Elimination of the Class A Scavenger Receptor Does Not Affect Amyloid Plaque Formation or Neurodegeneration in Transgenic Mice Expressing Human Amyloid Protein Precursors

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The class A scavenger receptor (SR) is expressed on reactive microglia surrounding cerebral amyloid plaques in Alzheimer's disease (AD). Interactions between the SR and amyloid β peptides ($A\beta$) in microglial cultures elicit phagocytosis of $A\beta$ aggregates and release of neurotoxins. To assess the role of the SR in amyloid clearance and $A\beta$ -associated neurodegeneration *in vivo*, we used the platelet-derived growth factor promoter to express human amyloid protein precursors (hAPPs) in neurons of transgenic mice. With increasing age, hAPP mice develop AD-like amyloid plaques. We bred heterozygous hAPP (hAPP^{+/−}) mice that were wild type for SR (SR^{+/+}) with SR knockout (SR^{−/−}) mice. Crosses among the resulting hAPP^{+/−}SR^{+/−} offspring yielded hAPP^{+/−} and hAPP^{−/−} littermates that were SR^{+/+} or SR^{−/−}. These second-generation mice were analyzed at 6 and 12 months of age for extent of cerebral amyloid deposition and loss of synaptophysin-immunoreactive presynaptic terminals. hAPP^{−/−}SR^{−/−} mice showed no lack of SR expression, plaque formation, or synaptic degeneration, indicating that lack of SR expression does not result in significant accumulation of endogenous amyloidogenic or neurotoxic factors. In hAPP^{+/−} mice, ablation of SR expression did not alter number, extent, distribution, or age-dependent accumulation of plaques; nor did it affect synaptic degeneration. Our results do not support a critical pathogenic role for microglial SR expression in neurodegenerative alterations associated with cerebral β amyloidosis. (*Am J Pathol* 1999, 155:1741–1747)

Because of the increasing age of many populations around the world and the lack of an effective cure, Alzheimer's disease (AD) represents an increasing medical and socioeconomic problem.^{1,2} While the pathological hallmarks of the disease have been known for almost a century, the pathogenetic mechanisms by which amyloid plaques form and neurons are injured in AD remain poorly understood. Because amyloid plaque formation and $A\beta$ -associated degeneration of neuronal processes can be simulated in transgenic mice expressing mutant forms of the human amyloid protein precursor (hAPP),^{3–7} these models make it possible to test specific hypotheses regarding the etiology of these neuropathological alterations.

Recent pathological and cell culture studies have raised the possibility that the class A scavenger receptor (SR) may be causally involved in the pathogenesis of AD. The SR is a transmembrane protein expressed on mononuclear phagocytes that mediates the internalization and lysosomal degradation of a wide variety of molecules.^{8,9} Among the molecules that bind the SR are acetylated low-density lipoproteins (LDLs), oxidized LDLs, polyribonucleotides, dextran sulfate, and the $A\beta$ peptide, which appears to play a key role in AD pathogenesis.^{10–13} Histopathological analysis of AD brain tissue revealed prominent expression of the SR on reactive microglia surrounding amyloid plaques.¹⁴ In cell culture, the SR mediates the adhesion of microglia to surfaces coated with $A\beta$ fibrils and the microglial internalization of $A\beta$ microaggregates, raising the possibility that the SR is involved in the clearance of $A\beta$ from the brain.^{11,15} If the SR is indeed involved in $A\beta$ clearance *in vivo*, enhancement of its function might help diminish accumulation of amyloid plaques in patients with AD.

Further support for a potential role of the SR in neurodegeneration comes from studies showing that exposure to $A\beta$ leads to the activation of cultured microglia and to microglial release of neurotoxins, such as reactive oxy-

Supported in part by a grant from the U.S. Public Health Service (AG11385 to LM) and a Research Fellowship from the Howard Hughes Medical Institutes (to FH).

Accepted for publication July 7, 1999.

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gen species and neurotoxic amines.^{16,17} These effects may be mediated, at least in part, by interactions between A β and the SR.¹¹ If confirmed *in vivo*, such pathogenic A β -SR interactions might constitute a useful therapeutic target in AD.

Here we show that the elimination of microglial SR expression does not affect amyloid deposition or neurodegeneration in hAPP transgenic mice. These findings suggest that the pharmacological manipulation of the SR may not be an effective strategy for preventing plaque formation or A β -induced neurodegeneration *in vivo*.

