Expression of Metallothionein-I, -II, and -III in Alzheimer Disease and Animal Models of Neuroinflammation

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In recent years it has become increasingly clear that the metallothionein (MT) family of proteins is important in neurobiology. MT-I and MT-II are normally dramatically up-regulated by neuroinflammation. Results for MT-III are less clear. MTs could also be relevant in human neuropathology. In Alzheimer disease (AD), a major neurodegenerative disease, clear signs of inflammation and oxidative stress were detected associated with amyloid plaques. Furthermore, the number of cells expressing apoptotic markers was also significantly increased in these plaques. As expected, MT-I and MT-II immunostaining was dramatically increased in cells surrounding the plaques, consistent with astrocytosis and microgliosis, as well as the increased oxidative stress elicited by the amyloid deposits. MT-III, in contrast, remained essentially unaltered, which agrees with some but not all studies, of AD. *In situ* hybridization results in a transgenic mouse model of AD amyloid deposits, the Tg2576 mouse, which expresses human Aβ precursor protein harboring the Swedish K670N/M671L mutations, are in accordance with results in human brains. Overall, these and other studies strongly suggest specific roles for MT-I, MT-II, and MT-III in brain physiology. Exp Biol Med 231:1450–1458, 2006

Key words: metallothionein-I; metallothionein-II; metallothionein-III; Alzheimer disease; transgenic mice; Tg2576; neuroinflammation

Introduction

In recent years an overwhelming number of studies have shown that metallothioneins (MTs) play a major role in brain physiology (1–3). There are four closely linked *MT* genes (*MT-1* through *MT-4*) present in rodents (4, 5). MT-I and MT-II are widely expressed in a coordinative manner (6–8), whereas MT-III and MT-IV show a much more restricted tissue expression (primarily localized in the central nervous system [CNS] and stratified squamous epithelia, respectively).

Results for MT-I and MT-II as brain injury responsive factors are clear-cut. Neuroinflammation seems to be a major condition promoting MT-I and MT-II up-regulation, including in humans. Indeed, increased levels of these proteins have been demonstrated in a number of human neurological diseases, including Alzheimer disease (AD) (9–13), Pick disease (9), short-course Creutzfeld-Jakob disease (14), amyotrophic lateral sclerosis (ALS) (15–17), and multiple sclerosis (MS) (18, 19). It is also well known that MT-I and MT-II isoforms are dramatically up-regulated in the brain following many types of insults (2, 3). Results obtained with transgenic mice have demonstrated a neuro-

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protective role for these proteins following mild focal cerebral ischemia and reperfusion (20, 21), kainic acid–induced seizures (22), 6-hydroxydopamine administration (23), damage to dopamine neurons (24), in models of ALS (25, 26) and MS (27, 28), traumatic brain injury (29–31), and transgenic interleukin (IL)-6–induced neuropathology (32–34).

Originally referred to as growth inhibitory factor because of its ability to inhibit neuronal growth *in vitro*, MT-III was discovered in the human brain and was suggested to be underlying the aberrant sprouting characteristic of AD (35). Several studies have examined MT-III expression in the brain of patients with AD, but unfortunately, the reported down-regulation in such brains is not a consistent finding (35–41). Also, in contrast to MT-I and MT-II, MT-III expression may be up-regulated or down-regulated in Down syndrome (42); Creutzfeld-Jakob disease (14); pontosubicular necrosis (43); and in Parkinson disease, meningitis, and ALS (41). In animal models of brain injury, MT-III regulation is complex, as indicated by up-regulation or down-regulation depending on the model, timing, and other factors (2). Results obtained in transgenic mice suggest MT-III has a neuroprotective role in models such as kainic acid–induced seizures (44) and increased oxidative damage (G93A SOD1 mouse) (26), but the opposite could be concluded following cortical cryolesion (45) and peripheral nerve injury (46). These discrepancies highlight the importance of further analyzing MT-I and MT-III expression both in human diseases and animal models, regardless of the somewhat clear fact that the roles of MT-III will differ substantially from those of MT-I and MT-II.

Materials and Methods

AD and Control Brains. Postmortem autopsy studies were performed in five patients with dementia and three control patients with no neurological symptoms in the Pathology Department of Vall d'Hebrón Hospital, Barcelona. Relevant clinical data and autopsy findings related to hypertensive vascular disease, considered as risk factors for vascular dementia in the elderly, were controlled. The brains of all patients and controls were removed 10–20 hrs after death, fixed in 10% formaldehyde buffered to pH 7, and stabilized with methanol. The neuropathological study was performed in paraffin-embedded tissue obtained from the frontal cortex and hippocampus, sectioned at a thickness of 5 μm, and mounted onto slides.

