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ORIGINAL ARTICLE

Analysis of Gene Expression in MOG-Induced Experimental Autoimmune Encephalomyelitis After Treatment With a Novel Brain-Penetrating Antioxidant

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Abstract

Accumulating data from experimental studies indicate that oxidative stress has a major role in the pathogenesis of multiple sclerosis (MS). It has been suggested that local production of reactive oxygen species, probably by macrophages, mediates axonal damage in both MS patients and the mouse model experimental autoimmune encephalomyelitis (EAE). We have shown previously that our novel brain-penetrating antioxidant, *N*-acetylcysteine amide (AD4), reduces the clinical and pathological symptoms, including inflammation and axonal damage in myelin oligodendrocyte glycoprotein (MOG)-induced chronic EAE in mice. The aim of this study was to examine the molecular mechanism by which AD4 exerts protection in MOG-induced EAE mice. Therefore, we analyzed gene-expression profile in the spinal cords of MOG-induced chronic EAE mice and compared them with MOG-induced mice treated with AD4, using a cDNA microarray. We found that MOG treatment up-regulated genes encoding growth factors, cytokines, death receptors, proteases, and myelin structure proteins, whereas MOG- and AD4-treated mice demonstrated gene expression profiles similar to that seen in naïve healthy mice. In conclusion, our study shows that chronic AD4 administration suppresses the induction of various pathological pathways that play a role in EAE and probably in MS.

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Index Entries: Multiple sclerosis (MS); experimental autoimmune encephalomyelitis (EAE); myelin oligodendrocyte glycoprotein (MOG); gene expression; antioxidants; AD4.

Background

Multiple sclerosis (MS) is a chronic, disabling autoimmune neurological disorder targeting the white and gray matter of the central nervous system (CNS). The etiology of MS has not yet been fully illuminated, but it is believed that immunological mechanisms and axonal damage operate in disease initiation and progression (Stinissen et al., 1997; Rieckmann and Mauser, 2002). The autoimmune attack includes autoreactive lymphocytes and local inflammatory production that causes demyelination and oligodendrocyte death. Recent evidence indicates that axonal damage and neural degeneration also occur

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in MS (Ferguson et al., 1997; Matthews et al., 1998; Trapp et al., 1998; Pike et al., 1999), as well as in experimental autoimmune ence-phalomyelitis (EAE), the animal model of MS (Gilgun-Sherki et al., 2003a, 2003b; Lev et al., 2004).

It is well known that inflammation might raise the level of reactive oxygen species (ROS) accumulation, which includes hydroxyl radical, hydrogen peroxide (H_2O_2), peroxynitrite, and nitric oxide ($NO^{\cdot}$), which was shown to be produced by reactive astrocytes via cytokine-mediated induction of NO synthase (Dawson et al., 1998). Oxygen- and nitrogen-free radicals might be important in the pathogenesis of MS and its animal model, EAE. Oxygen- and nitrogen-free radicals generated by macrophages have been implicated as mediators of demyelination and axonal injury in both MS and EAE (Koprowski et al., 1993; Bo et al., 1994; Van der Goes et al., 1998; Gilgun-Sherki, 2004). In addition, free radicals can activate certain transcription factors, such as nuclear transcription factor-κB, which up-regulate the expression of many genes involved in EAE and MS, such as tumor necrosis factor α (TNF)-α, nitric oxide synthase, intracellular adhesion molecule 1, and vascular cell-adhesion molecule 1 (Barnes and Karin, 1997; Winyard and Blake, 1997). Also, redox reactions are involved in the activity of matrix metalloproteinases, which are important to T-cell trafficking into the CNS, and therefore giving rise to ROS production (Romanic and Madri, 1994; Leppert et al., 1995; Merrill and Murphy, 1997).

Because of the pathogenic role of oxygen- and nitrogen-free radicals in MS, it seems reasonable to suggest that antioxidants might prevent free radical-mediated tissue destruction and inhibit some of the early proinflammatory events, such as T-cell activation and trafficking into the CNS, which lead to inflammation and tissue destruction in EAE and MS. The possible beneficial effects of antioxidant treatment on EAE and MS have been examined in several studies. However, very few antioxidants demonstrate any beneficial effect, probably because of their limited passage through the blood–brain barrier (BBB) (for review, *see* Gilgun-Sherki et al., 2004).

