consistent with the finding that only a tiny subset of these cells was PBR-immunoreactive. Rearrangement of the data for side-by-side comparison of PBR and GDNF immunolabeling in these animal models (supplemental Fig. 4, available at www.jneurosci.org as supplemental material) revealed good agreement between the expression profiles of the two molecules in astrocytes in these models of acute/subacute neurotoxicity.

Discussion

The elevated levels of PBR observed in tau and APP Tg mice that model the core tangle and plaque pathologies of AD by both radiographical and immunohistochemical assays here rationalize the application of PBR imaging for diagnostic and therapeutic assessments of patients with this illness. Significantly, our data also demonstrate distinct mechanisms regulating PBR expression in microglia and astrocytes reacting to tau and Aβ lesions. Of greater interest is the observation that PBR upregulation in astrocytes and microglia may reflect beneficial and deleterious consequences of gliotic changes, respectively, which critically differentiated the modest neurotoxic effects of Aβ versus the more profound neurodegenerative consequences of tau pathologies on hippocampal/entorhinal neurons in APP and tau Tg mice, respectively. Unlike APP Tg mice, patients with AD develop progressive and extensive neuron loss in CNS regions with abundant plaques and tangles, whereas some of the potential restorative processes associated with amyloid plaques, such as axonal sprouting and remodeling of neuritic and synaptic architectures frequently observed in mice (Phinney et al., 1999), are present but less evident in human AD brains (Masliah et al., 1991). Hence, we speculate that "neurotoxic gliosis" characterized by PBR-positive microglia in AD brains may overwhelm the potentially "neutoprotective gliosis" of PBR expressing astrocytes, although this hypothesis will require further testing. For example, this could be examined by developing antibodies that could be used for detecting human PBR in diverse neurodegenerative and other CNS disorders. Moreover, the therapeutic benefit of modulating microglial activity (Dodel et al., 2003; Marx, 2007) could be safely assessed in patients with AD and related tauopathies as well as in other neuroinflammatory CNS conditions by using PBR as a biomarker for microglial neurotoxicity. As a second application, the response to trophic factors could be monitored by detecting increased PBR signals in reactive astrocytes that exert neuroprotective effects. In this context, the implications of PBR changes largely depend on the mechanistic modes of treatments, and changes in PBR expression patterns can be clarified for a given therapeutic approach by preclinical studies using experimental animal models.

As the scope of our research was enlarged from AD pathology to non-AD neurodegenerative or toxic conditions, the common framework of the interplay between microglia and astrocytes after neuronal injury has been delineated with emphasis on PBR as a potentially informative imaging biomarker as schematically illustrated in Figure 8. Disorganization of myelin is a strong inducer of microglial activation, as observed in the midbrain of KA-treated rats and striatum of CZ-exposed mice and documented previously (Smith, 1999), which may involve transient upregulation of microglial PBR (Chen et al., 2004). However, irreversible impairments of neuronal structures are conceivably requisite for long-lasting upregulation of PBR in microglia, in light of our previous and present findings in ethanol-injected rats (Maeda et al., 2007a) and tau Tg mice (Yoshiyama et al., 2007). In contrast, activation of nondeleterious microglia is one of the significant cellular events that result from reversible neuronal injuries, and these glial cells barely express PBR at a high level. This property is exemplified by the CZ challenge in mice, during which spontaneous remyelination occurs at 5–7 weeks (Matsushima and Morell, 2001). As demonstrated by the transplantation of Ra2 cells into the hippocampus of mice, these nontoxic microglia are capable of locally driving PBR expression in astrocytes. Interestingly, our preliminary analysis of transplanted microglia has also indicated that PBR levels in Ra2 cells are notably lower

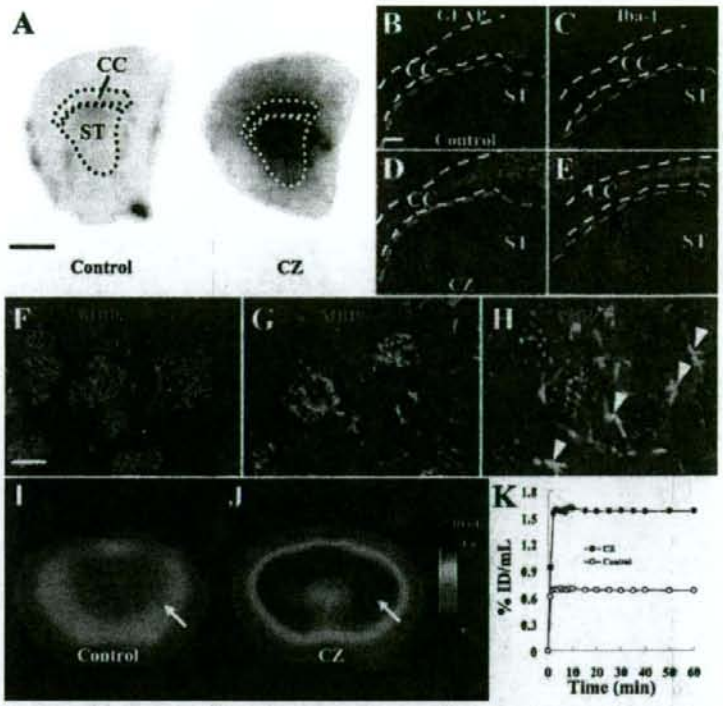

Figure 5. ***A***, Radiolabeling of PBR in brains of mice without treatment (Control) and treated with CZ. The corpus callosum (CC) and striatum (ST) are outlined by dots. ***B–E***, Immunofluorescence staining of GFAP (***B***, ***D***) and Iba-1 (***C***, ***E***) in brain sections of untreated (***B***, ***C***) and CZ-treated (***D***, ***E***) mice. Dashed lines define borders of CC and ST. ***F–H***, Double immunofluorescence labeling of MBP and Iba-1 (***F***), MBP and GFAP (***G***), and PBR (NP155) and GFAP (***H***) in striatal sections of a CZ-treated mouse. Arrowheads in ***H*** indicate astrocytes doubly positive for PBR and GFAP. ***I–K***, *In vivo* detection of PBR in CZ-treated mice. Coronal (1 mm anterior to the bregma) PET images in control (***I***) and CZ-treated (***J***) mice were generated by summation of dynamic data at 0–60 min after injection of [^{18}F]FE-DAA1106 and were overlaid on the MRI template. Marked increase of radioactivity was found primarily in the corpus callosum and striatum (arrows). Time-radioactivity curves (***K***) for striatal VOIs in control (open circles) and CZ-fed (closed circles) mice indicate ~2-fold increase of radioligand accumulation induced by CZ treatment. Scale bars: (***A***) 2 mm, (***B–E***) 150 μm, (***F–H***) 25 μm.

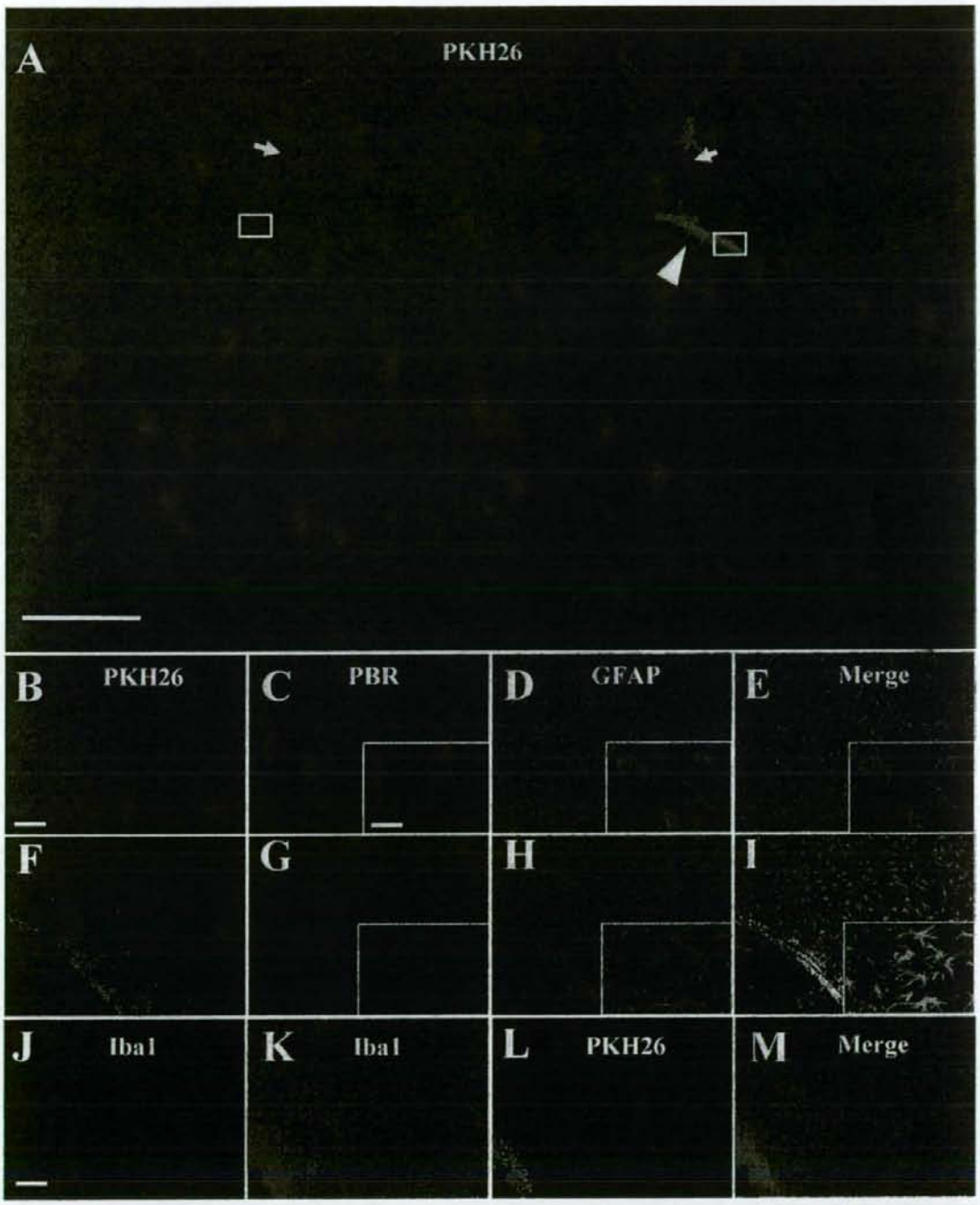

Figure 6. ***A***, Localization of PKH26-labeled Ra2 cells transplanted into unilateral hippocampus of a mouse (arrowhead). Regions of interest (squares) were defined in hippocampal areas sufficiently distant from needle tracts (detectable by weak autofluorescence; arrows) and are shown in ***B–M*** at high magnification. Fluorescence of PKH26 is converted to blue for the purpose of the multicolor display. ***B–I***, Fluorescence labeling for exogenous microglia (***B***, ***F***), PBR (***C***, ***G***) and GFAP (***D***, ***H***) along with three-channel images (***E***, ***I***) in the subregions of the hippocampus injected with vehicle (***B–E***) and Ra2 (***F–I***). Transplanted Ra2 cells (***F***) induced prominent upregulation of PBR (***G***) as well as hypertrophic changes (***H***) in neighboring astrocytes. High-power photomicrographs are shown in insets (***C–E***, ***G–I***). ***J–M***, Fluorescence mapping of microglia positive for Iba1 (***J***, ***K***) and PHK26 (***L***) along with a merged image (***M***) in the subregions of the hippocampus injected with vehicle (***J***) and Ra2 (***K–M***). Iba1-positive, PKH26-negative endogenous microglia were manifestly activated in the proximity of the transplantation site containing Iba1/PKH26 double positive exogenous Ra2 cells (***K–M***), compared with the vehicle-injected contralateral area (***J***). Scale bars: (***A***) 1 mm, (***B–I***) 100 μm, (***J–M***) 25 μm, (insets) 20 μm.