Tissue Processing and Histochemistry. Sections were processed as previously described (29–31). Hematoxylin and eosin staining of brain sections was performed according to standard procedures.

Immunohistochemistry. Sections were incubated overnight at 4°C with the following antibodies or detection systems, or both: mouse anti-human CD68 (Serotec, Kidlington, UK) (marks macrophages); mouse anti-human interleukin (IL)-1β 1:50 (Biogenesis, Kidlington, UK); rabbit anti-mouse tumor necrosis factor (TNF)-α 1:100 (Biosource, Cammarillo, CA); rabbit anti-NITT 1:100 (Alpha Diagnostic International, San Antonio, TX) (a marker for peroxynitrite-induced nitration of tyrosine residues); rabbit anti-MDA 1:100 (Alpha Diagnostic Intl.) (a marker for malondialdehyde [MDA] produced as a byproduct of fatty acid peroxidation); mouse anti-8-oxoguanine 1:100 (Chemicon Intl., Chandlers Ford, UK) (marks oxidative DNA damage); rabbit antineurofibrillary tangles 1:200 (Abcam, Cambridge, MA) (marks several of the insoluble proteins that form intracellular neurofibrillary tangles); goat anti-human amyloid precursor protein Frameshift Mutant (APP-FM) 1:50 (Chemicon Intl.); rabbit anti-human β-amyloid (peptide fragments 40, 42, and 43) 1:200 (Merck Biosciences, Nottingham, UK); rabbit anti-human (activated/cleaved) caspase-3 1:50 (Cell Signaling Technology, Inc., Medinova Scientific A/S, Denmark); mouse anti-horse MT-I+II 1:50 (DakoCytomation, Glostrup, Denmark); rabbit anti-rat MT-I+II 1:500 (47); and rabbit anti-rat MT-III 1:500 (39).

Secondary Antibodies and Detection Systems. Sections were incubated for 30 mins at room temperature with the following secondary antibodies: biotin-conjugated anti-rabbit IgG 1:400 (Sigma-Aldrich, St. Louis, MO) or biotin-conjugated anti-mouse IgG 1:200 (Sigma-Aldrich) or biotin-conjugated anti-goat IgG 1:20 (Amersham Biosciences, Little Chalfont, UK) or biotin-conjugated anti-rat IgG 1:1500 (Amersham Biosciences); or biotin-conjugated anti-mouse IgM 1:20 (Jackson Immunoresearch Laboratories, Inc., West Grove, PA). All the secondary antibodies were detected by StreptABComplex/horseradish peroxidase followed by biotinylated tyramide and streptavidin-peroxidase complex (tyramide signal amplification; TSA indirect) (NEN, Life Science Products, Boston, MA) prepared following the manufacturer's recommendations.

***In Situ* Detection of DNA F (TUNEL).** Terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP)-biotin nick end labeling (TUNEL) was performed using the Fragment End Labeling (FragEL) Detection Kit (Calbiochem, La Jolla, CA).

Fluorescence Histochemistry. Mouse anti-horse MT-I+II was incubated simultaneously overnight at 4°C with APP-FM, and the next day, they were incubated with a fluorescein isothiocyate (FITC)-linked TUNEL kit (Calbiochem). The primary antibodies were visualized by using donkey anti-mouse IgG linked with aminomethylcoumarin (AMCA) 1:20 (Jackson ImmunoResearch Laboratories) and donkey anti-goat IgG 1:30 linked with TexasRed (TXRD) (Jackson ImmunoResearch Laboratories).

We also performed double immunofluorescence for neurofibrillary tangles (as mentioned above) and goat anti-human GFAP 1:100 (sc-6170; Santa Cruz Biotechnology Inc., Santa Cruz, CA). The primary antibodies were visualized by using donkey anti-goat IgG 1:50 linked with FITC (Jackson ImmunoResearch Laboratories) and donkey

