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RESEARCH PAPER

Increase in hemokinin-1 mRNA in the spinal cord during the early phase of a neuropathic pain state

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Background and purpose: Substance P (SP), a representative member of the tachykinin family, is involved in nociception under physiological and pathological conditions. Recently, hemokinin-1 (HK-1) was identified as a new member of this family. Although HK-1 acts on NK_1 tachykinin receptors that are thought to be innate for SP, the roles of HK-1 in neuropathic pain are still unknown.

Experimental approach: Using rats that had been subjected to chronic constrictive injury (CCI) of the sciatic nerve as a neuropathic pain model, we examined the changes in expression of SP- and HK-1-encoding genes (TAC1 and TAC4, respectively) in the L4/L5 spinal cord and L4/L5 dorsal root ganglia (DRGs) in association with changes in pain-related behaviours in this neuropathic pain state.

Key results: The TAC4 mRNA level was increased on the ipsilateral side of the dorsal spinal cord, but not in DRGs, at day 3 after CCI. In contrast, the TAC1 mRNA level was significantly increased in the DRGs at day 3 after CCI without any changes in the dorsal spinal cord. Analysis of a cultured microglial cell line revealed the presence of TAC4 mRNA in microglial cells. Minocycline, an inhibitor of microglial activation, blocked the increased expression of TAC4 mRNA after CCI and inhibited the associated pain-related behaviours and microglial activation in the spinal cord.

Conclusions and implications: The present results suggest that HK-1 expression is increased at least partly in activated microglial cells after nerve injury and is clearly involved in the early phase of neuropathic pain.

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Keywords: chronic constrictive injury; dorsal root ganglion; hemokinin-1; neuropathic pain; microglial cell; spinal cord; substance P; TAC1; TAC4; tachykinin

Abbreviations: CCI, chronic constrictive injury; DRG, dorsal root ganglion; HK-1, hemokinin-1; PNS, peripheral nervous system; SP, substance P

Introduction

Mammalian tachykinins comprise a family of peptides with a common carboxyl terminal amide motif (Patacchini *et al.*, 2004). Substance P (SP), a representative member of this family, is mainly distributed in the peripheral nervous system (PNS) and CNS and serves as a neurotransmitter in higher brain functions such as emotion and pain perception (Otsuka and Yoshioka, 1993; Severini *et al.*, 2002). SP is expressed in nociceptive dorsal root ganglion (DRG) neurons with small cell bodies, and is released upon stimulation. The released SP activates its innate receptor NK_1 on second-order neurons in the spinal cord and transmits intense noxious signals to the higher CNS (Hill, 2000; Mantyh and Hunt, 2004). Studies using knockout mice and selective NK_1 receptor antagonists have revealed that the SP-NK_1 system contributes to a variety of pathological pain states, including inflammatory and neuropathic pain (Hill, 2000; Mantyh and Hunt, 2004). In neuropathic pain, which is characterized by severe chronic pain caused by damage to the PNS or CNS (Scholz and Woolf, 2007), SP and NK_1 play a crucial role in spinal neuron sensitization through modulation of NMDA receptor gating (Woolf and Salter, 2000).

Recently, hemokinin-1 (HK-1) has been identified as a novel tachykinin peptide involved in the development of the B cell lineage (Zhang *et al.*, 2000). HK-1 binds to NK_1 receptors with high affinity, similar to SP (Kurtz *et al.*, 2002; Page, 2004). The TAC1 gene encoding SP and neurokinin A is mainly expressed in the nervous systems, whereas the TAC4 gene-encoding HK-1 is expressed in various types of tissues

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and cells, including immune cells (Patacchini *et al.*, 2004). Although HK-1 and SP have been shown to cause similar, but distinct, pain-related behaviours (Endo *et al.*, 2006), no studies have examined how HK-1 behaves during inflammatory or neuropathic pain. Thus, the roles of HK-1 in physiological or pathological pain states, such as neuropathic pain, are still unknown.

Microglial cells are considered to be CNS-resident macrophages and are present in all regions of the CNS where they constantly screen its environment (Hanisch and Kettenmann, 2007). Upon peripheral nerve injury, microglial cells in the spinal cord transform from their resting phenotype to their activated phenotype (Tsuda *et al.*, 2005) and secrete a variety of molecules, thereby contributing to the development of neuropathic pain (Tsuda *et al.*, 2005; Hanisch and Kettenmann, 2007). As HK-1 is expressed in microglial cells (Nelson *et al.*, 2004), it may play a role in neuropathic pain through activated microglial cells.

In the present study, we examined TAC4 mRNA expression in a rat model of neuropathic pain and compared it with TAC1 mRNA expression so as to clarify the specific contribution of HK-1 to neuropathic pain.

Materials and methods

Animals

All experimental procedures were approved by the Nippon Medical School Animal Care and Use Committee (approval number H19-053) and carried out in accordance with the guidelines of the International Association for the Study of Pain (Covino *et al.*, 1980). Six-week-old male Sprague–Dawley rats, weighing 150–170 g at the time of surgery, were used for all experiments. The rats were housed singly in an animal house for at least 5 days before the experiments, and water and food were available *ad libitum*.

Chronic constrictive injury (CCI) was basically produced as described in a previous report (Bennett and Xie, 1988), except that 4–0 silk thread was used instead of chromic gut. Briefly, rats were deeply anaesthetized with sodium pentobarbital (50 mg kg^{-1}, i.p.). The left (ipsilateral) sciatic nerve was exposed at the mid-thigh level, and four 4–0 silk threads were loosely ligated around the nerve at intervals of approximately 1 mm. The incision was closed with a 4–0 silk suture. The right (contralateral) sciatic nerve was left intact. In sham-operated rats, the operation was performed in the same manner except that sciatic nerve ligation was not carried out. Naive control rats were housed without an operation until tissue sampling. After 1, 3 and 7 days, the animals were deeply anaesthetized and the portions of the ipsilateral and contralateral dorsal quadrants of the L4/L5 spinal cord and the L4/L5 DRGs were immediately dissected. Each sample was stored at −80 °C until use for RNA extraction.

Minocycline administration

Minocycline hydrochloride, an inhibitor of microglial activation (Yrjänheikki *et al.*, 1998), was dissolved in sterile saline and administered to CCI rats at a dose of 90 mg kg^{-1} (i.p.) immediately after the CCI operation and then at 45 mg kg^{-1} every 12 h for 3 consecutive days according to a previous study (Yune *et al.*, 2007). Saline alone was administered to CCI rats as a control. The rats were killed for mRNA quantification and immunohistochemistry immediately after the behavioural tests on day 3.

Behavioural tests

Mechanical allodynia and thermal hyperalgesia were examined on day 0 before the surgery and at days 1, 3 and 7 after CCI as previously described (Nagano *et al.*, 2003). Paw withdrawal in response to mechanical stimuli was measured using a set of von Frey filaments with bending forces ranging from 1.0 to 52.8 g. Each rat was placed on a metallic mesh floor covered with a plastic box and a von Frey monofilament was applied to the plantar surface of the contralateral or ipsilateral hind paw from underneath the mesh floor. Each paw was stimulated with each filament five times in an individual trial. The weakest force (g) inducing withdrawal of the stimulated paw at least three times in each trial was referred to as the paw withdrawal threshold. The Plantar Test (Ugo Basile, Varese, Italy) was used to examine thermal hyperalgesia. Each rat was placed on a glass plate with a radiant heat generator underneath. After an acclimatization period, radiation heat was independently applied to the pad of the contralateral or ipsilateral hind paw. The latency of paw withdrawal from the heat stimuli was measured three times at 5-min intervals, and the average value was used as the latency of the response.

Real-time PCR

Quantitative analyses of rat TAC1 and TAC4 mRNA levels were performed as previously described (Nagano *et al.*, 2006). Total RNA was extracted from the L4/L5 dorsal spinal cord and L4/L5 DRGs using RNAiso and purified using an RNeasy Mini Kit according to the manufacturers' instructions. To avoid contamination by genomic DNA, the extracted RNA was treated with 10 μL of DNase (0.125 u μL^{-1}) at 37 °C for 30 min while on the column of the RNeasy Mini Kit. First-strand cDNAs were synthesized using 0.5 μg of total RNA in each tube with Oligo (dT) primer, dNTP mix and Superscript II Reverse Transcriptase. For PCR amplification, 1 μL of the first-strand cDNAs was added to 19 μL of a reaction mixture containing TaqMan Gene Expression Master Mix, 900 nM of each primer and 250 nM of TaqMan Probe and amplified using a GeneAmp 5700 sequence detection system. PCR primers specific for rat TAC1 and TAC4 were designed based on the cDNA sequences deposited in GenBank (NM_012666 and AY471575, respectively) using Primer Express version 2.0. The forward primers, reverse primers and probes were 5′-CGCAATGCAGAACTACGAAAGA-3′, 5′-CGCGGACACAGAT GGAGAT-3′ and 5′-CGTAAATAAACCCTGTAACGCACTATCT AT-3′ for TAC1 and 5-AGGGCTCGATAAAGGAGTTA-3′, 5-TT CAGCCCTCTACCCAGCAT-3′ and 5′-TAGGCAGCTTCCTCA GC-3′ for TAC4, respectively. For quantification, the cDNA sequences of TAC1 and TAC4 were inserted into the pGEM-T easy vector and pBluescript II SK (+) vector, respectively. The plasmids were then serially diluted to concentrations of 1.0×10^{1}–1.0×10^{6} mol per reaction tube for use as standards. All PCRs using the standards and samples were

performed in triplicate at 50 °C for 2 min and 95 °C for 10 min, followed by 50 cycles of 95 °C for 15 s and 60 °C for 1 min. The number of cDNA copies was calculated using a standard curve obtained from the set of control plasmids in each assay.

