Overexpression of Monocyte Chemotactic Protein-1/CCL2 in β -Amyloid Precursor Protein Transgenic Mice Show Accelerated Diffuse β -Amyloid Deposition

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Microglia accumulation at the site of amyloid plaques is a strong indication that microglia play a major role in Alzheimer's disease pathogenesis. However, how microglia affect amyloid- β peptide ($A\beta$) deposition remains poorly understood. To address this question, we developed a novel bigenic mouse that overexpresses both amyloid precursor protein (APP) and monocyte chemotactic protein-1 (MCP-1; CCL2 in systematic nomenclature). CCL2 expression, driven by the glial fibrillary acidic protein promoter, induced mononuclear phagocyte (MP; monocyte-derived macrophage and microglial) accumulation in the brain. When APP/CCL2 transgenic mice were compared to APP mice, a fivefold increase in $A\beta$ deposition was present despite increased MP accumulation around hippocampal and cortical amyloid plaques. Levels of full-length APP, its C-terminal fragment, and $A\beta$ -degrading enzymes (insulin-degrading enzyme and neprilysin) in APP/CCL2 and APP mice were indistinguishable. Sodium dodecyl sulfate-insoluble $A\beta$ (an indicator of fibrillar $A\beta$) was increased in APP/CCL2 mice at 5 months of age. Apolipoprotein E, which enhances $A\beta$ deposition, was also increased (2.2-fold) in aged APP/CCL2 as compared to APP mice. We propose that although CCL2 stimulates MP accumulation, it increases $A\beta$ deposition by reducing $A\beta$ clearance through increased apolipoprotein E expression. Understanding the mechanisms underlying these events

could be used to modulate microglial function in Alzheimer's disease and positively affect disease outcomes. (Am J Pathol 2005, 166:1475–1485)

Accumulating evidence supports a prominent role for brain inflammation in the pathogenesis of Alzheimer's disease (AD). That nonsteroidal anti-inflammatory drugs (NSAIDs) can positively affect the onset and progression of AD supports this notion.¹ Indeed, NSAIDs suppress glial activation and regulate amyloid precursor protein (APP) processing.^{2–5} However, a recent AD cooperative study showed no clinical improvement in AD symptoms with either a cyclooxygenase-2 inhibitor (rofecoxib) or a nonselective NSAID (naproxen) when the drugs were administered for 1 year.⁶ Disease outcomes after longer NSAID treatment regimens await further study. The different reported clinical outcomes may result from differences in how the drugs affect microglial inflammation. Microglia play critical roles in inciting inflammation while serving to clear damaged brain tissue. Nonetheless, their precise role in disease pathogenesis remains uncertain.^{7–9} In addition, microglia accumulate at the site of senile plaques in AD brains, are activated by APP processing products, such as secreted APP and amyloid- β peptide ($A\beta$), and induce neurotoxicity.^{10,11} They also affect $A\beta$ deposition and neurodegenerative processes including synaptic and neuronal cell loss. Elucidation of microglial function and its effect on cognitive impairment

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in AD is critical for understanding the mechanisms underlying disease pathogenesis.

Tg2576 transgenic APP mice mimic many aspects of human disease including microglial-induced brain inflammation. Importantly, APP transgenic mice replicate many aspects of AD neuropathology including (A β plaque formation, dystrophic neurites, astrogliosis, and microglial activation) along with deficits in learning and memory.^{12,13} Tg2576 mouse lines have also been instrumental in clarifying the importance of microglial cells in AD. Tg2576 mice deficient for CD40 ligand, a signaling molecule participating in T-cell-microglial immune responses, show a marked reduction in A β deposition, microglial reactions, astrogliosis, and APP β -processing.¹⁴ However, APP mice deficient in complement component C1q show reduced microgliosis without significant changes in amyloid deposition.¹⁵ Furthermore, APP mice overexpressing C3 inhibitor, soluble complement receptor-related protein y, show reduced microglial activities and enhanced A β deposition.¹⁶