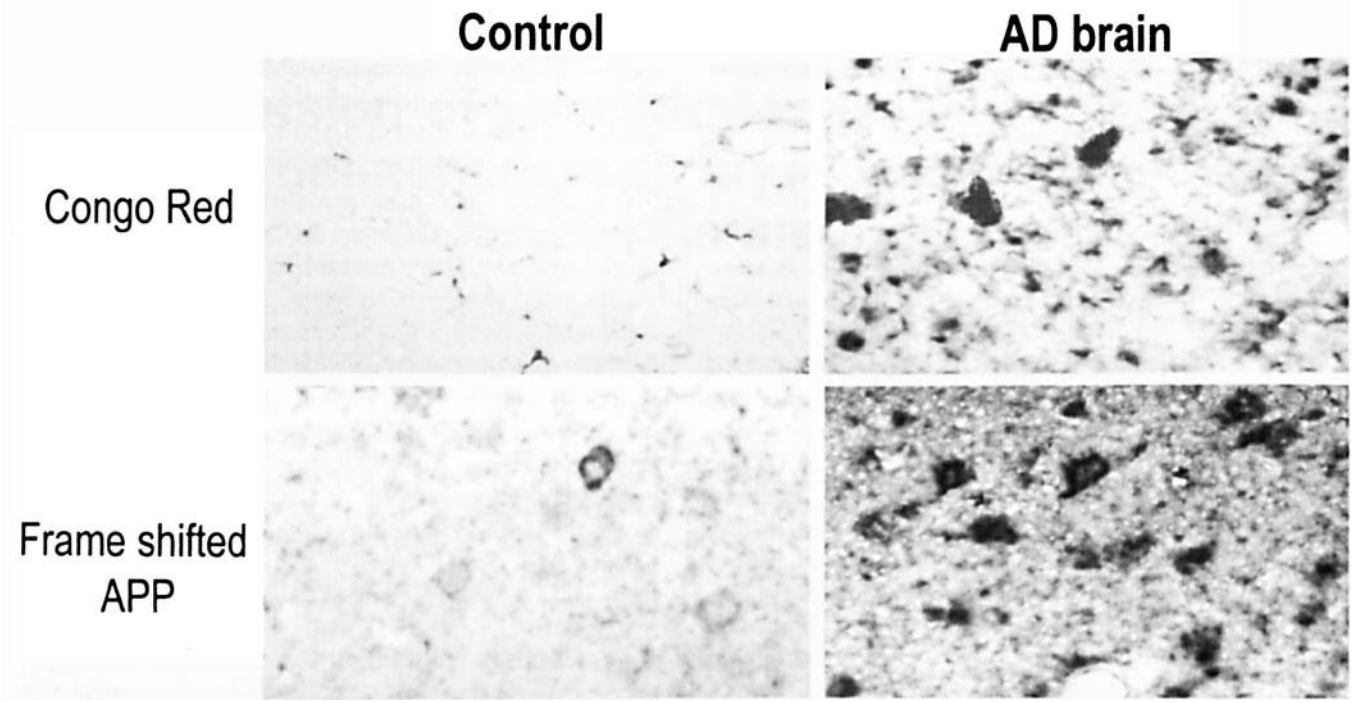

Figure 1. Demonstration of amyloid deposits by Congo red and frame-shifted staining. A representative sample is shown for normal brains (control) and brains affected by Alzheimer disease. Amyloid deposits were also demonstrated with other techniques; quantitative measurements are shown in Table 1. Color figure available in the on-line version.

anti-rabbit IgG 1:30 linked with AMCA (Jackson ImmunoResearch Laboratories).

MT-I+II and MT-III were studied by using mouse anti-MT-I+II and rabbit anti-rat MT III (39), which were visualized by using goat anti-mouse linked with AMCA 1:30 (Jackson ImmunoResearch Laboratories) and goat anti-rabbit linked with FITC 1:50 (Southern Biotechnology Associates, Birmingham, AL). Also, distribution of MT-I+II in relation to neurofibrillary tangles was examined by using mouse anti-MT-I+II and rabbit antineurofibrillary tangles (both as mentioned above). They were visualized by using goat anti-mouse linked with AMCA 1:30 (Jackson ImmunoResearch Laboratories) and goat anti-rabbit linked with TXRD 1:50 (Jackson ImmunoResearch Laboratories).

For the simultaneous examination and recording of the fluorescence stained sections, a Zeiss AxioImager Z1 microscope was used. The Zeiss AxioImager Z1 is fully motorized, freely configurable, and equipped with a triple band (FITC/TXRD/AMCA) filter.

Cell Counts and Statistical Analysis. In addition to morphological evaluation, quantitation (cellular counts) of some of the variables analyzed was carried out from 0.5 mm^2 matched areas of the brain sections for statistical analysis of the results. For each parameter analyzed in each individual AD biopsy (from one patient), cell counts were performed in four different areas (two hippocampal areas and two areas of the frontal cortex), and the mean value of these four areas per patient is shown for each patient (three patients were used). The healthy controls were processed similarly. Results shown are mean ± SE, and they were analyzed with the SPSS software.