Immunohistochemistry

The animals were deeply anaesthetized with pentobarbital (i.p.) and perfused transcardially with phosphate-buffered saline (PBS; pH 7.4) followed by freshly prepared 4% paraformaldehyde in PBS. The L4 spinal cord was dissected out, post-fixed in the same fixative at 4 °C overnight and then cryoprotected in 20% sucrose in PBS at 4 °C overnight. Subsequently, the spinal cord was rapidly frozen in dry ice/acetone and cut into 10-μm transverse sections using a cryostat. The sections were preincubated in PBS containing 5% normal donkey serum and 0.2% Triton X-100 at room temperature for 30 min, and then incubated with a mouse monoclonal anti-CD11b antibody (OX-42, a microglial activation marker (Ling *et al.*, 1990); 1:1000 dilution) at 4 °C overnight. After being washed in PBS, the sections were incubated with an Alexa Fluor 488-conjugated anti-mouse secondary antibody (1:1000 dilution). Images were captured using a high-resolution digital camera equipped with a computer (Olympus, Tokyo, Japan).

Microglial cell culture

An immortalized rat microglial cell line, GMI-R1, was originally established from a neonatal Fisher 344 rat brain primary culture using a non-enzymatic and non-virus-transformed procedure (Salimi *et al.*, 2002). These microglial cells were maintained in Eagle's minimal essential medium (M4655), supplemented with 0.2% glucose, 5 $\mu g\,mL^{-1}$ insulin (I5500) and 10% foetal calf serum at 37 °C under a 5% CO_2/95% air atmosphere. Mouse recombinant granulocyte-macrophage colony-stimulating factor (1 $ng\,mL^{-1}$) was added as a supplement in the culture medium to maintain the cells in a proliferative state. The culture media were renewed two times per week. For mRNA expression analyses, the cells were harvested by scraping in a lysis buffer (RLT buffer in the RNeasy Mini Kit) and stored at −80 °C until use. First-strand cDNAs were prepared as described above for the real-time PCR procedure. PCR was performed at 50 °C for 2 min and 95 °C for 10 min, followed by 50 cycles of 95 °C for 15 s and 60 °C for 1 min using forward and reverse primer pairs for TAC4 (forward, 5-AGGGCTCGATAAAGGAGTTA-3′; reverse, 5-TTCAGCCCTCTACCCAGCAT-3′; 62-bp product) or NK_1 (NM_012667; forward, 5′-CACCCGATACCTCCAGAC ACA-3′; reverse, 5′-GGAGCCGTTGGAGGTGAGA-3′; 148-bp product) and 25 mu μL^{-1} Ampli*Taq* Gold DNA polymerase. The amplified PCR products were electrophoresed in a 2% ($w\,v^{-1}$) agarose gel and stained with ethidium bromide. An image of the agarose gel was captured using a BioDoc-It System UV Transilluminator.

Materials

Minocycline hydrochloride was obtained from Sigma-Aldrich (St Louis, MO, USA); von Frey filaments, Muromachi Kikai (Tokyo, Japan); RNAiso, Takara (Shiga, Japan) RNeasy Mini Kit (Qiagen, Valencia, CA, USA); Dnase, Promega (Madison, WI, USA). Oligo (dT) primer, dNTP mix and Superscript II Reverse Transcriptase were from Invitrogen (San Diego, CA, USA). The TaqMan Gene Expression Master Mix, TaqMan Probe, GeneAmp 5700 sequence detection system and Primer Express version 2.0 were from Applied Biosystems (Foster City, CA, USA); pGEM-T easy vector, Promega and pBluescript II SK (+) vector, Stratagene (La Jolla, CA, USA). The cryostat was from Leica (Tokyo, Japan); mouse monoclonal anti-CD11b antibody, Serotec (Cambridge, UK); Alexa Fluor 488-conjugated anti-mouse secondary antibody, Invitrogen. Eagle's minimal essential medium and insulin, Sigma-Aldrich; mouse recombinant granulocyte-macrophage colony stimulating factor, Genzyme (Cambridge, MA, USA); Ampli*Taq* Gold DNA polymerase, Applied Biosystems. The BioDoc-It System UV Transilluminator was obtained from BM Equipment (Tokyo, Japan).

Statistical analysis

Values are expressed as means ± s.e.mean. In behavioural tests, differences in the threshold or latency values before and after surgery were analysed by one-way ANOVA, followed by individual *post hoc* multiple comparisons (Dunnett's test) and the paired *t*-test was used to compare these values between the hind paws on the ipsilateral and contralateral sides of each rat. In real-time PCR experiments, differences in the amounts of the products before and after surgery were analysed by one-way ANOVA, followed by individual *post hoc* multiple comparisons (Dunnett's test). For comparison of the amounts of PCR products between the ipsilateral and contralateral sides of minocycline- and saline-treated rats, the paired *t*-test was used. Differences in the amounts of the PCR products of the ipsilateral side between the minocycline- and saline-treated rats were analysed by the unpaired *t*-test. Values of $P<0.05$ were considered to indicate statistical significance.

Nomenclature

The drug/molecular target nomenclature conforms with BJP's Guide to Receptors and Channels (Alexander *et al.*, 2008).

Results

Behavioural studies

Before the CCI operation on day 0, the paw withdrawal thresholds of 6-week-old rats in response to stimulation with von Frey filaments were 24.3 ± 0.5 g for the hind paws on both sides ($n=6$; Figure 1a). The latencies of withdrawal from heat stimulation in intact rats were 14.8 ± 2.0 and 14.6 ± 1.4 s for the ipsilateral and contralateral hind paws, respectively ($n=6$; Figure 1b). After the CCI operation, the thresholds of paw withdrawal in response to mechanical and heat stimuli began to decrease significantly on the ipsilateral side, but not the contralateral side, from day 3 and day 1, respectively (Figure 1). The increased sensitivities to both stimuli persisted until at least day 7 after CCI (Figure 1). On day 3 after the sham operation, the paw withdrawal

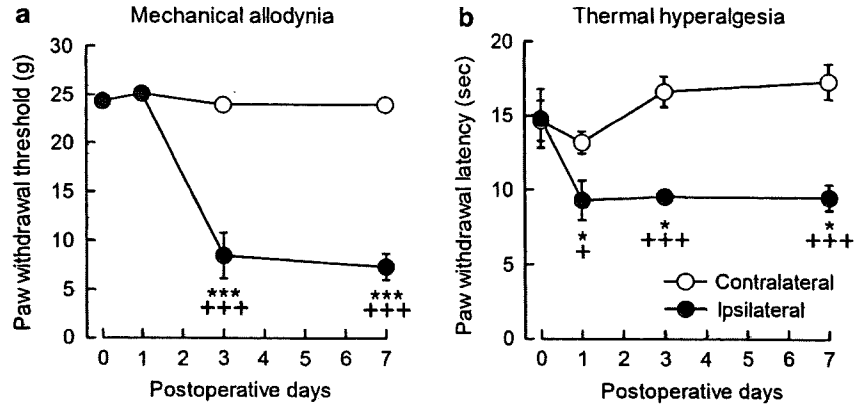

Figure 1 CCI-induced mechanical allodynia (**a**) and thermal hyperalgesia (**b**). The thresholds of paw withdrawal on the ipsilateral side and contralateral side in response to mechanical (**a**) or thermal (**b**) stimuli were measured in rats after CCI. $^{*}P<0.05$ and $^{***}P<0.001$ vs the values on day 0 by one-way ANOVA, followed by Dunnett's *post hoc* test; $^{+}P<0.05$ and $^{+++}P<0.001$ vs the values on the contralateral side at the corresponding days by the paired *t*-test; $n=6$.

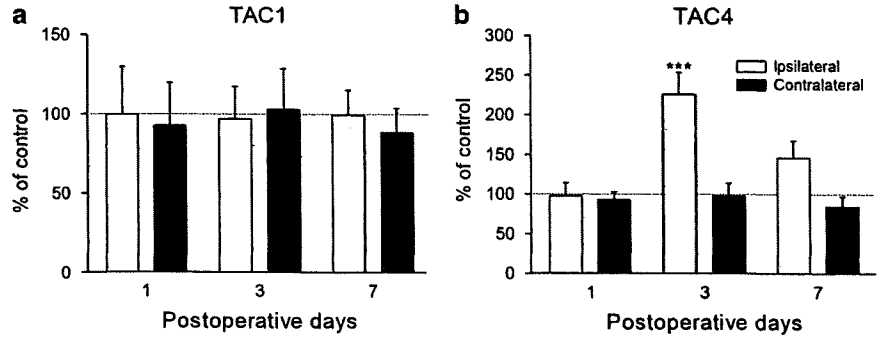

Figure 2 Real-time PCR analysis for TAC1 (**a**) and TAC4 (**b**) mRNA expression levels in the dorsal spinal cord. The levels of mRNA are expressed as percentages of the levels in intact rats. $^{***}P<0.001$ vs the values in intact rats by one-way ANOVA, followed by Dunnett's *post hoc* test; $n=6$.

thresholds were 24.0 ± 0.7 g for the hind paws on both sides ($n=4$), and the latencies of withdrawal from heat stimulation were 21.3 ± 2.0 and 22.3 ± 0.7 s for the ipsilateral and contralateral hind paws, respectively ($n=4$), indicating that the sham operation had no effects on the pain thresholds ($P=0.61$).