We have previously shown that *N*-acetylcysteine amide (AD4), our novel brain-penetrating antioxidant, reduces clinical and pathological symptoms, including inflammation and axonal damage, in myelin oligodendrocyte glycoprotein (MOG)-induced chronic EAE in mice (Offen et al., 2004). The aim of this study was to examine the molecular mechanism by which AD4 exerts its protection. Therefore, we analyzed gene-expression profile in the spinal cords of MOG-induced chronic EAE mice treated with AD4, using a cDNA microarray.

Materials and Methods

Animals

Six- to 8-wk-old C57/bl mice (n = 10–12 in each group), weighing 20 g, were obtained from Harl an Laboratories (Israel). The animals were housed under standard conditions—constant temperature (22 ± 1°C), humidity (relative, 25%), and a 12-h light/dark cycle—and were allowed free access to food and water. The animals and protocol procedures were approved and supervised by the Animal Care Committee at the Rabin Medical Center.

Induction of EAE

Experimental autoimmune encephalomyelitis (EAE) was induced by immunization with the peptide encompassing amino acids 35–55 of rat MOG (pMOG 35–55) (Kerlero de Rosbo et al., 1995). Myelin oligodendrocyte glycoprotein (MOG) synthesis was carried out by the Weizmann Institute Synthesis Unit, using a solid-phase technique on a peptide synthesizer (Applied Biosystems, Foster City, CA). Mice were injected subcutaneously at one site in the flank with a 200-μL emulsion containing 300 μg MOG in complete Freund's adjuvant (CFA) and 200 μg *Mycobacterium tuberculosis* (Sigma, Israel). An identical booster immunization was given at one site of the other flank 1 wk later.

Neurological Evaluation of EAE

Following the encephalitogenic challenge, mice were observed daily, and clinical manifestations of EAE were scored as follows: 0 = no clinical symptoms, 1 = loss of tail tonicity, 2 = partial hind limb pa ralysis, 3 = complete hind limb paralysis, 4 = paralysis of four limbs, 5 = total paralysis, and 6 = death (Mendel et al., 1998).

Treatment

N-acetylcysteine amide (AD4) was prepared and chemically characterized as published previously (Atlas et al., 1999). It was dissolved in saline and given chronically (250 mg/kg × 2/d) from the first day of MOG injection by intraperitoneal injection for the all-study period.

Isolation and Preparation of RNA

Specimens of six spinal cords from each of the followings groups were obtained at autopsy: untreated healthy mice, healthy mice treated with AD4 chronically for 24 d, mice injected with MOG only, and MOG-induced EAE mice treated with AD4 chronically for 24 d. Tissues were frozen in liquid nitrogen and stored at –80°C. Total mRNAs were extracted from mice spinal cords using the lithium chloride urea method (modified from Auffray and Rougeon, 1980). Briefly, harvested tissues were homogenized in 6 *M* urea/3 *M* lithium chloride, and the relatively insoluble lithium-RNA salts were precipitated overnight at 4°C. Much of the DNA remained in solution. The recovered lithium-RNA was phenol chloroform extracted to remove contaminating proteins. RNA was quantified by spectrophotometer and separated by 1% agarose formaldehyde-denaturing gel electrophoreses to verify its integrity.

cDNA Microarray Analysis

Two micrograms of DNase I-treated total mRNA pool from each experimental group was processed according to the Clontech protocol and hybridized with Atlas mouse 1.2 microarrays according to the manufacturer's instructions (Clontech, Palo Alto, CA). Each microarray includes 1176 mouse cDNAs immobilized on nylon membranes, 9 housekeeping cDNAs, and negative controls. The list of the cDNAs can be found at the Clontech web site (http://www.clontech.com/atlas/genelists/7853-1_mouse12.txt).

The hybridization signals were measured by Phosphorimager (Cyclone, Packard, UK) and Clontech Image Software. Calculation of each cDNA expression ratio was based on normalization to the global signal background.