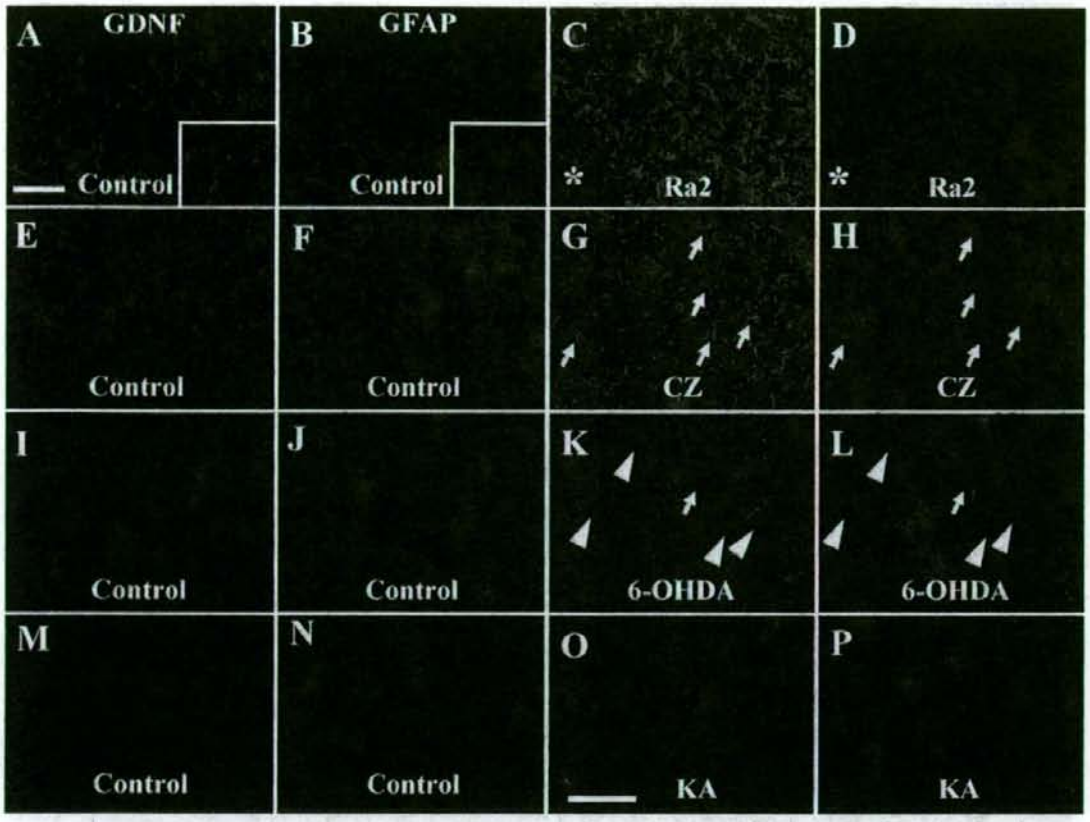

Figure 7. Expression of GDNF in astrocytes assessed by double immunofluorescence staining for GDNF (green) and GFAP (red). ***A–D***, Mouse hippocampi injected with vehicle (***A***, ***B***) and Ra2 cells (***C***, ***D***). Relative to astrocytes on the vehicle-injected side, the number, shape and GDNF levels of which were comparable with untreated condition (insets in ***A***, ***B***), astrocytes in the vicinity of the transplantation site (asterisks in ***C***, ***D***) exhibited pronounced activation accompanied by GDNF upregulation. ***E–H***, Striata of mice without treatment (***E***, ***F***) and at 4 weeks of CZ challenge (***G***, ***H***). ***E–H***, GDNF- and GFAP-immunoreactivities in the control striatum (***E***, ***F***) were much less than those in the hippocampus (insets in ***A***, ***B***), whereas markedly increased astrocytes in this region expressed GDNF at a significant level (arrows in ***G***, ***H***). ***I–L***, Striata of control (***I***, ***J***) and 6-OHDA-treated (***K***, ***L***) rats. GDNF signals were undetectable in the vast majority of activated astrocytes in the treated rat (arrowheads), and only a limited subset of these cells showed GDNF expression slightly above the detection threshold (arrows). ***M–P***, Midbrain of control (***M***, ***N***) and KA-treated (***O***, ***P***) rats. ***P***, ***O***, KA induced prominent astrogliosis (***P***) with only slight GDNF expression (***O***) in the region outlining the inflammatory core. Scale bars: (***A–L***) 50 μm, (***M–P***) 50 μm.

than those in HIV-1 Nef-incorporated Ra2 cells (data not shown), which exhibit dysregulated NADPH oxidase activity and ROS synthesis (Vilhardt et al., 2002), supporting a positive correlation between neurotoxic capability and PBR expression in microglia. This view is additionally supported by a previous demonstration that PK11195, a putative antagonist for PBR, strongly inhibited the lipopolysaccharide-induced production of tumor necrosis factor-α and ROS in primary microglial cells (Wilms et al., 2003). However, PBR-bearing astrocytes present enhanced production of GDNF (supplemental Fig. 4, available at www.jneurosci.org as supplemental material), highlighting the roles of these cells in neuroprotection. This is in line with the previous finding that several PBR ligands could facilitate the survival and regeneration of nerve cells after physical damage (Ferzaz et al., 2002; Mills et al., 2005). The roles of neurotrophins in chronic neurodegeneration may be more complicated, because restorative processes mediated by glial cells are possibly comingled with progressive neuronal injuries. This has been confirmed by the rise of astroglial GDNF expression in PS19 mice. However, GDNF upregulation in these animals was not associated with the emergence of PBR-positive astrocytes, thereby validating the notion that PBR in astrocytes serves as a marker for the repairability of neuronal integrity and sufficiency of neurotrophic support by glial cells.

Glial mediators leading astrocytes to express PBR could be identified by comparatively analyzing signals secreted by microglia in pathologies causing PBR-positive and PBR-negative astrogliosis. Indeed, a subset of secretory molecules, including IL-1 (O'Callaghan et al., 1990), was reported to be undetectable in striatal microglia responding to MPTP lesions of the nigrostriatal dopaminergic projection. As IL-1 receptors are known to be present in astrocytes (Ban et al., 1993), certain IL-1 family cytokines may be key players serving as functional correspondence from microglia to astrocytes, which consequently triggers PBR expression in astrocytes. Neurotrophic components, including IL-10 (Mizuno et al., 1994; Sawada et al., 1998), TGF-β (Constam et al., 1992; Suzumura et al., 1993), plasminogen (Nakajima et al., 1992), nerve growth factors (Elkabes et al., 1996), brain-derived neurotrophic factors (BDNF) and GDNF (Batchelor et al., 1999; Ziv et al., 2006) released from microglia are also plausible candi-

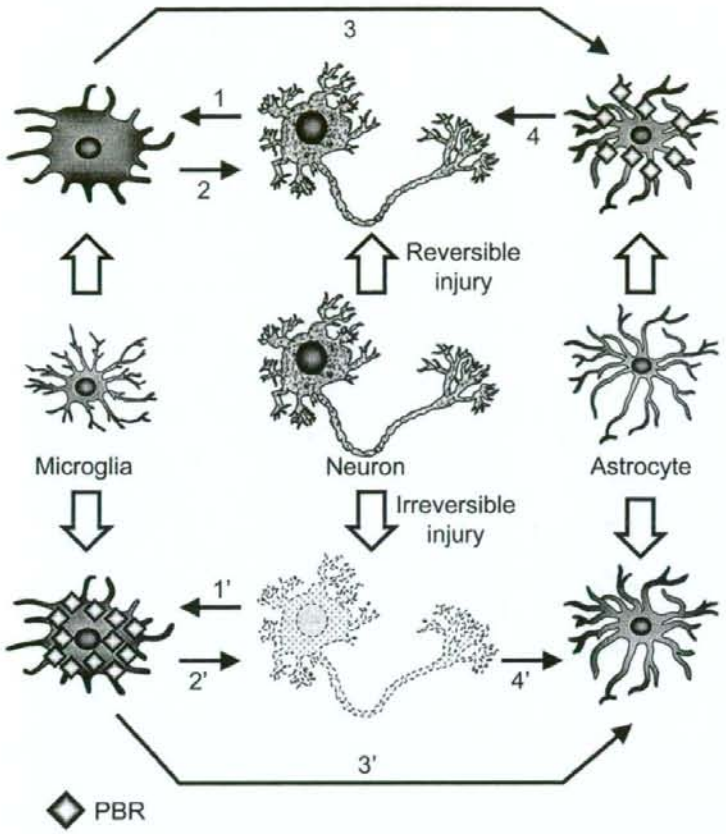

Figure 8. Schematic presentation of proposed intercellular network underlying the differential expression of PBR in microglia and astrocytes. Microglial cells are activated by signals from injured neurons, among which myelin components can be highly potent stimulants (1, 1'). Pathologies in which neuronal viability is protected are mutually linked to microgliosis with no or limited PBR expression (1, 2). These microglia trigger activation of PBR-positive astrocytes (3), which may act as neuroprotectors by releasing trophic elements including GDNF (4). Irreversibly damaged neurons have mutual interactions with microglia persistently expressing PBR (1', 2'). These microglial cells are in close association with activation of astrocytes devoid of significant PBR upregulation (3'), whereas PBR-negative astrogliosis is also inducible by degeneration of unmyelinated neurons without prominent microgliosis (4').

dates for direct or indirect regulators of astroglial PBR enabling PBR-positive astrocytes to promote the protection and repair of injured CNS neurons.

Our present data also suggest a lack of astroglial PBR and a paucity of microglia-astrocyte coupling in the striata of PD models, presumably in association with a paucity of neuroprotective factors in these tissues. In fact, reduced levels of BDNF and a lack of upregulated GDNF production in the nigrostriatal system of PD patients were reported in previous investigations (Mogi et al., 1999, 2001), despite the presence of prominent GFAP-positive astrogliosis in the same brain region. Feasible explanations for the absence of full-range activation of microglia in these conditions might be that dopaminergic terminals account for <15% of the entire synapses in the striatum (Pickel et al., 1981; Teismann et al., 2003), and that myelinated axons, the loss of which gives strong stimuli to microglia, are absent in the nigrostriatal dopaminergic pathway (Pickel et al., 1981). In line with this contention, MPTP-administered monkeys did not develop microgliosis at a detectable level in the striatum (Hurley et al., 2003). It is also noteworthy that intrastriatal injection of 6-OHDA to rats added a small but not striking enhancement to trauma-induced microgliosis (Depino et al., 2003). In humans, a significant number of studies using postmortem PD brains documented that microglial activation was less robust in the striatum than in the substantia nigra (McGeer et al., 1988; Mirza et al., 2000; Knott et al., 2002). In the context that beneficial coupling of microglia and astrocytes does not emerge in response to PD pathologies, pharmacological augmentation of the dialogue between these glial subtypes in the striatum could have a therapeutic value in PD, conceivably by a mechanism that transforms astrocytes into potent protectors of neuronal structures and functions capable of releasing sufficient neurotrophins. This assertion is supported by studies showing a beneficial outcome of GDNF delivery to the striatum of PD models and patients (Tomac et al., 1995; Gash et al., 1996; Kordower et al., 2000; Gill et al., 2003). Interglial communication to promote neuroprotection against these pathological conditions could also be stimulated by adopting a treatment with either locally or systemically administered exogenous microglia as used for an animal model of transient global brain ischemia (Imai et al., 2007), because this approach provokes neurons and glia to produce BDNF and GDNF (Suzuki et al., 2001; Imai et al., 2007). On the supposition that astroglial PBR is an index of the protective and reconstructive activities of glial cells, the present study highlights the usefulness of PET imaging with radioligands for PBR in monitoring interactions between microglia and astrocytes during the course of therapies to upregulate glial neurotrophins.

In conclusion, cytomolecular and intercellular dynamics governing differential PBR expression between microglia-dominant and astrocytes-dominant states may be relevant to mechanisms underlying major neurodegenerative disorders including AD and PD, and further insights into these phenomena could expand the utility of *in vivo* PBR imaging as a powerful tool for estimating the therapeutic and adverse effects of inflammatory modulators and glioactive agents in diverse CNS disorders.

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Neuroprotective and toxic changes in microglia in neurodegenerative disease

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Abstract

Microglia are macrophage-like cells in the CNS. As macrophages, activated microglia remove potentially deleterious debris and promote tissue repair. However, they can release potentially cytotoxic substances *in vitro*. So-called fully activated microglia, observed at the injury site in many neurodegenerative conditions, are neurotoxic. This suggests that some factor(s) may contribute to change microglial phenotype from protective to toxic, but details are not clear. Recently, we generated HIV-derived Nef protein-transduced microglia. They increase the potential to produce O_2^- and MPO-like peroxidase activity, resulting in neurotoxicity. Therefore, the target protein(s) of Nef might be involved in the control of microglial neurotoxicity.

