Trabajo 9

Role of interleukin-6 on metalothioneins response to brain injury

Manuscrito

Role of interleukin-6 on metalothioneins response to brain injury

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ABSTRACT

Focal cryo injury to the fronto-parietal cortex in 129/Sv mice elevated the Metalothionein (MT-I) but not MT-III mRNA expression in the ipsilateral cortex to the lesion. The lesions to the central nervous system (CNS) usually elicits an inflammatory response involving activation of microglia, brain macrophages and astrocytes, processes likely mediated by the release of proinflammatory cytokines. In order to determine the role of interleukin-6 (IL-6) in the control of MTs expression during the inflammatory response in the brain, we examined the effects of a focal cryo injury in interleukin-6 deficient (IL-6-/-) and normal (IL-6+/+) mice. In IL-6+/+ mice, in a similar way to 129/Sv mice, brain injury resulted in up-regulation of MT-I+II mRNA levels, while the brain specific MT-III was transiently downregulated by the lesion. In IL-6-/- mice, however, MT-I+II expression was markedly depressed compared to IL-6+/+ mice, but no effect was noted on MT-III mRNA. The present results demonstrate that IL-6 is crucial for MT-I expression. The lack of effect of IL-6 on MT-III mRNA levels in the damaged brain suggest MT isoform specific functions.

Key Words: IL-6 deficiency, brain injury, metallothionein-I+II, metallothionein-III.

INTRODUCTION

Injury to the CNS elicits a characteristic inflammatory response. Ramified microglia and bone marrow-derived monocytes are activated and transform to amoeboid brain macrophages (Andersson *et al*., 1992; Stevens and Bähr, 1993; Perry *et al*., 1995; Calvo *et al*., 1996) Regulation of the inflammatory response is mediated by several cytokines including interleukin-1 (IL-1), (IL-6), and tumour necrosis factor-α (TNF-α), with microglia, brain macrophages and astrocytes as the most abundant cellular sources (Giulian *et al*., 1994a; Hopkins and Rothwell, 1995; Calvo *et al*., 1996; Gebicke-Haerter *et al*., 1996)

IL-6 is a proinflammatory cytokine and a neuropoietin (Hopkins and Rothwell, 1995). IL-6 affects CNS by stimulating the activity of the hypothalamus-adrenocortical axis, inducing fever or neuronal differentiation (Pousset, 1994; Mandrup-Poulsen *et al*., 1995; Chai *et al*., 1996). IL-6 has a survival promoting action on cultured mesencephalic, catecholaminergic and septal neurons (Hama *et al*., 1991) and protects cultured neurons from the toxic effects of NMDA and glutamate (Toulmond *et al*., 1992; Yamada and Hatanaka, 1994). IL-6 is important for the inflammatory response and is involved in reactive gliosis, (Campbell *et al*., 1993; Chiang *et al*., 1994; Klein *et al*., 1997).

IL-6 induces expression of acute-phase proteins such as MTs (De *et al*., 1990; Schroeder and Cousins, 1990). In CNS, MTs occur in isoforms MT-I, MT-II and MT-III (Palmiter *et al*., 1992). In the brain, MTs are espressed in glial, ependymal, neuronal, and choroid plexus cells (Uchida *et al*., 1991; Masters *et al*., 1994; Penkowa and Moos, 1995; Vela *et al*., 1997). The physiological roles of the different MT isoforms in the brain still remain to be established; presumably MT-III functions differ from those of MT-I+II, as suggested from in vitro studies in that only MT-

III has inhibitory effects on rat neonatal neuronal survival and is involved in the pathogenesis of Alzheimer's disease (Uchida *et al*., 1991; Erickson *et al*., 1994; Sewell *et al*., 1995). In this study we examine the expression of MT-I and MT-III in normal and IL-6 deficient mice following focal cryo injury. Our results demonstrate IL-6 as a crucial regulator of MT expression. IL-6 appears to up regulate MT-I+II but not MT-III, which suggests different physiological roles for these MT isoforms.

MATERIAL AND METHODS

Production of IL-6 deficient mice.

The generation and development of the IL-6 deficient mice (IL-6-/-) were described previously (Kopf *et al*., 1994). The mice were maintained on a C57BL/6x129/Sv genetic background and provided by F. Hoffmann-LaRoche (Basel, Schwitzerland). Wildtype (IL-6+/+) C57BL/6 and 129/Sv mice were used as controls (Jackson, Germany).