Real-Time Quantitative RT-PCR

mRNA samples (0.5 μg), which were extracted from each spinal cord, were transferred to cDNA using 10 units of enzyme RT-Superscript II (GIBCO-BRL) in a mixture containing: 1.3 μ*M* random primer (Invitrogen, Paisley, UK), 1× buffer supplied by the manufacturer, 10 m*M* DTT, 20 μ*M* dNTPs, and RNase inhibitor (RNAguard, Amersham Pharmacia Biotech). Reverse transcription–polymerase chain reaction (RT-PCR) was performed at 25°C for 10 min at 42°C for 2 h, followed by 70°C for 15 min and 95°C for 5 min. Reverse transcription–polymerase chain reaction (RT-PCR) of the desired genes was performed in an ABI Prism 7000 sequence detection system (Applied Biosystems) using Syber green PCR master mix and specific primers (*see* Table 1) at final concentrations of 500 nmol/L. We used amplification of 18S rRNA and GAPDH for stimulated conditions for sample normalization. The amplification protocol was 40 cycles of 94°C for 15 s, followed by 60°C for 1 min each. Quantification of gene expression relative to GAPDH or 18S rRNA was calculated by the protocol's ΔΔCT method and from the standard curve method.

Statistical Analysis

The differences in the clinical severity of EAE following pMOG 35–55 induction between the control and AD4-treated mice groups were evaluated daily using two-tail Student's *t*-test. Real-time PCR results are presented as mean ± S.E.M. The differences in gene expression between the groups were assessed using one-way ANOVA, followed by Tukey's *post hoc* multiple comparisons test. $p < 0.05$ was considered significant.

Results

Experimental autoimmune encephalomyelitis (EAE) was induced in C57/bl mice by using two injections of MOG. The immunized mice developed severe clinical manifestations, starting on day 14 and peaking on day 24. Most of the animal group (7/10) that were immunized with MOG were affected and developed mild hind limb paralysis (clinical score of 1.6 ± 0.39 [Fig. 1A]). In contrast, all the mice that were immunized with MOG and treated daily with AD4 were resistant, and none of them showed any clinical signs (Fig. 1A,B).

To identify possible differentiations in gene expression in the spinal cords of EAE mice treated or untreated with AD4, compared with healthy mice, we used the Clontech microarray system. We found that in extracts of RNA pools from the three experimental mouse groups, >269 expressed genes were detected in various levels (out of 1176, Fig. 2). Comparison of the general pattern of gene expression revealed that AD4 treatment alone in untreated healthy mice did not change the pattern (Fig. 2A). However, MOG immunization altered the expression of several genes compared with that in untreated healthy mice (Fig. 2B). Interestingly, the gene-expression pattern of the MOG-immunized group that was chronically treated with AD4 was similar to the untreated healthy mouse group (Fig. 2C).

Table 1
Primer Sequence for Quantitative PCR

Gene	Gene ID	Primer	Sequence	Product size (bp)
Thymosin β-4	X16053	F	AGCAAGCTGGCGAATCGTAATGAGG	226
		R	CTGCTAGCCAGACCATCAGATGGG	
CNTF	U05342	F	CAGTGGACTGTGAGGTCTATCCATG	217
		R	TGACGAAATATGCCTGTGGTCTAAGG	
BLBP	S69799	F	TCAGCTGACTAGGCGGTTAAGGATG	193
		R	TGTGCATTGTGTCCGGATCACCAC	
E3	U29539	F	GTGCCCACCTACCTCAACTTC	56
		R	TGGCTTGGGAGGTAATTCATG	
Cathepsin D	X53337	F	CTCCTGGCTTCGTCCTCCTT	51
		R	GAACTTGCGCAGAGGGATTCT	
OPN	J04806	F	ACCATGCAGGGAGCGAGGATTCTG	294
		R	GGGCAACAGGGATGACATCGAGG	
TNFR2	M59378	F	AAGTGTCCTCCTGGCCAATATG	51
		R	GTCCGAGGTCTTGTTGCAGAA	
Clusterin	L08235	F	TCTGGATGAACGGCGACCGCATCG	229
		R	CTGCGGACCAAGCGGGACTTGGG	
MOG	U64572	F	CCTGGTTGCCTTGATCATCTGCTAC	209
		R	TCTACTCGGTATCCAGAATGTGTCTG	
MOBP	U81317	F	GACAAGCGGAGACCAGAGTTCTGG	194
		R	ATGCTGTACTTGCGGTCCACGATC	
PMCA	AF053471	F	AGATCCCTGTGGCCGAGAT	66
		R	CGGGAAGAAGGTCACCATATTT	