Mouse Models of AD Amyloid Deposits. Tg2576 male mice (48) were purchased from Taconic Europe A/S (Lille Skensved, Denmark), and crossed with C57BL/6 females. Offspring at age 14–18 months were used in this study. All mice were killed by decapitation, and the brains were immediately removed and frozen in liquid nitrogen. Brains were stored at –80°C.

***In Situ* Hybridization.** Serial sagittal sections (20 μm in thickness) were cut on a cryostat and mounted on poly-L-lysine coated slides. All sections were then maintained at –80°C until the day of analysis. The *in situ* hybridization analysis of the MT-I and MT-III isoforms was carried out as previously described (33, 49). Because the MT-I and MT-II isoforms are coordinately regulated in both the periphery (7) and the brain (8, 50), we assume that the results for MT-I are representative of those of MT-II. Autoradiography was performed by exposing the slides to the film (hyperfilm-MP, Amersham) for several days. All sections to be compared were prepared simultaneously and exposed to the same autoradiographic film. MT-I and MT-III messenger RNA (mRNA) levels were semiquantitatively determined in several sections per brain area by measuring the optical densities and the number of pixels in defined areas with a Leica Q 500 MC system. The mRNA values shown are expressed in arbitrary units (number of pixels × optic density).

Microautoradiography. After macroautoradiography was performed, the slides were coated with Hypercoat LM-1 emulsion (Amersham) following the instructions of