Experimental Procedures

Adult mice were lesioned under tribromethanol anaesthesia. The skull above the right fronto-parietal cortex was exposed, and a focal cryo injury on the surface of the brain was produced with dry ice (-78°C). The animals were housed in cages with free access to food and water. The handling of the animals were approved by the proper National Committees of Animal Research in Spain.

We have used IL-6 deficient and control male mice for the experiment. Animals were killed at 0, 6 and 24 hours after lesion (n=3 except unlesioned IL-6 mice (n=4). Brains were dissected and processed for MT-I and MT-III *in situ* hybridization.

MT-I and MT-III in situ hybridization

For in situ hybridization studies, unlesioned and lesioned mice were killed by cervical dislocation and the brains immediately frozen in liquid nitrogen and stored at –80°C. Lesioned mice were killed 6 and 24 hours after the injury.

Brain MT-I and MT-III mRNA levels were assayed by in situ hybridization in control and cryo lesioned mice who were killed 6 and 24 hours after the lesion. Serial coronal 20 μm sections were cut on a cryostat and mounted on poly-L-lysine coated slides. For MT-I mRNA studies, we used the mouse cDNA kindly provided by Dr. R.D. Palmiter (University of Washington, Seattle, WA, USA). MT-I and MT-II are isoforms regulated co-ordinately and thus we assume that MT-I mRNA levels are representative of the MT-I+II isoforms (Yagle and Palmiter, 1985). 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 aminoacids and the 3´untranslated region until the poly G stretch of MT-III mRNA generously provided by Dr. G.K. Andrews (The University of Kansas Medical Center, Kansas City, Kansas, USA). Both the MT-I and MT-III cDNA were labelled with (^{35}S) α–UTP using a SP6/T7 transcription kit (Boehringer Mannheim, Mannheim, Germany). Preparation of sense and antisense probes and the in situ hybridization procedure were performed as previously described (Carrasco *et al*., 1998a). Autoradiography was performed exposing the film (Hyperfilm-MP, Amersham) to the slides for several days. All sections to be compared were prepared simultaneously and exposed to the same autoradiographic film. MT-I or MT-III mRNA levels were semiquantitatively determined in 4 sections per brain area and animal, by measuring the optical densities and the number of pixels in defined areas with a Leica Q 500 MC system. The MT-I and MT-III mRNA values shown are expressed in arbitrary units (number of pixels x optic density).

Statistical analysis

Results were evaluated by two-way analysis of variance (ANOVA), with strain and freeze lesion as main factors. Separate ANOVA were carried out for the ipsilateral and contralateral hemispheres of the lesioned mice. When the interaction was significant, it was interpreted to be the consequence of a specific effect of the IL-6 deficiency during the lesion.

RESULTS

Effect of IL-6 deficiency on MT-I expression

Fig. 1 shows a representative MT-I and MT-III in situ hybridization analysis of IL-6+/+ and IL-6-/- mice of control and lesioned (24 h after the lesion) mice. In IL-6+/+ mice, the MT-I isoform showed significantly increased MT-I mRNA levels in the parenchyma surrounding the lesion but also in other areas of the ipsilateral hemisphere. A smaller but significant upregulation was also observed in the contralateral hemisphere. In IL-6-/- mice, MT-I mRNA levels were also significantly increased; however, the MT-I mRNA levels were not as increased as those of IL-6+/+ mice were. Fig. 2 shows the quantification carried out in several

mice of each experimental period (0, 6 and 24 h after the lesion). MT-I mRNA levels were increased in the ipsilateral cortex in a time-dependent manner ($p<0.001$); the induction was lower in IL-6-/- mice ($p<0.01$). A small but significant ($p<0.001$) increase was also observed in the cortex of the contralateral hemisphere of lesioned IL-6+/+ mice, and again the induction tended to be lower in IL-6-/- mice ($p<0.072$).

Effect of IL-6 deficiency on MT-III expression

In IL-6+/+ mice, in situ hybridization analysis of the MT-III isoform indicated that the lesion (24 h) did not significantly affect MT-III mRNA levels in the cortex surrounding the lesion (Fig. 1). However, in Fig. 2 the results of several mice shows a transient decrease of the MT-III mRNA levels at 6 h after the lesion, and an increase at 24 h compared to the 6 h lesioned mice. No clear difference in this response was observed in IL-6-/- mice, although the transient decrease was of higher magnitude than in IL-6+/+ mice.