Keywords: Microglia; Toxic transformation; HIV Nef; ROS

1. Microglia in Parkinson's brain

The presence of oxidative stress and inflammatory activity is one of the significant pathological features of Parkinson's disease (PD) [1,2]. It has been shown that the levels of cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and interferon (IFN)-γ are elevated in the substantia nigra of patients with PD [3]. Since microglia are a principal source of these cytokines, the data support microglial involvement in the pathogenesis of PD. However, the role of activated microglia is controversial. The characteristic pathological features of the PD brain are a selective and progressive loss of dopamine neurons of the substantia nigra and their terminals in the caudate-putamen, along with focal accumulation of activated microglia in the substantia nigra and caudate-putamen. The traditional view is that microglia act merely as scavengers and their activation is secondary to the neuronal damage. However, activated microglia have been observed in the limbic system and neocortex, where there are few or no degenerating neurons, in significantly higher numbers in PD brains than in brains from normal controls [4].

Activation of microglia has also been identified in the substantia nigra and/or striatum of parkinsonian animal models, such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)–induced parkinsonism [5–7]. The link between microglia activation and selective neuronal vulnerability has led many researchers to suggest that microglia activity participates in neuronal demise. In this respect, microglial cytotoxicity may contribute to, or even promote neuronal damage. Activated microglia are capable of releasing numerous cytotoxic agents, including proteolytic enzymes, cytokines, complement proteins, reactive oxygen intermediates, NMDA-like toxins, and nitric oxide [8]. In fact, β-amyloid, the senile plaque-derived component found in Alzheimer's disease, appears to elicit neurotoxic responses through the activation of microglia [9]. However, this suggestion relies solely on *in vitro* data and as yet, no evidence has been presented that indicates that activated microglia destroy neurons under *in vivo* conditions.

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2. Existence of non-toxic activated form of microglia in the brain

Recently we showed that highly activated microglia treated with lipopolysaccharide (LPS) may have neurotrophic potential toward dopamine neurons in neonatal mice administered MPTP [10]. Tyrosine hydroxylase activity and the levels of dopamine and dihydroxyphyenylacetic acid (DOPAC), as well as those of the pro-inflammatory cytokines IL-1β and IL-6, were elevated in the midbrain of LPS-MPTP-treated neonatal mice. The viability of dopamine (A9) neurons was preserved in neonatal mice of the LPS-MPTP group compared with the MPTP group. In contrast, the viability of these neurons in aged mice dropped significantly. These results may suggest that activated microglia show different phenotypes; i.e., microglia activated by LPS in the neonatal brain may be neuroprotective for dopaminergic neurons in MPTP-treated mice, whereas in aged mice they may be neurotoxic for dopaminergic neurons. The dissociation between injury-induced microglial activation and neuronal degeneration in TNF receptor and colony stimulating factor-1 (CSF-1) knockout mice suggests that microglial activation is not a determinate event in dopaminergic neuronal damage in brain. Furthermore, there is a growing body of evidence that microglia may play a beneficial role in ischemia by secreting factors (growth factors or cytokines) that promote survival of neurons. Therefore, activated microglia may produce not only neurotoxic effects, but also neuroprotective ones, depending upon their environmental situation.

3. Direct evidence of neuroprotection of microglia in the brain

We have reported that exogenous microglia enter the brain parenchyma through the blood–brain barrier and migrate to ischemic hippocampal lesions when they are injected into the circulation. By applying this brain-targeted delivery technique, we investigated the effect of exogenous microglia on ischemic pyramidal neurons [11]. To this end, we isolated microglia from neonatal mixed brain cultures, labeled them with a fluorescent dye PKH26, and injected them into the artery of Mongolian gerbils subjected to ischemia reperfusion neuronal injury. PKH26-labeled microglia migrated to the ischemic hippocampal lesion and increased the survival of neurons, even when the cells were injected 24 h after the ischemic insult. Stimulation of isolated microglia with IFN-γ enhanced the neuroprotective effect on the ischemic neurons. Microglia also protected ischemia-induced learning disability. As migratory microglia increased the expression of brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) in the ischemic hippocampus, they might induce neurotrophin-dependent protective activity in damaged neurons. These results represent the first experimental demonstration of neurotrophic effects of microglia on transient global ischemia *in vivo*. Since peripherally injected microglia exhibit specific affinity for ischemic brain lesions and protect against ischemic neuronal injury in the present model, we suggest that microglia may have the potential to be used as a candidate for cell therapy for central nervous system (CNS) repair following transitory global ischemia and other neurodegenerative diseases including PD.

4. Heterogeneity of microglia

Microglial cells show a rather uniform distribution of cell numbers throughout the brain with only minor preponderance in some regions. Their in situ morphologies, however, may vary markedly from elongated forms observed in apposition with neuronal fibers to spherical cell bodies with sometimes extremely elaborate branching. This heterogeneity gave rise to the hypothesis that these cells are differentially conditioned by their microenvironment and, therefore, also display specific patterns of differential gene expression. The distinct phenotypes in activated forms of microglia might result from this heterogeneity.

Recently, we identified two distinct sub-populations of microglia in the normal mouse brain: type I and type II. Both types had similar phagocytic activity, but they showed distinct phenotypes such as cell-surface markers, mRNA expression, and growth factor dependency. Type I, but not type II, microglia expressed the hallmarks of microglia such as CD14 and C3b receptors, and production of Il-1β. Type II expressed surface markers for immature bone marrow cells, such as ER-MP12 and 20, and one tenth the amount of those present on mature monocytes/macrophages, such as C3b and F4/80 antigen. Type I microglia exhibited M-CSF-dependent growth, while the type II were M-CSF-independent. Microglia produce superoxide [12] and express all components of the superoxide generating phagocyte NADPH oxidase [13]. Isolated microglia were analyzed for superoxide production by DCFH-DA fluorescence with FACS. Purified microglia indicated low and high superoxide production activity. Two-parameter analysis showed that these activities resulted from distinct cell populations of microglia. The high superoxide-producing population had a toxic effect on cholinergic neurons and induced cell death of cholinergic neurons in culture. Since this toxic effect was blocked by superoxide dismutase, it seemed to be caused by superoxide from activated microglia. The low superoxide-producing population did not have a toxic effect but rather a protective effect on cholinergic neurons. These observations indicate that there are at least two distinct populations of microglia in the brain, which are probably of different origins and have different functions.

5. Toxic transformation of microglia by Nef protein introduction

Nef, a multifunctional HIV protein, activates the Vav/Rac/p21-activated kinase (PAK) signaling pathway. Given the potential role of this pathway in the activation of the phagocyte NADPH oxidase, we have investigated the effect of the HIV-1 Nef protein on microglia superoxide production

and toxicity for neurons [13]. Microglia were transduced with lentiviral expression vectors to produce a high level of Nef protein. Expression of Nef did not activate the NADPH oxidase by itself, but led to a massive enhancement of the responses to a variety of stimuli (Ca^{2+} ionophore, formyl peptide, endotoxin) and induction to produce a large amount of superoxide. These effects were not caused by up-regulation of phagocyte NADPH oxidase subunits. Nef mutants lacking motifs involved in the interaction with plasma membrane and/or other cytosolic proteins failed to reproduce the effects of wild-type Nef, suggesting involvement of a certain signaling pathway in microglia for their trophic-toxic control. Our results suggest a key role for Rac activation in the priming for microglia toxic activation, which is enhanced by Nef introduction in the non-toxic form of microglia. Rac activation is not sufficient to induce the toxic form of microglia accompanied by stimulation of the phagocyte NADPH oxidase; however, it markedly enhances the NADPH oxidase response to other stimuli and might be involved in the trophic-toxic control of microglia.

6. Conclusion

Microglia, macrophage-like cells in the CNS, are multifunctional cells; they play an important role in the removal of dead cells or their remnants by phagocytosis in CNS degeneration, as well as being one of the important cell populations in the CNS cytokine network. They are thought to originate from mesoderm, and to be similar cells to other tissue-resident macrophages. As macrophages, activated microglia have been shown to remove potentially deleterious debris and promote tissue repair by secreting neurotrophic factors at the neuronal injury sites. However, they can release potentially cytotoxic substances *in vitro*, and at least the so-called fully activated form of microglia, which are observed at the injury site in many neurodegenerative conditions such as PD, Alzheimer's disease, and AIDS dementia, are neurotoxic. This suggests that some factor(s) may contribute to changing the microglial phenotype from protective to toxic, but details are not clear. Recently we generated HIV-derived Nef protein transduced microglia. They increase the potential to produce both O_2^- and MPO-like peroxidase activity, resulting in neurotoxicity. Therefore, the target protein(s) of Nef might be involved in the control of microglial neurotoxicity.

Conflict of interest

The author has no conflict of interest to report. No funding applicable.

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IL-4-Induced Selective Clearance of Oligomeric -Amyloid Peptide$_{1-42}$ by Rat Primary Type 2 Microglia[1]

Eisuke Shimizu,* Kohichi Kawahara,* Makoto Kajizono,* Makoto Sawada,† and Hitoshi Nakayama[2]*

A hallmark of immunopathology associated with Alzheimer's disease is the presence of activated microglia (MG) surrounding senile plaque deposition of -amyloid (A) peptides. A peptides are believed to be potent activators of MG, which leads to Alzheimer's disease pathology, but the role of MG subtypes in A clearance still remains unclear. In this study, we found that IL-4 treatment of rat primary-type 2 MG enhanced uptake and degradation of oligomeric A $_{1-42}$ (o-A $_{1-42}$). IL-4 treatment induced significant expression of the scavenger receptor CD36 and the A -degrading enzymes neprilysin (NEP) and insulin-degrading enzyme (IDE) but reduced expression of certain other scavenger receptors. Of cytokines and stimulants tested, the anti-inflammatory cytokines IL-4 and IL-13 effectively enhanced CD36, NEP, and IDE. We demonstrated the CD36 contribution to IL-4-induced A clearance: Chinese hamster ovary cells overexpressing CD36 exhibited marked, dose-dependent degradation of ^{125}I-labeled o-A $_{1-42}$ compared with controls, the degradation being blocked by anti-CD36 Ab. Also, we found IL-4-induced clearance of o-A $_{1-42}$ in type 2 MG from CD36-expressing WKY/NCrj rats but not in cells from SHR/NCrj rats with dysfunctional CD36 expression. NEP and IDE also contributed to IL-4-induced degradation of A $_{1-42}$, because their inhibitors, thiorphan and insulin, respectively, significantly suppressed this activity. IL-4-stimulated uptake and degradation of o-A $_{1-42}$ were selectively enhanced in type 2, but not type 1 MG that express CD40, which suggests that the two MG types may play different neuroimmunomodulating roles in the A -overproducing brain. Thus, selective o-A $_{1-42}$ clearance, which is induced by IL-4, may provide an additional focus for developing strategies to prevent and treat Alzheimer's disease. *The Journal of Immunology,* **2008, 181: 6503–6513.**

A pathological hallmark of Alzheimer's disease (AD)[3] is the presence of senile plaque deposition of -amyloid (A) peptides. Preventing or reversing A deposition in AD is therefore considered to be a major therapeutic goal. Fibrillar A is observed in senile plaques and A $_{1-42}$ forms aggregates and A fibrils more easily than does A $_{1-40}$. In addition to forming such fibrils, oligomeric A (o-A) was revealed to be more neurotoxic (1–3), which suggests that it plays crucial roles during AD development or progression. Therefore, blocking formation or promoting clearance of o-A is of prime importance for prevention or treatment of AD.

Activated microglia (MG) were demonstrated to surround senile plaque deposition of A peptides. A peptides were thought to be potent activators of MG, and many articles reported the induction of neuronal damage by means of an unrestrained inflammatory response (for reviews, see Refs. 4 and 5) involving the proinflammatory cytokines TNF- , IFN- , and IL-1 , as well as NO (4, 6). Participation of scavenger receptors was also reported, because their levels increased on MG around the compacted plaques and they could mediate many proinflammatory outcomes. Although Glabe's group (7) reported that the increase in membrane permeability induced by oligomeric A may be receptor independent, the proposed participant microglial receptors included scavenger receptor class AI/AII (SRA) (8) and receptor for advanced glycation end products (RAGE) (9). Data provided in studies of SRA knockout mice (10), however, do not support a role for SRA. MG have also long been thought to elicit neuroinflammatory reactions (4, 6).