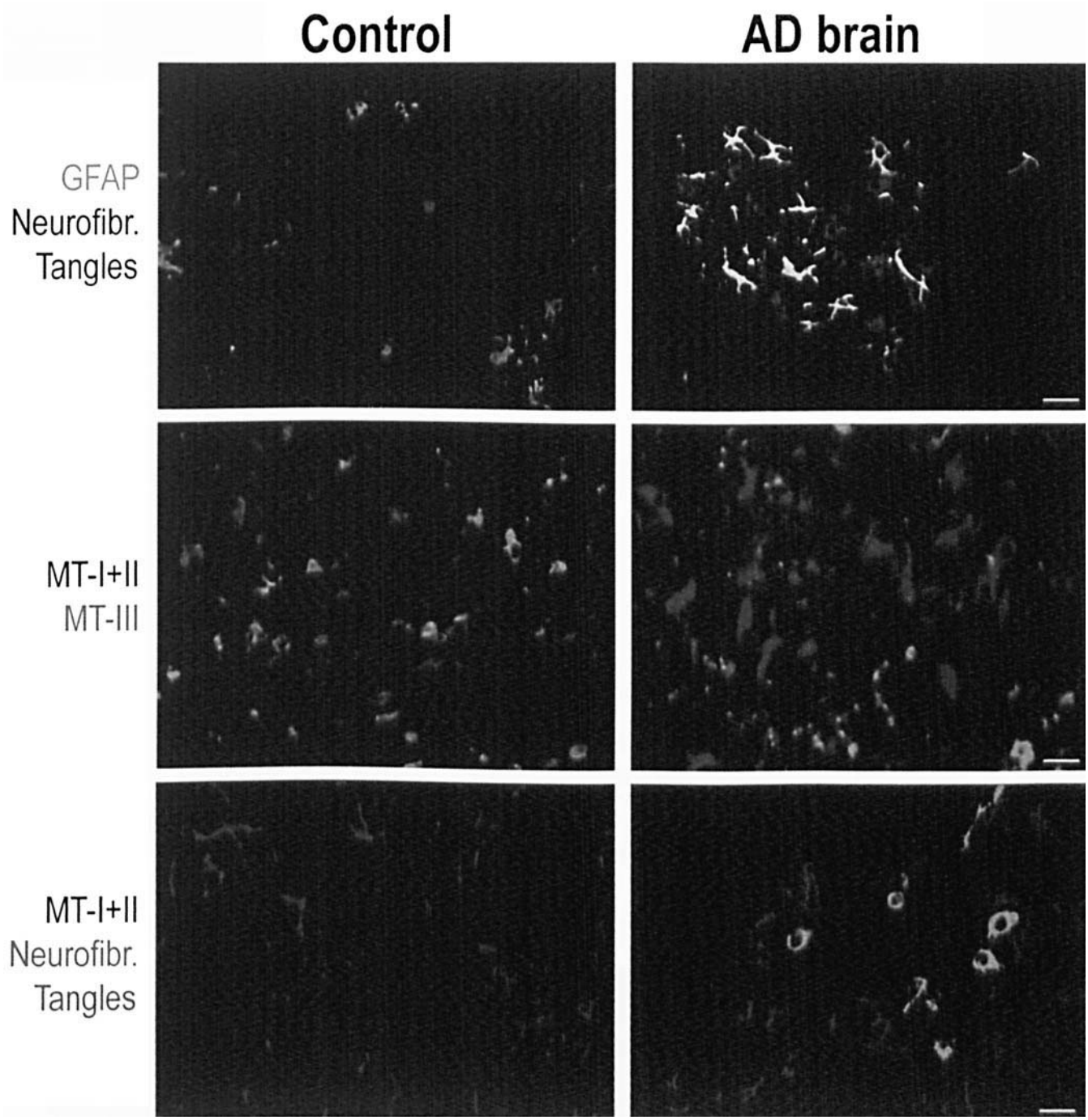

Figure 2. Double immunofluorescence staining. Top: GFAP (green) and neurofibrillary tangles (blue) staining in representative normal brains and brains affected by Alzheimer disease. Middle: MT-I and MT-II (blue) and MT-III (red) staining. Bottom: MT-I and MT-II (blue) and neurofibrillary (red) staining. MT-I and MT-II but not MT-III was increased in brains affected by Alzheimer disease. Color figure available in the on-line version.

the manufacturer. The slides were then exposed for 3 weeks at 4°C and subsequently developed in D-19 (Kodak, Rochester, NY). For microscope observation the slides were counterstained with hematoxylin and eosin.

Plaques. Amyloid plaques were demonstrated by the Congo red method using the Sigma HT60 kit following the recommendations of the manufacturer. On some occasions Congo red staining was performed after the *in situ* hybridization was carried out, followed by microautoradiography to simultaneously demonstrate MT-I or MT-III expression surrounding the amyloid plaques.

Results

Figure 1 shows one of the hallmarks of AD, amyloid plaques, as identified by Congo red and frame-shifted

Table 1. Cell counts carried in the normal brains and brains affected by Alzheimer disease[a]

	Alzheimer disease	Control	Probability
Neurofibrillary tangles	54.33 (5.24)	2.33 (1.20)	0.001
AD-mutated APP	70.33 (4.63)	1.00 (0.58)	0.000
Amyloid beta	76.67 (5.24)	2.00 (1.15)	0.000
CD68	97.00 (8.96)	9.00 (1.15)	0.001
IL-1	65.00 (4.16)	6.33 (1.45)	0.000
TNF-α	77.33 (6.49)	7.33 (4.51)	0.001
MDA	47.00 (5.69)	3.67 (0.88)	0.002
NITT	50.67 (8.01)	2.67 (1.76)	0.004
8-Oxoguanin	52.33 (5.55)	3.00 (1.15)	0.001
TUNEL	65.67 (3.93)	1.67 (0.33)	0.000
Caspase-3	54.67 (3.18)	1.33 (0.88)	0.000
MT-I+II	83.00 (3.06)	14.67 (2.73)	0.000

[a] Cells were counted from frontal cortex and hippocampus as cells/0.5 mm^2. From each individual AD biopsy, cell counts were performed in four different areas (two hippocampal areas and two areas of the frontal cortex) with abnormalities/plaque, and the mean value of these four areas was obtained for each patient (three patients were used). We also used three normal brains as controls. Statistical analysis was performed using the Student's *t* test. Due to the number of comparisons, Bonferroni correction was applied (0.05/12). A significant difference was considered when $P < 0.0042$. Data are expressed as mean of positive cells (± SE). AD, Alzheimer disease; APP, amyloid precursor protein; IL, interleukin; TNF, tumor necrosis factor; MDA, malondialdehyde; NITT, nitrotyrosine; MT, metallothionein.

stainings. Figure 2 shows the second main feature, the neurofibrillary tangles, and Table 1 shows quantitations carried out in three brains of individuals who had AD and three control brains, showing statistically significant increases of several markers of amyloid deposits and neurofibrillary tangles. These features were observed primarily in hippocampus and cortex.

