Expression of Macrophage Colony-Stimulating Factor Receptor Is Increased in the A β PP^{V717F} Transgenic Mouse Model of Alzheimer's Disease

Greer M. Murphy, Jr.,* Feifei Zhao,* Lan Yang,* and Barbara Cordell†

From the Department of Psychiatry and Behavioral Sciences,* Stanford University School of Medicine, Stanford; and Scios, Inc.,† Sunnyvale, California

Inflammation is an important neuropathological change in Alzheimer's disease (AD). However, the pathophysiological factors that initiate and maintain the inflammatory response in AD are unknown. We examined A β PP^{V717F} transgenic mice, which show numerous brain amyloid- β (A β) deposits, for expression of the macrophage colony-stimulating factor (M-CSF) and its receptor (M-CSFR). M-CSF is increased in the brain in AD and dramatically augments the effects of A β on cultured microglia. A β PP^{V717F} animals 12 months of age showed large numbers of microglia strongly labeled with an M-CSFR antibody near A β deposits. M-CSFR mRNA and protein levels were also increased in brain homogenates from A β PP^{V717F} animals. Dystrophic neurites and astroglia showed no M-CSFR labeling in the transgenic animals. A M-CSF antibody decorated neuritic structures near hippocampal A β deposits in transgenic animals. M-CSF mRNA was also increased in A β PP^{V717F} animals in comparison with wild-type controls. Simultaneous overexpression of M-CSFR and its ligand in A β PP^{V717F} animals could result in augmentation of A β -induced activation of microglia. Because chronic activation of microglia is thought to result in neuronal injury, the M-CSF system may be a potential target for therapeutic intervention in AD. (*Am J Pathol* 2000, 157:895-904)

Inflammation is thought to be important in neuronal injury in Alzheimer's disease (AD).¹⁻³ Microglia, intrinsic immune effector cells of the brain, may be key in this process.⁴ Cell-culture studies suggest that the amyloid- β peptide (A β) is likely to be an important stimulus for microglial activation in AD.⁵⁻¹⁰ Further, transgenic mouse models for AD based on expression of mutant human amyloid precursor proteins (A β PP) show microgliosis in the vicinity of A β deposits in a pattern reminiscent of the AD brain.¹¹⁻¹³ However, in cell-culture systems A β alone has often been found to be a relatively weak activator of

microglia, with pro-inflammatory augmenting agents often required for a robust response.^{6,9,14} This suggests that in mutant A β PP transgenic mouse brain, factors other than the direct actions of A β may be important in inducing microglial activation.

We recently demonstrated that macrophage colony-stimulating factor (M-CSF) strongly augments the pro-inflammatory effects of A β on cultured microglial cells.¹⁵ M-CSF, a hematopoietic cytokine also produced by neurons and glia,¹⁶⁻¹⁸ has powerful activating effects on microglia and on other macrophage-like cells.^{19,20} The receptor for M-CSF (M-CSFR) is encoded by the *c-fms* proto-oncogene, is expressed on microglia and on cells of the monocyte/macrophage lineage, and has tyrosine kinase activity.^{21,22} M-CSF is elevated in cerebrospinal fluid of AD patients,¹⁸ and there is increased M-CSFR expression on microglia surrounding A β deposits in the AD brain.²³ Thus, unlike other microglial activators such as lipopolysaccharide and interferon- γ that augment the effects of A β on microglia *in vitro*,^{5,6,24,25} clinical and neuropathological data suggest that M-CSF and its receptor have pathophysiological relevance to AD.

To further clarify the role of M-CSF and its receptor in microglial activation in AD, we examined their expression in a mouse model of AD that shows extensive A β deposits, neuritic dystrophy, and astrogliosis.^{12,26} Microgliosis has also been reported in these animals,²⁷ although little information is available on the phenotypic features of the microglia. Our results demonstrate that there is extensive microglial activation in A β PP^{V717F} mouse brain characterized by dramatic up-regulation of M-CSFR expression.

Materials and Methods

Animals and Tissue Processing

Development of animals carrying the A β PP^{V717F} transgene and basic characterization of neuropathological changes have been described previously.^{12,26} In the

Supported by National Institute of Mental Health grants MH01239, MH 57833, and MH40041.

Accepted for publication May 31, 2000.

Address reprint requests to Greer M. Murphy, Jr., M.D., Ph.D., Neuroscience Research Laboratories, Department of Psychiatry and Behavioral Sciences, Stanford University School of Medicine, MSLS P-104, Stanford, CA 94305-5485. E-mail: gmurphy@leland.stanford.edu.

present study, A β PP^{V717F} transgenic mice and wild-type animals were anesthetized with Fatal-Plus (0.1 ml/mouse, i.p.; Vortech) and transcardially perfused with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in Sorensen's buffer. Brains were immediately removed and placed in ice-cold 4% paraformaldehyde. After 2.5 hours of fixation at 4°C, the brains were sectioned at 50 μ m into ice-cold Tris-buffered saline (TBS) on a Vibroslice 752 (Campden Instruments, London, UK). Free-floating sections were used immediately for immunocytochemistry. Other transgenic and control brains were removed without perfusion, frozen immediately on dry-ice, and stored at -80°C until processed for RNA and protein. Transgenic ($n = 16$) and wild-type ($n = 12$) animals used in this study were between 4 and 23 months of age. For immunohistochemistry, 10 transgenic and six control animals were used. For reverse transcription (RT) and polymerase chain reaction (PCR), three transgenic and three wild-type animals were used, and for Western blotting, another three transgenic and three wild-type animals were used. All animal procedures were approved by institutional review boards.