We found that out of 1176 genes, 269 were expressed; and we selected genes whose expression was altered by at least twofold between the MOG-treated group and naive healthy mice. The genes were analyzed and classified according to the best-characterized function of the protein that they encode (Table 2). We found that MOG immunization up-regulated several genes, including ciliary neurotrophic factor (CNTF), thymosin β-4, retinoic acid (RA)-inducible E3 protein (E3), brain lipid-binding protein (BLBP), cathepsins A, B, and D, osteopontin (OPN), TNF receptor 2 (TNFR2) precursor, and clusterin (*see* Table 1). In addition, MOG treatment also reduced the expression of others genes, including myelin-associated oligodendrocytic basic protein (MOBP), plasma membrane calcium ATPase (PMCA), and MOG (*see* Table 2). However, chronic administration of AD4 after MOG immunization preserved their normal gene expression (*see* Tables 2 and 3). Thus, cDNA microarray profiling provided an initial and global insight into potential cellular mechanisms that might be affected during a defined stage of the disease.

Because of the fact that the array is based on one-time hybridization in each treatment and pool sample, we further corroborated our findings by quantitative RT-PCR, which emphasizes the variability within each experimental group, utilizing the same source of mRNAs as those used for cDNA microarray analysis. Specific primers were selected for genes, which were alerted by at least 1.7-fold between the groups. Genes that were up-regulated after MOG treatment compared with healthy control mice include CNTF (×3.3, $p < 0.05$), thymosin β-4 (×4.8, $p < 0.001$), E3 (×12.1, $p < 0.005$), BLBP (×5, $p < 0.008$), cathepsin D (×7.7, $p < 0.015$), OPN (×2.3, $p < 0.04$), TNFR2 (×15.7, $p < 0.002$), and clusterin (×1.7, $p < 0.015$) (Fig. 3A–H). In addition, MOG treatment also reduced the expression of MOBP (×0.5, $p < 0.01$) (Fig. 3I). However, chronic administration of AD4 normalized the expression of MOG-induced, up-regulated genes ([Fig. 3A-H] $p < 0.05$) and also the MOBP gene, which was down-regulated after MOG immunization, though it was not significant (Fig. 3I), similar to the levels of the untreated healthy group.

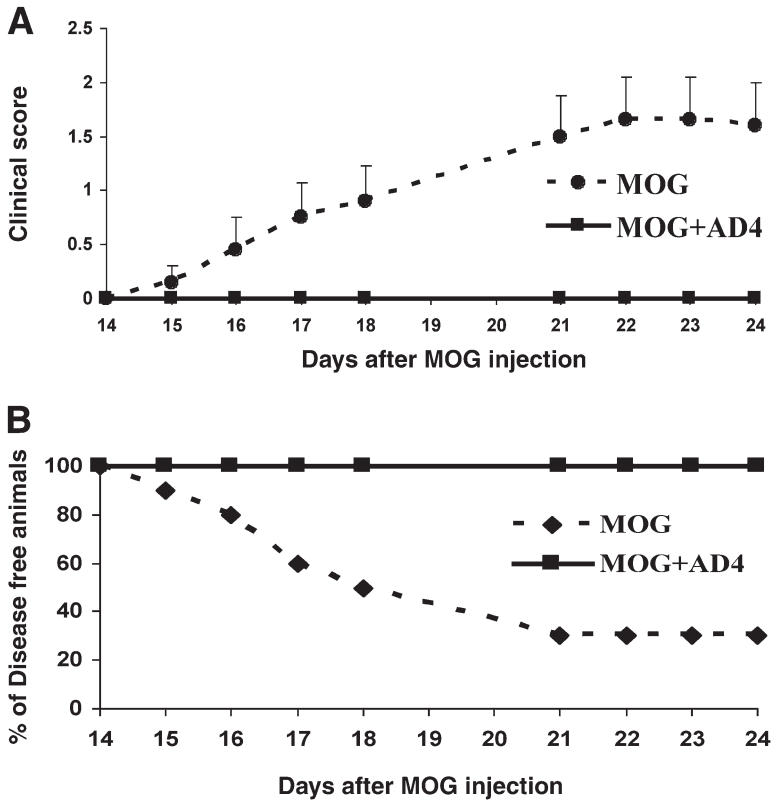

Fig. 1. Induction of EAE in C57/bl mice with pMOG 35–55. Ten MOG-treated and 10 AD4-treated mice were injected with pMOG 35–55 in CFA supplemented with *Mycobacterium tuberculosis*. **(A)** The mean daily clinical score ± S.E. is shown for each group of mice: 0 = no clinical symptoms, 1 = loss of tail tonicity, 2 = partial hind limb paralysis, 3 = complete hind limb paralysis, 4 = paralysis of four limbs, 5 = total paralysis, 6 = death. **(B)** Percent of disease-free animals.