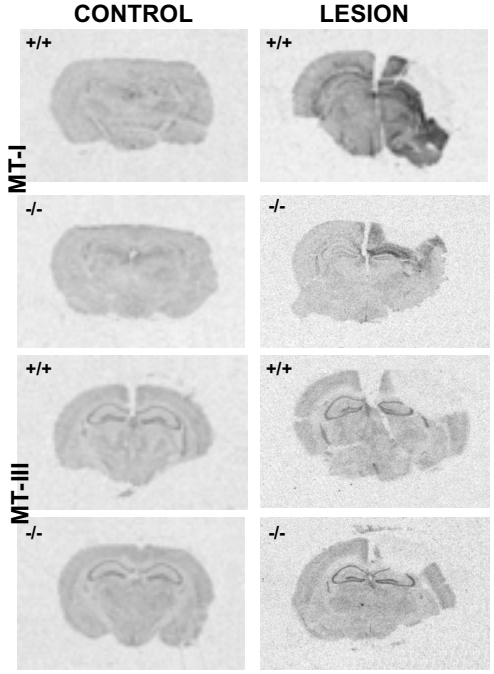

Fig. 1. Representative in situ hybridization analysis of MT-I and MT-III mRNA levels in IL-6+/+ and IL-6-/- mice. Unlesioned (control) and lesioned (24 hours) mice are shown. The cryo lesion significantly increased MT-I mRNA levels in both control and IL-6 deficient mice, but the increase was decreased in the latter. In contrast, MT-III mRNA levels transiently decreased (see Fig. 7) and at 24 h were similar to unlesioned mice. IL-6 deficiency did not have clear effects.

DISCUSSION

In the CNS, MTs occur in isoforms MT-I, MT-II and MT-III; the latter is also referred to as Growth Inhibitory Factor (GIF) because it has an inhibitory role on the survival of rat neurons cultured with human brain extracts in vitro (Uchida *et al*., 1991). MTI+II are widely expressed, while MT-III is primarily confined to the brain (Uchida *et al*., 1991; Palmiter *et al*., 1992; Kobayashi *et al*., 1993). The actual physiological role(s) of the different MT isoforms in the brain still remains to be established, but presumably they will be related to their high affinity binding capacities for the essential metals, zinc and copper and/or their antioxidant functions (Aschner *et al*., 1997; Hidalgo *et al*., 1997). In vitro studies suggest that MT-III functions will differ from those of MTI+II (Erickson *et al*., 1994; Sewell *et al*., 1995; Faller and Vasák, 1997), but we must await in vivo studies to verify such differences.

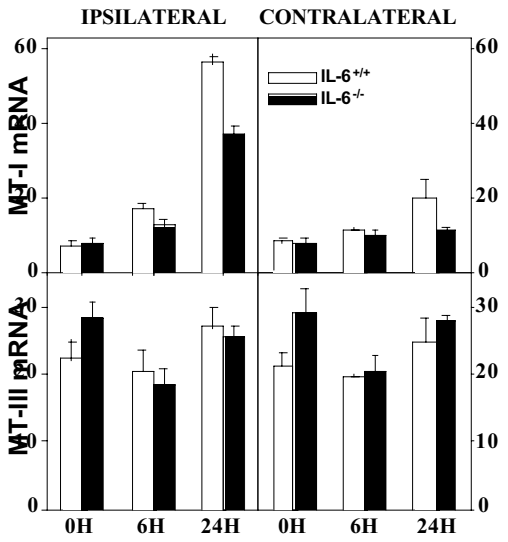

Fig. 2 In situ hybridization analysis of MT-I and MT-III mRNA levels. Three mice of each experimental group (unlesioned-except IL-6-/-, where n=4-, and 6 and 24 hours after the lesion) and strain were analysed as shown in Fig. 3. Specific measurements were carried out in the ipsilateral and contralateral cortex, and the results were analysed with two-way ANOVA. In the ipsilateral cortex, MT-I was up regulated by the lesion in a time-dependent manner ($p<0.001$), and the induction was lower in the IL-6 deficient mice ($p<0.01$). In contrast, MT-III mRNA was transiently downregulated ($p<0.05$), and IL-6 deficiency had no significant effect ($p<0.07$). Similar

trends were observed in the contralateral cortex. Results are mean±SE.