Several articles have claimed a neuroprotective role of MG in AD (11, 12; for review, see Ref. 13). They showed that activated MG increased A clearance and that A vaccination (14–16) or recruitment of bone marrow-derived MG (17, 18) could result in neuroprotection. M-CSF receptor (19) was also proposed to be a factor in clearance of A $_{1-40}$. In addition to participation of MG in clearance of A from the CNS, certain receptors important to the blood-brain barrier (BBB) are involved. These receptors include low-density lipoprotein-related protein (LPR) 1 and LRP-2 (megalin or gp330). LRP-1 may promote the export of soluble A from the brain. However, these same receptors on different cells, e.g., neurons, may increase intraneuronal uptake and toxicity of A (20). Similarly, carrier proteins such as apolipoprotein E, apolipoprotein J, and $_2$-macroglobulin (21) may deliver A either to MG, where it may help A clearance, or to neurons, where it may aid neurodegeneration. The effect of insulin-like growth factor 1 (22) on decreasing the A burden is believed to be mediated by enhancement of A carriers such as albumin and the thyroid carrier protein transthyretin. Another finding confirming

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[3] Abbreviations used in this paper: AD, Alzheimer's disease; A , -amyloid; BBB, blood-brain barrier; CHO, Chinese hamster ovary; IDE, insulin-degrading enzyme; MG, microglia; NEP, neprilysin; o-A $_{1-42}$, oligomeric A $_{1-42}$; RAGE, receptor for advanced glycation end product; SRA, scavenger receptor class AI/AII; SR-BI, scavenger receptor class B type I.

MG-associated neuroprotection is suppression of the microglial inflammatory response with assistance of anti-inflammatory cytokines such as IL-4, IL-10, and IL-13 (23).

IL-4 and other anti-inflammatory cytokines have been shown to promote alternative activation of MG (24–26). However, Landreth and colleague (27) reported that, in both BV-2 cells and mouse primary MG, IL-4 and other anti-inflammatory cytokines neither stimulated nor inhibited phagocytosis induced by phagocytic ligands including fibrillar A . They suggested that these anti-inflammatory cytokines may oppose harmful effects of IL-1 , which stops phagocytic microspheres from forming inside cells and IFN- from preventing phagocytosis.

These studies, however, did not identify which type of MG possessed these neuroprotective functions. MG, as macrophage-like cells, are a heterogeneous population and several subsets were characterized as presenting different alloantigen molecules (e.g., CD40, MHC class II) during the establishment of cell lines (28). Alternative activation of MG to Th2- responsive cells is promoted by IL-4 and other anti-inflammatory cytokines (24–26). For greater understanding of the mechanisms important to amyloid vaccine approaches and alternatives to these approaches, it is necessary to ensure a Th2-type response as opposed to a Th1-type response. The prevailing working hypothesis in AD research, especially of groups studying amyloid vaccines, is that a shift to a Th2-type response may correct microglial dysfunction, reduce chronic inflammation, and enhance A clearance and neuroprotective mechanisms.

We describe here a novel A clearance mechanism involving type 2 MG, not type 1 MG, in primary culture after IL-4 treatment. We found clearance of o-A $_{1-42}$ with increased expression of scavenger receptor CD36 and the A -degrading enzymes neprilysin (NEP) and insulin-degrading enzyme (IDE). We also discuss here the potential usefulness of these findings for developing anti-inflammatory therapeutic strategies against AD.

Materials and Methods

Chemicals and materials

The chloride form of A $_{1-42}$ was purchased from American Peptide. Thiorphan, fucoidan, and bovine insulin solution were purchased from Sigma-Aldrich. Proteinase K was obtained from Merck Biosciences. Na^{125}I (3.7 GBq/ml) was from Amersham Pharmacia Biotech. Iodo-Gen was from Pierce Biotechnology. A mouse monoclonal anti-human A $_{1-17}$ was purchased from Chemicon International. Goat polyclonal anti-human CD36 Ab (L-17), rabbit polyclonal anti-human NEP/CD10 (H-321), goat anti-mouse CD40 Ab (T-20), and rabbit anti-mouse IL-4R Ab (S-20) were from Santa Cruz Biotechnology. A mouse monoclonal anti-IDE Ab (9B12) was from Covance. A rabbit polyclonal anti-mouse scavenger receptor class B type 1 (SR-BI) Ab (RED-1) was from Novus Biologicals. Rabbit polyclonal anti-mouse RAGE (5503) was from Transgenic. Mouse monoclonal anti-human SRA Ab (SRA-E5) described by Tomokiyo et al. (29) was a gift from Prof. M. Takeya (Kumamoto University, Kumamoto, Japan). Mouse monoclonal anti-human CD36 Ab (FA6-152) was from Immunotech. Goat anti-B7-2 (CD86) polyclonal Ab was purchased from Genzyme Techne. Mouse IgG (MOPC21) was obtained from Sigma-Aldrich. Chinese hamster ovary (CHO) cells overexpressing human CD36 (CD36-CHO) cells were provided by Prof. H. Arai (Tokyo University, Tokyo, Japan). Other chemicals were of the best grade available from commercial sources.

Preparation of oligomeric A $_{1-42}$

Oligomeric A was prepared as previously reported (30), with a slight modification. The chloride form of A $_{1-42}$ peptide was initially dissolved in hexafluoroisopropanol (Sigma-Aldrich) to achieve a concentration of 1 mM, and the solution was then separated into aliquots in sterile microcentrifuge tubes. Hexafluoroisopropanol was removed under vacuum in a SpeedVac concentrator and the peptide film was stored in desiccated form at −20°C. For the aggregation protocol on a preparative scale, the peptide was first resuspended in dry DMSO to a concentration of 5 mM. Ham's F-12 (Invitrogen) was then added to bring the peptide to a final concentration of 500 M, and the samples were rotated on a rotary shaker at 4°C for 24 h. o-A $_{1-42}$ prepared as above (200 g in PBS) was radioiodinated by the Iodo-Gen method with Na^{125}I at 18.5 MBq. Excess ^{125}I was equilibrated with PBS. The specific activity of the ^{125}I-labeled A $_{1-42}$ was 856-1324 cpm/ng. Analytical scale aggregation protocols are described in Fig. 1 legend.

Cell culture and treatment

Rat primary MG were harvested from primary mixed glial cell cultures prepared from neonatal Wistar rats, spontaneously hypertensive rats (SHR) (NCrj), Wistar-Kyoto (WKY) (NCrj) rats, or ddY mice as previously reported (31, 32). In brief, after meninges were carefully removed, the neonatal brain was dissociated by pipetting. The cell suspension was added to 75-cm^2 culture flasks at a density of five brains per 12 flasks (for rats) or one brain per flask (for mice) in 10 ml of Eagle's MEM supplemented with 10% bovine serum, 5 g/ml bovine insulin, and 0.2% glucose. Type 1 MG were isolated on days 14–16 by the "shaking off" method previously described (32). Type 2 MG were isolated on days 19–21 by harvesting with 5 mM EDTA in phosphate buffer solution via a modification of the mild trypsinization method (33). For some experiments, mixed MG that included both type 1 and type 2 cells were separately harvested on day 16 from a mixed glial cell culture with 5 mM EDTA in PBS via a modification of the mild trypsinization method (33). All MG preparations (mixed MG, type 1 MG, and type 2 MG) did not contain O_2-A progenitors, oligodendrocytes, or astrocytes. CHO-K1 cells and human CD36-CHO cells were maintained as described previously (34). Rat primary MG were treated with *Escherichia coli* LPS (1 g/ml, serotype 0127:B8; Sigma-Aldrich), rat IFN- (100 U/ml; PeproTech), mouse TNF- (150 U/ml; Sigma-Aldrich), mouse M-CSF (50 U/ml; Sigma-Aldrich), rat IL-4 (5 ng/ml; PeproTech), rat IL-13 (5 ng/ml; PeproTech), or human TGF- 1 (2 ng/ml; PeproTech). Mouse primary MG were treated with mouse IL-4 (5 ng/ml; PeproTech).

Immunoblot analysis

Rat primary MG, mouse primary MG, and rat elicited macrophages (5 × 10^6 cells/100-mm dish) were homogenized in 10 mM Tris-HCl (pH 7.6) containing 1% w/v SDS, 2% w/v sucrose, 4 mM EDTA, 1 mM PMSF, 1 g/ml pepstatin A, 1 g/ml leupeptin, and 10 g/ml soybean trypsin inhibitor. Aliquots of homogenates were saved for protein determination and, after addition of 25 mM DTT, samples were incubated at 60°C for 10 min. The samples (50 g) were then subjected to SDS-PAGE and proteins were electrotransferred to polyvinylidene difluoride membranes. These membranes were incubated with TBS (pH 7.4) containing 2% dehydrated skimmed milk to block nonspecific protein binding. Membranes were then incubated with Abs to A $_{1-17}$ (6E10, 1/500 dilution), CD36 (L-17, 1/30 dilution), SRA (SRA-E5, 1/2000 dilution), SR-BI (RED-1, 1/1000 dilution), RAGE (5503, 1/560 dilution), NEP/CD10 (H-321, 1/100 dilution), IDE (9B12, 1/1000 dilution), CD40 (T-20, 1/100 dilution), CD86 (421340, 1/500 dilution), or -actin (AC15, 1/2000 dilution), followed by secondary Ab: HRP-linked Abs against rabbit, goat, or mouse Ig (each diluted 1/1000). Bound HRP-labeled Abs were then detected via chemiluminescence (ECL kit).

Cell assays

For the A degradation assay in medium, rat primary MG (see Fig. 2), rat primary type 2 MG (see Figs. 7 and 9), and mouse primary type 2 MG (see Fig. 10) were cultured in 24-well plates for 96 h in MEM/10% FCS in the presence or absence of IL-4 (5 ng/ml). Human CD36-CHO cells (34) and the control CHO-K1 cells (see Fig. 6) were grown in 24-well plates for 2–4 days before the start of the experiment. The cells were washed twice with labeling medium (DMEM containing 3% BSA) and then incubated with ^{125}I-labeled o-A $_{1-42}$ for 6 h at 37°C in a 5% CO_2-humidified air atmosphere. After the 6-h incubation, medium was removed from each well, and soluble radioactivity in TCA (degraded and extracellularly released peptide fragments) was determined as an index of A degradation in medium as described previously (34). After cells were washed three times with 1 ml of labeling medium and then three more times with PBS, they were lysed with 1 ml of 0.1 M NaOH for 30 min at 37°C, and cell proteins were determined with the BCA Protein Assay Reagent (Pierce).

The A degradation activity may thus include both receptor-mediated phagocytic activity and extracellular protease-mediated degradation. For inhibition assays, cells were incubated with or without Abs (10 g/ml FA6-152 or control IgG for human CD36-CHO cells; see Fig. 6) or fucoidan (100 g/ml), thiorphan (30 M), or insulin (100 g/ml) (see Figs. 7, 9, and 10). Radioactivity of these background dishes was subtracted from values for control experiments. Results are represented as the means ± SD (n = 3).

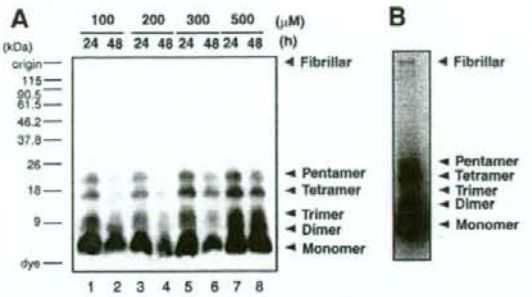

FIGURE 1. Preparation of the o-A $_{1-42}$ and ^{125}I-labeled A $_{1-42}$. *A*, Results of Western blot analysis. o-A $_{1-42}$ was prepared via a previously reported method (30), with a slight modification, and as described in *Materials and Methods*. For aggregation protocols, the stored peptide was resuspended in DMSO to a concentration of 5 mM. Then Ham's F-12 was added to bring the peptide to final concentrations of 100–500 M, and samples were rotated at 4°C for either 24 or 48 h. The o-A $_{1-42}$ preparations were separated by SDS-PAGE (15% gels) and were subjected to immunoblot analysis using anti-A $_{1-17}$ Ab. Besides oligomeric forms, monomeric and fibrillar forms of A $_{1-42}$ were detected. *B*, Electrophoretic and radioluminographic findings for ^{125}I-labeled A $_{1-42}$. A portion of the oligomer preparation (*A*, *lane 7*) was further iodinated with Na^{125}I via Iodo-Gen and was then subjected to SDS-PAGE. The dried gel was contacted with an imaging plate and luminography was evaluated with a FUJIX BAS 1800 analyzer (Fuji Photo Film).