TAC1 and TAC4 mRNA expression levels in the dorsal spinal cord following CCI

We examined the expression levels of TAC1 and TAC4 mRNAs in the dorsal spinal cord following CCI. In the intact rats, the numbers of TAC1 and TAC4 mRNA molecules in the dorsal spinal cord were $5.60\times10^{6}\pm9.66\times10^{4}$ and $2.55\times10^{3}\pm4.76\times10^{2}$ mol μg^{-1} total RNA, respectively ($n=6$), indicating that TAC1 mRNA was more abundantly expressed than TAC4 mRNA. After CCI, a significant increase in TAC4 mRNA expression was observed in the dorsal spinal cord on the ipsilateral side at day 3 (225.96 ± 27.92% expressed as a percentage of the value for intact rats; $P<0.001$, vs the value for intact rats; $n=6$; Figure 2b) in association with the development of mechanical allodynia (Figure 1). The increase in TAC4 mRNA expression had almost returned to the baseline level at day 7 after CCI (146.93 ± 21.83%; $n=6$; Figure 2b). In contrast, no significant changes were observed for TAC1 mRNA expression on any of the days examined ($n=6$; Figure 2a). In the sham-operated group, the TAC1 and TAC4 mRNA expression levels on the ipsilateral side at day 3 were 110.31 ± 15.48 and 114.32 ± 17.81%, respectively (expressed as percentages of the values for the contralateral side; $n=4$). There were no significant differences in the TAC1 and TAC4 mRNA expression levels between the two sides of the dorsal spinal cord ($P=0.55$ and $P=0.48$, respectively).

TAC1 and TAC4 mRNA expression levels in DRGs

We examined the expression levels of TAC1 and TAC4 mRNAs in the DRGs. In the intact rats, the numbers of TAC1 and TAC4 mRNA molecules in the L4/L5 DRGs were $6.10\times10^{6}\pm3.97\times10^{5}$ and $2.99\times10^{4}\pm3.47\times10^{3}$ mol μg^{-1} total RNA, respectively ($n=9$). In contrast to the dorsal spinal cord, there were no significant changes in TAC4 mRNA expression during the first 7 days after CCI (Figure 3b). In contrast, the TAC1 mRNA expression level was significantly increased at day 3 after CCI (121.50 ± 3.56% expressed as a percentage of the value for intact rats; $P<0.05$, vs the value for intact rats; $n=9$; Figure 3a). The increase in TAC1 mRNA expression in the DRGs had declined to the baseline level at day 7 after CCI (Figure 3a). The sham operation did not affect the TAC1 and TAC4 mRNA

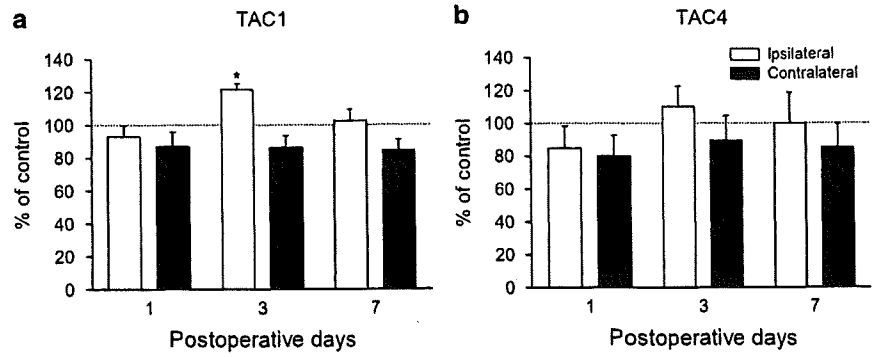

Figure 3 Real-time PCR analysis for TAC1 (a) and TAC4 (b) mRNA expression levels in the DRGs. The levels of mRNA are expressed as percentages of the levels in intact rats. *$P<0.05$ vs the values in intact rats by one-way ANOVA, followed by Dunnett's *post hoc* test; $n=9$.

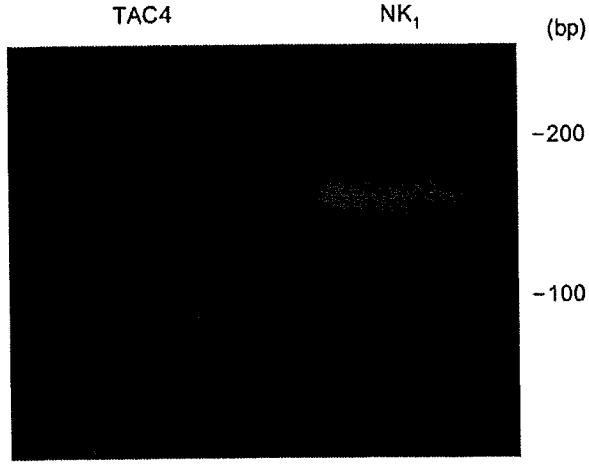

Figure 4 A representative gel of the PCR products for TAC4 and NK_1 mRNAs in microglial cells *in vitro*. The sizes of the PCR products for the TAC4 and NK_1 mRNAs are 62 and 148 bp, respectively.

expression levels on the ipsilateral side at day 3 (TAC1, 105.07 ± 18.96% expressed as a percentage of the value for the contralateral side, $P=0.81$; TAC4, 101.70 ± 6.81%, $P=0.82$; $n=4$).

TAC4 mRNA expression in microglial cells

Next, using a cultured microglial cell line, GMI-RI, we determined whether microglial cells express TAC4 mRNA. As shown in Figure 4, RT-PCR analysis revealed that TAC4 mRNA was expressed in this microglial cell line. In addition, mRNA expression of NK_1, a high affinity receptor for HK-1 and SP, was observed in the same cell line.

Effects of minocycline on the behaviour, microglial activation and spinal cord TAC4 mRNA expression of CCI rats

We further examined the effects of minocycline, an inhibitor of microglial activation, on rats treated with CCI. In the behavioural experiments, repeated administration of minocycline after the CCI operation inhibited the development of both mechanical allodynia and thermal hyperalgesia in CCI rats on day 3 ($n=5$; Figure 5a). Saline administration alone had no effect on neuropathic pain development ($n=5$; Figure 5a). Immunohistochemistry revealed that CCI increased the number and fluorescence intensity of CD11b-immunoreactive cells in the ipsilateral dorsal horn of the L4 spinal cord ($n=3$; Figure 5b) on day 3. These CD11b-positive cells also changed their morphology from ramified to amoeboid, indicating that microglial activation was induced by the CCI operation. Minocycline treatment blocked the activation of microglial cells in the ipsilateral dorsal horn of the L4 spinal cord on day 3 ($n=3$; Figure 5b). Intraperitoneal injection of saline or minocycline had no apparent effects on the microglial cells on the contralateral side of the dorsal horn, as evaluated by immunohistochemistry. In association with the suppression of both neuropathic pain development and microglial activation by minocycline, the above-described increase in TAC4 mRNA expression observed after the CCI operation was abolished in the ipsilateral L4 dorsal spinal cord of rats treated with CCI and minocycline on day 3 after CCI compared with that in rats treated with CCI and saline (CCI and saline-treated rats, 247.51 ± 31.91% expressed as a percentage of the value for the contralateral side; CCI and minocycline-treated rats, 172.0 ± 28.59%; $n=6$; $P<0.05$; Figure 5c). On the contralateral side of the dorsal spinal cord of CCI rats, minocycline treatment had no effect on the level of TAC4 mRNA expressed. In contrast to its effect on the expression of TAC4 mRNA in the spinal cord, minocycline did not suppress the increase in TAC1 mRNA expressed in the L4/L5 DRGs on day 3 after CCI compared with saline treatment (saline treatment, 114.66 ± 5.70% expressed as a percentage of the value for the contralateral side, $n=12$; minocycline treatment, 118.99 ± 6.34%, $n=12$; $P=0.62$; Figure 5c).

Discussion and conclusion

Differential expression of TAC1 and TAC4 mRNAs in the neuropathic pain state

In the present study, we have demonstrated differences in the mRNA expressions of TAC1 and TAC4 during the development of a neuropathic pain state. Specifically, TAC4 mRNA expression was increased in the dorsal spinal cord, but not the DRGs, on day 3 after CCI, whereas TAC1 mRNA expression was significantly increased in the DRGs, but not the dorsal spinal cord, on day 3 after CCI.