CCL2 is a member of the β chemokine subfamily and a signaling ligand for the seven-transmembrane spanning G-protein-coupled receptor, CCR2.¹⁷ Activated astrocytes and mononuclear phagocytes (MP; perivascular and parenchymal macrophages and microglia) express CCL2 in the brain.^{18,19} CCL2 is present in senile plaques, reactive microglia,^{20,21} and microvessels²² in AD brains. Because astrocytes are the main source of CCL2,²³ a transgenic mouse (JE-95) expressing the murine CCL2 under the control of the human glial fibrillar acidic protein (GFAP) promoter was generated. The constructed animals overexpress CCL2 in an astroglial activation-dependent manner.²⁴ JE-95 mice develop a pertussis toxin-induced reversible encephalopathy dependent on CCL2 overexpression.²⁴ With chronic overexpression of CCL2, aged JE-95 mice develop delayed spontaneous neurodegeneration dependent on MCP-1 (CCL2) overexpression (DESMO), characterized by motor impairment and a reduced life span.²⁵ The pathological features of DESMO include modest perivascular cell infiltrates and blood-brain barrier compromise, with widespread microglia activation shown by ionized calcium-binding adaptor molecule 1 (IBA-1) immunohistochemistry. In the central nervous system of DESMO mice there was no demyelination, and a reduction in the numbers of neurons, axons, and synapses. The great majority of MP cells in the central nervous system of aged JE-95 mice were CD45^{dim} microglia. Despite their activated morphology, these microglia did not express the surface markers of antigen-presenting cells, and were grossly deficient in acquiring amoeboid morphology in cortical slice preparations. We demonstrate that APP/CCL2 mice show increased numbers of MP and diffuse A β plaque deposition in both the hippocampus and the cortex. Biochemical studies demonstrate that sodium dodecyl sulfate (SDS)-insoluble A β accumulates in the hippocampus by 5 months of age. In contrast, A β production, APP processing, and A β -degrading enzyme levels remain unchanged. These data support the idea that enhanced A β deposition is due to reduced A β clearance. To investigate how brain MP may affect amyloid deposition during

AD, APP/CCL2 bigenic mice were generated and analyzed.

Materials and Methods

APP/CCL2 Bigenic Mice

Tg2576 mice expressing the Swedish mutation of human APP₆₉₅ were obtained from Drs. G. Carlson and K. Ashe through Mayo Medical Venture.¹² Male Tg2576 mice were crossed with CCL2 (JE-95 strain) females generating APP/CCL2 mice and wild-type (WT) littermates, and then backcrossed at least four times to 129/Sve mice to develop a 129/Sve strain. Animals used for this study were WT (APP and CCL2 transgene-negative, 14 months; three males and three females), CCL2 (APP transgene-negative and CCL2 transgene-positive, 14 months; three males and three females), APP (APP transgene-positive and CCL2 transgene-negative, 5 months, two males and one female; 14 months, three males and three females), and APP/CCL2 (APP and CCL2 transgene-positive, 5 months, two males and one female; 14 months, three males and three females) mice. The APP, APP/CCL2, WT, and CCL2 mice are littermates.

Genotyping Protocol

DNA samples were prepared from cut tail tips (<1.0 cm) of individual pups, and genomic DNA was extracted using the Easy DNA kit (Invitrogen, Carlsbad, CA). Polymerase chain reaction (PCR) was performed as described previously to identify APP transgene-positive mice.²⁶ The human GFAP promoter murine CCL2 transgene was confirmed by PCR of genomic DNA with primers JE-5 (5'-TTC CTG GGC ACA GGC TGA ATA GAG) and JE-3 (5'-ATT GAG CAG GGG GCT TGC ATT G), which amplify a 150-bp DNA fragment from the transgene, but not the endogenous murine CCL2 gene.

Protein Extraction and Enzyme-Linked Immunosorbent Assay (ELISA)

For immunoblotting and CCL2 ELISA, brain tissues were homogenized in solubilization buffer [50 mmol/L Tris-Cl, pH 7.5, 100 mmol/L NaCl, 2 mmol/L ethylenediamine tetraacetic acid-Na, 1% Triton X-100, and protease inhibitor cocktails (Roche Applied Science, Indianapolis, IN)] and centrifuged at 100,000 $\times$ g for 1 hour at 4°C. The protein concentration of the supernatant was quantified by BCA assay kit (Pierce, Rockford, IL) and subjected to immunoblotting and CCL2 ELISA (mouse JE/CCL2; R&D Systems, Minneapolis, MN).²⁷ For total A β ELISA, brain tissues were homogenized in 5 mol/L guanidine isothiocyanate to prepare a protein extract²⁸ and subjected to A β 40 and A β 42 ELISA (Biosource, Camarillo, CA). For SDS-insoluble A β ELISA, brain tissue was homogenized in 2% SDS-containing solubilization buffer, followed by centrifugation at 100,000 $\times$ g for 1 hour. The supernatant fraction was used to measure SDS-soluble A β . The pellet