Discussion

Our study shows that AD4-treated C57/bl mice were highly resistant to MOG-induced chronic EAE. The incidence of the disease and its clinical severity were markedly reduced in AD4-treated mice, as compared with MOG-treated mice. In addition, the pattern of gene expression in the MOG-treated mice was drastically altered, whereas gene expression in the AD4-treated group was similar to the untreated healthy group.

Several genes were up-regulated in MOG-induced EAE mice, whereas their expression in the AD4-treated group was similar to the untreated healthy group. Those selected genes belong to different families and biological functions that are probably involved in the pathological pathways of the disease.

The gene thymosin β-4, which was unregulated in MOG-treated mice, is a family member of heat-stable, polypeptide hormones, secreted by the thymus gland. Its biological activities include lymphocytopoiesis, restoration of immunological competence, and enhancement of expression of T-cell characteristics and function. In addition, it was also reported to have therapeutic potential in patients having primary or secondary immunodeficiency diseases (Baxevanis et al., 1990).

Another gene that was up-regulated is CNTF, which is a polypeptide hormone whose actions appear to be restricted to the nervous system, where it promotes neurotransmitter synthesis and neurite outgrowth in certain neuronal populations. The protein is a potent survival factor for neurons and oligodendrocytes and might be relevant in reducing tissue destruction during inflammatory attacks. In addition, it is thought to enhance myelin formation by favoring final maturation of oligodendrocytes (Linker et al., 2002; Stankoff et al., 2002).

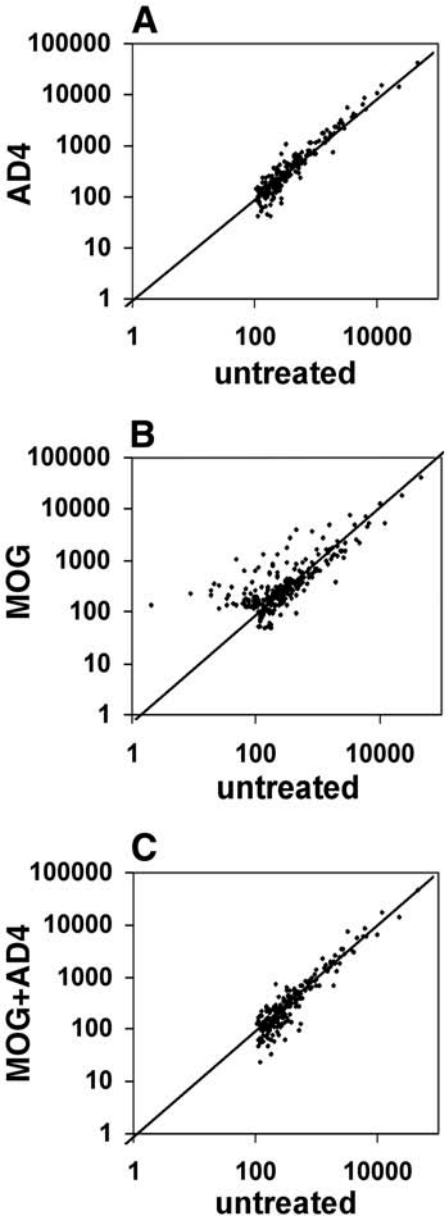

Fig. 2. Scatter plot analysis of expression data for EAE mice treated or not treated with AD4. **(A)** AD4-treated mice vs naive. **(B)** MOG-treated mice vs naive **(C)** MOG-treated mice vs MOG + AD4 mice.