As expected, GFAP (Fig. 2, top) and CD68 (Table 1) staining (markers of astrocytes and microglia/macrophages, respectively) were dramatically increased in areas associated with the amyloid deposits/tangles, which is indicative of the gliosis and inflammation well known to occur in brains of patients with AD. This was also obvious from the analysis of the expression of several proinflammatory cytokines such as IL-1β and TNF (Table 1). At least in part because of this scenario, clear signs of oxidative stress (MDA, NITT) and apoptosis (TUNEL, 8-oxoguanine, and activated caspase-3) were observed in the plaque areas (Table 1).

Figure 2 (middle) shows MT-I, MT-II, and MT-III staining. MT-I and MT-II immunostaining was clearly increased in brains of individuals who had AD (also see Table 1), whereas MT-III remained essentially unaltered. MT-I and MT-II immunoreactivity was increased in cells surrounding cells that were positive for neurofibrillary

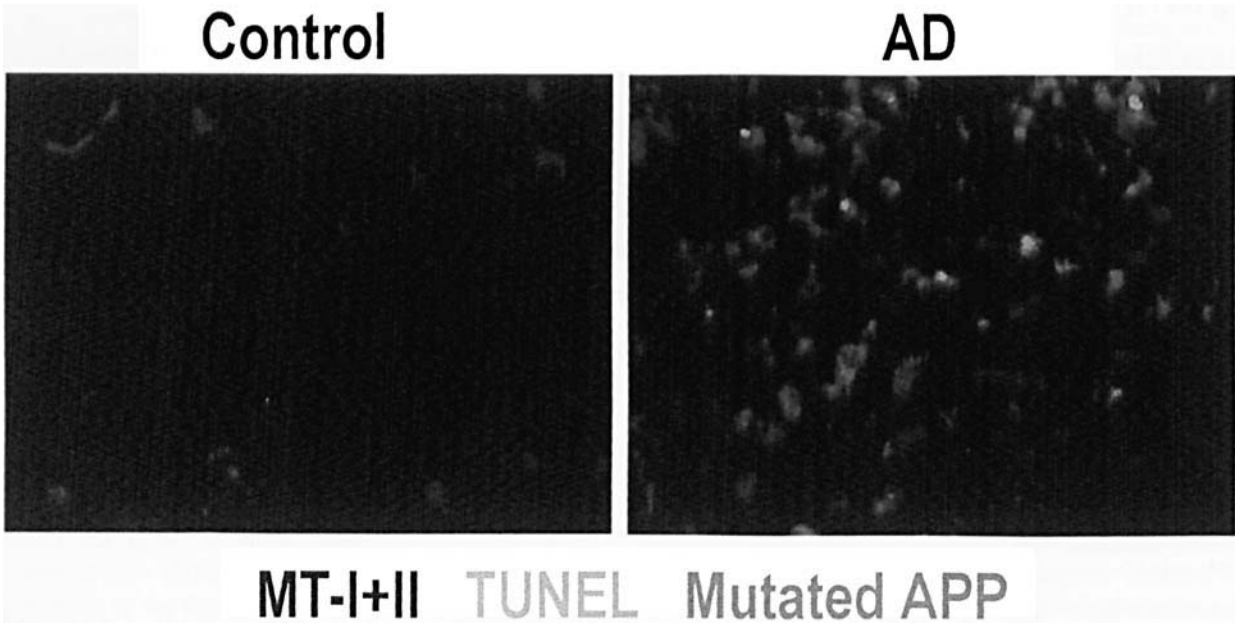

Figure 3. Triple immunofluorescence stainings: MT-I and MT-II (blue), TUNEL (green), and mutated APP (red) staining are shown. TUNEL staining occurred in cells devoid of MT-I and MT-II. Color figure available in the on-line version.