Antibodies

Antibody reagents included a polyclonal reagent to the extracellular domain of mouse M-CSFR (diluted 1:1,000; Upstate, Lake Placid, NY), a polyclonal reagent to the intracellular domain of M-CSFR (1:200; Santa Cruz Biotechnology, Santa Cruz, CA), a polyclonal reagent to human M-CSF (1:100; Santa Cruz Biotechnology), an antibody to phosphotyrosine (1:200; Sigma Chemical Co., St. Louis, MO), an F4/80 antibody (1:30; Harlan, Indianapolis, IN), an antibody to CD11b (5C6, 1:750; Serotec, Raleigh, NC), a polyclonal reagent to glial fibrillary acidic protein (GFAP) (1:1,000; a gift from Dr. L. Eng), an antibody to neurofilament proteins (SMI312, 1:1,000; Sternberger Monoclonals, Baltimore, MD), and a monoclonal reagent that reacts with all full-length A β species²⁸ (mAb 4.1; 1:150). For comparison, normal rabbit serum (Jackson ImmunoResearch, West Grove, PA) and normal goat serum (Zymed, South San Francisco, CA) were also used as primary antibodies. Secondary antibodies included Cy5-conjugated and Cy3-conjugated affinity-purified IgG reagents raised in goat, sheep, horse, or donkey and diluted to 1:1,000 (all from Jackson ImmunoResearch).

Immunocytochemistry

Free-floating sections were reacted with 10% normal blocking serum in TBS for 1 hour at room temperature, and then washed three times for 5 minutes each in TBS. The sections were then incubated with the primary antibody diluted in TBS at 4°C overnight with gentle shaking. Sections were then washed three times for 10 minutes each in TBS at room temperature. Secondary antibody (diluted in TBS) was applied to the sections and incubated at 37°C for 1 hour with gentle shaking. The sections were washed four times for 10 minutes each with PBS and twice with equilibration buffer (from SlowFade Anti-

fade kit; Molecular Probes, Eugene, OR) for 3 minutes. Sections were then mounted on glass slides with the anti-fade reagent in glycerol/PBS. The coverslips were sealed with nail polish and stored in the dark at 4°C. All images were obtained within 24 hours after immunostaining.

For double-label immunocytochemistry, sections were reacted with 10% normal blocking serum (for the first secondary antibody) in TBS at room temperature for 1 hour, and then washed three times for 5 minutes each in TBS. They were then incubated overnight with the first primary antibody at 4°C with gentle shaking. After washing in TBS three times for 10 minutes each, sections were reacted with the first secondary antibody diluted in TBS at 37°C for 1 hour with gentle shaking. Sections were then washed in TBS four times for 10 minutes each, and incubated with the second normal blocking serum (for the second secondary antibody) in TBS at room temperature for 1 hour. After washing in TBS three times for 5 minutes each, the sections were reacted with the second primary antibody diluted in serum overnight at 4°C with gentle shaking. Sections were washed three times in TBS for 10 minutes each, and then reacted with the second secondary antibody at 37°C for 1 hour. Finally, sections were washed in TBS four times for 10 minutes each and then mounted as described above. Immunohistochemical results illustrated below are representative of data obtained from a minimum of three transgenic or wild-type animals. For a given primary antibody, immunohistochemistry was performed on at least three sections per animal.

Confocal Microscopy

Images were collected on a Molecular Dynamics (Sunnyvale, CA) MultiProbe 2010 laser-scanning confocal microscope with an argon/krypton laser and a Nikon Diaphot 200 inverted microscope. The microscope was interfaced with a Silicon Graphics Indigo2 system (Mountain View, CA) running Molecular Dynamics ImageSpace software. The instrument was configured for excitation at 568 nm (Cy3 secondary antibody), or 647 and 568 nm (Cy5 and Cy3). For all images a 50- μ m pinhole aperture was used to produce true confocal images with maximum z axis resolution. Images were collected using $\times 10$ and $\times 20$ objectives or $\times 40$ and $\times 60$ oil immersion objectives. Contrast and hue were adjusted on some images using the ImageSpace software or Adobe Photoshop to provide optimal resolution of structures. However, in all cases adjustments were applied to the entire image and in no case was selective editing performed on a subregion of the image. Between two and 10 confocal images were collected from each section. A total of 603 confocal images of immunostained transgenic and wild-type sections were acquired during the course of the study.