The understanding of the regulation of brain MT isoforms could help in the elucidation of their physiological roles. A number of studies have shown that the intracerebral expression of MTI+II is clearly up regulated by a number of insults and in several human adult neurodegenerative disorders (Hidalgo *et al*., 1990; Sillevis Smitt *et al*., 1992; Dalton *et al*., 1995; Penkowa and Moos, 1995; Penkowa *et al*., 1997). On the other hand, MT-III, which was discovered unexpectedly when studying the AD pathogenesis (Uchida *et al*., 1991), has also been demonstrated to be significantly altered by a number of animal models of brain damage (Hozumi *et al*., 1995; Hozumi *et al*., 1996; Inuzuka *et al*., 1996; Yuguchi *et al*., 1997). Taken together, these studies strongly suggest that MTs are important proteins in the brain for coping with the tissue damage elicited by a wide array of factors and diseases. However, the responses of the brain MT isoforms have not yet been studied simultaneously during brain trauma, and the factors responsible for the control of their induction are unknowns.

Injury to the CNS leads to a well-regulated inflammatory response, which is important for tissue regeneration (Giulian *et al*., 1988; Unsicker *et al*., 1992; Gebicke-Haerter *et al*., 1996). Cytokines such as IL-6 and GM-CSF are considered as the signalling molecules during inflammation (Unsicker *et al*., 1992; Giulian *et al*., 1994b; Hopkins and Rothwell, 1995; Gebicke-Haerter *et al*., 1996; Ridet *et al*., 1997). The relative importance of each cytokine, however, has been difficult to ascertain since they are pleiotropic factors with significant overlapping functions. In the present study we examine mice carrying a null mutation in the IL-6 gene, a unique approach to determine the role of IL-6 on the regulation of MTs response to lesion.

In normal mice, injury was followed by a MT-I mRNA upregulation but not MT-III in the ipsilateral cortex to lesion in both the vicinity of the lesion and in deeper layers. The MT-I mRNA is also induced in the contralateral cortex but to a lower extent suggesting that the response of MTs is dependent of tissue injury and/or inflammation and not merely of a stress associated response. Cytokines are putative factors that control the expression of MTs. IL-6 its a major cytokine and one of its hallmarks functions is the induction of acute-phase proteins in liver and brain (Heinrich *et al*., 1990; Campbell *et al*., 1993), and is induced in the brain parenchyma after stimulus like LPS (Schöbitz *et al*., 1993; Laye *et al*., 1994) or brain trauma (Hariri *et al*., 1994). It is well known that IL-6 is a major regulator of the liver MT-I+II isoforms (De *et al*., 1990; Schroeder and Cousins, 1990), and these proteins belong to the acute-phase response (Karin, 1985). We have recently shown that IL-6 is also involved in the regulation of brain MTs. The i.c.v. Injection of IL-6 in rats increases MT-I+II levels in some brains areas (Hernández and Hidalgo, 1998), and transgenic mice overexpressing IL-6 in the CNS also shows increased MT-I+II expression (Hernández *et al*., 1997; Carrasco *et al*., 1998b). We have previously shown that IL-6 participate in the regulation of MT-I but not MT-III in the inflammation elicited by the LPS administration (Carrasco *et al*., 1998a), confirming that this cytokine is a inducer of MTs when administered directly but also in the inflammation context. The present results confirm and extend these studies. The IL-6 deficiency has no effect in basal levels of MT-I mRNA but decreased significantly its induction in injury brains. We conclude that IL-6 is a positive regulator of the synthesis of MT-I in inflammatory condition like that elicited in the focal cryo-injury. In contrast, IL-6 deficiency has no effect on MT-III RNA levels suggesting that this isoform it is not controlled by IL-6, at least in our brain injury model.

Increased MT levels could exert a significant protection, since MT-I+II have antioxidant properties (Sato and Bremner, 1993), and neutralise free metals ions released from dying cells and BBB damage, in that excess free metal ions are toxic to neurons (Hidalgo *et al*., 1994; Koh and Choi, 1994; Aschner *et al*., 1996). MT-I+II induction was severely reduced in IL-6-/- mice, and these animals showed more neuronal death in the vicinity of the lesion (data not shown), supporting a neuroprotective role of MT-I+II. Some results in MT-I+II null mice support such a role since the MT-I+II deficiency leads to a more severe hallmarks of injury and a delayed healing of the lesion (Penkowa *et al*., 1999).

In conclusion, MT-I mRNA levels are elevated during brain injury and IL-6 is a major inducer of MT-I in damaged brain. MT-I and MT-III probably have different functions in virtue of the findings exposed above.