Table 1. Summary of Antibodies Used for Immunohistochemistry and Immunoblotting

Antibody	Epitope protein/amino acids	Type	Dilution	Company	Reference
A β	A β 1-30	P	1:200	Zymed	52
β -Actin	Slightly modified synthetic β -cytoplasmic actin N-terminal peptide	M	1:100,000	Sigma	56
ApoE	Purified mouse apoE from pooled mouse plasma high- and very low-density lipoproteins	P	1:1000	Biodesign	55
APP C-terminal	C-terminal 20 amino acids residue (751 to 770) of human APP	P		Calbiochem	57
CD10	Prokaryotic recombinant fusion protein corresponding to the external domain of the CD10 glycoprotein	M	1:100	Novocastra	58
CD11b/Mac-1	T cell-enriched splenocytes from B10 mice	R	1:50	Serotec	15
CD11c	Purified from mouse ascites fluid	H	1:100	eBioscience	59
CD45	Purified B cells from mouse lymph nodes	R	1:500	Serotec	41
6E10	A β 3-8	M	1:500	Signet	56
GFAP	GFAP isolated from cow spinal cord	P	1:1000	DAKO	53
IBA-1	C-terminal portion of IBA-1	P	1:500	*	54
IDE	Recombinant GST-fusion protein containing amino acids 97 to 273 of IDE	P	1:1000	Oncogene	60
MHC-II (I-A/I-E)	Activated C57BL/6 mouse spleen cells	M	1:100	BD PharMingen	15
PY-20	phosphotyrosine-protein conjugate	M	1:400	Sigma	56
Synaptophysin	synaptosome preparation from rat retina	M	1:1000	Sigma	57

*Antibody from Dr. S. Kohsaka.

M, mouse monoclonal antibody; P, rabbit polyclonal antibody; R, rat monoclonal antibody; H, hamster polyclonal antibody.

was resuspended in 5 mol/L guanidine isothiocyanate and used for SDS-insoluble A β measurements.²⁸

Quantitative Real-Time Reverse Transcriptase (RT)-PCR for CCL2 mRNA Levels

Total RNA was isolated from brain tissues using TRIzol (Invitrogen), and subjected to real-time RT-PCR tests using TaqMan predeveloped murine CCL2 gene expression assay reagents and the ABI Prism 7000 sequence detection system (both from Applied Biosystems, Foster City, CA) and the mRNA levels were quantified as previously described.²⁹

Immunohistochemistry

Animals were euthanized with isoflurane and perfused transcardially with 25 ml of normal (0.9%) saline as described.² The brains were rapidly removed and bisected sagittally. The left hemispheres were immersed in freshly depolymerized 4% paraformaldehyde for 48 hours and the frontal cortex were dissected and frozen. The left hemispheres were cryoprotected by successive 24-hour immersions in 10%, 20%, and 30% sucrose in Sorenson's phosphate buffer immediately before sectioning. Fixed, cryoprotected brains were frozen and sectioned in the horizontal plane at 10 μ m using a Cryostat (Leica, Bannockburn, IL), with sections collected serially. The right hemispheres were embedded in paraffin block and sectioned as well. Immunohistochemistry was performed using specific antibodies to identify cellular and molecular markers for A β , GFAP, CD11b/Mac-1, CD11c, IBA-1,³⁰ MHC class II (MHC-II), CD45, phosphotyrosine, and synaptophysin antibodies as listed in Table 1, which also includes the antibodies, specificities, dilutions, and references. Brain sections stained for microglial markers were

additionally stained with thioflavin-S (TS; Sigma, St. Louis, MO) to localize deposits of amyloid in a β -sheet configuration. Two regions were examined quantitatively using a stereological system: the hippocampus and the cortex. Alternatively, paraffin-embedded brains were sectioned at 5 μ m for A β , TS, or GFAP staining. Systematic uniform random sets of sections with 300- μ m spacing were used for staining. All immunohistochemistry was visualized using avidin-biotin-horseradish peroxidase with 3,3'-diaminobenzidine for color development (Vector Laboratories, Burlingame, CA). Envision Plus (DAKO, Carpinteria, CA) was used instead of avidin-biotin-horseradish peroxidase for A β staining.

Image Analysis

Images were captured with a digital camera (DVC-1310C; DVC Company) attached to an Eclipse TE-300 Nikon microscope using C-View v1.2 software (DVC).³¹ Ten to twenty images, covering the entire cortical and hippocampal areas, were taken per each 5- or 10- μ m section (10 sections per brain, 300- μ m spacing) at $\times$ 200 magnification. Because the measured outcome of total volume occupied by TS, A β , GFAP, Mac-1, and IBA-1 immunohistochemical reaction were readily discernable by visual inspection. These were quantified by image software (NIH Image 1.62). The number of A β plaques and immunopositive cells (GFAP, Mac-1, and IBA-1 staining) were counted manually. The intensity of synaptophysin was determined by semiquantification of digitally captured image by Image-Pro Plus 4.0 (Media Cybernetics, Inc., Silver Spring, MD). Data are reported as a percentage of the total stained area divided by the total cortical or hippocampal area, or the total number of immunostained cells divided by the total cortical or hippocampal

area for each animal. Immunohistochemical tests were analyzed by M.Y. and M.H. in a blind manner.