We also found increased expression of another well-known group of lysosomal/endosomal proteinase/endoproteinase genes called cathepsins. The cathepsins are marker enzymes for the activity of lysosomal protein degradation, and in MS, especially for myelin peptides like myelin basic protein (MBP) (Pritzker et al., 2000; Beck et al., 2001); and their expression was shown to increase in the MS tissue compared with controls (Bever et al., 1994; Bever and Garver, 1995). These genes were also found to be elevated in several studies that profiled gene expression in EAE and MS (Chabas et al., 2001; Ibrahim et al., 2001).

Brain lipid-binding protein (BLBP) was also found to be overexpressed in EAE spinal cords. This protein is a member of the fatty acid-binding proteins (FABPs), required for radial glial morphological differentiation (antibodies to BLBP can block glial cell differentiation [Xu et al., 1996; Anton et al., 1997]). Members of this family, such as cellular RA-binding protein and ApoE, which is the major FABP (Sabo et al., 2000), are thought to play roles in fatty acid uptake, transport, and metabolism between cellular compartments (Feng et al., 1994).

An additional gene that was found to be elevated is E3/lysosome-associated protein, transmembrane-5. The E3 protein is regulated by RA, which is by itself a regulator of gene expression derived from vitamin A and is known to promote the development of T-helper cell-2-like human MBP (Lovett-Racke and Racke, 2002) during myelopoiesis (Scott et al., 1996). The E3 protein is assumed to play a role in the differentiation of B-cells during the later stages, and its homologous gene product GCD-10 is specifically expressed in microglia and is up-regulated during neuronal apoptosis (Origasa et al., 2001). The E3 protein was found to be elevated in other studies that analyzed gene expression in EAE (Ibrahim et al., 2001) and MS (Lock et al., 2002).

Osteopontin (OPN), also called early T-cell activation gene-1 (Oldberg et al., 1986; Fisher et al., 2001), is another gene of interest that belongs to the cell-adhesion receptor and protein family. It has pleiotropic functions (Denhardt and Guo, 1993; Rittling and Denhardt, 1999; O'Regan and Berman, 2000), including roles in inflammation and immunity to infectious diseases (O'Regan and Berman, 2000). Osteopontin (OPN) costimulates T-cell proliferation (O'Regan and Berman, 2000) and is classified as T-helper cell-1 cytokine, because of its ability to enhance γ-interferon (IFN-γ) and interleukin 12 (IL-12) production and diminish IL-10 (Ashkar, et al., 2000). Osteopontin (OPN) protein levels significantly increased in the plasma of relapsing-remitting MS patients. In contrast, OPN protein levels in primary progressive and secondary progressive MS patients were similar to healthy control levels. In addition, OPN-deficient mice were resistant to progressive EAE and had fre-

Table 2
Genes With High Expression in MOG-Induced EAE Mice and Normal-Like Expression After AD4 Administration (Compared With Untreated Mice)

Gene	MOG	MOG + AD4	Function
CNTF	10.00	0.85	Growth factors, cytokines, and chemokines
Thymosin β-4	3.13	0.92	
E3	9.17	1.29	Functionally unclassified proteins
BLBP	2.72	0.66	
Cathepsin D	7.85	1.24	Aspartic proteases
Cathepsin B	4.16	0.66	Cysteine proteases
Cathepsin A	2.86	1.06	Amino- and carboxy-peptidases
OPN	4.6	0.8	Cell-adhesion receptors and proteins
TNFR2	2.21	0.31	Death receptors
Clusterin	1.64	0.86	Other apoptosis-associated proteins

Table 3
Genes With Down Expression in MOG-Induced EAE Mice and Normal-Like Expression After AD4 Administration (Compared With Untreated Mice)

Gene	MOG	MOG + AD4	Function
MOBP	0.42	1.37	Extracellular matrix proteins
PMCA	0.48	1.2	ATPase transporters
MOG	0.66	0.93	Cell-surface antigens

quent remissions (Vogt et al., 2003). Osteopontin (OPN) was also found to increase in a study that analyzed gene expression in EAE (Chabas et al., 2001).

We also found that the gene for TNFR2 was up-regulated. The gene belongs to the death receptor family, and it serves as a key cytokine in the pathogenesis of MS and EAE. Both TNF and TNFR were demonstrated with immunohistochemistry in MS plaques (Raine et al., 1998). Tumor necrosis factor receptor 2 (TNFR2) precursor has also been suggested as a susceptibility gene in linkage analysis in human MS patients (Croxford et al., 1997). This gene was found to be elevated in EAE and MS in several studies (Ibrahim et al., 2001; Lock et al., 2002; Mix et al., 2002; Bomprezzi et al., 2003).