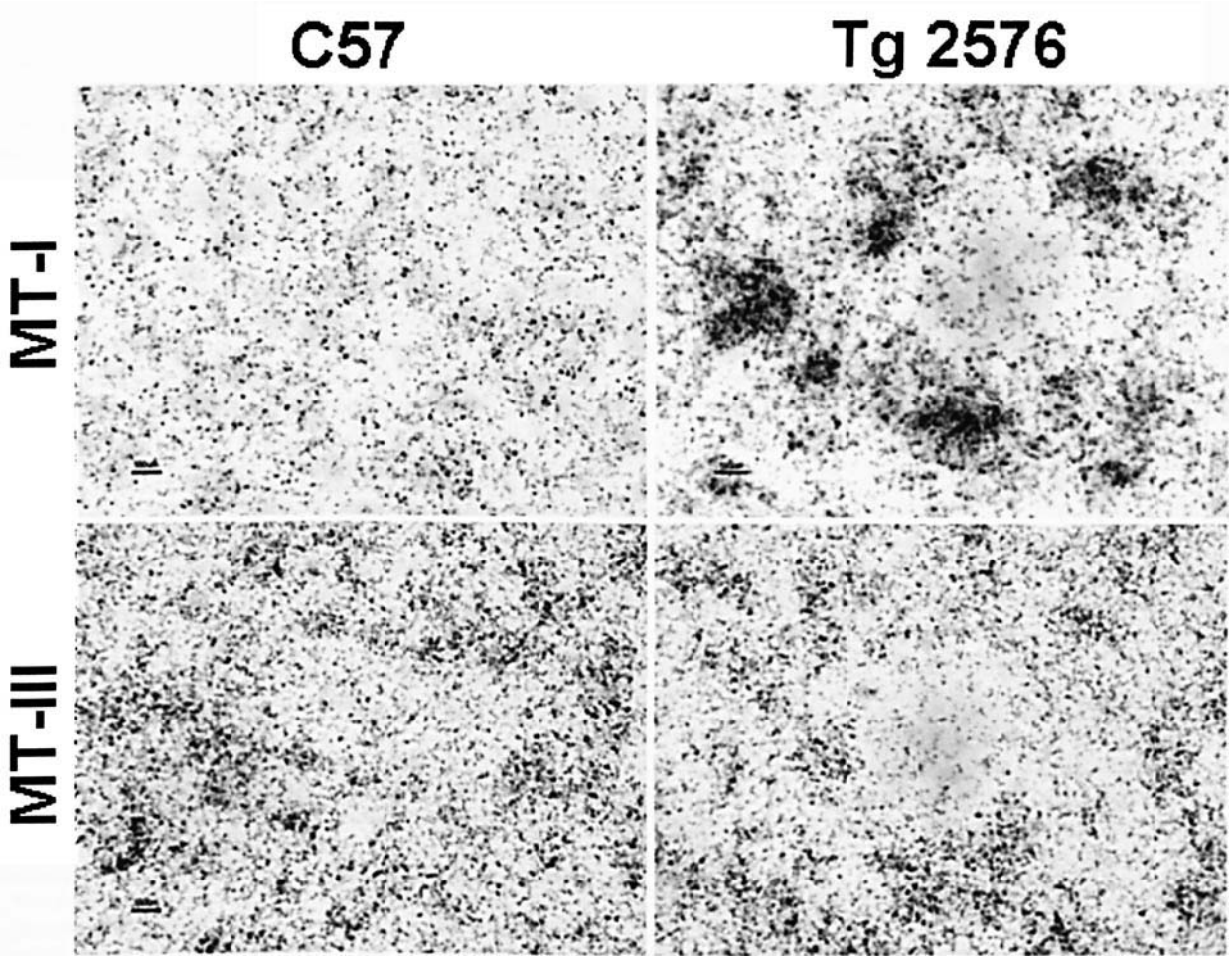

Figure 4. Representative microautoradiographies for MT-I and MT-III *in situ* hybridizations obtained in Tg2576 and wild-type (C57) mice. Congo red staining was also carried out. MT-I but not MT-III signal was clearly increased in cells surrounding Congo red–positive plaques. Color figure available in the on-line version.

tangles (namely, damaged neurons; Fig. 2, bottom). According to their morphology, the majority of cells appeared to be reactive astrocytes. Most of the cells that were suggestive of entering the apoptotic process (TUNEL-positive) were positive for mutated APP but negative for MT-I and MT-II (Fig. 3).

Figure 4 shows representative microautoradiographies for MT-I and MT-III *in situ* hybridizations obtained in the Tg2576 AD mouse model. MT-I mRNA levels were clearly increased in cells surrounding Congo red–positive plaques. MT-III showed the expected different pattern of expression compared with MT-I; namely, a primary neuronal expression especially prominent in the hippocampus (not shown). MT-III expression tended to be decreased in the cortex of the Tg2576 mice (not shown), but this was a general phenomenon because the MT-III signal in the vicinity of the amyloid deposits was not affected.

Discussion

Alzheimer disease is a progressive neurodegenerative disease of the CNS and constitutes a major clinical and social problem in Western countries. AD is characterized by senile plaques, neurofibrillary tangles, and neuronal loss. Following the discovery that β-amyloid is the major constituent of plaques, the so-called amyloid cascade/neuroinflammation hypothesis has been established. Thus suggests that deposits of Aβ are responsible for causing tau phosphorylation and neurofibrillary tangle formation, as well as for activating microglia. In turn, activated microglia would release proinflammatory cytokines and other inflammatory mediators, as well as oxidative species, that altogether would bring about the neurodegenerative changes and eventually, neuronal death and dementia in patients with AD (51, 52).