Immunoblots

Brain lysates (30 μ g) were precleared with 10 μ l of protein G-Sepharose FF (Amersham Pharmacia, Arlington Heights, IL) to remove endogenous mouse IgG and subjected to standard immunoblotting using 4 to 15% Pre-cast gel SDS-polyacrylamide gel electrophoresis (Bio-Rad, Richmond, CA).¹¹ Proteins were transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA), and subjected to immunoblotting using primary antibodies (listed in Table 1). Alkaline phosphatase-conjugated secondary antibodies were used against mouse and rabbit IgG (1:2000 dilution; Vector Laboratories, Burlingame, CA), and developed using NBT/BCIP solution (Roche Applied Science).¹¹ The images were digitally captured by a computer scanner (Canon), and the band intensities of apoE, APP, APP C-terminal fragments (CTF), β -actin, GFAP, IBA-1, insulin-degrading enzyme (IDE), and neprilysin were measured by NIH Image 1.62 software program. Data were presented as a ratio of target band intensity/ β -actin band intensity.

Tissue Culture and Recombinant Adenovirus Infection

PC12 cells (1×10^5 cells/well in poly-D-lysine-coated 24-well plates) were differentiated by nerve growth factor (100 ng/ml, R&D Systems) for 5 days, and infected with recombinant adenovirus expressing Swedish APP mutation (multiplicity of infection = 10) as described previously.¹¹

Statistics

All data were normally distributed. In case of multiple mean comparisons, data were analyzed by analysis of variances, followed by Newman-Keuls multiple comparison test using statistics software (Prism 3.0 and 4.0; Graphpad Software, Inc., San Diego, CA). In case of single mean comparison, data were analyzed by Student's *t*-test. A *P* value of <0.05 was regarded as significant.

Results

CCL2 Enhances Diffuse Amyloid Deposition

Neuropathological changes and clearance of A β in the APP/CCL2 bigenic mice were followed throughout time and compared to APP and CCL2 transgenics and control littermates (WT). A 1000-fold increase in levels of CCL2 protein was observed in the frontal cortex of APP/CCL2 and CCL2 mice (34.6 and 18.1 ng/mg, respectively) compared to APP and WT mice (3.0 and 21.6 pg/mg, respectively; Figure 1A, left). Murine CCL2 mRNA levels, analyzed by quantitative real-time RT-PCR, were significantly

Figure 1. CCL2 levels and A β deposition in APP/CCL2 mice. **A:** Frontal cortex of APP/CCL2, APP, CCL2, and WT mice at 14 months of age ($n = 6$) were dissected and subjected for protein extraction in solubilization buffer or total RNA preparation. Murine CCL2 protein and mRNA levels were determined using ELISA and real-time RT-PCR as described in the Material and Methods, respectively. *, $P < 0.0001$ between APP/CCL2 versus APP or CCL2 versus WT; #, $P < 0.05$ between APP/CCL2 versus CCL2. **B:** Ten slides of 5- μ m-thick brain sections of APP or APP/CCL2 mice were immunostained with anti-A β antibody, developed with DAB, and counterstained with hematoxylin. The arrows indicate immunopositive A β deposits. **C:** Average percent area occupied by A β deposits in the hippocampus (**left**) and the cortex (**right**) as quantified by image analyses of immunostained slides ($n = 6$). * and **, $P < 0.05$ and $P < 0.01$ versus APP mice as determined by Student's *t*-test, respectively. Original magnifications, $\times 40$. Scale bar, 250 μ m.

cantly increased in APP/CCL2 and CCL2 mice (10,127 and 9048 copies/ng total RNA, respectively) compared to APP and WT mice (3.7 and 2.3 copies/ng total RNA, respectively; Figure 1A, right). APP/CCL2 mice showed significantly more A β deposition than did their APP littermates as determined by A β immunostaining of both hippocampal and cortical diffuse plaques at 14 months of age (Figure 1B). Quantitative immunohistochemical assays showed a 5.6- and a 5.0-fold increase in A β deposition in the hippocampus and cortex, respectively, of APP/CCL2 compared to APP mice (Figure 1C). Measures of total A β 40 and A β 42 by ELISA confirmed these results (Table 2). In both SDS-soluble and -insoluble fractions, A β 40 and A β 42 were significantly increased in APP/CCL2 mice as compared to their APP littermates, although the A β 42/40 ratio remained unchanged. There was no difference in the number of compact plaques stained by TS between APP and APP/CCL2 animals (Figure 2; A to C). This suggested that the A β deposition of only diffuse plaques was increased in APP/CCL2 mice. In addition, TS-positive plaques at 10 and 12 months of age were detected in both APP and APP/CCL2 mice (data not