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Trabajo 10

Metallothionein (MT)-I and –III response to cryo injury and the effect of MT-I+II genetic deficiency on MT-III

Manuscrito

Metalothionein (MT)-I and –III response to cryo injury and the effect of MT-I+II genetic deficiency on MT-III

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ABSTRACT
Focal cryo injury to the fronto-parietal cortex in 129/Sv mice elevates the Metalothionein (MT-I) and MT-III mRNA expression in the ipsilateral but not in the contralateral cortex to the lesion. However, the dynamic and spatial responses of the two isoforms are different. MT-I is up- regulated in the vicinity of the lesion and in deeper layers of the cortex. MT-I RNA levels are maintained elevated in the border of the lesion at least for 13 days post lesion (dpl). MT-III mRNA levels are affected by the injury but only in the vicinity of the lesion. Initially, the MT-III expression decreases (1 day after lesion) and it is induced for the next days, reaching a maximum at 13 days post lesion. The MT-I+II genetic deficiency has no effect on the MT-III response to injury suggesting that there are not compensatory mechanisms between the two isoforms. The present results demonstrate that MT-I and MT-III are differently regulated and suggest MT isoform specific functions.

Key Words: brain injury, metallothionein-I+II, metallothionein-III.

INTRODUCTION

Metallothioneins (MTs) are a family of low molecular weight, heavy metal binding, cysteine-rich proteins. In the mouse, there are four isoforms, MT-I to MT-IV (Palmiter *et al*., 1992; Quaife *et al*., 1994). In the CNS, MTs occur in the isoforms MT-I, MT-II and MT-III. MT-I+II are expressed in virtually all tissues, and in the brain they are localised mainly in astrocytes, microglia, leptomeningeal cells, ependyma and choroid plexus epithelium (Young *et al*., 1991; Masters *et al*., 1994b; Penkowa and Moos, 1995; Penkowa *et al*., 1997). MT-III is primarily confined to the brain, but the data regarding its cellular localisation are conflicting since in situ hybridization analysis suggest neurons as the main site of expression (Masters *et al*., 1994b), while immunocytochemistry studies suggest microglia/macrophages and astrocytes as the cells with the highest MT-III protein content (Hozumi *et al*., 1996; Yamada *et al*., 1996).

The intracerebral expression of MT-I+II is clearly up regulated during pathological conditions induced by trauma (Penkowa and Moos, 1995), immobilisation stress (Hidalgo *et al*., 1990), kainic acid-induced seizures (Dalton *et al*., 1995; Zheng *et al*., 1995), excitotoxic NMDA cortex damage (Hidalgo *et al*., 1997), and administration of 6-aminonicotinamide (Penkowa *et al*., 1997). Furthermore, MT-I+II expression is increased in several human adult neurodegenerative disorders such as Alzheimer's disease (AD) and Pick's disease (Duguid *et al*., 1989), and amyotrophic lateral sclerosis (Sillevis Smitt *et al*., 1992), as well as in ageing (Suzuki *et al*., 1994) and after brain ischaemia (Neal *et al*., 1996). MT-III was discovered unexpectedly as a factor decreased in AD (Uchida *et al*., 1991), and a number of animal models have shown that MT-III mRNA or protein levels are significantly altered during CNS damage (Hozumi *et al*., 1995; Hozumi *et al*., 1996; Yamada *et al*., 1996; Yuguchi *et al*., 1997).

Taken together, these studies strongly suggest that MTs are important proteins in the brain for

coping with the tissue damage elicited by a wide array of factors and diseases. However the specific role of each isoform remains to be established. Those for MT-I+I could be related to their putative antioxidant functions as well as zinc and/or copper metabolism (Sato and Bremner, 1993; Aschner, 1996; Kelly *et al.*, 1996; Hidalgo *et al.*, 1997). Presumably the MT-III functions will differ from those of their normal counterparts MT-I+II, as suggested from in vitro (Uchida *et al.*, 1991; Erickson *et al.*, 1994) and in vivo studies (Quaife *et al.*, 1998).

The understanding of the regulation of brain MT isoforms could help in the elucidation of their physiological roles. However, the responses of the brain MT isoforms have not yet been studied simultaneously during brain trauma. In this study we examine the expression of MT-I and MT-III in normal and MT-I+II genetically deficient mice (MT-I+II-KO) following focal cryo injury in order to asses possible compensatory mechanism between the two isoforms.

MATERIAL AND METHODS

Production of MT-I+II deficient mice.

Homozygous MT-I+II knockout (KO) mice were generated as previously described (Masters *et al.*, 1994a). The KO mice were raised on the 129/Sv genetic background; therefore mice from this strain were used as controls.

Experimental Procedures

Adult mice were lesioned under tribromethanol anaesthesia. The skull above the right fronto-parietal cortex was exposed, and a focal cryo injury on the surface of the brain was produced with dry ice (-78°C). The animals were housed in cages with free access to food and water. The handling of the animals were approved by the proper National Committees of Animal Research in Spain.