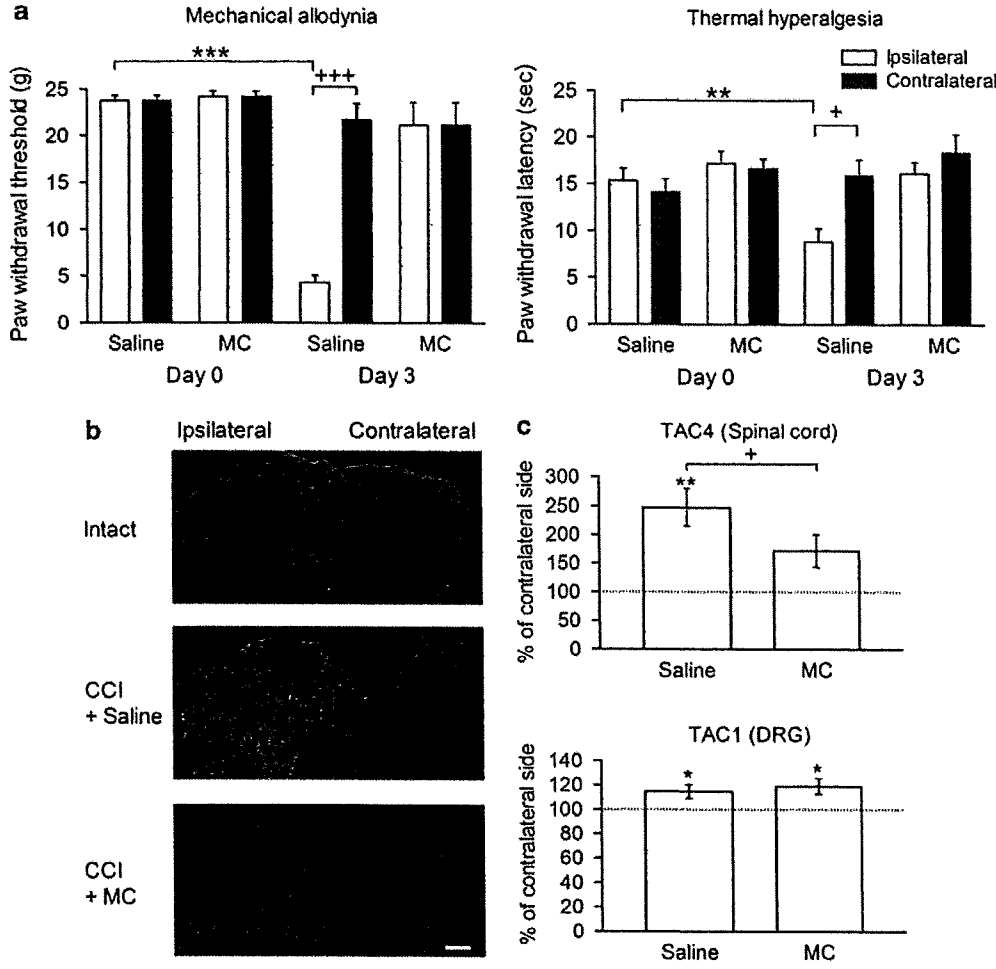

Figure 5 Effects of minocycline on rats at day 3 after the CCI operation. Minocycline hydrochloride, an inhibitor of microglial activation, was administered to CCI rats, i.p., every 12 h for 3 consecutive days. Saline alone was administered to CCI rats as a control. (**a**) The thresholds of paw withdrawal on the ipsilateral side and contralateral side in response to mechanical (left panel) or thermal (right panel) stimuli were measured in CCI rats treated with minocycline (MC) before (day 0) and 3 days after (day 3) the CCI operation. $^{**}P<0.01$ and $^{***}P<0.001$ vs the values on day 0 by one-way ANOVA, followed by Dunnett's *post hoc* test; $^{+}P<0.05$ and $^{+++}P<0.001$ vs the values on the contralateral side at the corresponding days by paired *t*-test; $n=5$. (**b**) Representative images of immunohistochemistry for activated microglial cells in the L4 spinal dorsal horn. L4 spinal cords were obtained from intact rats, as well as rats treated with CCI and saline (CCI + saline) and rats treated with CCI and minocycline (CCI + MC) at day 3 after CCI. The spinal cords were stained with an anti-CD11b antibody (OX-42), a microglial activation marker. Scale bar, 50 μm. The experiments for each treatment were repeated in three rats. (**c**) Real-time PCR analysis for TAC4 mRNA expression in the dorsal spinal cord (top) and TAC1 mRNA expression in the DRGs (bottom). Data are presented as percentages of the levels on the contralateral side. $^{*}P<0.05$ and $^{**}P<0.01$ vs the values of the contralateral side by the paired *t*-test; $n=6$ (spinal cord) and $n=12$ (DRG). $^{+}P<0.05$ vs the values in the saline-treated rats.

Consistent with our present observations, increased expression levels of SP peptide and its mRNA (TAC1) in the DRGs have been observed in the early phase after nerve injury (Marchand *et al.*, 1994). SP released from central axon terminals of DRG neurons in the spinal cord binds to NK_1 receptors expressed in nociceptive projection neurons, and sensitizes these neurons to cause hyperalgesia (Basbaum, 1999; Nichols *et al.*, 1999; Snijdelaar *et al.*, 2000). HK-1 can also bind to NK_1 receptors with a similar potency to SP (Kurtz *et al.*, 2002). Therefore, in addition to SP, HK-1 produced in the spinal cord may contribute to such sensitization of second-order nociceptive neurons. In fact, Endo *et al.* (2006) demonstrated that intrathecal administration of HK-1 to naive rats induced a pain-related behaviour (scratching), which was similar to the effect of SP administration. On the other hand, they also showed that intrathecal SP administration evoked another pain-related behaviour (thermal hyperalgesia), whereas HK-1 did not (Endo *et al.*, 2006). Although the mechanisms underlying this discrepancy in the effects of SP and HK-1 on nociception are still unclear, the existence of innate receptors for HK-1 other than NK_1 receptors has been suggested as a possible explanation (Zhang and Paige, 2003; Naono *et al.*, 2007). Collectively, these two tachykinin family members, HK-1 and SP, may provide different contributions to neuropathic pain. This possibility may be worthy of further investigation from the point of view of developing anti-nociceptive drugs, as NK_1 antagonists have so far failed to produce analgesia in clinical trials (Rupniak and Kramer, 1999; Hill, 2000).

In the present study, we were unable to confirm which cells expressed TAC4 mRNA in the spinal cord, as the level of TAC4 mRNA expression was lower than the detection limit

of the non-radioisotopic *in situ* hybridization method adopted in our preliminary experiments. It is also unclear whether the translated HK-1 peptide is indeed present and increased in the spinal cord in the neuropathic pain state, as no specific antibodies against HK-1 are available. Therefore, further studies are needed to confirm the role of HK-1 in neuropathic pain.

TAC4 mRNA expression in microglial cells

Hemokinin-1 was originally identified as a regulator of B lymphopoiesis and is thought to act in an autocrine and paracrine manner (Zhang *et al.*, 2000). SP expression is almost completely restricted to the nervous systems, whereas HK-1 is expressed not only in the nervous systems but also in many peripheral organs and immunocompetent cells, such as microglial cells, macrophages and dendritic cells (Kurtz *et al.*, 2002; Duffy *et al.*, 2003; Nelson *et al.*, 2004). In the present study, we demonstrated that microglial cells expressed TAC4 and NK_1 mRNAs *in vitro*. Consistent with these findings, the increase in TAC4 mRNA expression in the spinal cord after CCI was effectively blocked by i.p. administration of minocycline, which inhibits microglial activation both *in vivo* and *in vitro* (Tikka and Koistinaho, 2001; Tikka *et al.*, 2001; Wang *et al.*, 2005). This blocking effect of minocycline seemed to be specific for TAC4 mRNA expression in the spinal cord, as the increase in TAC1 mRNA expression in the L4/L5 DRGs on day 3 after CCI was not affected by the minocycline treatment. Taken together, these results suggest that the increased expression of TAC4 mRNA in neuropathic pain occurs at least partly in activated microglial cells. Recently, the dynamic and active roles of microglial cells have been studied (Hanisch and Kettenmann, 2007). Upon peripheral nerve injury, microglial cells in the spinal cord change from their resting to their reactive phenotype (Tsuda *et al.*, 2005) and secrete a variety of molecules, thereby contributing to neuropathic pain (Tsuda *et al.*, 2005; Hanisch and Kettenmann, 2007). Activation of spinal microglial cells has been shown to peak at around day 3 after peripheral nerve injury (Gehrmann and Banati, 1995) and to be crucial for the development of neuropathic pain (Jin *et al.*, 2003; Milligan *et al.*, 2003; Zhuang *et al.*, 2007). It is noteworthy that, in the present study, the increase in TAC4 mRNA expression in the dorsal spinal cord was observed on day 3 in association with the development of pain, and then declined by day 7 after nerve injury. Therefore, HK-1 may be secreted by activated microglial cells and play a role in the early phase of a neuropathic pain state.

Given that HK-1 is secreted by activated microglial cells, it could act on NK_1 receptor-bearing cells. As discussed above, NK_1 receptors are expressed on nociceptive spinothalamic neurons in spinal cord laminae I and V (Nakaya *et al.*, 1994), and the increased HK-1 may, therefore, sensitize these projection neurons in concert with SP, resulting in a modification of pain perception. Alternatively, microglial cells may be another target of HK-1. Microglial cells themselves express NK_1 receptors as shown in the present and a previous study (Pocock and Kettenmann, 2007). SP has been proposed to activate the transcriptional factor NF-κB (Rasley *et al.*, 2002) and NADPH oxidase (Block *et al.*, 2006) in microglial cells through the NK_1-mediated pathway. Therefore, HK-1 released from microglial cells appears to act in an autocrine and/or paracrine manner, as observed in B lymphopoiesis (Zhang *et al.*, 2000). Additional studies are required to investigate these possibilities further.

