of the downstream proteins controlled by this cytokine. In this report we have studied for the first time the effect of astrocyte-targeted expression of TNF-α on brain MT-I–III regulation. We have analyzed symptomatic (with clear clinical signs of ataxia) as well as presymptomatic mice. The results clearly demonstrate that a significant effect of the transgene expression of TNF-α is seen mainly in symptomatic animals and in a

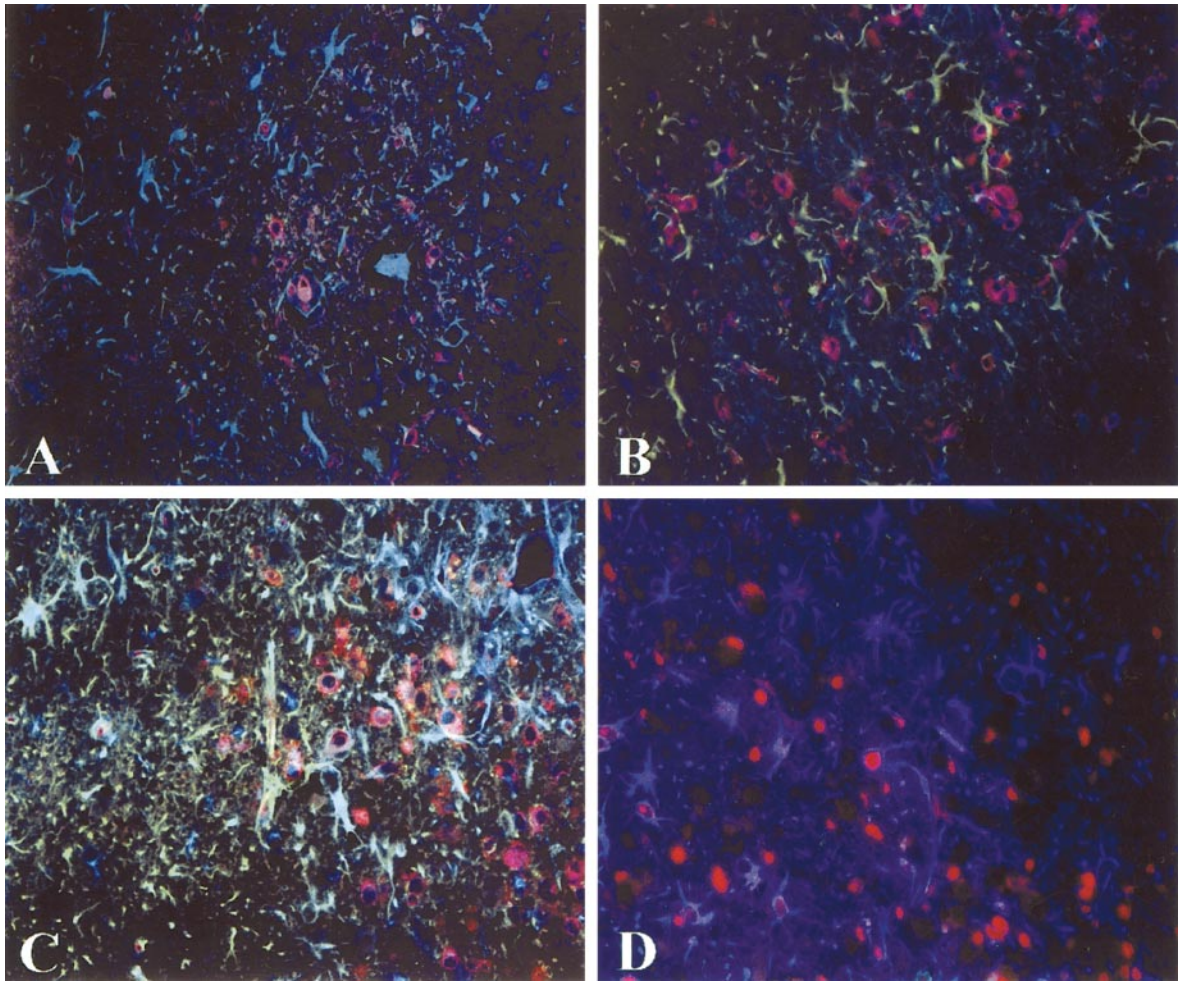

FIG. 5. Triple and double immunofluorescence histochemistry, showing vimentin (blue), MT-I + II (green), and MT-III (red) in A–C, and MT-I + II (blue) and MT-III (red) in D. A: Normal mice show a very few MT+ cells. B: In presymptomatic GFAP-TNF mice the number of MT-I + II+ cells and MT-III+ cells is increased. C: In symptomatic GFAP-TNF mice the number of MT-I + II+ cells and MT-III+ cells is dramatically increased compared with presymptomatic mice seen in B. In both presymptomatic and symptomatic GFAP-TNF mice, MT-I + II are mainly seen in stellate astrocytic cells, while MT-III primarily is located in round microglia/macrophages. D: In order to colocalize MT isoforms, double stainings were performed, showing MT-I + II (blue) and MT-III (red) in symptomatic GFAP-TNF mice. Many amoeboid or round macrophages showed MT-III only, while some of the MT-I + II+ astrocytes coexpressed MT-III. Original magnification: 525×.

brain-area- and MT-isoform-specific manner. Thus, the MT-I + II isoforms were dramatically increased by TNF-α-induced CNS damage, while only moderate effects were observed for the MT-III isoform. For the sake of clarity, we will discuss first the MT-I + II results while those of MT-III will be discussed below.