Male mice (129/Sv (n=15) and MT-I+II KO mice (n=15)) were lesioned as described and sacrificed along 0, 1, 3, 7 and 13 days post lesion (n=3 per group). Brains were dissected and treated for *in situ* hybridization with MT-I and MT-III probes.

MT-I and MT-III in situ hybridization

For in situ hybridization studies, unlesioned and lesioned mice were killed by cervical dislocation and the brains immediately frozen in liquid nitrogen and stored at –80°C.

Brain MT-I and MT-III mRNA levels were assayed by in situ hybridization in control and cryo lesioned mice. Serial coronal 20 μm sections were cut on a cryostat and mounted on poly-L-lysine coated slides. For MT-I mRNA studies, we used the mouse cDNA kindly provided by Dr. R.D. Palmiter (University of Washington, Seattle, WA, USA). MT-I and MT-II are isoforms regulated co-ordinately and thus we assume that MT-I mRNA levels are representative of the MT-I+II isoforms (Yagle and Palmiter, 1985). 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 aminoacids and the 3´untranslated region until the poly G stretch of MT-III mRNA generously provided by Dr. G.K. Andrews (The University of Kansas Medical Center, Kansas City, Kansas, USA). Both the MT-I and MT-III cDNA were labelled with (^{35}S) α–UTP using a SP6/T7 transcription kit (Boehringer Mannheim, Mannheim, Germany). Preparation of sense and antisense probes and the in situ hybridization procedure were performed as previously described (Carrasco *et al.*, 1998a). Autoradiography was performed exposing the film (Hyperfilm-MP, Amersham) to the slides for several days. All sections to be compared were prepared simultaneously and exposed to the same autoradiographic film. MT-I or MT-III mRNA levels were semiquantitatively determined in 4 sections per brain area and animal, by measuring the optical densities and the number of pixels in defined areas with a Leica Q 500 MC system. The MT-I and MT-III mRNA values shown are expressed in arbitrary units (number of pixels x optic density). For more detailed analysis of the expression, hybridized sections were dipped in autoradiographic emulsion (LM-1 emulsion, Amersham), exposed to the proper time, developed and counterstained for microscopy examination.

Statistical analysis

MT-I results were evaluated by one-way analysis of variance (ANOVA), with dpl as grouping factor. MT-III results were evaluated by two-way ANOVA, with strain and dpl as main factors. Separate ANOVA were carried out for the ipsilateral and contralateral hemispheres of the lesioned mice.

RESULTS AND DISCUSSION

Figure 1 shows representative MT-I and MT-III in situ hybridization analysis of 129/Sv mice in unlesioned and lesioned animals. Quantitative analysis of several mice from each group is shown in figure 2.

MT-I mRNA levels were significatively increased ($p<0.001$) in the ipsilateral cortex to lesion at all the time-points examined but not in the contralateral cortex. The lack of induction in contralateral cortex suggests that the response of MTs is dependent of tissue injury and/or inflammation and not merely of a stress associated response. The expression of MT-I mRNA raised up at the first day post lesion and remained elevated up to the last time-point studied (13 days after lesion). Initially, days 1 and 3 post-lesion, the MT-I mRNA was induced in the vicinity of the lesion and in deeper layers of the ipsilateral cortex. Induction was also observed in the contralateral corpus callosum. Others structures, like hippocampus layers, were positive for MT-I mRNA but the degree of induction was dependent of extend of the lesion, being maximal if the necrotic area raised the boundaries of the hippocampus (data not shown). Progressively induction of the MT-I mRNA expression was limited to the cells around the lesion, presumibly in the cells that form the glial scar.