Protein Extraction and Western Blotting

Brains were removed rapidly, the cortex and cerebellum separated from the rest of the brain by dissection, and then samples were frozen immediately in liquid nitrogen. Membrane protein extracts were prepared by chopping

samples with a razor blade, suspending in lysis buffer (50 mmol/L mannitol, 5 mmol/L Hepes, pH 7.4, 1 mmol/L phenylmethyl sulfonyl fluoride, 1 μ g/ml leupeptin, and 1 μ g/ml pepstatin A), followed by 10 passages through a 25-gauge needle, after which CaCl₂ was added with vortexing to a final concentration of 10 mmol/L. The resulting homogenate was centrifuged in a 1.5-ml tube at 15,600 $\times g$ for 1 minute. The supernatant was then centrifuged at 430,000 $\times g$ for 6 minutes in a TL-100 ultracentrifuge (Beckman, Palo Alto, CA). The resulting pellet was resuspended in lysis buffer. All protein extraction procedures were performed at 4°C using ice-cold reagents. Protein concentrations were determined using the bicinchoninic acid protein assay (Pierce, Rockford IL). For protein electrophoresis, the stacking gel was 5% sodium dodecyl sulfate-polyacrylamide, whereas the resolving gel was 8% sodium dodecyl sulfate-polyacrylamide. Five hundred μ g of membrane protein extract was loaded per lane. Proteins were transferred from gels to polyvinylidene difluoride membranes (Millipore, Bedford, MA), and immunodetection was performed by incubating membranes with 10% normal goat serum in 3% nonfat milk (w/v) in PBS (PBS-milk) at room temperature for 1 hour. After two washes with PBS, the membranes were incubated overnight with agitation at 4°C with the anti-serum against the extracellular domain of mouse M-CSFR in 3% PBS-milk (1:1,000; Upstate). Then the membranes were washed three times with PBS and incubated with goat anti-rabbit IgG linked to horseradish peroxidase in 3% PBS-milk (1:500; Santa Cruz Biotechnology) for 1.5 hours at room temperature with agitation. After three washes with PBS, bound antibody was detected with diaminobenzidine and hydrogen peroxide.

RNA Extraction

Frozen hemibrains were pulverized in liquid nitrogen and transferred to a 50-ml centrifuge tube along with Trizol reagent (1 ml per 100 mg tissue; Life Technologies, Inc., Gaithersburg, MD). Tissue was homogenized using a Polytron (Brinkman, Westbury, NY), and total RNA extraction was performed according to the manufacturer's instructions. The concentration of total RNA samples dissolved in RNase-free water was determined using a spectrophotometer.

RT-PCR

Methods for semiquantitative measurement of mRNA using RT-PCR have been previously described.²⁹ Briefly, total RNA was reverse-transcribed using Superscript II (Life Technologies, Inc.), primed by random hexamers. To equalize samples for RNA loading, the level of hypoxanthine phosphoribosyl transferase (HPRT) mRNA was determined in each sample as previously described,²⁹ and cDNA concentrations for subsequent PCR reactions were adjusted accordingly. For quantification of M-CSFR and M-CSF mRNA, the primers described by Raivich et al.³⁰ were used. For M-CSFR mRNA, after a 5-minute

denaturing incubation at 95°C, 35 cycles of PCR were performed in a Perkin-Elmer 9600 thermal cycler (Perkin-Elmer-Cetus, Emeryville, CA) consisting of 95°C for 45 seconds, 59°C for 45 seconds, and 72°C for 90 seconds, followed by an extension phase of 72°C for 5 minutes. For M-CSF mRNA, RT-PCR included a denaturing phase at 95°C for 5 minutes, then 30 cycles of 95°C for 45 seconds, 65°C for 45 seconds, 72°C for 1 minute and 30 seconds, and then a 5-minute 72°C extension phase. For HPRT mRNA, PCR was performed by denaturing at 96°C for 2 minutes, followed by 30 cycles of 94°C for 20 seconds, 60°C for 60 seconds, and 72°C for 2 minutes and 20 seconds.

To quantify PCR products, amplified DNA samples were transferred to a nylon membrane in a slot-blot apparatus and visualized using radiolabeled oligonucleotide probes as previously described.²⁹ For M-CSF mRNA detection, the probe was 5'-TCGGTGGCGTTAGCATTGGGGGTGT-3' (bases 464 to 488, GenBank MUSCSFA, antisense orientation). For M-CSFR mRNA detection, the probe was 5'-TTTATCTGTGGGGGCTCTGGGTGG-3' (bases 669 to 693, GenBank MMFMSCR, antisense orientation). The HPRT probe has been previously described.²⁹ Autoradiograms were quantified using a densitometer. To assure that PCR amplifications had occurred in the exponential phase of the reaction, serial dilutions of cDNA were performed and quantified for each PCR reaction as described.²⁹

Results

Immunocytochemistry

Antibodies to M-CSFR strongly labeled cells associated with A β deposits in hippocampus and cerebral cortex in sections from A β PP^{V717F} transgenic mice. Labeling with the antibody to the extracellular domain of mouse M-CSFR is shown in Figure 1A. Cells decorated with the extracellular M-CSFR reagent had the morphology of activated microglia (Figure 1C). In control brain this antibody reacted weakly with cells having the morphology of resting microglia (Figure 1B). The reagent to the intracellular domain of M-CSFR also labeled cells with the morphology of activated microglia in transgenic mouse brain (Figure 1D). No labeling was observed with the nonimmune sera. Likewise, the secondary antibodies showed no reactivity when the primary antibodies were omitted.