Consistent with the hypothesis proposed above, we have observed in our cases of AD clear symptoms of inflammation, oxidative stress, and apoptosis in the plaque areas. As expected, MT-I and MT-II immunostainings were consistently increased in cells surrounding Congo red–positive plaques and cells that were positive for neurofibrillary tangles. A significant up-regulation of these proteins around amyloid deposits is consistent with the

inflammatory scenario normally found in them; namely, gliosis, inflammation, oxidative stress, and metals (i.e., zinc) accumulation. It is well known that reactive astrocytes and microglia up-regulate MT-I and MT-II synthesis in a number of injury models (2) and that inflammatory cytokines such as IL-6 and TNF are major inducers of these MT isoforms (49, 53–55). Also, oxidative stress is normally coupled with MT-I and MT-II synthesis (56). Finally, heavy metals such as zinc and copper are known to accumulate in plaques (57), and these are primary inducers of the MT-I and MT-II genes through the metal response element-binding transcription factor-1 (MTF-1) (56). Teleologically, considering the neuroprotective properties demonstrated by these MT isoforms in animal models, it is logical to conclude that such a response in the AD brain is an attempt to cope with the damage elicited by the plaques, regardless of the mechanism or mechanisms involved.

The identification of Aβ as a key factor in patients with AD has led to the generation of transgenic mice that overexpress human APP with specific mutations normally found in patients with familial AD (48, 58–60). The Tg2576 mouse model is one of the best characterized strains of APP transgenic mice, with a clear phenotype at 9–12 months of age, when they begin to form β-amyloid deposits in cortical and hippocampal areas that are accompanied by clear signs of inflammation, behavioral deficits, and learning disabilities (48). In this study we used mice that were 14–18 months old, and thus with a very mature amyloid phenotype. MT-I was up-regulated in the vicinity of the Congo red–positive plaques, but not in areas away from plaques, ruling out an unspecific induction caused by stress factors such as glucocorticoids (61, 62). These results are clearly in accordance with those obtained in brains of individuals with AD, highlighting the usefulness of this mouse model for providing insight into the mechanisms underlying AD. Further studies characterizing what the outcome would be of crossing the Tg2576 mice with MT knockout or MT-overexpressing mice (or both) are warranted.

In contrast to MT-I and MT-II, results for MT-III expression in AD are not consistent (see Introduction), which creates uncertainties regarding its putative physiological role. We herewith report in brains with AD that MT-III expression is not altered. Importantly, MT-III expression was not altered either by the amyloid deposits in the Tg2576 mice, strongly suggesting that the above scenario known to affect MT-I expression (gliosis, oxidative stress, inflammation, metals) is not important for MT-III regulation. We noticed, however, a tendency of the cortex MT-III signal to be decreased in these mice, in line with the reported results for the MT-III protein (63). Regardless of the mechanisms underlying such a decrease, the present results do not support a plaque-specific effect because the MT-III signal surrounding the Congo red–positive plaques was just the same than that of areas away from plaques. Whether or not this reflects a rather unspecific, stress-like phenomenon remains to be established.

As already indicated, MT-III regulation is in many instances of a biphasic nature (2), and in a chronic situation like this (AD, Tg2576 mouse model), it may be a significant confounding factor for understanding how this protein is regulated. But yet, this is the physiological model for understanding AD, where acute, transient responses are of dubious importance. Nevertheless, that could have to do with the discrepancies between different studies on MT-III and AD, as it is also very likely the use of different detection systems. To add complexity to this research area, the physiological roles of MT-III not only appear to differ from those of MT-I and MT-II, but also may depend on its concentration (64, 65), timing (30, 53, 66), brain area (44, 67), and quite likely, on its putative partners (68–70).

Thus, many shortcomings are still present, and many efforts have yet to be devoted. The putative therapeutic use of these proteins deserves our continuous attention. Not only are MT-I and MT-II-types of proteins as efficient as neuroprotective factors when exogenously administered in animal models of MS (71, 72) or trauma (31, 73, 74), but exogenous MT-III also demonstrates neurobiological effects (64, 65). Thus, to investigate the neurobiology of MTs is worth the effort.

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