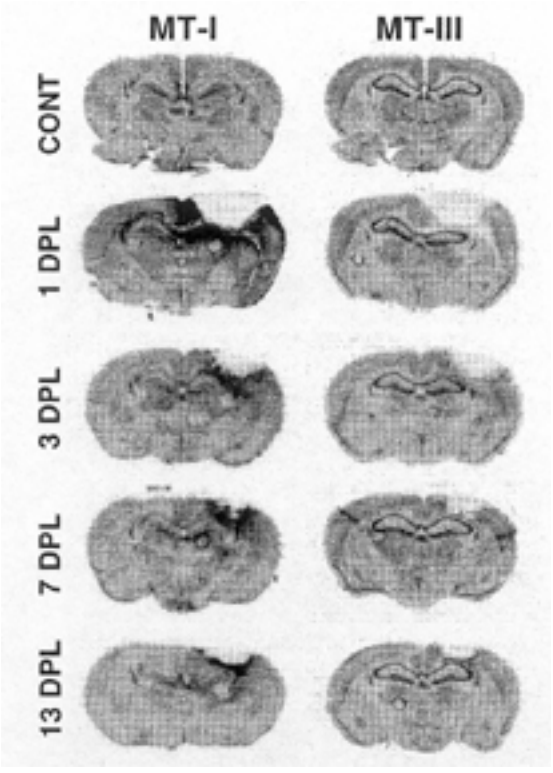

Figure1. Representative in situ hybridization analysis of MT-I and MT-III mRNA levels in 129/Sv mice. Unlesioned (Control) and lesioned (1, 3, 7 and 13 dpl) are shown. The cryo lesion significantly increased MT-I and MT-III mRNA levels, but the time-response of each isoform was different. MT-I expression was elevated in all the times examined after lesion. In contrast, MT-III was only up regulated at 7 and 13 dpl.

The temporal pattern of MT-I+II induction suggests that they form part of the plethora of mechanisms involved in protection against CNS inflammation. Consistent with such a role, MT-I+II levels have been observed to be increased in several human neurodegenerative disorders as well as in a number of experimental models (Hidalgo *et al*., 1997). Furthermore, in transgenic mice with IL-6 expression targeted to astrocytes, which show profound microgliosis and astrogliosis and upregulation of several inflammatory and other host-response genes (Campbell *et al*., 1993), MT-I+II were also dramatically induced (Hernández *et al*., 1997; Carrasco *et al*., 1998b). These and other preliminary results from our laboratory suggests that MT-I+II upregulation during brain damage is mediated by cytokines such as IL-6. Thus, the above studies suggest that MT-I+II appear to behave as acute-phase proteins in the brain, but their physiological roles remain uncertain.

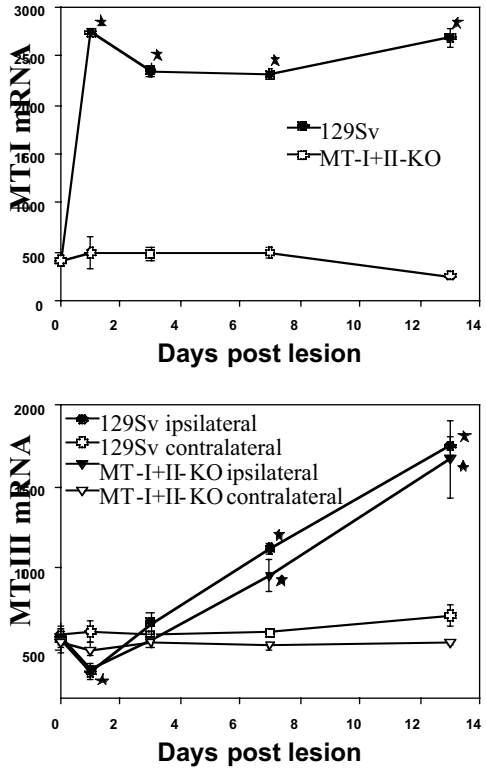

Figure 2. In situ hybridization analysis of MT-I and MT-III mRNA in control and MT-KO mice. MT-I and MT-III results are shown in the top and bottom panels respectively. Three mice of each experimental group and strain were analysed. Specific measurements were carried out in the ipsilateral and contralateral cortex. The results were analysed with one-way ANOVA for MT-I (time after

lesion as group factor) and two-way ANOVA for MT-III (with strain and time after lesion as main factors). * p<0.001 vs. control.

In injured brain the damaged cells relase free radicals to the medium, that can be deletereous to surrounding cells, especially to neurons (Coyle and Puttfarcken, 1993). In this contex the MT-I upregulation could exert protective roles by scavenging the free radicals (Sato and Bremner, 1993). In some injury models it has been proposed a preponderant extracelular Zn effect on neuronal death (Frederickson *et al.*, 1989; Koh *et al.*, 1996). MT-I could neutralize free Zn released during the injury from dying cells. In fact, MT-I+II deficient mice have more severe injury hallmarcks and retarded healing after cryo injury, supporting a protective role for MT-I+II in damaged brain (Penkowa *et al.*, 1999).