For the uptake assay, rat primary type 2 MG were cultured in 3.5-cm dishes (Western blotting; see Figs. 4 and 7) or 24-well plates (autogamma counter; see Fig. 9) for 96 h in MEM/10% FCS in the presence or absence of IL-4 (5 ng/ml). The cells were washed with labeling medium (DMEM containing 3% BSA) and then incubated with o-A $_{1-42}$ (see Figs. 4 and 7) or ^{125}I-labeled o-A $_{1-42}$ (see Fig. 9) for 3 h at 37°C in the presence or absence of fucoidan (100 g/ml). The cells were rinsed with ice-cold PBS, treated with proteinase K (100 g/ml) or trypsin (2.5 mg/ml) at 4°C for 15 min, and then washed again with PBS, to remove all extracellular A , as previously reported (35). For Figs. 4 and 7, proteins (10 g) in the cell lysate were subjected to Western blot analysis using anti-A $_{1-17}$ Ab or anti- -actin Ab. For Fig. 9, cells were lysed with 1 ml of 0.1 M NaOH for 30 min at 37°C, and cell-incorporated radioactivity and cell proteins were determined with an autogamma counter and the BCA Protein Assay Reagent (Pierce), respectively. Radioactivity of these background dishes was subtracted from values for control experiments.

Results

Preparation of o-A $_{1-42}$ and ^{125}I-labeled A $_{1-42}$

To determine the microglial clearance of A , o-A $_{1-42}$ was prepared by a method similar to that of Dahlgren et al. (30), but with a modification of incubation conditions. Fig. 1*A* shows that A oligomers were formed as dimers to pentamers after 24 h of incubation at 4°C with the chloride form of A $_{1-42}$ at initial concentrations of 100–500 M. After 48 h of incubation, the proportion of oligomers and monomers decreased, but fibrillar formation increased. Among the conditions tested, incubation of A at 500 M for 24 h gave the highest yield (~30–40%) of A oligomers. A portion of the oligmer preparation was further iodinated with Na^{125}I via Iodo-Gen, and the resultant sample (namely, ^{125}I-labeled o-A $_{1-42}$) had a similar electrophoretic pattern on SDS-PAGE (Fig. 1*B*). Starting with the trifluoroacetate form of A $_{1-42}$ under the same conditions (data not shown) resulted in a less efficient oligomer yield.

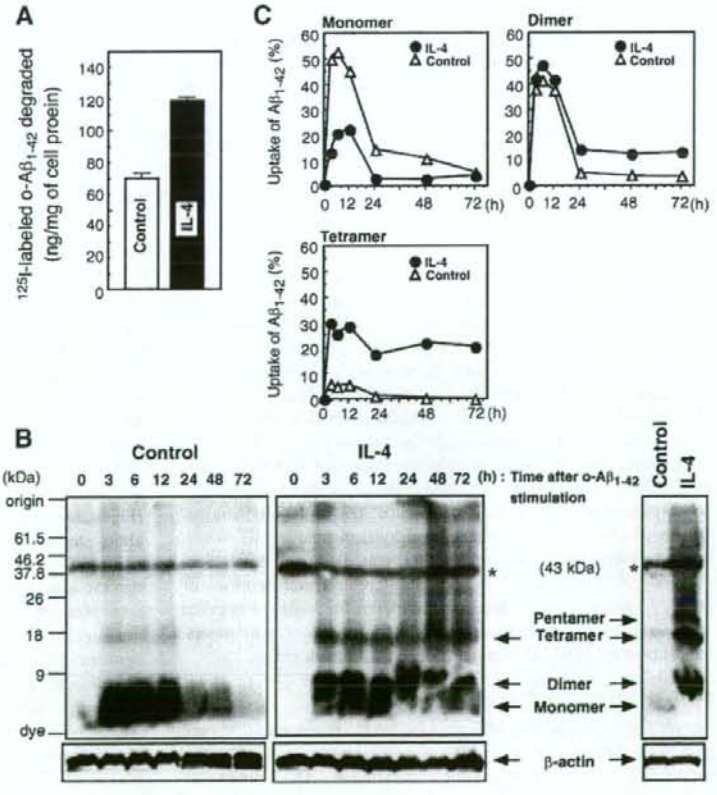

FIGURE 2. Uptake and clearance of o-A $_{1-42}$ by rat primary MG. *A*, Rat primary MG were cultured for 96 h in MEM/10% FCS in the presence or absence of IL-4 (5 ng/ml) and were then incubated for 6 h with ^{125}I-labeled o-A $_{1-42}$ (1 g/ml). Amounts of degraded ^{125}I-labeled o-A $_{1-42}$ were determined as described in *Materials and Methods*. Values are means ± SD (n = 3). *B*, Rat MG were cultured as described in *A* and were then incubated with o-A $_{1-42}$ (2 g/ml) for the indicated periods (*left panel*) or for 72 h (*right panel*). Proteins (30 g) in the cell lysate were subjected to Western blot analysis using anti-A $_{1-17}$ Ab or anti- -actin Ab. The asterisk indicates a nonspecific band detected by the anti-A Ab. *C*, Each A species was measured by densitometric scanning and the amount was plotted as a percent value of the total amount of A at 6 h.

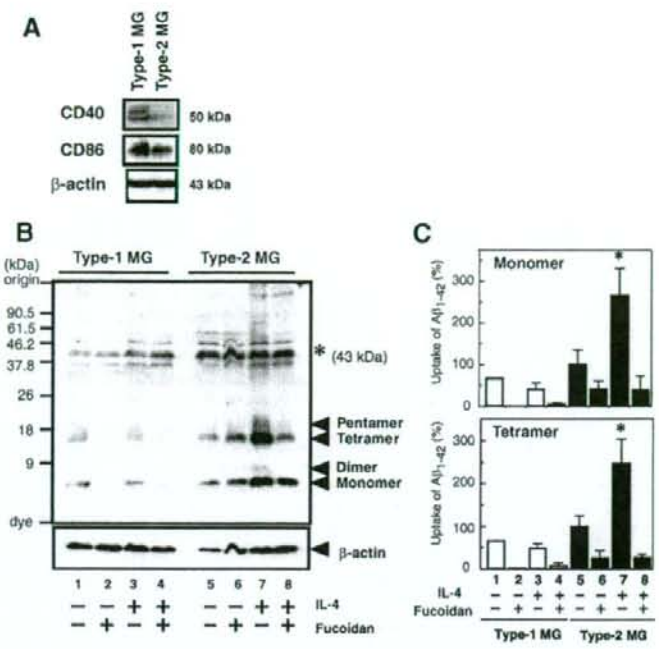

FIGURE 3. Comparison of uptake of o-A $_{1-42}$ by type 1 and type 2 MG. *A*, Rat primary type 1 and type 2 MG were isolated as described in *Materials and Methods*. Cell proteins (40 g) were subjected to immunoblot analysis with Abs against CD40, CD86, or -actin. *B*, Rat primary type 1 and type 2 MG were cultured for 96 h in MEM/10% FCS in the presence or absence of IL-4 (5 ng/ml) and were then incubated for 6 h with o-A $_{1-42}$ (2 g/ml) in the presence or absence of fucoidan (100 g/ml). Proteins (30 g) in the cell lysate were subjected to Western blot analysis with anti-A $_{1-17}$ Ab or anti- -actin Ab. The asterisk indicates a nonspecific band detected by the anti-A Ab. *C*, Monomer and tetramer A levels in *B* were measured by densitometric scanning and the results were plotted. The value for unstimulated type 2 MG (*lane 5*) was set at 100%. Values are means ± SD ($n = 3$). *, $p < 0.05$, significantly different from unstimulated type 2 MG.

IL-4 enhances uptake of o-A $_{1-42}$ by MG

Fig. 2*A* shows endocytic degradation of ^{125}I-labeled o-A $_{1-42}$ by primary mixed MG before and after treatment with IL-4. The degraded amount reached 120 ng/mg cell protein after treatment, which was 170% of the untreated control.

To determine which molecular species was incorporated into cells, cell lysate was subjected to Western blot analysis with anti-A $_{1-17}$ Ab (Fig. 2*B*). Control MG incorporated mainly the A monomer (4 kDa) and dimer (8 kDa) for 3–12 h. In contrast, IL-4-treated MG predominantly incorporated the dimer and tetramer (16 kDa) during the same period, with only slight incorporation of monomer.

The amount of uptake of each species was determined by densitometric scanning, normalized to the total amounts of the samples (control and IL-4 treated at 72 h) on the same blot sheet (Fig. 2*B*, *right panel*), and was expressed as a value relative to the total amounts incorporated at 6 h (Fig. 2*C*). Uptake of monomer reached a maximum around 50% of the total amounts in untreated MG during 3–12 h, which was 2.5 times greater than that of the IL-4-treated MG, and the uptake markedly decreased after 12 h in both samples. During the same period, uptake of dimer in both MG samples reached 40–50% of total incorporation and decreased considerably afterward. In contrast, uptake of tetramer was mainly observed in IL-4-treated MG and reached 30% of the total amount incorporated in 3 h. The uptake level by IL-4-treated MG, which was five times that of untreated MG, was maintained after 3 h. Overall, IL-4-treated MG exhibited preferential uptake of o-A $_{1-42}$ tetramers and dimers to monomers for 3–12 h, compared with control MG that had a preference for monomers and dimers, and uptake by both MG samples decreased afterward.

Selective uptake of o-A $_{1-42}$ by type 2 MG

Several subpopulations of MG are believed to exist in the CNS and may have different functions (36). We separated type 1 MG and type 2 MG from a mixed glial cell culture by using a previously described method (33) and compared uptake of o-A $_{1-42}$ by both cell types. Successful separation of MG types was confirmed, because the type 2 MG obtained expressed essentially no CD40 and weakly expressed CD86 (Fig. 3*A*), as previously described (28). Using these MG preparations, we found greater uptake of the tetramer and monomer forms of A $_{1-42}$ by IL-4-treated type 2 MG than type 1 MG (Fig. 3*B*, *lane* 7 vs *lane 3*). Fig. 3*C* gives quantified results after densitometric scanning. Uptake that increased 2.5-fold after IL-4 treatment was apparently suppressed by addition of fucoidan, a ligand for scavenger receptors, which suggests that this process involves a specific scavenger receptor.