Table 2. A β Level in APP and APP/CCL2 Transgenic Mouse Brain

Genotype	Age (months)	n	A β	2% SDS A β ng/mg protein*	5 mol/L guanidine A β ng/mg protein†	Total A β ng/mg protein‡	Total A β 40 + A β 42 ng/mg protein§
APP	14	6	A β 40	38.3 $\pm$ 8.4	130.7 $\pm$ 17.0	54.1 $\pm$ 8.6	61.0 $\pm$ 9.0
			A β 42	7.1 $\pm$ 2.2	8.1 $\pm$ 0.8	6.9 $\pm$ 0.6	
			A β 42/A β 40	21.2 $\pm$ 3.7	6.8 $\pm$ 1.2	14.0 $\pm$ 2.0	
APP/CCL2	14	6	A β 40	70.5 $\pm$ 13.1 [§]	298.5 $\pm$ 54.6 [§]	102.5 $\pm$ 15.4 [§]	117.3 $\pm$ 17.1 [§]
			A β 42	13.7 $\pm$ 2.1 [¶]	22.6 $\pm$ 2.8 [¶]	14.8 $\pm$ 2.1 [¶]	
			A β 42/A β 40	18.7 $\pm$ 1.7	8.3 $\pm$ 1.0	14.5 $\pm$ 1.3	

Brain cerebrum has been homogenized in 2% SDS buffer and ultracentrifuged as described in the Material and Methods. SDS-soluble A β 40 and A β 42 levels in the supernatant fraction were presented as 2% SDS values. The SDS-insoluble fraction was solubilized in 5 mol/L guanidine buffer and its A β 40 and A β 42 values were presented as 5 mol/L guanidine values.

*Total protein in SDS soluble fraction.

†Total protein in SDS insoluble fraction.

‡Total protein in SDS soluble and insoluble fraction.

§, ¶, and ¶ in APP/CCL2 group denotes $P < 0.05$, 0.01, and 0.001 versus corresponding A β value of APP group as determined by Student's t -test, respectively.

shown), suggesting that the onset of amyloid plaque formation remained unchanged by CCL2 transgene expression.

CCL2 Enhances Mononuclear Phagocyte Accumulation in Brain

Increased A β deposition correlated with increased microglial numbers in both the cortex and the hippocampus of APP/CCL2 mice when assessed by IBA-1 immunostaining (Figure 3, A and B). In APP/CCL2 bigenic mice,

MP accumulated at the TS-positive compact plaques in both the hippocampus and the cortex to a significantly greater extent than what was observed in APP mice (Figure 3, C and D). Similar results were obtained for microglia by Mac-1 staining (data not shown). These data demonstrated that not just the total number (Figure 3B) but also the plaque-associated microglia (Figure 3D) were increased in APP/CCL2 mice as compared to APP mice in both the hippocampus and the cortex. Such microglia showed a nonactivated phenotype as determined by diminished CD45, MHC class II, CD11c, or phosphotyrosine immunostaining (data not shown). The intensity of astrogliosis, as determined by quantitative immunohistochemistry and immunoblotting of GFAP, was similar in APP and APP/CCL2 mice (Figure 4, A and B). GFAP immunoblotting showed increased astrogliosis in APP and APP/CCL2 mice when compared to WT and CCL2 mice (Figure 4C). No differences in synaptic density were seen in APP/CCL2 and APP mice by synaptophysin immunostaining (Figure 5). Such findings showed that MP numbers, rather than the level of astrogliosis, were enhanced by CCL2 and associated with A β deposition.

Figure 2. TS staining of APP/CCL2 mouse brains. **A:** Mice at 14 months of age were tested for compact amyloid plaques by TS staining. Images show 10- μ m-thick coronal sections of the hippocampal region. **B:** Quantitative analysis of the number of TS-positive plaques in hippocampus and cortex. **C:** Quantitative analysis of the area occupied by TS-positive plaques in hippocampus and cortex. N.S. in **B** and **C** denotes no statistical significance as determined by Student's t -test. Original magnifications, $\times 40$.