To conclude, our results suggest that HK-1 plays a role in neuropathic pain, which is different from that of the conventional tachykinin SP and occurs in a different place. Therefore, a comprehensive assessment of the contributions of the various tachykinins to neuropathic pain would be useful for the development of more effective tachykinin-related analgesics.

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Conflict of interest

The authors state no conflict of interest.

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

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

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A hallmark of immunopathology associated with Alzheimer's disease is the presence of activated microglia (MG) surrounding senile plaque deposition of β-amyloid (Aβ) peptides. Aβ peptides are believed to be potent activators of MG, which leads to Alzheimer's disease pathology, but the role of MG subtypes in Aβ 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 Aβ$_{1-42}$ (o-Aβ$_{1-42}$). IL-4 treatment induced significant expression of the scavenger receptor CD36 and the Aβ-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 Aβ clearance: Chinese hamster ovary cells overexpressing CD36 exhibited marked, dose-dependent degradation of ^{125}I-labeled o-Aβ$_{1-42}$ compared with controls, the degradation being blocked by anti-CD36 Ab. Also, we found IL-4-induced clearance of o-Aβ$_{1-42}$ 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 Aβ$_{1-42}$, because their inhibitors, thiorphan and insulin, respectively, significantly suppressed this activity. IL-4-stimulated uptake and degradation of o-Aβ$_{1-42}$ 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 Aβ-overproducing brain. Thus, selective o-Aβ$_{1-42}$ 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 β-amyloid (Aβ) peptides. Preventing or reversing Aβ deposition in AD is therefore considered to be a major therapeutic goal. Fibrillar Aβ is observed in senile plaques and Aβ$_{1-42}$ forms aggregates and Aβ fibrils more easily than does Aβ$_{1-40}$. In addition to forming such fibrils, oligomeric Aβ (o-Aβ) 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-Aβ is of prime importance for prevention or treatment of AD.

Activated microglia (MG) were demonstrated to surround senile plaque deposition of Aβ peptides. Aβ 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-α, IFN-γ, and IL-1β, 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 Aβ 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 Aβ clearance and that Aβ 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 Aβ$_{1-40}$. In addition to participation of MG in clearance of Aβ 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 Aβ from the brain. However, these same receptors on different cells, e.g., neurons, may increase intraneuronal uptake and toxicity of Aβ (20). Similarly, carrier proteins such as apolipoprotein E, apolipoprotein J, and α_2-macroglobulin (21) may deliver Aβ either to MG, where it may help Aβ clearance, or to neurons, where it may aid neurodegeneration. The effect of insulin-like growth factor I (22) on decreasing the Aβ burden is believed to be mediated by enhancement of Aβ 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; Aβ, β-amyloid; BBB, blood-brain barrier; CHO, Chinese hamster ovary; IDE, insulin-degrading enzyme; MG, microglia; NEP, neprilysin; o-Aβ$_{1-42}$, oligomeric Aβ$_{1-42}$; 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 $A\beta$. They suggested that these anti-inflammatory cytokines may oppose harmful effects of IL-1β, which stops phagocytic microspheres from forming inside cells and IFN-γ 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 $A\beta$ clearance and neuroprotective mechanisms.

We describe here a novel $A\beta$ clearance mechanism involving type 2 MG, not type 1 MG, in primary culture after IL-4 treatment. We found clearance of o-$A\beta_{1-42}$ with increased expression of scavenger receptor CD36 and the $A\beta$-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 $A\beta_{1-42}$ 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 $A\beta_{1-17}$ 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-4Rα 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 I (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 $A\beta_{1-42}$

Oligomeric $A\beta$ was prepared as previously reported (30), with a slight modification. The chloride form of $A\beta_{1-42}$ 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 μM, and the samples were rotated on a rotary shaker at 4°C for 24 h. o-$A\beta_{1-42}$ prepared as above (200 μg 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 $A\beta_{1-42}$ 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 μg/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 μg/ml, serotype 0127:B8; Sigma-Aldrich), rat IFN-γ (100 U/ml; PeproTech), mouse TNF-α (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-β1 (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 μg/ml pepstatin A, 1 μg/ml leupeptin, and 10 μg/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 μg) 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 $A\beta_{1-17}$ (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 β-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 $A\beta$ 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-$A\beta_{1-42}$ 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 $A\beta$ 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 $A\beta$ 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 μg/ml FA6-152 or control IgG for human CD36-CHO cells; see Fig. 6) or fucoidan (100 μg/ml), thiorphan (30 μM), or insulin (100 μg/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 ($n = 3$).

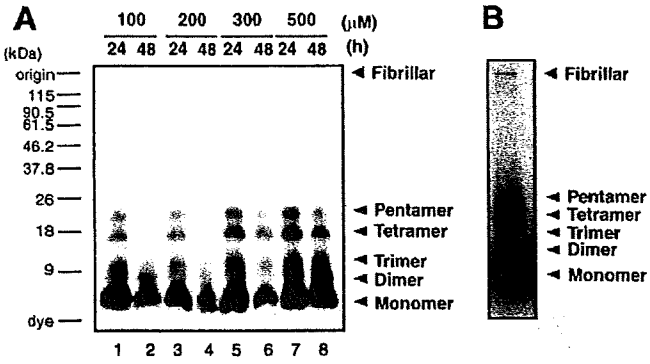

FIGURE 1. Preparation of the o-$A\beta_{1-42}$ and ^{125}I-labeled $A\beta_{1-42}$. *A*, Results of Western blot analysis. o-$A\beta_{1-42}$ was prepared via a previously reported method (30), with a slight modification, and as described in *Materials and Methods*. For aggregation protocols, the stored 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 μM, and samples were rotated at 4°C for either 24 or 48 h. The o-$A\beta_{1-42}$ preparations were separated by SDS-PAGE (15% gels) and were subjected to immunoblot analysis using anti-$A\beta_{1-17}$ Ab. Besides oligomeric forms, monomeric and fibrillar forms of $A\beta_{1-42}$ were detected. *B*, Electrophoretic and radioluminographic findings for ^{125}I-labeled $A\beta_{1-42}$. 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-$A\beta_{1-42}$ (see Figs. 4 and 7) or ^{125}I-labeled o-$A\beta_{1-42}$ (see Fig. 9) for 3 h at 37°C in the presence or absence of fucoidan (100 μg/ml). The cells were rinsed with ice-cold PBS, treated with proteinase K (100 μg/ml) or trypsin (2.5 mg/ml) at 4°C for 15 min, and then washed again with PBS, to remove all extracellular Aβ, as previously reported (35). For Figs. 4 and 7, proteins (10 μg) in the cell lysate were subjected to Western blot analysis using anti-$A\beta_{1-17}$ Ab or anti-β-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-$A\beta_{1-42}$ and ^{125}I-labeled $A\beta_{1-42}$

To determine the microglial clearance of Aβ, o-$A\beta_{1-42}$ 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 Aβ oligomers were formed as dimers to pentamers after 24 h of incubation at 4°C with the chloride form of $A\beta_{1-42}$ at initial concentrations of 100–500 μM. After 48 h of incubation, the proportion of oligomers and monomers decreased, but fibrillar formation increased. Among the conditions tested, incubation of Aβ at 500 μM for 24 h gave the highest yield (~30–40%) of Aβ 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-$A\beta_{1-42}$) had a similar electrophoretic pattern on SDS-PAGE (Fig. 1*B*). Starting with the trifluoroacetate form of $A\beta_{1-42}$ under the same conditions (data not shown) resulted in a less efficient oligomer yield.