Refinement of cellular uptake of oligomeric A $_{1-42}$ by IL-4-treated type 2 MG

The apparent uptake shown in Fig. 2 may incorporate some nonspecific binding of A $_{1-42}$ species to the cell surface, as mentioned by other investigators (35). To exclude nonspecific bound fractions, we performed additional experiments. Brief proteinase K treatment (4°C for 15 min) of type 2 MG, which had been preincubated with o-Ab$_{1-42}$ (for 3 h at 37°C), markedly reduced the amounts of tetramer (85% reduction of control) and monomer (75% reduction of control) in IL-4-untreated control cells, as Fig. 4*A* (*lane 3*) shows. In IL-4-treated MG treated with proteinase K (Fig. 4, *lane 4*), however, the amounts of the A components were almost unchanged (90% of control for the monomer) or weakly reduced (30% of control for the tetramer). These results indicate that the unfavorable nonspecific fractions can be removed by protease treatment and that the A components observed after such treatment are believed to reflect cellular uptake. This result was confirmed by subsequent experiments (Fig. 4*B*). Treatments with proteinase K (Fig. 4, *lanes 1–4*) and trypsin (Fig. 4, *lanes 5–8*) gave similar patterns of o-A $_{1-42}$ uptake: tetramer and monomer were the main uptake components, and IL-4 treatment enhanced uptake (Fig. 4, *lane 3*). Addition of fucoidan markedly suppressed uptake of both tetramer and monomer with either proteinase K or

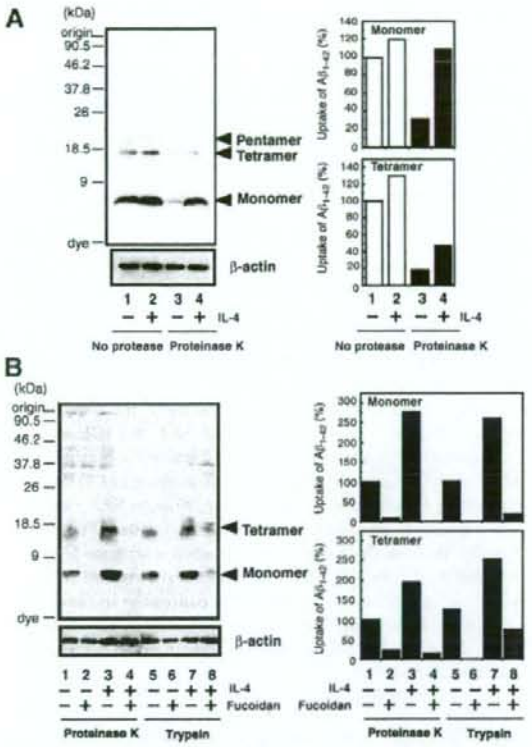

FIGURE 4. Refined uptake of o-A $_{1-42}$ by type 2 MG after protease treatment. *A*, Rat primary type 2 MG were cultured for 96 h in MEM/10% FCS in the presence or absence of IL-4 (5 ng/ml). The cells were incubated with o-A $_{1-42}$ (2 g/ml) for 3 h at 37°C and then treated with proteinase K (100 g/ml) at 4°C for 15 min or were untreated, as described in *Materials and Methods*. Proteins (10 g) in the cell lysate were subjected to Western blot analysis using anti-A $_{1-17}$ Ab or anti- -actin Ab. Monomer and tetramer A levels were measured by densitometric scanning and the results were plotted (*right panel*). The value for nonstimulated type 2 MG (*lane 1*) was set at 100%. *B*, Rat type 2 primary MG were cultured as described in *A* and were then incubated with o-A $_{1-42}$ (2 g/ml) for 3 h at 37°C. The cells were treated with proteinase K (100 g/ml) or trypsin (2.5 mg/ml) at 4°C for 15 min as described in *Materials and Methods*. Proteins (10 g) in the cell lysate were subjected to Western blot analysis using anti-A $_{1-17}$ Ab or anti- -actin Ab. Monomer and tetramer A levels were measured by densitometric scanning and the results were plotted (*right panel*). The value for nonstimulated type 2 MG (*lane 1*) was set at 100%.

trypsin treatment (Fig. 4, *lanes 2* and *6*), even with the addition of IL-4 (Fig. 4, *lanes 4* and *8*), which suggests that cellular uptake is mediated by scavenger receptor(s).

Change in expression of CD36 and SRA in type 1 and type2 MG stimulated with specific cytokines or stimulants

We investigated the effects of other cytokines and cell modulators on expression of the scavenger receptors CD36 and SRA and compared these effects in both types of MG. Among the molecules tested, CD36 was strongly induced by IL-4 in type 2 MG but moderately induced in type 1 MG (Fig. 5). Other modulators tested had no effect on the CD36 expression level. With regard to SRA expression, both MGs had similar responses to the effectors. IL-4 suppressed SRA expression to 82 and 83% of untreated controls in type 1 and type 2 MG, respectively. IFN- , M-CSF, and LPS

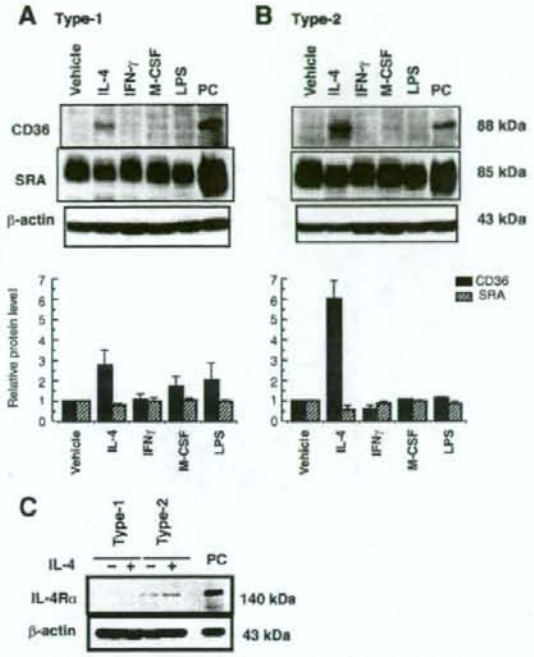

FIGURE 5. Change in expression of CD36 and SRA in type 1 and type 2 MG stimulated with various cytokines or stimulants. Type 1 (*A*) and type 2 (*B*) MG were incubated for 96 h with IL-4 (5 ng/ml), IFN- (100 U/ml), M-CSF (50 U/ml), or LPS (1 g/ml). Cell proteins (50 g) were subjected to immunoblot analysis with Abs against CD36, SRA, or -actin. The positive controls (PC) for CD36 and SRA were proteins (50 g) from IL-4-treated and untreated rat primary macrophages, respectively. Results were quantified by means of densitometric scanning. Vehicle values were set at 1. Values are means ± SD (n = 3). *C*, Type 1 and type 2 MG were incubated with or without IL-4 (5 ng/ml) for 96 h. Cell proteins (30 g) were subjected to immunoblot analysis with Abs against IL-4R or -actin. Protein (30 g) from IL-4-treated mouse microglial MG5 cells served as the positive control (PC).

caused little change in the SRA level. These results suggest that the two cell types regulate expression of CD36 and SRA differently. The effect of IL-4 on expression of other scavenger receptors (SR-BI and RAGE) by type 2 MG is illustrated later in Fig. 7. The level of IL-4R expressed was higher in type 2 MG than in type 1 MG, but IL-4 treatment did not change these levels (Fig. 5*C*).

Selective uptake as related to CD36 function

To demonstrate the contribution of CD36 to IL-4-induced clearance of o-A $_{1-42}$, we first evaluated CD36-CHO cells. The amount of ^{125}I-labeled o-A $_{1-42}$ degraded by these cells was much higher than that of wild-type CHO cells (Fig. 6*A*). This dose-dependent increase to 600 ng/mg protein for 10 g of ^{125}I-labeled o-A $_{1-42}$ added was six times the level of the wild-type cells. Anti-CD36 Ab (FA6-152) inhibited this endocytic degradation by CD36-CHO cells by 55%, to a level similar to that of wild-type cells (Fig. 6*B*). No inhibition was observed after addition of control IgG, which demonstrated that the endocytic degradation of ^{125}I-labeled o-A $_{1-42}$ was mediated by CD36.

To confirm the contribution of CD36 to phagocytic clearance of o-A $_{1-42}$, type 2 MG from SHR rats with dysfunctional CD36 expression were compared with cells from wild-type WKY rats.

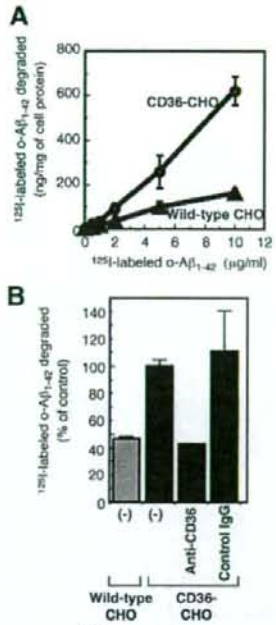

FIGURE 6. Clearance of ^{125}I-labeled o-A $_{1-42}$ by human CD36-CHO cells and effect of anti-CD36 Ab. *A*, CD36-CHO (E) and wild-type CHO (,) cells were incubated for 6 h with ^{125}I-labeled o-A $_{1-42}$ at 0–10 g/ml. The amounts of ^{125}I-labeled o-A $_{1-42}$ degradation products were determined as described in *Materials and Methods*. Values are means ± SD (n = 3). *B*, CD36-CHO and wild-type CHO cells were incubated for 6 h with ^{125}I-labeled o-A $_{1-42}$ (1 g/ml) in the presence or absence of anti-human CD36 Ab (FA6-152: 10 g/ml) or control Ab (MOPC21: 10 g/ml).

First, expression levels of CD36, SRA, SR-BI, and RAGE by both types of MG were examined via Western blotting (Fig. 7*A*). CD36 was clearly absent in SHR MG and was not induced even after IL-4 treatment, whereas it was significantly induced in WKY MG by IL-4. Among other scavenger receptors, SRA and RAGE were present in both MG types and were suppressed considerably after IL-4 treatment. SR-BI was absent in both MG types and was not induced by IL-4.

In a Western blot analysis of cells after proteinase K treatment (Fig. 7*B*), selective, marked uptake of o-A $_{1-42}$ (tetramer and monomer forms) was observed in IL-4-treated compared with untreated type 2 MG from WKY rats (Fig. 7*B*, *lanes 7* vs *5*) after 3 h of incubation with the o-A $_{1-42}$ preparation, but IL-4-treated and untreated MG from SHR rats did not differ in uptake (Fig. 7*B*, *lanes 3* vs *1*). IL-4-induced uptake of o-A $_{1-42}$ was markedly suppressed by addition of fucoidan (Fig. 7*B*, *lane 8*), which suggests again that a scavenger receptor, most likely CD36, is involved in cellular uptake. The degradation step was quantitatively evaluated by using ^{125}I-labeled o-A $_{1-42}$ and type 2 MG from WKY and SHR rats were compared (Fig. 7*C*). After IL-4 treatment, MG from WKY rats increased degradation of o-A $_{1-42}$ 1.5-fold (Fig. 7*C*, *rows 7* vs *5*), whereas MG from CD36-dysfunctional SHR showed no change (Fig. 7*C*, *rows 3* vs *1*). Addition of fucoidan also significantly suppressed degraded amounts of o-A $_{1-42}$, which suggests that a scavenger receptor (or receptors), including CD36, is also involved in the degradation step.

Effects of cytokines on expression of CD36, NEP, and IDE in type 2 MG

As a consequence of the finding that IL-4 induced expression of CD36 (Figs. 5*B* and 7*A*), and the concomitant increase in uptake of o-A $_{1-42}$ by type 2 MG, we examined whether IL-4 affected expression and activity of the A -degrading enzymes NEP and IDE. We also examined whether the effect would be selectively induced in type 2 MG by particular cytokines. As shown in Fig. 8*A*, CD36 expression was similarly induced by IL-4 and IL-13, but less effectively by TNF- . NEP expression was also enhanced by IL-4 and IL-13 but was suppressed by TNF- and TGF- 1. IL-4 and TGF- 1 showed high potency for induction of IDE expression, followed by IL-13; TNF- had the lowest potency. It is noteworthy that IL-4 and IL-13 enhanced CD36, NEP, and IDE, which were involved in phagocytosis and catabolism of A $_{1-42}$.

The effect of IL-4 on expression of NEP and IDE was further investigated and compared with expression of the scavenger receptors CD36 and SRA. Expression of NEP and IDE increased considerably after addition of IL-4 and reached a maximum at 5 and 2 ng/ml, respectively (Fig. 8*B*). The increase in CD36 expression showed a similar dose dependence, whereas SRA expression was conversely decreased at 2–5 ng/ml IL-4 added. The effect of 5 ng/ml IL-4 on their expression changed over time (Fig. 8*C*): expression of both NEP and IDE uniformly increased in a time-dependent manner until day 10; CD36 expression increased more rapidly to reach a maximum at day 4, followed thereafter by a slight decrease; and SRA expression continued to decrease after addition of IL-4.

Effects of various ligands on uptake and degradation of ^{125}I-labeled o-A $_{1-42}$ in type 2 MG before and after IL-4 treatment

To evaluate the contribution of NEP, IDE, and CD36 to o-A $_{1-42}$ uptake and degradation, we investigated the effects of their inhibitors on cellular uptake and degradation of ^{125}I-labeled o-A $_{1-42}$ in type 2 MG. Effects before and after IL-4 treatment were analyzed and compared (Fig. 9).