CCL2 Does Not Alter APP Processing but Enhances A β Aggregation

Whether A β deposition occurs is determined by the balance between three processes: A β production, aggregation, and clearance. Although A β production was up-regulated in APP/CCL2 mice at 14 months of age, no significant differences were found in APP expression between APP and APP/CCL2 mice in the cortex (Figure 6A). APP C-terminal fragments are processing products of APP at the α -site (α -CTF) or β -site (β -CTF, Figure 6B). Neither α/β -processing nor the ratio of α/β processing was altered in APP/CCL2 as compared to APP mice. The SDS-insoluble fraction of A β 40 was increased in the hippocampus of APP/CCL2 mice as compared to APP mice at 5 months of age (Figure 7C). Total amounts of A β 40, A β 42, or the A β 42/40 ratio were unchanged in brains at 5 months of age (Figure 7; A to C). Although there is no difference in GFAP expression,

Figure 3. Microglial accumulation in APP/CCL2 mice. **A:** Ten- μm -thick slices of cortical (C) and hippocampal (H) regions of mice at 14 months of age ($n = 6$) were immunostained with anti-IBA-1 antibody and counterstained with hematoxylin. **Arrows** indicate IBA-1-positive cells in the brain regions. **B:** Quantification of IBA-1-positive cells in brain regions. *, $P < 0.05$ versus other groups as determined by analysis of variance and Newman-Keuls post hoc. **C:** TS-positive amyloid plaques (AP) were surrounded by IBA-1-positive microglial cells in both the hippocampal and cortical regions of APP/CCL2 or APP mice. **D:** Quantification of IBA-1-positive cells per μm^2 AP. *, $P < 0.05$ versus APP mice ($n = 6$) as determined by Student's t -test. Original magnifications: $\times 200$ (**A**); $\times 400$ (**C**). Scale bars, 25 μm .

there is a significant increase in IBA-1 expression in APP/CCL2 mice at 5 months of age (Figure 8, A and B), suggesting increased MP accumulation in brain tissues. CCL2 had no direct effect on APP processing when PC12 cells expressing the Swedish APP mutant were treated with increasing doses of CCL2 (Figure 9). Taken together, these data suggest that increased $A\beta$

deposition in APP/CCL2 mice was not secondary to enhanced APP or $A\beta$ production. Thus, we next investigated whether $A\beta$ clearance was affected in the APP/CCL2 mice. In these experiments the $A\beta$ degradation enzymes, IDE and neprilysin,^{32,33} were examined. The levels for each enzyme in the frontal cortex of APP/CCL2, APP, CCL2, and WT mice were not significantly

Figure 4. Astrogliosis in APP/CCL2 mouse brains. **A:** APP and APP/CCL2 mice at 14 months of age ($n = 3$) were tested for astrogliosis by anti-GFAP staining. Images show 5- μ m-thick coronal sections of the hippocampal region. **B:** Quantitative analysis of the number of GFAP-positive astrocytes in the hippocampus and the cortex of APP/CCL2 and APP mice. No statistical significance was observed. **C:** Immunoblotting analysis of GFAP at 14 months of age ($n = 3$ per group). *, Statistical differences ($P < 0.05$) between WT versus APP and CCL2 versus APP/CCL2 as determined by analysis of variance and Newman-Keuls post hoc. Original magnifications, $\times 40$.

different (Figure 10, A and B). Because this reflects the extracellular levels of A β -degrading enzyme, the data suggested that A β degradation was not affected by CCL2 expression in the various regions of the brain. Finally, we examined the expression of apolipoprotein E (apoE), which has been shown to be important in A β clearance. The apoE4 allele has been genetically linked to the incidence of sporadic and late-onset AD.^{34,35} Although there was no significant difference in apoE levels between APP/CCL2 and APP mice at 5 months of age, there was a 2.2-fold increase of apoE in

Figure 6. APP expression and processing. **A:** Protein extracts from the cortex and the hippocampus of APP/CCL2 and APP mice ($n = 3$) were subjected to immunoblotting for APP and β -actin using anti-A β (6E10) and anti- β -actin monoclonal antibodies. The APP-immunoreactive band intensity was normalized by β -actin band intensity. No statistical significance was observed. **B:** APP α - and β -CTF protein levels were quantified by immunoblotting using anti-APP 751-770 antibody and normalized by β -actin protein levels in cortex ($n = 3$). No statistical significance was observed.

APP/CCL2 mice as compared to APP mice at 14 months of age (Figure 11, A and B). Because endogenous murine apoE has been shown to induce A β deposition *in vivo*,³⁶ and all apoE isoforms can propagate A β aggregation *in vitro*,³⁷ this suggests that the accumulation of A β in APP/CCL2 mice may be linked to increased apoE levels. Because apoE is mainly secreted from microglia instead of astrocytes *in vitro*,³⁸ the increased apoE level is likely because of the enhanced accumulation of microglia seen in the APP/CCL2 mouse brain. These data suggest that chronic up-regulation of CCL2 enhances MP accumulation and amyloid deposition due to the reduced amyloid clearance. This reduced clearance may be because of increased A β aggregation caused by increased apoE expression in the mouse brain.