Materials and Methods

Transgenic Mice

The generation of mice expressing hAPP from the platelet-derived growth (PDGF) factor B chain promoter has been described previously.³ The PDGF-hAPP line J9⁷ selected for this study expresses an alternatively spliced minigene,¹⁸ hAPP, bearing the amyloidogenic V717F¹⁹ and K670M/N671L²⁰ mutations that have been linked to familial AD (FAD). The line has been maintained by crossing heterozygous transgenic mice with nontransgenic (C57BL/6J $\times$ DBA/2) F1 breeders. The generation of SR^{-/-} mice on a C57BL/6 $\times$ ICR hybrid background has also been described.²¹ Compared with SR^{+/+} mice, SR^{-/-} mice have an increased susceptibility to infection by *Listeria monocytogenes* and herpes simplex virus type 1, but they show no obvious neurological or behavioral phenotype.²¹

Heterozygous hAPP transgenic mice (hAPP^{+/-}) were crossed with homozygous SR knockout (SR^{-/-}) mice, and the resulting offspring (hAPP^{+/-}SR^{+/-}) were intercrossed through brother-sister mating to obtain the genotypes used in this study. The resulting groups of littermates contained comparable random mixtures of the C57BL/6, DBA/2, and ICR strains. All mice used in this study were wild type for the mouse APP gene. Genomic DNA was extracted from tail biopsies and analyzed for the presence of the hAPP transgene and the endogenous SR gene, as outlined in Figure 1.

Quantitation of Amyloid Plaques

Mice were euthanized by transcardial saline perfusion under anesthesia with chloral hydrate. Brains were removed rapidly, drop-fixed in phosphate-buffered 4% paraformaldehyde at 4°C for 72 h, and sectioned with a vibratome at 40 μ m for neuropathological analysis. Plaques were quantitated by staining with thioflavin S or anti-A β antibody. For thioflavin-S staining, vibratome sections were prepared from murine brain tissue as described,^{3,22} air-dried overnight on Superfrost slides (Fisher), fixed to the slides with 4% paraformaldehyde in 0.1 M phosphate buffer, and stained with a 1% thioflavin-S solution for 8 min. Sections were rapidly washed once in 100% ethanol and twice in 80% ethanol/water, rinsed for 10 min with water, and mounted with Vectashield fluorescent mounting medium (Vector). Sections were then an-

Figure 1. Transgene constructs and polymerase chain reaction (PCR) analysis of genotypes. **(a)** PDGF-hAPP transgene and SR knockout strategy. The PDGF-hAPP transgene with which line J9 was generated contains the PDGF β -chain promoter, an alternatively spliced hAPP minigene encoding the amyloidogenic V717F and K670M/N671L mutations, and SV40 sequences providing a polyadenylation signal.^{7,18} The SR knockout was achieved by inserting the neomycin resistance gene (*Neo*) into an *EcoRI* site disrupting exon 4.²¹ Elements are not drawn to scale. The locations of primers for analysis of genotypes by PCR are indicated.

Primers:

- A (5'-GGCATGCGGATCGAGACATGATAAG-3')
- B (5'-GCTTTAAAAACCTCCACACCTCCCC-3')
- C (5'-AGAGAATCCAAAGCATTTCA-3')
- D (5'-TGAACATCAAGCAGTGTTCAT-3')
- E (5'-GCTCAGAAGAACTCGTCAAG-3')
- F (5'-CGAATATCATGGTGGAAAT-3')

(b) PCR analysis of genotypes. Tail tissues were digested with proteinase K, and genomic DNA was amplified by a touch-down PCR protocol.⁴⁸ Amplicons were separated electrophoretically on a 2% agarose gel and detected by staining with ethidium bromide. The PDGF-hAPP transgene was identified by amplifying the SV40 sequence at its 3' end. Amplification of DNA with GFAP primers (forward: 5'-GCGCGCTCGTGACACATTATCAGAC-3', reverse: 5'-CTGCCCTGACTTCCTGGAAGCAC-3') was used to ensure the presence of DNA and efficiency of the *Taq* polymerase reaction in samples without an SV40 amplicon (not shown). SR^{-/-} mice had a *Neo* amplicon but no SR amplicon, whereas the opposite was found in SR^{+/+} mice. SR^{+/-} mice (not analyzed in this study) had amplification of *Neo* and SR sequences. No band was produced with primers C and D (see **a**) from the SR knockout allele because amplification of larger DNA fragments was relatively ineffective under the PCR conditions used.

alyzed by fluorescence light microscopy, using a fluorescein isothiocyanate (FITC) filter. For each mouse, thioflavin-S-positive plaques were counted in 10 sections (spaced 240 μ m apart) from one hemibrain.