Clusterin, which is a serum glycoprotein that plays an important role in lipid transport and maturation, was found to be elevated in MOG-induced EAE. Also known as an inhibitor of complement, clusterin is expressed in many tissues in cell injury and death, including promotion of amyloid plaque formation and neuritic toxicity in Alzheimer's disease (DeMattos et al., 2002). Clusterin has been identified in normal and pathological brain tissue and is a component of normal human CSF. The levels of clusterin were found to be elevated in patients with demyelination (Polihronis et al., 1993), and its expression by astrocytes was found to be influenced by transforming growth factor-β (Morgan et al., 1995).

Several genes were down-regulated in MOG-induced EAE mice, whereas their expression in the AD4-treated group was similar to the untreated healthy group. One of those genes belongs to the extracellular matrix family and is called MOBP. This protein is the major component of both the CNS/PNS myelin found in the periaxonal membrane (Yool et al., 2002). Myelin-associated oligodendrocytic basic protein (MOBP) was shown to modulate the arrangement of radial growth of the axon and myelin (Yoshikawa, 2001) and was reported to decrease in EAE mice (Carmody et al., 2002).

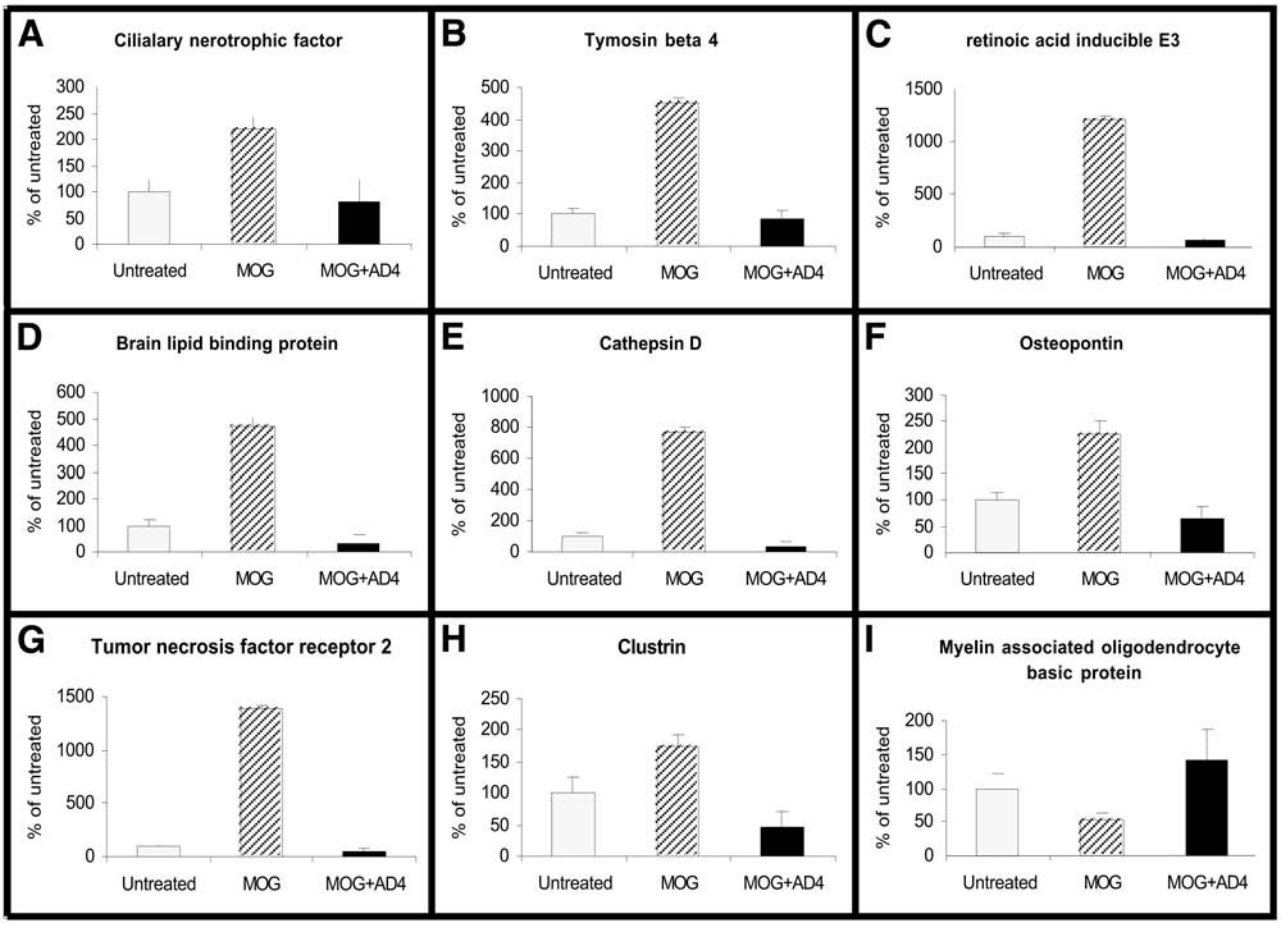

Fig. 3. Quantitative PCR assessment of gene expression using RT-PCR revealed up-regulation of several genes: **(A)** CNTF; **(B)** thymosin β-4; **(C)** E3; **(D)** BLBP; **(E)** cathepsin D; **(F)** OPN; **(G)** TNFR2; **(H)** Clusterin; and **(I)** down-regulation of MOBP in the MOG-treated group and normal-like expression in the MOG +AD4 group.

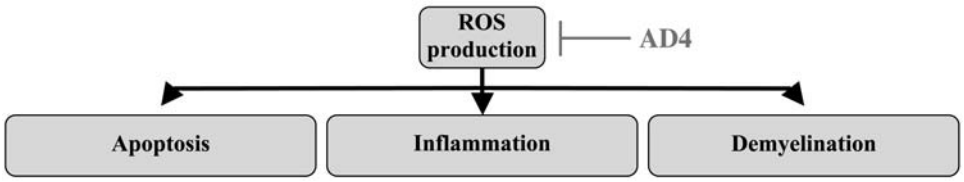

Fig. 4. Proposed molecular mechanism of AD4 in EAE. Inhibition of ROS production by AD4 restrains the pathological pathways involved in EAE and probably MS pathogenesis, including inflammation, demyelination, and apoptosis.