Figure 5. Synaptic density in APP/CCL2 mouse brains. **A:** Mice at 14 months of age were tested for synaptic density by anti-synaptophysin staining. Images show 5- μ m-thick coronal section of hippocampal region. **B:** Quantitative analysis of the density of synaptophysin staining in the hippocampus and the cortex. No statistical significance was observed among the group in either brain subregion. Original magnifications, $\times 40$.

Figure 7. APP and Aβ in young mouse brains. **A** and **B**: APP, total Aβ40, Aβ42, and Aβ42/Aβ40 ratio in cortex quantified by specific Aβ ELISA at 5 months of age ($n = 3$ per group). No statistical significance was observed. **C**: SDS-insoluble Aβ40 ELISA in hippocampus and cortex of APP/CCL2 and APP mice at 5 months of age ($n = 3$). * $P < 0.05$ versus APP mice as determined by Student's t -test.

Discussion

These data show a significant role for CCL2 in the development of the AD-like pathology seen in APP mouse brain tissue. This role for CCL2 is supported by a number of experimental observations. First, CCL2 enhances MP

Figure 8. GFAP and IBA-1 expression in young mouse brains. **A**: Protein extracts (30 μg/lane) from the frontal cortex of APP/CCL2 and APP mice at 5 months of age ($n = 3$ per group) were subjected to immunoblotting for β-actin, GFAP, and IBA-1. **B**: Quantification of immunoreactive bands in **A**. * $P < 0.05$ versus APP as determined by Student's t -test.

Figure 9. Effect of CCL2 on APP processing *in vitro*. Nerve growth factor-treated differentiated PC12 cells (1×10^5 cells/well in poly-D-lysine-coated 24-well plate) were infected with recombinant adenovirus expressing Swedish FAD APP₆₉₅ mutant¹¹ and subsequently stimulated with increasing doses of recombinant murine CCL2 for 24 hours. Media was collected and both Aβ40 and Aβ42 levels were determined by specific Aβ ELISA. Data were presented as pg/ml, ng/ml, or percent ratio of Aβ42/Aβ40.

accumulation, although astrogliosis remains unchanged. Second, MP accumulation is accompanied by enhanced Aβ deposition. Third, the differences in Aβ deposition were due to an increase in diffuse plaques in APP/CCL2 mice because the numbers of TS-positive compact plaques were similar between APP/CCL2 and APP mice at 14 months of age. This indicated that microglial accumulation enhances diffuse but not compact plaque formation. Such observations were noted in both the hippocampus and the cortex. Fourth, CCL2 overexpression significantly enhanced the formation of SDS-insoluble Aβ in the hippocampus at 5 months of age although its amount is slightly but not significantly increased in the cortex. This suggested that Aβ deposition in the hippocampus precedes its deposition in the cortex, probably due to the perforant pathway-mediated hippocampal amyloid deposition mechanism.³⁹ Both diffuse and compact plaque formation starts as early as 10 months of age (data not shown), although the number of TS-positive compact plaques was similar to age-matched APP mice at 14 months of age. Thus, CCL2 overexpression enhanced SDS-insoluble Aβ formation but did not accelerate the onset of Aβ deposition in the APP mouse brain. Because there is no difference in APP expression, α/β-processing, or Aβ-degrading enzyme levels between

Figure 10. Aβ-degrading enzymes in brains. **A** and **B**: Protein extracts (30 μg/lane) from the frontal cortex of four mouse groups at 14 months of age ($n = 3$) were subjected to immunoblotting using anti-IDE (**A**), anti-nephrilysin (**B**), and anti-β-actin monoclonal antibodies (**top**). The band intensity of IDE and nephrilysin was normalized by the β-actin signal and the average intensity ratios were presented (**bottom**). No statistical significance was observed.

Figure 11. ApoE levels in brains of young and aged animals. **A:** Protein extracts (30 μ g/lane) from the frontal cortex of APP and APP/CCL2 mice at 5 and 14 months of age ($n = 3$ per group per age) were subjected to immunoblotting using anti-apoE (Bioscience) and anti- β -actin monoclonal antibodies. **B:** The band intensity of apoE was normalized by the β -actin signal and the average intensity ratios were presented (filled bar, APP/CCL2 group; open bar, APP group). *, $P < 0.05$ versus APP at 14 months of age as determined by Student's *t*-test.