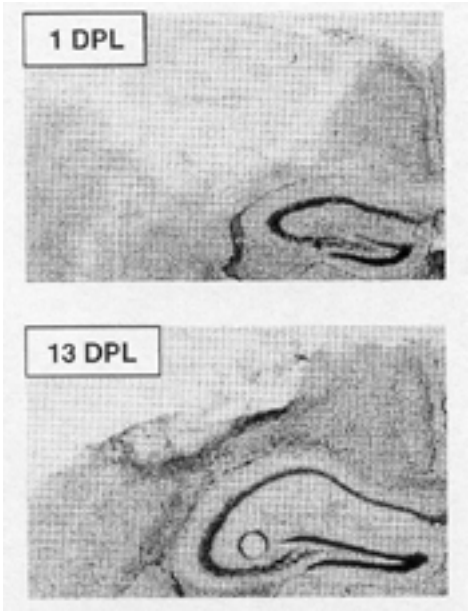

Figure 3. MT-III mRNA expression in ipsilateral cortex to lesion in representatives animals at 1 and 13 days post lesion. MT-III RNA signal is visualised as black precipitate and the cells nuclei are stained in blue with tionin. MT-III expression is enhanced in the vicinity of the lesion at 13 dpl but not at 1 dpl. In contrast, the lesion does not affect MT-III levels in the pyramidal layer of hippocampus (major area of MT-III expression in normal mice).

The MT-III mRNA levels were affected by the lesion to a lower extent than MT-I mRNA but significant changes were observed (p<0.001). Only the cells located in the border of the lesion respond to the injury, and the effect is difficult to note in an autoradiogram (figure 1). For more detailed analysis, sections deeped in autoradiographic emulsion and developed are shown in figure 3. The spatial induction pattern of MT-III suggest that, in this contex, the MT-III expressing cells are glial cells from glial scar as noted in other injury model (Acarin *et al.*, 1999).

In control mice, the brain specific isoform, MT-III, has a different dynamic response to injury. Initially, MT-III expression was decreased at 1 day post lesion, restored to normal after 3 days and increased from this point to the last day of the experiment (bottom panel of figure 2). Obviously, MT-III is controlled in a different way that MT-I. We have previously shown that MT-I and MT-III response to differents stimulus and the factors involved in their control are not the same. In fact, MT-I, but not MT-III, is upregulated by stress and LPS (Carrasco *et al.*, 1998a). Furthermore, transgenic IL-6 overexpression in the CNS leads to increased MT-I, but not MT-III, levels (Hernández *et al.*, 1997; Carrasco *et al.*, 1998b). In addition, we demonstrate in the present work that in lesioned brain the MT-I+II deficiency has no effect on the MT-III expression (figure 2). This result demonstrates that there are no compensatory mechanisms between MT-I and MT-III in the cryolesioned brain. Preliminary results from our lab aimed from other brain lesion models are consistent with the data presented here. Our results suggest that the MT-I and MT-III regulatory factors are different and that probably MTs have isoform specific functions.

The pattern of MT-III expression showed in this work has been reported for this isoform in others injury models, including stab wounds (Hozumi *et al.*, 1995; Hozumi *et al.*, 1996), cortical ablation (Yuguchi *et al.*, 1995a), ischemia (Inuzuka *et al.*, 1996; Yuguchi *et al.*, 1997), nerve transection (Yuguchi *et al.*, 1995b) and excitotoxicity (Acarin *et al.*, 1999). MT-III has a neuron growth inhibitory action in culture in the presence of brain extract (Uchida *et al.*, 1991; Erickson *et al.*, 1994). In virtue of this action sometimes it is referred as growth inhibitory factor (GIF)(Uchida *et al*, 1991). It has been proposed that decreases of MT-III could facilitate the neuronal regeneration after lesion and MT-III increases during brain damage could reflect attempts to limit neuronal sprouting promoted by neurotrophic factors produced in response to tissue injury (Yuguchi *et al.*, 1995a; Hozumi *et al.*, 1998). MT-III expression is also afected in astrocyte culture with cell density, being minimal in a confluent monolayer (Uchida, 1999). The results presented here add more circumstantial evidence to the putative role of MT-III as neurotrophic factor. In addition, MT-III may bind zinc and copper and scavenge free oxygen radicals comparably to MT-I+II (Aschner, 1996; Hidalgo *et al.*, 1997). Data obtained in MT-III deficient mice also suggest a protective role of MT-III in neuronal death induced with kainic acid injections (Erickson *et al.*, 1997). A MT-III role as antioxidant or regulating de metal metabolism in the cryo-lesion can not be ruled out.

In conclusion, MT-I and MT-III are induced during brain injury but are regulated in different way. The fact that MT-I and MT-III have different expression dynamics and there are no compensatory mechanism between them suggest that they have different functions.

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