Inhibitors of A -degrading enzymes, thiorphan as the NEP inhibitor and insulin as the IDE inhibitor, had no effects on the uptake step before and after IL-4 treatment (Fig. 9*A*). In the degradation step, however, both thiorphan and insulin considerably inhibited degradation of o-A $_{1-42}$, at a level similar to that of fucoidan inhibition, in IL-4-treated cells (Fig. 9*B*, *lanes 8* and *10*). Without IL-4 treatment, thiorphan and insulin also inhibited the degradation, which is likely due to inhibition of activities of NEP and IDE expressed at the original level.

Fucoidan, an inhibitor of various scavenger receptors, inhibited both cellular uptake and degradation of o-A $_{1-42}$, but strong inhibition was observed for the uptake step (see also Fig. 7*B*) before and after IL-4 treatment. These data indicate that scavenger receptors function differently in both steps. However, details of this inhibition, such as which scavenger receptor is involved and the extent of its contribution, differ before and after IL-4 treatment. Before IL-4 treatment (control), fucoidan seemed to suppress SRA-mediated phagocytic uptake and degradation, because SRA was expressed at a high level but no CD36 was expressed (Figs. 5 and 8, *B* and *C*). In the IL-4-treated MG, however, cellular uptake and degradation increased 2.1 and 1.7 times, respectively (Fig. 9), and CD36-mediated uptake and degradation were suppressed by fucoidan, because expression of SRA was markedly reduced, whereas that of CD36 was enhanced (see also Fig. 8, *B* and *C*).

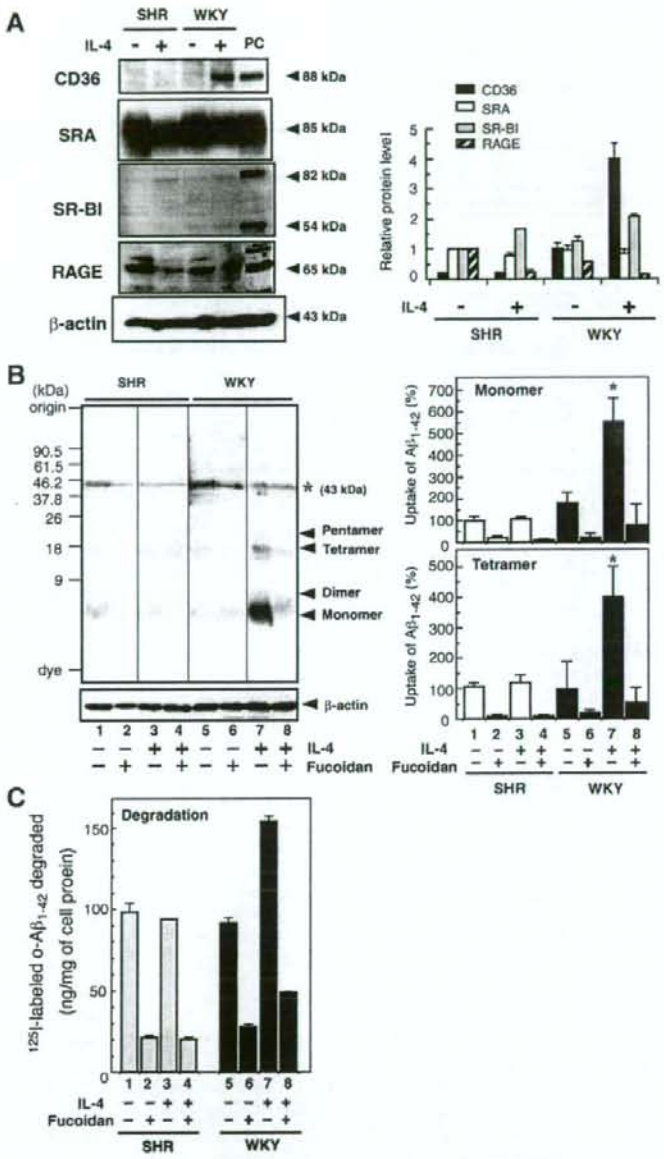

FIGURE 7. Comparison of o-A $_{1-42}$ endocytosis by type 2 MG derived from SHR/NCrj rats with cells derived from WKY/NCrj rats after IL-4 stimulation. *A*, Cells were cultured for 96 h in MEM/10% FCS in the presence or absence of IL-4 (5 ng/ml). Cell proteins (50 g) were subjected to immunoblot analysis as described in *Materials and Methods*. Positive controls for CD36, SRA, SR-BI, and RAGE were proteins (50 g) from IL-4-treated rat primary macrophages, proteins (50 g) from rat primary macrophages, extracts (30 g of protein) from rat liver, and extracts (30 g of protein) from RAGE-CHO cells, respectively. Results were quantified by means of densitometric scanning (*right panel*). Values are means ± SD ($n = 3$). *B*, Cells were cultured for 96 h in MEM/10% FCS in the presence or absence of IL-4 (5 ng/ml) and were then incubated with o-A $_{1-42}$ (2 g/ml) for 3 h in the presence or absence of fucoidan (100 g/ml). The cells were treated with proteinase K (100 g/ml) at 4°C for 15 min as described in *Materials and Methods*. Cell proteins (10 g) were subjected to immunoblot analysis with anti-A $_{1-17}$ Ab or anti--actin Ab. The asterisk indicates a nonspecific band detected by anti-A Ab. Results were quantified by means of densitometric scanning (*right panel*). Values are means ± SD ($n = 3$). *, $p < 0.05$, significantly different from nonstimulated type 2 MG derived from WKY/NCrj rats (*lane 5*). The two values for nonstimulated (*lane 1*) and IL-4-stimulated (*lane 3*) type 2 MG derived from SHR/NCrj rats were not statistically different ($p > 0.1$). *C*, Cells were cultured for 96 h in MEM/10% FCS in the presence or absence of IL-4 (5 ng/ml) and were then incubated with ^{125}I-labeled o-A $_{1-42}$ (1 g/ml) for 6 h in the presence or absence of fucoidan (100 g/ml). Amounts of degraded ^{125}I-labeled o-A $_{1-42}$ were determined as described in *Materials and Methods*. Values are means ± SD ($n = 3$).

Addition of fucoidan resulted in a marked inhibitory effect on cellular uptake of o-A $_{1-42}$ and some inhibitory effect on its degradation in IL-4-treated type 2 MG, whereas thiorphan and insulin inhibited only degradation. These results indicated that both CD36-mediated uptake followed by intracellular degradation and A catabolism by NEP and IDE are important for IL-4-induced clearance of o-A $_{1-42}$.

Effect of IL-4 on mouse type 2 MG

The effect of IL-4 on mouse MG was also assessed and compared with that on rat MG described above. IL-4 treatment markedly increased expression of CD36 but only slightly increased NEP expression (Fig. 10*A*). IL-4 also increased degradation of ^{125}I-labeled o-A $_{1-42}$ 1.8-fold. This degradation seen with IL-4 treatment was markedly inhibited by addition of fucoidan, whereas it was moderately inhibited by thiorphan (Fig. 10*B*). In contrast, addition of fucoidan or thiorphan resulted in partial or no inhibition of degradation, respectively, in control MG. These observations were very similar to data for type 2 MG from rat brains described above.

Discussion

Many observations have shown that activated MG may contribute significantly to the neuropathology of AD and other neurodegenerative diseases by induction of inducible NO synthase or production of TNF- (for reviews, see Refs. 4–6). Recently, increasing

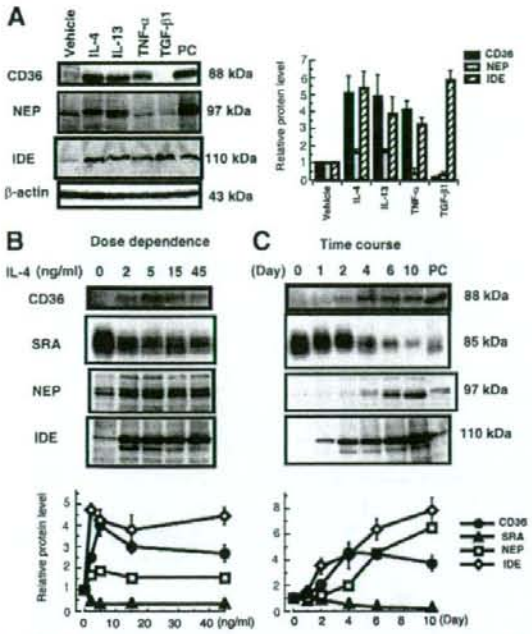

FIGURE 8. Effects of various cytokines on expression of NEP and IDE in rat type 2 MG. *A*, Rat type 2 MG were incubated for 96 h with IL-4 (5 ng/ml), IL-13 (5 ng/ml), TNF- (150 U/ml), or TGF- 1 (2 ng/ml). Cell proteins (50 g) were subjected to immunoblot analysis by using anti-CD36 Ab (1/30 dilution), anti-NEP Ab (1/100 dilution), anti-IDE Ab (1/1000 dilution), or anti- -actin Ab (1/2000 dilution). Positive controls (PC) for CD36 and NEP were proteins (50 g) from IL-4-treated rat primary macrophages, and the positive control for IDE was protein (30 g) from rat brain extracts. Vehicle values were set at 1. Values are means ± SD (n = 3). *B*, Rat type 2 MG were treated with various concentrations of IL-4 for 96 h. Cell proteins (50 g) were subjected to immunoblot analysis for CD36, SRA, NEP, IDE, and -actin. *C*, Rat type 2 MG were treated with IL-4 (5 ng/ml) for 0–10 days. Cell proteins (50 g) were subjected to immunoblot analysis for CD36, SRA, NEP, IDE, and -actin. All results were quantified by means of densitometric scanning, with vehicle values set at 1. Values are means ± SD (n = 3).

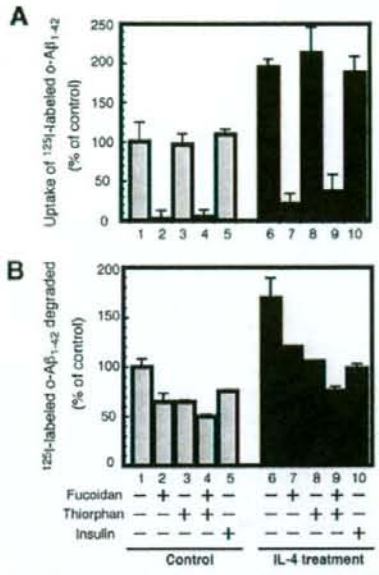

FIGURE 9. Effects of various ligands on cellular uptake (*A*) and degradation (*B*) of ^{125}I-labeled o-A $_{1-42}$ in type 2 MG before and after IL-4 treatment. *A*, Cells were cultured for 96 h in MEM/10% FCS in the presence or absence of IL-4 (5 ng/ml) and were then incubated for 3 h with ^{125}I-labeled o-A $_{1-42}$ (1 g/ml) in the presence or absence of fucoidan (100 g/ml), thiorphan (30 M), or insulin (100 g/ml). Cells were treated with proteinase K (100 g/ml) at 4°C for 15 min as described in *Materials and Methods*. The amounts of uptake of ^{125}I-labeled o-A $_{1-42}$ were determined as described in *Materials and Methods*. Values are means ± SD (n = 3). *B*, Cells were cultured as described in *A* and were then incubated for 6 h with ^{125}I-labeled o-A $_{1-42}$ (1 g/ml) in the presence or absence of fucoidan (100 g/ml), thiorphan (30 M), or insulin (100 g/ml). The amounts of cell-associated or degradation products of ^{125}I-labeled o-A were determined as described in *Materials and Methods*. Values are means ± SD (n = 3).

numbers of articles have claimed a neuroprotective role of MG in AD: for example, microglial activation by A vaccination (14–16) or recruitment of bone marrow-derived MG (17, 18) contributed to A clearance and concomitant neuroprotection. Among possible beneficial roles of MG in AD, we demonstrated here a novel A clearance mechanism that was induced selectively in type 2 MG by IL-4 treatment.