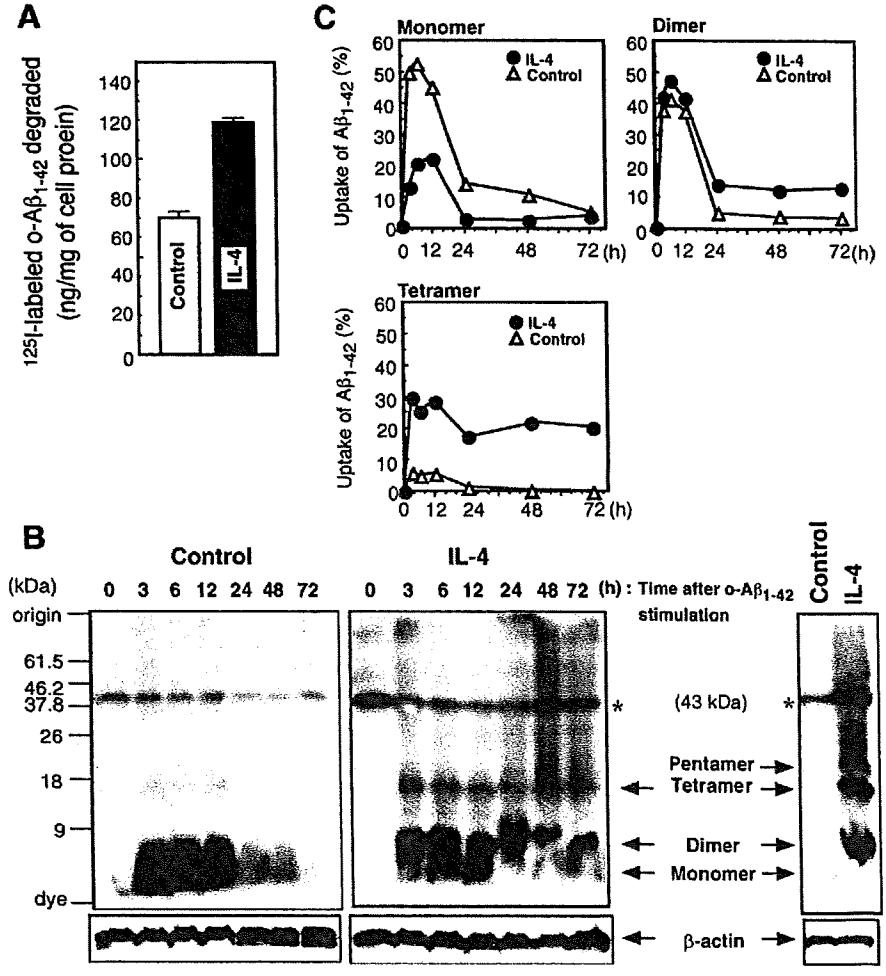

FIGURE 2. Uptake and clearance of o-$A\beta_{1-42}$ by rat primary MG. *A*, Rat primary MG were cultured for 96 h in MEM/10% FCS in the presence or absence of IL-4 (5 ng/ml) and were then incubated for 6 h with ^{125}I-labeled o-$A\beta_{1-42}$ (1 μg/ml). Amounts of degraded ^{125}I-labeled o-$A\beta_{1-42}$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

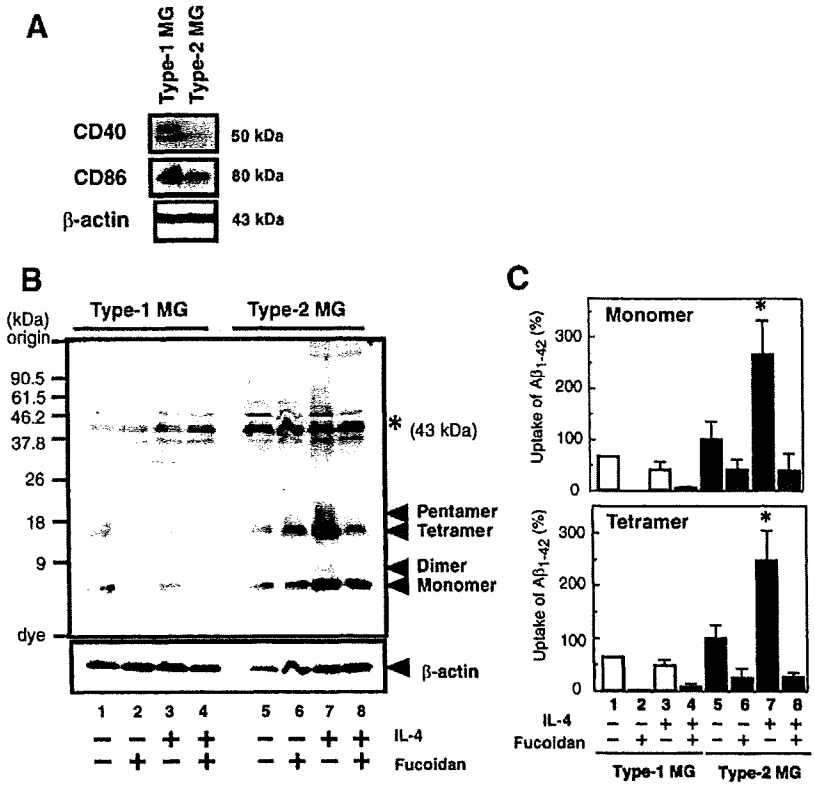

FIGURE 3. Comparison of uptake of o-Aβ_{1-42} by type 1 and type 2 MG. *A*, Rat primary type 1 and type 2 MG were isolated as described in *Materials and Methods*. Cell proteins (40 μg) were subjected to immunoblot analysis with Abs against CD40, CD86, or β-actin. *B*, Rat primary type 1 and type 2 MG 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-Aβ_{1-42} (2 μg/ml) in the presence or absence of fucoidan (100 μg/ml). Proteins (30 μg) in the cell lysate were subjected to Western blot analysis with anti-Aβ_{1-17} Ab or anti-β-actin Ab. The asterisk indicates a nonspecific band detected by the anti-Aβ Ab. *C*, Monomer and tetramer Aβ 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-Aβ_{1-42} by MG

Fig. 2*A* shows endocytic degradation of ^{125}I-labeled o-Aβ_{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-Aβ_{1-17} Ab (Fig. 2*B*). Control MG incorporated mainly the Aβ 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-Aβ_{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-Aβ_{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-Aβ_{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 Aβ_{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 Aβ_{1-42} by IL-4-treated type 2 MG

The apparent uptake shown in Fig. 2 may incorporate some nonspecific binding of Aβ_{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 Aβ 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 Aβ 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-Aβ_{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

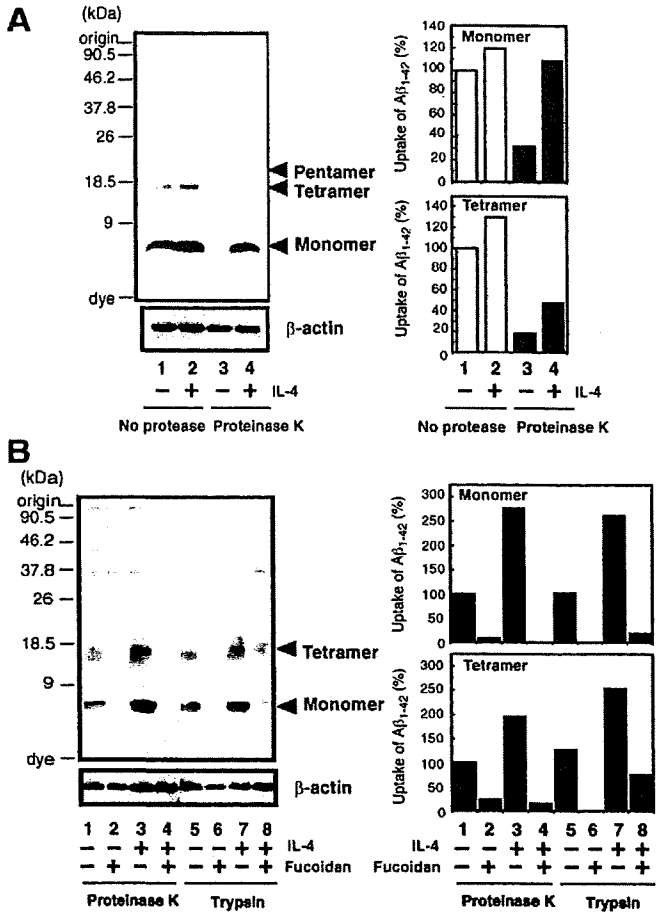

FIGURE 4. Refined uptake of o-Aβ_{1-42} by type 2 MG after protease treatment. *A*, Rat primary type 2 MG were cultured for 96 h in MEM/10% FCS in the presence or absence of IL-4 (5 ng/ml). The cells were incubated with o-Aβ_{1-42} (2 μg/ml) for 3 h at 37°C and then treated with proteinase K (100 μg/ml)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

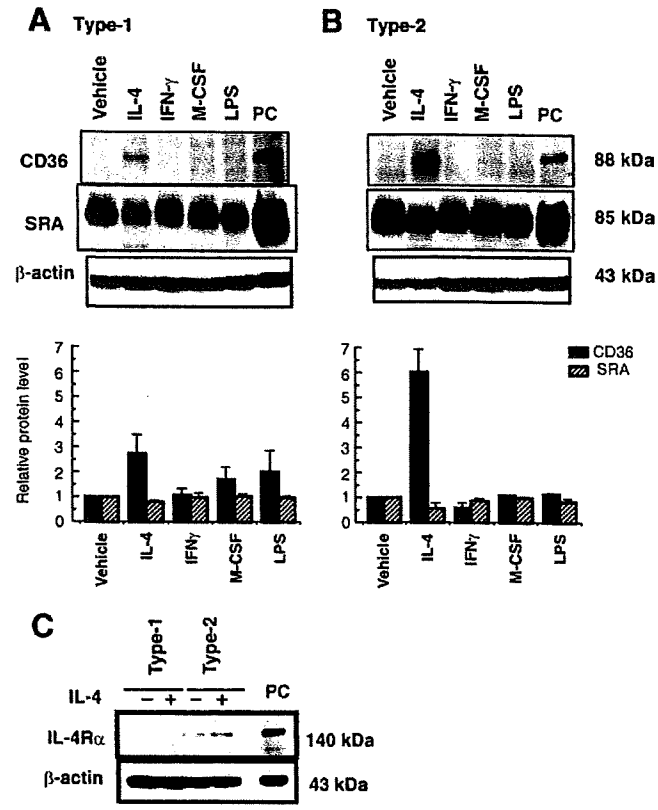

FIGURE 5. Change in expression of CD36 and SRA in type 1 and type 2 MG stimulated with various cytokines or stimulants. Type 1 (*A*) and type 2 (*B*) MG were incubated for 96 h with IL-4 (5 ng/ml), IFN-γ (100 U/ml), M-CSF (50 U/ml), or LPS (1 μg/ml). Cell proteins (50 μg) were subjected to immunoblot analysis with Abs against CD36, SRA, or β-actin. The positive controls (PC) for CD36 and SRA were proteins (50 μg) 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 μg) were subjected to immunoblot analysis with Abs against IL-4Rα or β-actin. Protein (30 μg) from IL-4-treated mouse microglial MG5 cells served as the positive control (PC).