Double-labeling experiments with M-CSFR reagents and antibodies to established microglial markers^{31,32} demonstrated that the M-CSFR-reactive cells were indeed microglia. Thus, in transgenic mouse brain microglia labeled with M-CSFR reagents surrounding plaques were also labeled with CD11b, phosphotyrosine, and F4/80 antibodies (Figure 2, A–C). In contrast, the antibody to GFAP-labeled cells with the morphology of reactive astrocytes, but never cells decorated by the M-CSFR reagents (Figure 2, D and E). Similarly, in transgenic brain the antibody to neurofilament proteins reacted with dystrophic neurites, many of which were adjacent to

Figure 1. Overexpression of M-CSFR in $A\beta PP^{V717F}$ transgenic mice. **A:** Low-power confocal image of occipital cortex in a 13-month-old $A\beta PP^{V717F}$ transgenic mouse reacted with antibody to M-CSFR, showing aggregates of activated microglia. Scale bars on this and other images are in micrometers. **B:** Confocal image from corresponding region of a 13-month-old, wild-type animal, showing absence of strong M-CSFR expression. **Inset:** microglial cells in wild-type animal weakly labeled with M-CSFR antibody. **C:** High-power confocal image of activated microglial cells in hippocampal formation of a 13-month-old $A\beta PP^{V717F}$ mouse labeled with antibody to extracellular domain of M-CSFR. **D:** Confocal image from another 13-month-old $A\beta PP^{V717F}$ mouse showing activated microglia in the hippocampal formation labeled with antibody to the intracellular domain of M-CSFR.

M-CSFR-labeled cells. However, co-localization of neurofilament and M-CSFR labeling was not observed (Figure 3, A and B).

In the posterior hippocampal formation of transgenic animals, plaques tended to be large with numerous M-CSFR-positive microglia clustered around the central $A\beta$ core (Figure 3C). Dystrophic neurites labeled with the neurofilament antibody were found at approximately the same distance from the plaque core as the M-CSFR-labeled microglia (Figure 3B). GFAP-positive astrocytes were generally found outside of the ring of M-CSFR-

positive microglia (Figure 2, D and E). In the occipital cortex, $A\beta$ plaques tended to be small and compact. Here microglial cells intensely labeled with the M-CSFR antibody were often located at the center of the plaque, with multiple cells forming a cluster one on top of the other (Figure 3D). These aggregates of microglial cells resembled the cap-like microglial cells described in AD brain.³³ In 4-month-old animals, cells with the morphology of activated microglia were occasionally detected with the M-CSFR reagents (Figure 3E). These cells colocalized with $A\beta$ deposits (Figure 3F).

Figure 2. M-CSFR is expressed by microglia but not by astrocytes. **A:** Confocal image of hippocampal section from a 21-month-old A β PP^{V717F} mouse showing microglia co-labeled with antibodies to M-CSFR (red) and CD11b (green). **B:** Confocal image of microglia in hippocampal formation of a 20-month-old A β PP^{V717F} mouse labeled with M-CSFR antibody (red) and antibody to phosphotyrosine (green). **C:** Confocal image of microglia in cortex of a 21-month-old A β PP^{V717F} mouse decorated with M-CSFR antibody (red) and F4/80 antibody (green). In **A–C** yellow indicates areas of co-labeling. **D:** Low-power confocal image of hippocampal formation in a 20-month-old A β PP^{V717F} mouse showing plaques surrounded by reactive astrocytes (green; GFAP antiserum) and microglia labeled with M-CSFR reagent (red). **E:** Higher power confocal image of hippocampal plaques in 20-month-old A β PP^{V717F} mouse showing GFAP-labeled astrocytes (green) and microglia expressing M-CSFR (red).

Figure 4. M-CSF immunoreactivity in A β PP^{V717F} mice. **A:** Confocal image of plaque in posterior hippocampal formation of 14-month-old A β PP^{V717F} mouse reacted with reagent to M-CSF. Structures with the morphology of dystrophic neurites are strongly labeled. **B:** Low-power image of dorsal posterior cerebral cortex in a 14-month-old A β PP^{V717F} mouse reacted with antibody to M-CSF. **C:** Cortical field similar to that in **B** in a 13-month-old wild-type animal labeled with antibody to M-CSF. **D:** Confocal image of piriform cortex in 21-month-old A β PP^{V717F} mouse showing M-CSF-positive cells at higher magnification.