Another myelin-associated protein, localized on the surface of the oligodendrocyte, is MOG, which is also a member of the immunoglobulin superfamily encoded within the major histocompatibility complex (Pham-Dinh et al., 1993; Slavin et al., 1997). Although it was decreased by only 1.5-fold, we chose to evaluate it by PCR because of its importance in the pathophysiology of both EAE and MS (Carmody et al., 2002).

PMCA is an essential ion pump expressed exclusively in gray matter and involved in Ca^{2+} extrusion (Nicot et al., 2003). The reduction in the PMCA gene raises the possibility of ion dyshomeostasis as one

of the mechanisms leading to neural dysfunction during EAE.

All of the above-mentioned genes play a role in apoptosis, inflammation, and demyelination, which are well-documented pathways in the pathogenesis of MS and EAE (Miller and Galboiz, 2002). Some of these genes have been found previously in profiled gene expression of EAE and MS (Ibrahim et al., 2001; Chabas et al., 2001; Carmody et al., 2002; Bomprezzi et al., 2003), thus supporting their role in the pathogenesis of MS.

We have shown recently that AD4 crosses the BBB and breaks down within the brain to *N*-acetylcysteine, a well-known antimucotyic agent, and cysteine, the rate-limiting factor in the synthesis of reduced glutathione (GSH). In addition, it also increased GSH levels and protected against oxidative stress in vitro (Grinberg et al., 2005) and in vivo (Offen et al., 2004). Additionally, it exhibited strong neuroprotective properties against Parkinson-related neurotoxins in vitro and in vivo (Bahat-Stroomza et al., 2005), and reduced the clinical and pathological symptoms in MOG-induced EAE mice (Offen et al., 2004).

In conclusion, our current study demonstrates that chronic administration of AD4 preserved the healthy gene expression of MOG-induced EAE mice. Therefore, we assume that the mechanism by which AD4 exerts its protection includes, at least in part, inhibition of various pathological pathways, related to the observed genes (*see* Fig. 4), involved in the pathophysiology of EAE and probably MS.

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