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-γ, M-CSF, and LPS 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-4Rα 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-Aβ_{1-42}, we first evaluated CD36-CHO cells. The amount of ^{125}I-labeled o-Aβ_{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 μg of ^{125}I-labeled o-Aβ_{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-Aβ_{1-42} was mediated by CD36.

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

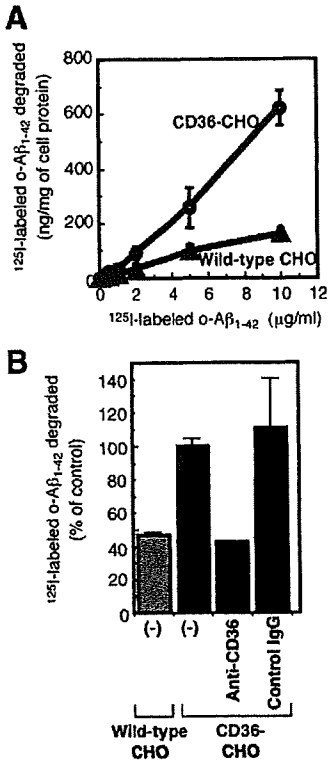

FIGURE 6. Clearance of ^{125}I-labeled o-$A\beta_{1-42}$ by human CD36-CHO cells and effect of anti-CD36 Ab. *A*, CD36-CHO (○) and wild-type CHO (△) cells were incubated for 6 h with ^{125}I-labeled o-$A\beta_{1-42}$ at 0–10 μg/ml. The amounts of ^{125}I-labeled o-$A\beta_{1-42}$ degradation products 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-$A\beta_{1-42}$ (1 μg/ml) in the presence or absence of anti-human CD36 Ab (FA6-152: 10 μg/ml) or control Ab (MOPC21: 10 μg/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-$A\beta_{1-42}$ (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-$A\beta_{1-42}$ 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-$A\beta_{1-42}$ 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-$A\beta_{1-42}$ 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-$A\beta_{1-42}$ 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-$A\beta_{1-42}$, 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-$A\beta_{1-42}$ by type 2 MG, we examined whether IL-4 affected expression and activity of the Aβ-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-α. NEP expression was also enhanced by IL-4 and IL-13 but was suppressed by TNF-α and TGF-β1. IL-4 and TGF-β1 showed high potency for induction of IDE expression, followed by IL-13; TNF-α 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 $A\beta_{1-42}$.

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-$A\beta_{1-42}$ in type 2 MG before and after IL-4 treatment

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

Inhibitors of Aβ-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-$A\beta_{1-42}$, 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-$A\beta_{1-42}$, 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*).

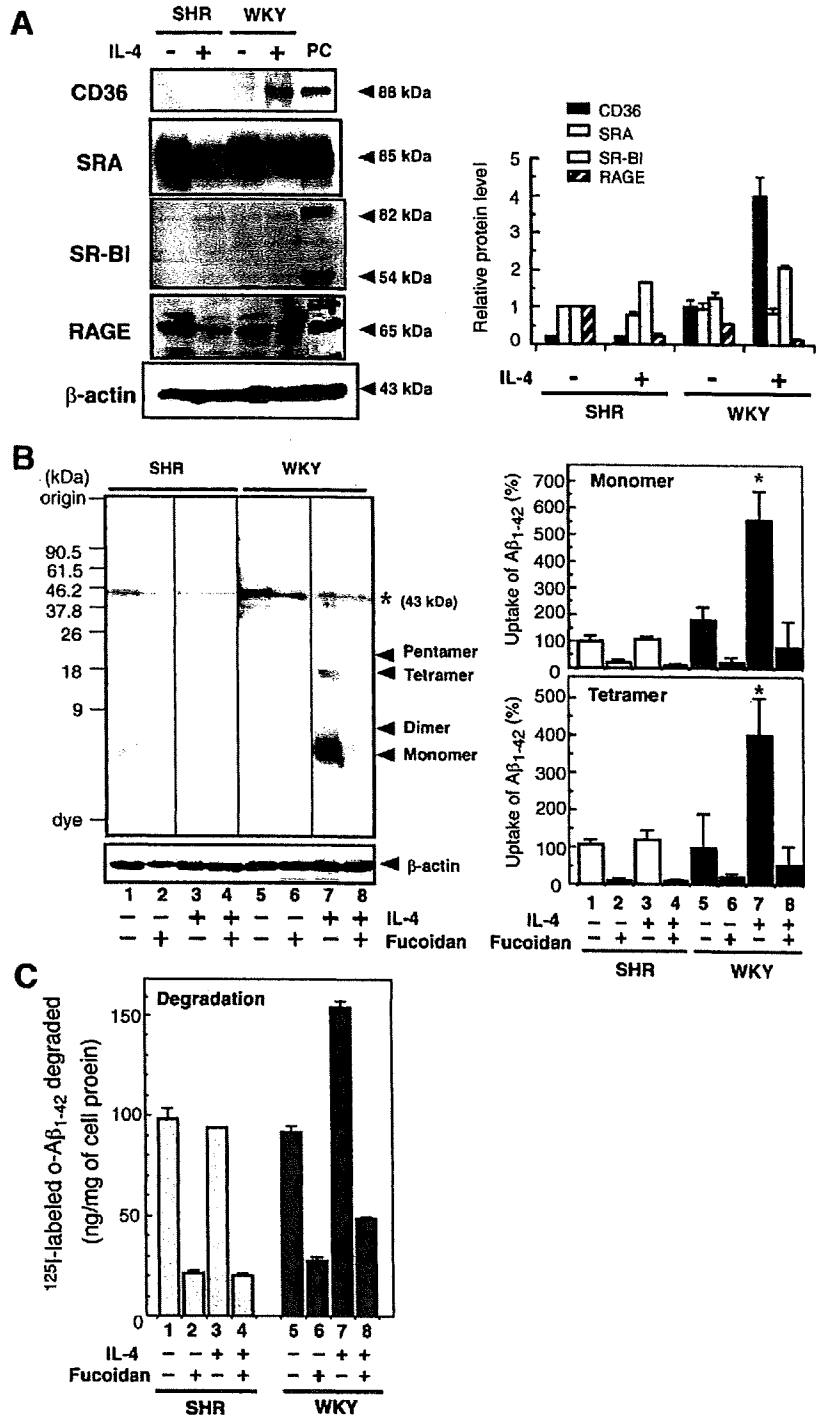

FIGURE 7. Comparison of o-Aβ_{1-42} endocytosis by type 2 MG derived from SHR/NCrj rats with cells derived from WKY/NCrj rats after IL-4 stimulation. *A*, Cells were cultured for 96 h in MEM/10% FCS in the presence or absence of IL-4 (5 ng/ml). Cell proteins (50 μg) were subjected to immunoblot analysis as described in *Materials and Methods*. Positive controls for CD36, SRA, SR-BI, and RAGE were proteins (50 μg) from IL-4-treated rat primary macrophages, proteins (50 μg) from rat primary macrophages, extracts (30 μg of protein) from rat liver, and extracts (30 μg 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-Aβ_{1-42} (2 μg/ml) for 3 h in the presence or absence of fucoidan (100 μg/ml). The cells were treated with proteinase K (100 μg/ml) at 4°C for 15 min as described in *Materials and Methods*. Cell proteins (10 μg) were subjected to immunoblot analysis with anti-Aβ_{1-17} Ab or anti-β-actin Ab. The asterisk indicates a nonspecific band detected by anti-Aβ 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-Aβ_{1-42} (1 μg/ml) for 6 h in the presence or absence of fucoidan (100 μg/ml). Amounts of degraded ^{125}I-labeled o-Aβ_{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-Aβ_{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 Aβ catabolism by NEP and IDE are important for IL-4-induced clearance of o-Aβ_{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-Aβ_{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-α (for reviews, see Refs. 4–6). Recently, increasing