Structures with the morphology of dystrophic neurites were labeled by the M-CSF reagent in large plaques in the hippocampi of A β PP^{V717F} transgenic mice (Figure 4A), whereas no neuritic labeling was observed in the

wild-type animals. No labeling of plaque structures with a nonimmune serum was observed. The antibody to M-CSF also labeled cells with neuronal morphology in both A β PP^{V717F} transgenic mice and control animals. Figure 4,

Figure 3. No M-CSFR on dystrophic neurites; microglia overexpressing M-CSFR co-localize with A β . **A:** Low-power confocal image of posterior cerebral cortex in 19-month-old A β PP^{V717F} mouse showing microglia labeled with antibody to M-CSFR (red) and an antibody to neurofilament proteins (green). Note the absence of neuronal labeling by the M-CSFR antibody. **B:** Confocal image of plaque in posterior cortex of a 21-month-old A β PP^{V717F} mouse labeled with antibody to M-CSFR (red) and an antibody to neurofilament proteins (green). **C:** Confocal image of large plaques in the hippocampal formation of a 20-month-old A β PP^{V717F} mouse labeled with antibody to M-CSFR (red) and antibody to A β (green). The dense plaque core is not strongly labeled with A β reagent, as is often seen in AD brain. **D:** Confocal image of microglia in occipital cortex of a 20-month-old A β PP^{V717F} mouse labeled with antibody to M-CSFR (red) and antibody to A β (green). Here the microglia appear as aggregates closely associated with compact deposits of A β . **E:** Confocal image of cell with morphology of activated microglial cell in frontal cortex of a 4-month-old A β PP^{V717F} mouse labeled with reagent to M-CSFR. **F:** Same cell as in **E** but with antibody to A β visualized second channel (green). The yellow signal indicates co-localization of A β and M-CSFR.

Figure 5. Western blot and RT-PCR for M-CSFR and M-CSF. **A:** Western blot of membrane extracts from cerebral cortex and cerebellum from a 12-month-old wild-type animal (WT) and from a 12-month-old $A\beta PP^{V717F}$ mouse (Tg) reacted with antibody to M-CSFR. The mature form of the M-CSFR protein is shown at ~165 kD. The M-CSFR protein is increased in the cortex of the Tg animal in comparison with WT cortex. In contrast, there is no difference between Tg and WT animals in the level of cerebellar M-CSFR. **B:** Autoradiogram of slot-blot showing RT-PCR products detected with probes for M-CSFR (lanes 1 and 2), M-CSF (lanes 3 and 4), and HPRT (lanes 5 and 6). PCR using M-CSFR-, M-CSF-, and HPRT-specific primers was performed on cDNA derived from total brain RNA from a 4-month-old, wild-type animal (WT) and from a 4-month-old $A\beta PP^{V717F}$ mouse (Tg). Lane 1 shows WT cDNA dilutions of 1:80 to 1:320, whereas lane 2 shows identical cDNA dilutions for Tg cDNA. An increase in M-CSFR mRNA in the Tg brain is apparent. Lanes 3 and 4 show WT cDNA dilutions of 1:320 and 1:640, and Tg cDNA dilutions of 1:640 and 1:1280, demonstrating an increase in M-CSF mRNA because of the transgene. Lanes 5 and 6 show serial dilutions (1:20 to 1:320) for WT and Tg cDNA demonstrating equal levels of HPRT mRNA in WT and Tg samples. **C:** Autoradiogram of slot-blot showing RT-PCR products detected with probes for M-CSFR (lanes 1 and 2), M-CSF (lanes 3 and 4), and HPRT (lanes 5 and 6). PCR using M-CSFR-, M-CSF-, and HPRT-specific primers was performed on cDNA derived from total brain RNA from 12-month-old, wild-type animal (WT) and from a 12-month-old $A\beta PP^{V717F}$ mouse (Tg). Lane 1 shows WT cDNA dilutions of 1:160 and 1:320, whereas lane 2 shows identical cDNA dilutions for Tg cDNA. An increase in M-CSFR mRNA in the Tg brain is apparent. Lanes 3 and 4 show WT cDNA dilutions of 1:160 and 1:320, and Tg cDNA dilutions of 1:160 and 1:320, demonstrating an increase in M-CSF mRNA in the transgenic animal. Lanes 5 and 6 show serial dilutions (1:40 to 1:320) for WT and Tg cDNA demonstrating equal levels of HPRT mRNA in WT and Tg samples.

B and C, shows representative sections from transgenic and wild-type brains. There was no obvious difference in the intensity of immunostaining between the transgenic and control brains. Figure 4D shows the morphology of M-CSF-labeled cortical cells at higher power.

Immunoblotting

Immunoblots from 4- and 12-month-old $A\beta PP^{V717F}$ transgenic mice showed that M-CSFR protein was increased in membrane protein extracts from cerebral cortices in comparison with levels in wild-type samples. In contrast, there was no increase in M-CSFR expression in cerebellum in transgenic animals in comparison with wild-type cerebellar M-CSFR levels. The protein detected by the intracellular M-CSFR antibody corresponded to the mo-

lecular weight of the mature posttranslationally modified form (~165 kD) of M-CSFR. Results from 12-month-old wild-type and transgenic animals are shown in Figure 5A. Similar results were obtained with a second pair of 12-month-old animals.