APP and APP/CCL2 groups, it is likely that the enhanced A β deposition was due to reduced A β clearance possibly caused by deficits in phagocytosis or in the vascular endothelial export from central nervous system.

How MP accumulation leads to reduce A β clearance is unknown. It is known that suppression of inflammatory reaction by NSAIDs reduces the A β load in APP mouse brain.^{2,3,40,41} NSAIDs have also been shown to directly lower A β 42 when administered at high concentrations.^{4,42} On the other hand, lipopolysaccharide stimulation activates microglia and enhances A β clearance in APP/PS-1 mouse brain,⁴³ whereas nonactivated microglia (by dexamethasone) are inefficient in clearing A β plaques even after passive immunization with A β antibody.^{44,45} The accumulated MP around A β plaques in APP/CCL2 are incompletely activated despite their morphology because they are negative for microglial activation markers, such as MHC class II, phosphotyrosine; further, these cells were deficient for acquiring amoeboid morphology in a cortical slice preparation assay (data not shown). Thus, it is likely that the nonactivated status of MP contributes to the reduced clearance seen in the APP/CCL2 mouse brain. Our data suggest that the effect of NSAIDs on APP mice may also be due to suppression of CCL2 and/or MP accumulation. Another APP bigenic mouse model that overexpresses transforming growth factor- β 1 (TGF- β 1) showed enhanced microglial A β

clearance and reduced deposition in the brain parenchyma.⁴⁶ The major difference between the APP/CCL2 and the APP/TGF- β 1 mouse model is the lower level of microglial activation seen in APP/CCL2 mice, as compared to the high level of microglial phagocytosis seen in APP/TGF- β 1 mice.⁴⁶ These studies suggest that the microglia associated with plaques require specific stimulating factors, such as antibodies or TGF- β 1, to initiate A β phagocytosis. Without this response, microglia are inefficient in the clearance of A β . There are several studies in the literature that support this idea. It is known that degradation of ingested A β by primary cultured, unstimulated, microglia *in vitro* is slow as compared to acetylated low-density lipoprotein.⁴⁷ There was little evidence of internalized A β found in the microglia of AD brains.⁴⁸ A similar observation is seen in Tg2576, where activated microglia may not be engaged in A β removal or plaque degradation, but may be enhancing plaque formation.⁴⁹ Three-dimensional reconstitution of an ultrastructural study showed that microglia do not engulf A β inclusions in APP23 mice.⁵⁰ Thus, microglial amyloid plaque phagocytosis is not evident in AD or APP mouse brains without administration of amyloid immune therapy. Another possible explanation for this finding is that accumulated microglia increase A β deposition to reduce its toxicity. It is known that soluble oligomer A β is detrimental to neurophysiological function, and that aggregation of the soluble oligomer A β as condensed A β deposits might be beneficial to the brain. To address this issue, we are examining the effect of CCL2 expression on APP mice by behavioral and electrophysiological analyses.

Accumulating evidence suggests that endogenous apoE in mouse brain enhances A β deposition in APP animal models.^{36,37} Although many studies concluded that astrocytes are the main apoE-producing cell in brain,⁵¹ recent reports suggest that microglial cells are also a significant source of apoE.^{38,52} ApoE secretion does not require microglial activation, because lipopolysaccharide stimulation rather reduces apoE secretion *in vitro*.³⁸ Thus, although microglia could contribute to the increase in apoE in the double-transgenic mice, astrocytes are an abundant source of apoE in the central nervous system and they might also contribute.^{53,54} The more than twofold increase of apoE levels seen in APP/CCL2 mice is significant, because APP/apoE^{-/+} mice showed more than an 80% reduction in A β deposition as compared to APP/apoE^{+/+} mice.³⁶ ApoE has also been shown to enhance A β aggregation *in vitro*.⁵⁵ These data indicate that microglia may enhance A β aggregation through apoE secretion. Thus, the accumulated microglia around TS⁺ plaques may be involved in the acceleration of diffuse plaque enlargement *in situ*. It would be worthwhile to examine apoE levels in the other APP transgenic animal models to see if alterations in A β deposition correlate with changes in apoE levels in the brain.

In summary, we have demonstrated that APP/CCL2 bigenic mice show enhanced A β plaque deposition, MP accumulation, and increased apoE levels. APP/CCL2 bigenic mice also show SDS-insoluble A β accumulation and increased IBA-1 levels at a younger age. Our data suggests that MP plays a critical role not only in the

regulation of A β clearance but as a central player in a novel mechanism for AD progression, in which chronic accumulation of MP leads to the acceleration of amyloid deposition because of reduced phagocytic function and increased apoE levels. Anti-chemokine interventions that reduce MP accumulation may prove useful as a way to prevent or treat this aspect of AD.

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