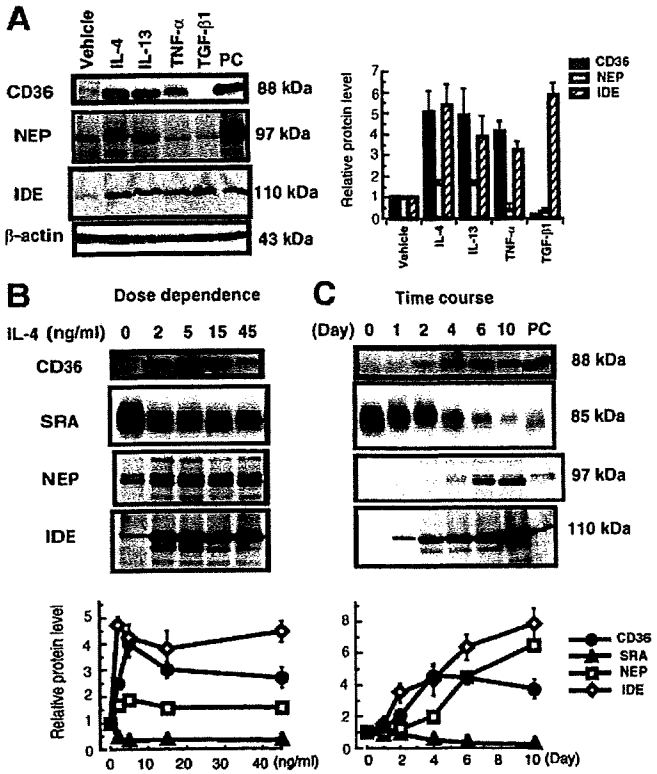

FIGURE 8. Effects of various cytokines on expression of NEP and IDE in rat type 2 MG. *A*, Rat type 2 MG were incubated for 96 h with IL-4 (5 ng/ml), IL-13 (5 ng/ml), TNF-α (150 U/ml), or TGF-β1 (2 ng/ml). Cell proteins (50 μg) were subjected to immunoblot analysis by using anti-CD36 Ab (1/30 dilution), anti-NEP Ab (1/100 dilution), anti-IDE Ab (1/1000 dilution), or anti-β-actin Ab (1/2000 dilution). Positive controls (PC) for CD36 and NEP were proteins (50 μg) from IL-4-treated rat primary macrophages, and the positive control for IDE was protein (30 μg) 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 μg) were subjected to immunoblot analysis for CD36, SRA, NEP, IDE, and β-actin. *C*, Rat type 2 MG were treated with IL-4 (5 ng/ml) for 0–10 days. Cell proteins (50 μg) were subjected to immunoblot analysis for CD36, SRA, NEP, IDE, and β-actin. All results were quantified by means of densitometric scanning, with vehicle values set at 1. Values are means ± SD (n = 3).

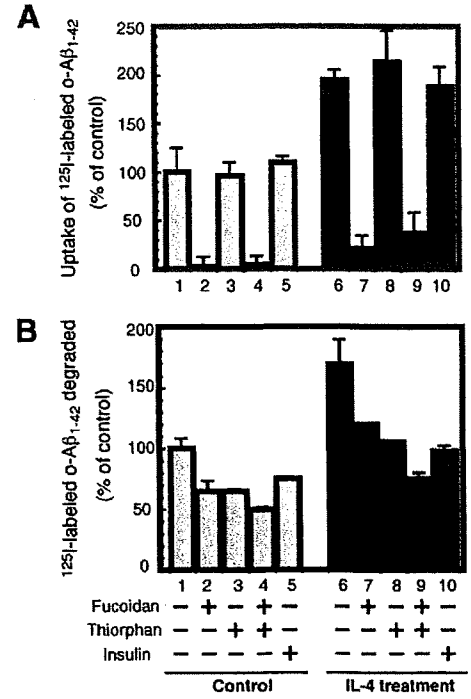

FIGURE 9. Effects of various ligands on cellular uptake (*A*) and degradation (*B*) of ^{125}I-labeled o-Aβ_{1-42} in type 2 MG before and after IL-4 treatment. *A*, Cells were cultured for 96 h in MEM/10% FCS in the presence or absence of IL-4 (5 ng/ml) and were then incubated for 3 h with ^{125}I-labeled o-Aβ_{1-42} (1 μg/ml) in the presence or absence of fucoidan (100 μg/ml), thiorphan (30 μM), or insulin (100 μg/ml). Cells were treated with proteinase K (100 μg/ml) at 4°C for 15 min as described in *Materials and Methods*. The amounts of uptake of ^{125}I-labeled o-Aβ_{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-Aβ_{1-42} (1 μg/ml) in the presence or absence of fucoidan (100 μg/ml), thiorphan (30 μM), or insulin (100 μg/ml). The amounts of cell-associated or degradation products of ^{125}I-labeled o-Aβ 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 Aβ vaccination (14–16) or recruitment of bone marrow-derived MG (17, 18) contributed to Aβ clearance and concomitant neuroprotection. Among possible beneficial roles of MG in AD, we demonstrated here a novel Aβ clearance mechanism that was induced selectively in type 2 MG by IL-4 treatment.

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

The clearance selectivity for o-Aβ_{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 Aβ_{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-Aβ_{1-42} was bound to CD36, because it was difficult to remove the considerable amount of nonspecific binding of ^{125}I-labeled o-Aβ_{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-Aβ_{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-Aβ_{1-42} even after IL-4 treatment (Fig. 7, *B* and *C*), which strongly suggests that o-Aβ_{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 Aβ fibrils, but this role of CD36 apparently differs from our Aβ clearance function.

Several other scavenger receptors have been proposed to contribute to Aβ clearance by MG. SRA is one candidate (8), but the report that SRA knockout mice suppressed uptake of Aβ_{1-40} by only 40% (10) suggested the participation of other molecules in Aβ 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-Aβ_{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

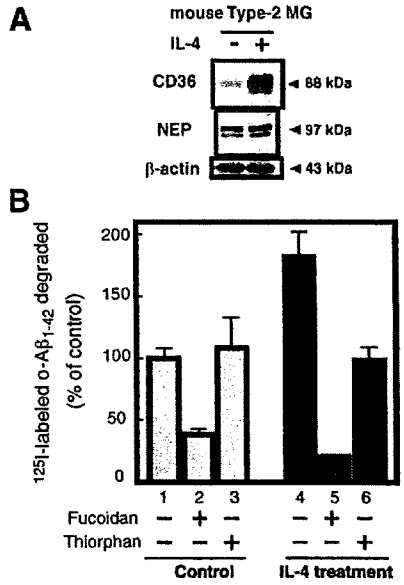

FIGURE 10. Clearance of ^{125}I-labeled o-Aβ_{1-42} by mouse primary type 2 MG. *A*, Mouse primary type 2 MG were isolated as described in *Materials and Methods*. Cells were cultured for 96 h in MEM/10% FCS in the presence or absence of IL-4 (5 ng/ml). Cell proteins (30 μg) were subjected to immunoblot analysis with Abs against CD36, NEP, or β-actin. *B*, Cells were cultured as described in *A* and were then incubated for 6 h with ^{125}I-labeled o-Aβ_{1-42} (1 μg/ml) in the presence or absence of fucoidan (100 μg/ml) or thiorphan (30 μM). The amounts of degradation products of ^{125}I-labeled o-Aβ 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 Aβ transport across the BBB and accumulation of Aβ in the brain (38). RAGE may have functions different from o-Aβ_{1-42} clearance, given that its expression was down-regulated by IL-4 in this study. Transcytosis of Aβ_{1-40} across brain endothelium of the BBB, mediated by low-density lipoprotein receptor-related protein 1, was also thought to be an Aβ_{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 Aβ_{1-40} (19) when it is overexpressed. It is not clear, however, whether macrophage scavenger receptors engage in uptake and degradation of Aβ_{1-42} as they do for Aβ_{1-40}.

As evidence of participation of the Aβ-degrading enzymes NEP and IDE (41, 42) in extracellular Aβ 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 Aβ, 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 Aβ 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 Aβ. For human neprilysin, it is reported to degrade Aβ 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 Aβ 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 Aβ (27). They suggested that these anti-inflammatory cytokines may oppose the harmful effects of IL-1β, which stops phagocytic microspheres from forming inside cells and IFN-γ from blocking phagocytosis. However, our data clearly show that IL-4 treatment enhanced degradation of ^{125}I-labeled o-Aβ_{1-42}, by CD36-mediated intracellular phagocytosis and extracellular proteolysis involving Aβ-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 Aβ 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 Aβ 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 Aβ peptide: it was not clear whether one MG subtype had an inflammatory reaction to Aβ, whereas another subtype exhibited an anti-inflammatory response to Aβ 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-Aβ_{1-42}. With regard to protection from neurotoxic o-Aβ, 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-4Rα 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 Aβ 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-γ-induced MHC class II (46), TNF-α, 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-1β (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 Aβ APCs and induced an adaptive immune response. They also demonstrated that Aβ-reactive Th1 cells underwent apoptosis after stimulation, accompanied by increased levels of IFN-γ, NO, and caspase 3. In contrast, MG-mediated proliferation of Aβ-reactive Th2 cells led to expression of the Th2 cytokines IL-4 and IL-10, which counteracted the toxic levels of NO induced by Aβ. 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 Aβ_{1-42} (58).

Akiyama and McGeer (59) demonstrated involvement of MG in immunotherapeutic clearance of Aβ 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 Aβ, 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 Aβ clearance by the anti-inflammatory cytokine IL-4, 2) simultaneous induction of three molecules (CD36, NEP, and IDE) that participate in clearance of Aβ_{1-42}, and 3) preferential clearance of the highly neurotoxic o-Aβ_{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 Aβ clearance. In addition to the innovative method of mucosal Aβ 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 Aβ_{1-42} presented here, may spur development of new anti-inflammatory therapeutic strategies for AD.

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Disclosures

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