RT-PCR

Semiquantitative RT-PCR using total RNA extracts from hemibrains showed an increase in the mRNA encoding M-CSFR in $A\beta PP^{V717F}$ animals at both 4 months (Figure 5B; 2.5-fold increase) and 12 months (Figure 5C; twofold increase) of age in comparison with wild-type animals. RT-PCR also showed an increase in M-CSF mRNA in total RNA extracts from 4- and 12-month-old $A\beta PP^{V717F}$ mice compared to wild-type animals (Figure 5, B and C; two-

fold increase at both ages). Similar results were obtained with a second pair of 12-month-old animals.

Discussion

These results demonstrate increased expression of the M-CSFR on microglia in transgenic A β PP^{V717F} mice. M-CSF was recently shown to augment the activating effects of A β on cultured microglia.¹⁵ Specifically, combined treatment with M-CSF and A β induced much larger increases in the production of interleukin-1, interleukin-6, and nitric oxide than did either agent alone. Increased expression of M-CSFR on microglia in transgenic A β PP^{V717F} mice could act synergistically with increased brain levels of M-CSF to powerfully augment A β -induced microglial activation. M-CSFR expression is increased in the AD brain, too.²³ Microglial activation in disease is thought to lead to production of potentially neurotoxic agents such as cytokines, nitric oxide, reactive oxygen species, and prostaglandins.³⁴

The close proximity of the M-CSFR-positive microglia to plaques suggests that aggregated A β could be the stimulus for increased M-CSFR expression. However, in this animal model A β deposits are but one change induced by the A β PP^{V717F} transgene. Other changes include increased expression of mutant A β PP, neuritic dystrophy, and astrogliosis.¹² The proximity of M-CSFR-reactive microglia to dystrophic neurites and reactive astrocytes in A β PP^{V717F} transgenic mice might also indicate that neurons and astrocytes play a role in inducing microglial M-CSFR expression.

We observed an increase in M-CSF mRNA and labeling of structures with the morphology of dystrophic neurites by an M-CSF antibody in the A β PP^{V717F} transgenic brain. These findings may be consistent with a report that M-CSF is increased in cerebrospinal fluid from patients with AD.¹⁸ Although we found that the M-CSF antibody labeled neuronal cell bodies, we did not observe an obvious increase in M-CSF immunostaining in the transgenic animals in comparison with controls. This finding is at variance with the observation of Du Yan et al¹⁸ that neuronal M-CSF immunoreactivity in the AD brain is markedly greater than that in control brain. Future studies using quantitative image analysis of M-CSF immunoreactivity in transgenic and wild-type brains might detect an increase in staining intensity in the transgenic neurons. Some reports have localized M-CSF expression to astroglia rather to neurons.^{16,17,35} We found no immunostaining of astrocytes with the M-CSF antibody. However, M-CSF exists in a variety of forms, including membrane bound, soluble, and bound to the extracellular matrix.³⁶ It is possible that the antibody we used did not detect the predominant form expressed by astrocytes. *In situ* hybridization studies of A β PP^{V717F} transgenic mice would contribute toward clarifying this issue. Regardless of the source of M-CSF in A β PP^{V717F} transgenic brain, increased expression of this cytokine and its receptor could act synergistically to activate microglia.

Extensive microglial activation has been reported in two other transgenic mouse models for AD characterized

by A β deposits in the brain.^{11,13} It will be interesting to determine whether expression of M-CSFR and/or M-CSF is altered in either of these two models. Increased M-CSFR expression by brain microglia has recently been observed in mouse mechanical injury and ischemia models.^{30,37} Indeed, it has been proposed that M-CSFR is a highly specific indicator of activation of all macrophage-like cells.³⁸ It is conceivable that a variety of neurological insults lead to activation of microglia via the M-CSF/M-CSFR system. Interestingly, mechanical injury resulted in a increase in M-CSFR expression but did not change the constitutive expression of M-CSF,³⁰ in contrast to our findings in the A β PP^{V717F} transgenic model. In the ischemia model, neuronal expression of M-CSF was detected near the lesion,³⁷ whereas in the mechanical injury model³⁰ and in the A β PP^{V717F} transgenic animals no neuronal M-CSFR expression was found. These contrasting results may reflect important underlying differences in the regulation of M-CSFR and its ligand among models of neurological disease.

Little is known about the specific signals that regulate M-CSFR expression in microglia. In other cell types, M-CSFR expression has been shown to be regulated by growth factors and cytokines such as heparin-binding epidermal growth factor-like growth factor, interleukin-10, and transforming growth factor- β .^{39–41} The neuritic plaque in AD is rich in a variety of growth factors and cytokines thought to be produced by neurons, astrocytes, and microglia.³⁴ One or more of these agents could induce M-CSFR expression by microglia, ultimately leading to an augmentation of the microglial activating effects of A β . Determining the signals for increased M-CSFR expression in AD and in animal models for AD may lead to new strategies for preventing the microglial-mediated neuronal injury in AD.

Acknowledgments

We thank Dr. Linda Higgins, Xiaorong Ou, and Dr. Susan Palmieri for their valuable assistance; and Lilly Neuroscience for kindly providing the A β PP^{V717F} transgenic mice.