Relevance of IL-4-induced clearance of o-A $_{1-42}$ to expression of CD36, NEP, and IDE

The clearance selectivity for o-A $_{1-42}$ is of great importance for prevention or treatment of AD, because this molecular species is believed to be more neurotoxic (1–3) than fibrillar A $_{1-42}$. We demonstrated that CD36, NEP, and IDE were responsible, in their own ways, for clearance activity induced by IL-4. Two lines of evidence proved that CD36 played a major role: First, CD36-CHO cells, a CD36-overexpressing cell line, exhibited significant endocytic degradative activity that was suppressed to the control level by addition of anti-CD36 Ab. Second, MG from SHR rats in which CD36 was dysfunctional did not show this clearance. We were unable to demonstrate directly that o-A $_{1-42}$ was bound to CD36, because it was difficult to remove the considerable amount of nonspecific binding of ^{125}I-labeled o-A $_{1-42}$ to CD36-CHO cells (data not shown). Instead, however, intracellular degradation was effectively suppressed by addition of anti-CD36 Ab (Fig. 6*B*), which suggests that the Ab inhibited o-A $_{1-42}$ binding to CD36 and the subsequent uptake and degradation process. In addition, MG from CD36-dysfunctional SHR rats showed no apparent uptake and degradation of o-A $_{1-42}$ even after IL-4 treatment (Fig. 7, *B* and *C*), which strongly suggests that o-A $_{1-42}$ clearance was mediated by CD36. Coraci et al. (37) reported that CD36 was expressed by MG in AD brains and could mediate production of reactive oxygen species in response to A fibrils, but this role of CD36 apparently differs from our A clearance function.

Several other scavenger receptors have been proposed to contribute to A clearance by MG. SRA is one candidate (8), but the report that SRA knockout mice suppressed uptake of A $_{1-40}$ by only 40% (10) suggested the participation of other molecules in A clearance. Our finding of down-regulated SRA expression in IL-4-treated MG leads us to propose that SRA may not play a major role in IL-4-induced clearance of o-A $_{1-42}$ as described here. This proposal is more likely in view of the effect of IL-4 treatment, which induced CD36 expression and led to higher clearance compared with no treatment. However, considerable SRA expression was retained, and we therefore do not exclude the possibility that CD36 may have an important function and cooperates

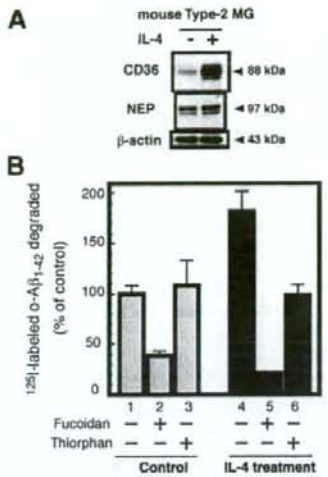

FIGURE 10. Clearance of ^{125}I-labeled o-A $_{1-42}$ by mouse primary type 2 MG. *A*, Mouse primary type 2 MG were isolated as described in *Materials and Methods*. Cells were cultured for 96 h in MEM/10% FCS in the presence or absence of IL-4 (5 ng/ml). Cell proteins (30 g) were subjected to immunoblot analysis with Abs against CD36, NEP, or -actin. *B*, Cells were cultured as described in *A* and were then incubated for 6 h with ^{125}I-labeled o-A $_{1-42}$ (1 g/ml) in the presence or absence of fucoidan (100 g/ml) or thiorphan (30 M). The amounts of degradation products of ^{125}I-labeled o-A were determined as described in *Materials and Methods*. Values are means ± SD (n = 3).

with SRA during IL-4 stimulation. SR-BI is not a candidate, because IL-4 treatment did not induce its expression. RAGE has been reported to mediate A transport across the BBB and accumulation of A in the brain (38). RAGE may have functions different from o-A $_{1-42}$ clearance, given that its expression was down-regulated by IL-4 in this study. Transcytosis of A $_{1-40}$ across brain endothelium of the BBB, mediated by low-density lipoprotein receptor-related protein 1, was also thought to be an A $_{1-40}$ clearance route (39). M-CSF receptor, which is up-regulated by MG in and around plaques in AD and the PDAPP mouse, has also reportedly induced accelerated phagocytosis of nonaggregated (40) and aggregated A $_{1-40}$ (19) when it is overexpressed. It is not clear, however, whether macrophage scavenger receptors engage in uptake and degradation of A $_{1-42}$ as they do for A $_{1-40}$.

As evidence of participation of the A -degrading enzymes NEP and IDE (41, 42) in extracellular A clearance, we demonstrated a 4- to 10-fold increase, dose and time dependent, in their expression in IL-4-treated type 2 MG. Their expression (on the cell surface) contributed to degradation of A , because use of their inhibitors, thiorphan and insulin, significantly inhibited degradation after IL-4 treatment (Fig. 9*B*). Insulin inhibited only the degradation process, suggesting cell surface receptors on which insulin may bind are not involved in the A clearance. Insulin is considered to be an inhibitor for IDE but not specific for IDE, and other proteases that could be inhibited by insulin may not exclude the role for degrading A . For human neprilysin, it is reported to degrade A peptide not only in the monomeric form but also in the pathological oligomeric form (43). However, our data on degradation by IDE seem in conflict with data from Selkoe's group (44) that showed that IDE can degrade monomer but not oligomer. Although our data on IL-4 treatment suggest it may lead to uptake of more oligomers, we assume that our A preparation contains some monomer (Fig. 1). Together, these results suggest that IL-4 may be modulating extracellular degradation of monomer here, which was not exactly measured.

Landreth and colleague (27) previously showed that, in both BV-2 cells and mouse primary MG, IL-4, and other anti-inflammatory cytokines neither stimulated nor inhibited phagocytosis elicited by any phagocytic ligands tested including fibrillar A (27). They suggested that these anti-inflammatory cytokines may oppose the harmful effects of IL-1 , which stops phagocytic microspheres from forming inside cells and IFN- from blocking phagocytosis. However, our data clearly show that IL-4 treatment enhanced degradation of ^{125}I-labeled o-A $_{1-42}$, by CD36-mediated intracellular phagocytosis and extracellular proteolysis involving A -degrading enzymes such as NEP, even in mouse primary type 2 MG (see Fig. 10). This discrepancy may have two explanations. One is the difference in molecular species of A used: Landreth and colleague (27) used primarily the fibrillar form, whereas we used mainly oligomers (monomer to pentamer), not the fibrillar form (see Fig. 1). The second is the difference between the MG preparations: Landreth and colleague (27) prepared samples by the simple shaking-off method, which is thought to provide mainly type 1 MG, whereas we prepared samples by using a modified mild trypsinization method, thereby producing predominantly type 2 MG (see Fig. 3*A*).

Selective A clearance by type 2 MG

We demonstrated that two microglial preparations, type 1 and type 2, had different responses to IL-4 stimulation. Heterogeneous microglial populations are known. For example, Kanzawa et al. (28) reported no and lower expression of CD40 and CD86, respectively, in cell lines that were established as type 2 MG, which was in sharp contrast to type 1 MG. Although MG have been long believed to have functions in neuroinflammatory reactions as macrophage-like cells in the brain or in neuroimmunomodulation as APCs, neuroprotective roles of MG were claimed relatively recently (11, 12). However, it is still unclear whether all MG or only a certain subtype (or subtypes) is responsible for such neuroprotective activity. A similar situation pertains for the microglial response to A peptide: it was not clear whether one MG subtype had an inflammatory reaction to A , whereas another subtype exhibited an anti-inflammatory response to A or clearance of the peptide. In the present study, we demonstrated that type 2 MG induced CD36 more selectively than did type 1 MG after IL-4 stimulation and that type 2 MG showed clearance for o-A $_{1-42}$. With regard to protection from neurotoxic o-A , type 2 MG seem to play a more protective role than do type 1 MG. This finding may be compared with the result that type 2 Ra2 cells but not type 1 MG had neuroprotective effects against oxidative stress in a co-culture system with neurons (45) We also found that type 2 MG expressed IL-4R at a much higher level than type 1 MG, with or without IL-4 (Fig. 5*C*). This result seems to be consistent with the observation that type 1 MG were less responsive to IL-4.

Type 2 MG are distinct from type 1 MG in terms of at least two cell surface molecules expressed: type 2 MG express no CD40 or less CD86, both of which are expressed by APCs to function in association and communication with CD4$^+$ Th cells. We recently developed a mAb that selectively recognizes type 1 MG and the Ag molecule is distinct from CD40 and CD86 (K. Kawahara, H. Hirata, K. Ohbuchi, K. Nishi, A. Kuniyasu, M. Sawada, and H. Nakayama, manuscript in preparation). It is not clear as yet whether or how these characteristics relate to the different induction ability of CD36. Further differentiation of type 1 and type 2 MG would be intriguing and is necessary.

Pathophysiological significance of IL-4-induced A clearance and source of IL-4 in the CNS

MG express a receptor for IL-4. IL-4 was shown in vitro to suppress microglial production of IFN- -induced MHC class II (46), TNF- , NO (47), and IL-6 (23), but relatively little is known about its role in microglial function in the CNS, probably because generation of IL-4 in the CNS has not been proved. mRNA for IL-4 was reportedly expressed in human brain (23), and expression levels of mRNA and proteins were markedly decreased in Tg2576 mice, which served as an AD model (48). Decreased hippocampal IL-4 concentration and IL-4-stimulated signaling were also found in aged rats compared with young rats (49). Although the amount of IL-4 found (20–40 pg/mg homogenate) in the hippocampal tissue was smaller than the amount of IL-1 (400–800 pg/mg homogenate), it is noteworthy that the concentration was comparable to that (5 ng/ml) applied in our study.

Another feasible source of IL-4 in AD brains is Th2 cells that infiltrate the brain. Activated lymphocytes have been demonstrated to enter the CNS in the absence of overt inflammatory disease (50). Other reports further suggest that T cells are activated in AD patients and that these cells exist both in the periphery and as infiltrates in the brain (Refs. 51–53; for review, see Ref. 54) via a rather leaky BBB (55, 56). Their numbers, however, can be lower than those found in the neurodegenerative disorders multiple sclerosis and experimental autoimmune encephalitis, in which the CNS is severely injured and the BBB is disrupted.

With respect to cross-talk between MG and T cells, Monsonego et al. (57) showed in cultured cells that activated MG served as A APCs and induced an adaptive immune response. They also demonstrated that A -reactive Th1 cells underwent apoptosis after stimulation, accompanied by increased levels of IFN- , NO, and caspase 3. In contrast, MG-mediated proliferation of A -reactive Th2 cells led to expression of the Th2 cytokines IL-4 and IL-10, which counteracted the toxic levels of NO induced by A . As an aid to understanding the in vivo T cell response in the CNS of patients with AD, a typical example of the response is induction of meningoencephalitis after immunization of AD patients with A $_{1-42}$ (58).

Akiyama and McGeer (59) demonstrated involvement of MG in immunotherapeutic clearance of A plaques. This report suggested that cross-talk between MG and T cells does exist and that therefore, in addition to the anti-inflammatory action of IL-4, IL-4-induced clearance by MG can be expected, if this IL-4 is derived from Th2 cells that infiltrate the AD brain. As a novel therapeutic method in AD, therefore, the use of agents to stimulate IL-4 production by either neuronal/glial cells or Th2 cells in the CNS, to augment MG-associated clearance activity, may be useful. However, IL-4 appears to contribute to pathogenesis of allergies and brain tumors, such as asthma (60) and glioma (61), respectively, and these effects should be kept in mind during development of IL-4 agonists for use as therapeutic agents in AD.

In conclusion, IL-4-stimulated microglial clearance of A , which is mediated by induced CD36, NEP, and IDE, has potential utility for development of therapeutic strategies for AD, as evidenced by the following points: 1) induction of A clearance by the anti-inflammatory cytokine IL-4, 2) simultaneous induction of three molecules (CD36, NEP, and IDE) that participate in clearance of A $_{1-42}$, and 3) preferential clearance of the highly neurotoxic o-A $_{1-42}$, which may prevent development and/or progression of AD. Among the different microglial phenotypes, type 2 MG was demonstrated to be sensitive to IL-4 and to have beneficial roles in A clearance. In addition to the innovative method of mucosal A vaccination (62), the technique of using agents to stimulate IL-4 production in the brain parenchyma or around the BBB, as based on the novel clearance mechanism of A $_{1-42}$ presented here, may spur development of new anti-inflammatory therapeutic strategies for AD.

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Disclosures

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