In rodents, the MT-I + II isoforms are regulated coordinately (66) and thus we assume that the MT-I RNA measurements are representative of the two isoforms. Indeed, the MT-I + II protein analysis typically confirms the RNA results, which was also the case in this study. MT-I + II are multiregulated proteins, and a wide set of factors including metals, hormones, free radicals, and cytokines have been shown to alter their expression. Some studies have been carried out with TNF-α in peripheral tissues which demonstrate a role of this cytokine on MT-I + II regulation. *In vivo,* liver MT-I + II have been shown to be increased by the exogenous administration of TNF-α (19, 26, 44, 56–58, 62). Results in cultured hepatocytes, however, are less clear (15, 16, 29) and indeed suggest that in the liver TNF-α is not affecting MT-I + II synthesis directly but in conjunction with other factors. To our knowledge, no studies have been carried out analyzing brain MT regulation after the exogenous administration of TNF-α, neither peripherally nor centrally. *In vitro* studies with brain cells are also lacking. The only study aimed at characterizing the putative role of TNF-α was carried out with TNF-α type 1 receptor-deficient mice (10). This study demonstrated that this cytokine is involved in MT-I regulation during endotoxin-elicited acute inflammatory response in some CNS areas, but not during the stress response.

The present results with the GFAP-TNFα mice indicated that either TNF-α per se or the associated inflammatory response and CNS damage upregulate

MT-I + II expression. This induction was dramatic in the cerebellum, thus paralleling the expression of the GFAP-TNFα gene, which is the highest in the cerebellum (59). Moreover, the endogenous TNF-α gene is also coordinately induced in the GFAP-TNFα mice (59). Thus, both the endogenous and the transgenic TNF-α could be driving MT-I + II induction in these transgenic mice. However, such upregulation mainly occurred in clinically affected animals. This could be somewhat surprising since the presymptomatic mice also have TNF-α production, which is lower than that of symptomatic mice but substantially higher than that of control mice (59). It is feasible to think of a threshold effect of the TNF-α concentration on MT-I + II induction, but considering the magnitude of the difference in the TNF-α expression of the presymptomatic and the symptomatic mice and the above-discussed results in liver cells, it seems likely that TNF-α would act in concert with other factors rather than exert a direct effect on MT synthesis. The CNS of symptomatic mice suffers a dramatic infiltration of lymphocytes and macrophages (59), which could produce a number of factors driving MT synthesis. Moreover, during the inflammatory response in the CNS an increased oxidative stress could be expected (27), which, in turn, could be coupled to MT-I + II upregulation (17, 18). Taken together, the results suggest that the brain MT-I + II isoforms are induced in conditions of significant injury to the CNS and that this induction could be coupled to the increased oxidative stress produced. Recent results with MT-I + II KO mice (49) give a rationale for such upregulation, demonstrating a significant role of these proteins for CNS wound healing.

Regarding the MT-III isoform, the GFAP-TNFα mice provided a good opportunity to ascertain the putative role of this protein in a situation of significant CNS damage. The discovery of MT-III (or growth inhibitory factor) raised great expectations given its apparent relationship with Alzheimer's disease (64), but as stated above this remains unclear. Significant changes of MT-III expression and/or protein levels have been observed in a number of animal models of brain damage such as stab wounds, cortical ablation, and ischemia (see (33, 36) for review). Increases, decreases, and no alterations of MT-III have been described depending on whether mRNA or protein has been analyzed, the type of damage caused, and/or the time at which the measurement is done, complicating the interpretation of the results obtained. Moreover, each particular system of CNS damage could elicit a unique set of factors responsible for the changes in MT-III regulation.

We have shown previously in the GFAP-IL6 mice (11) that the transgenic expression of IL-6 causes a highly specific pattern of MT-III RNA changes throughout the brain, with significant increases in the cerebellum and decreases, for instance, in some hippocampal areas and the occipital cortex. MT-III immunoreactivity matches consistently the RNA levels measured by *in situ* hybridization (unpublished data). This pattern of expression is noteworthy since significant damage is caused by IL-6 in all those areas, and thus the relationship of MT-III with tissue injury is not as clear-cut as with the MT-I + II isoforms, which systematically are upregulated by CNS damage. The results obtained with the GFAP-TNFα mice are in line with those of the GFAP-IL6 mice, since despite significant CNS injury, MT-III RNA levels were not increased dramatically, although in some mice a modest induction was observed, especially in the white matter of the cerebellum. Immunocytochemical analysis of the brains confirmed the small increase of the MT-III isoform and indicated that the sources of the protein were mainly amobeoid or round microglia/macrophages.

In summary, the present results demonstrate that MT-I + II are dramatically upregulated in the cerebellum of transgenic mice with astrocyte-targeted TNF-α expression only when significant damage has occurred and clinical symptoms are evident. MT-III expression was only moderately increased. The results demonstrate a MT-isoform-specific regulation in these mice and suggest that MT induction reflects the inflammatory response and damage associated to the cytokine rather than a direct effect on MT expression.

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