References

1. Eikelenboom P, Rozemuller JM, van Muiswinkel FL: Inflammation and Alzheimer's disease: relationships between pathogenic mechanisms and clinical expression. *Exp Neurol* 1998, 154:89–98
2. Griffin WS, Sheng JG, Royston MC, Gentleman SM, McKenzie JE, Graham DI, Roberts GW, Mrak RE: Glial-neuronal interactions in Alzheimer's disease: the potential role of a 'cytokine cycle' in disease progression. *Brain Pathol* 1998, 8:65–72
3. McGeer PL, McGeer EG: Inflammation of the brain in Alzheimer's disease: implications for therapy. *J Leukoc Biol* 1999, 65:409–415
4. Gonzalez-Scarano F, Baltuch G: Microglia as mediators of inflammatory and degenerative diseases. *Annu Rev Neurosci* 1999, 22:219–240
5. Van Muiswinkel FL, Veerhuis R, Eikelenboom P: Amyloid beta protein primes cultured rat microglial cells for an enhanced phorbol 12-myristate 13-acetate-induced respiratory burst activity. *J Neurochem* 1996, 66:2468–2476
6. Meda L, Cassatella MA, Szendrei GI, Otvos Jr L, Baron P, Villalba M,

- Ferrari D, Rossi F: Activation of microglial cells by beta-amyloid protein and interferon-gamma. *Nature* 1995, 374:647-650
7. Li M, Sunamoto M, Ohnishi K, Ichimori Y: Beta-amyloid protein-dependent nitric oxide production from microglial cells and neurotoxicity. *Brain Res* 1996, 720:93-100
8. Giulian D, Haverkamp LJ, Yu J, Karshin W, Tom D, Li J, Kazanskaia A, Kirkpatrick J, Roher AE: The HHQK domain of beta-amyloid provides a structural basis for the immunopathology of Alzheimer's disease. *J Biol Chem* 1998, 273:29719-29726
9. Araujo DM, Cotman CW: Beta-amyloid stimulates glial cells *in vitro* to produce growth factors that accumulate in senile plaques in Alzheimer's disease. *Brain Res* 1992, 569:141-145
10. Bitting L, Naidu A, Cordell B, Murphy Jr GM: Beta-amyloid peptide secretion by a microglial cell line is induced by beta-amyloid-(25-35) and lipopolysaccharide. *J Biol Chem* 1996, 271:16084-16089
11. Frautschy SA, Yang F, Irrizarry M, Hyman B, Saido TC, Hsiao K, Cole GM: Microglial response to amyloid plaques in APPsw transgenic mice. *Am J Pathol* 1998, 152:307-317
12. Masliah E, Sisk A, Mallory M, Mucke L, Schenk D, Games D: Comparison of neurodegenerative pathology in transgenic mice overexpressing V717F beta-amyloid precursor protein and Alzheimer's disease. *J Neurosci* 1996, 16:5795-5811
13. Stalder M, Phinney A, Probst A, Sommer B, Staufenbiel M, Jucker M: Association of microglia with amyloid plaques in brains of APP23 transgenic mice. *Am J Pathol* 1999, 154:1673-1684
14. Yates SL, Burgess LH, Kocsis-Angle J, Antal JM, Dority MD, Embury PB, Piotrkowski AM, Brunden KR: Amyloid beta and amylin fibrils induce increases in proinflammatory cytokine and chemokine production by THP-1 cells and murine microglia. *J Neurochem* 2000, 74:1017-1025
15. Murphy Jr GM, Yang L, Cordell B: Macrophage colony-stimulating factor augments beta-amyloid-induced interleukin-1, interleukin-6, and nitric oxide production by microglial cells. *J Biol Chem* 1998, 273:20967-20971
16. Hao C, Guilbert LJ, Fedoroff S: Production of colony-stimulating factor-1 (CSF-1) by mouse astroglia *in vitro*. *J Neurosci Res* 1990, 27:314-323
17. Lee SC, Liu W, Roth P, Dickson DW, Berman JW, Brosnan CF: Macrophage colony-stimulating factor in human fetal astrocytes and microglia. Differential regulation by cytokines and lipopolysaccharide, and modulation of class II MHC on microglia. *J Immunol* 1993, 150:594-604
18. Du Yan S, Zhu H, Fu J, Yan SF, Roher A, Tourtellotte WW, Rajavashisth T, Chen X, Godman GC, Stern D, Schmidt AM: Amyloid-beta peptide-receptor for advanced glycation endproduct interaction elicits neuronal expression of macrophage-colony stimulating factor: a proinflammatory pathway in Alzheimer disease. *Proc Natl Acad Sci USA* 1997, 94:5296-5301
19. Kloss CU, Kreutzberg GW, Raivich G: Proliferation of ramified microglia on an astrocyte monolayer: characterization of stimulatory and inhibitory cytokines. *J Neurosci Res* 1997, 49:248-254
20. Suzumura A, Sawada M, Yamamoto H, Marunouchi T: Effects of colony stimulating factors on isolated microglia *in vitro*. *J Neuroimmunol* 1990, 30:111-120
21. Hamilton JA: CSF-1 signal transduction: what is of functional significance? *Immunol Today* 1997, 18:313-317
22. Sawada M, Suzumura A, Yamamoto H, Marunouchi T: Activation and proliferation of the isolated microglia by colony stimulating factor-1 and possible involvement of protein kinase C. *Brain Res* 1990, 509:119-124
23. Akiyama H, Nishimura T, Kondo H, Ikeda K, Hayashi Y, McGeer PL: Expression of the receptor for macrophage colony stimulating factor by brain microglia and its upregulation in brains of patients with Alzheimer's disease and amyotrophic lateral sclerosis. *Brain Res* 1994, 639:171-174
24. Goodwin JL, Uemura E, Cunnick JE: Microglial release of nitric oxide by the synergistic action of beta-amyloid and IFN-gamma. *Brain Res* 1995, 692:207-214
25. Lorton D, Kocsis JM, King L, Madden K, Brunden KR: Beta-amyloid induces increased release of interleukin-1 beta from lipopolysaccharide-activated human monocytes. *J Neuroimmunol* 1996, 67:21-29
26. Games D, Adams D, Alessandrini R, Barbour R, Berthelette P, Blackwell C, Carr T, Clemens J, Donaldson T, Gillespie F, et al: Alzheimer-type neuropathology in transgenic mice overexpressing V717F beta-amyloid precursor protein. *Nature* 1995, 373:523-527
27. Chen KS, Masliah E, Grajeda H, Guido T, Huang J, Khan K, Motter R, Soriano F, Games D: Neurodegenerative Alzheimer-like pathology in PDAPP 717V→F transgenic mice. *Prog Brain Res* 1998, 117:327-334
28. Murphy Jr GM, Forno LS, Higgins L, Scardina JM, Eng LF, Cordell B: Development of a monoclonal antibody specific for the COOH-terminal of beta-amyloid 1-42 and its immunohistochemical reactivity in Alzheimer's disease and related disorders. *Am J Pathol* 1994, 144:1082-1088
29. Murphy JR GM, Jia XC, Yu AC, Lee YL, Tinklenberg JR, Eng LF: Reverse transcription and polymerase chain reaction technique for quantification of mRNA in primary astrocyte cultures. *J Neurosci Res* 1993, 35:643-651
30. Raivich G, Haas S, Werner A, Klein MA, Kloss C, Kreutzberg GW: Regulation of MCSF receptors on microglia in the normal and injured mouse central nervous system: a quantitative immunofluorescence study using confocal laser microscopy. *J Comp Neurol* 1998, 395:342-358
31. Perry VH, Hume DA, Gordon S: Immunohistochemical localization of macrophages and microglia in the adult and developing mouse brain. *Neuroscience* 1985, 15:313-326
32. Tillotson ML, Wood JG: Phosphotyrosine antibodies specifically label ameboid microglia *in vitro* and ramified microglia *in vivo*. *Glia* 1989, 2:412-419
33. Wegiel J, Wisniewski HM: The complex of microglial cells and amyloid star in three-dimensional reconstruction. *Acta Neuropathol* 1990, 81:116-124
34. McGeer PL, McGeer EG: Mechanisms of cell death in Alzheimer disease—immunopathology. *J Neural Transm Suppl* 1998, 54:159-166
35. Calvo CF, Dobberty A, Gelman M, Glowinski J, Mallat M: Identification of CSF-1 as a brain macrophage migratory activity produced by astrocytes. *Glia* 1998, 24:180-186
36. Fixe P, Praloran V: Macrophage colony-stimulating-factor (M-CSF or CSF-1) and its receptor: structure-function relationships. *Eur Cytokine Netw* 1997, 8:125-136
37. Wang Y, Berezovska O, Fedoroff S: Expression of colony stimulating factor-1 receptor (CSF-1R) by CNS neurons in mice. *J Neurosci Res* 1999, 57:616-632
38. Chan J, Leenen PJ, Bertoncello I, Nishikawa SI, Hamilton JA: Macrophage lineage cells in inflammation: characterization by colony-stimulating factor-1 (CSF-1) receptor (c-fms), ER-MP58, and ER-MP20 (Ly-6C) expression. *Blood* 1998, 92:1423-1431
39. Inaba T, Ishibashi S, Harada K, Osuga J, Yagyu H, Ohashi K, Yazaki Y, Yamada N: Synergistic effects of transforming growth factor-beta on the expression of c-fms, macrophage colony-stimulating factor receptor gene, in vascular smooth muscle cells. *FEBS Lett* 1996, 399:207-210
40. Inaba T, Ishibashi S, Harada K, Ohsuga J, Ohashi K, Yagyu H, Yazaki Y, Higashiyama S, Kawata S, Matsuzawa Y, Yamada N: Induction of macrophage colony-stimulating factor receptor (c-fms) expression in vascular medial smooth muscle cells treated with heparin binding epidermal growth factor-like growth factor. *J Biol Chem* 1996, 271:24413-24417
41. Rieser C, Ramoner R, Bock G, Deo YM, Holtl L, Bartsch G, Thurnher M: Human monocyte-derived dendritic cells produce macrophage colony-stimulating factor: enhancement of c-fms expression by interleukin-10. *Eur J Immunol* 1998, 28:2283-2288