For A β antibody staining, free-floating vibratome sections were stained with a monoclonal antibody against A β (3D6; Elan Pharmaceuticals, South San Francisco, CA). A β -bound primary antibody was visualized with a FITC-labeled secondary antibody. Sections were analyzed by laser scanning confocal microscopy with a Bio-Rad MRC-1024 mounted on a Nikon Optiphot-2 microscope. Digitized images were transferred to a Macintosh computer and analyzed with Image 1.5 (public domain program of W. Rasband) to determine the average percentage of the hippocampal area occupied by 3D6-positive amyloid deposits in three hippocampal sections per mouse. A similar approach has been used previously to quantitate amyloid plaque load in diseased human brains.²³

Semiquantitative Assessment of Immunolabeled Presynaptic Terminals and Neuronal Dendrites

Immunolabeling of brain sections for synaptophysin (a marker of presynaptic terminals) and for microtubule-associated protein 2 (MAP-2) (a marker of neuronal cell bodies and dendrites), analysis of labeled sections with laser scanning confocal microscopy, and computer-aided semiquantitative analysis of confocal images were carried out essentially as described.^{22,24} Neuronal integrity was assessed in the outer molecular layer of the hippocampus. This brain region was chosen because it was previously found to exhibit neurodegenerative alterations in line J9 and other lines of FAD-mutant hAPP mice.^{3,7} Binding of primary antibodies (Boehringer Mannheim) was detected with an FITC-labeled secondary antibody (Vector). Sections were assigned code numbers to ensure objective assessment, and codes were not broken until the analysis was complete. For each mouse, we analyzed two brain sections per marker by laser scanning confocal microscopy and obtained four confocal images (two per section). Digitized images were transferred to a Macintosh computer and analyzed with Image 1.5. The density of MAP-2-immunoreactive dendrites or by synaptophysin-immunoreactive presynaptic terminals was determined and expressed as a percentage of the total image area as described.^{22,24}

Statistical Analysis

All quantitative results are expressed as means $\pm$ SEM. Differences between means were assessed by Mann-Whitney U test. Differences among multiple means were evaluated by analysis of variance followed by Dunnett's or Tukey-Kramer post hoc tests as appropriate. The null hypothesis was rejected at the 0.05 level. Analyses were made with Statview and SuperANOVA software (Abacus).

Results

Elimination of the SR in hAPP Transgenic Mice

Mutations linked to FAD increase the production of the amyloidogenic A β 42 peptide (see refs. 13 and 25 for reviews), and transgenic mice expressing FAD-mutant hAPPs develop amyloid plaques and neurodegenerative changes resembling in several respects those found in humans with AD.³⁻⁶ To test *in vivo* whether the SR is critical to amyloid plaque formation and neurodegeneration, we crossed FAD-mutant hAPP mice that show high levels of human A β expression⁷ with SR^{-/-} mice,²¹ as described in Materials and Methods. The following genotypes were selected (Figure 1) for histopathological analysis: hAPP^{+/+}-SR^{+/+}, hAPP^{+/+}-SR^{-/-}, hAPP^{-/-}-SR^{+/+}, and hAPP^{-/-}-SR^{-/-}.

Lack of the SR Does Not Affect Amyloid Plaque Deposition in hAPP Mice

Amyloid deposition in hAPP^{+/+}-SR^{+/+} mice begins around 6–8 months of age.^{3,7,26} To assess whether lack of SR impairs A β clearance and thereby accelerates amyloid deposition, we compared amyloid deposition in 6- and 12-month-old hAPP^{+/+} mice that were wild type or knockout for the SR. Thioflavin S staining identified no plaques in hAPP^{-/-} mice and revealed similar numbers and distributions of amyloid plaques in hAPP^{+/+}-SR^{+/+} and hAPP^{+/+}-SR^{-/-} mice (Figures 2, 3a, 3b, and 4), indicating that the absence of the SR did not accelerate or otherwise enhance amyloid deposition in hAPP^{+/+} mice.

Labeling of brain sections with the anti-A β antibody 3D6 and measurements of the hippocampal area occupied by A β -immunoreactive deposits confirmed the results of thioflavin-S staining: no significant difference was detected between hAPP^{+/+} mice with or without SR expression (Figure 3c). A substantial but comparable variability in plaque load was seen within the two groups of mice (Figure 3), consistent with observations at early stages of plaque formation in other lines of hAPP^{+/+}-SR^{+/+} mice.^{5,7,27}

The regional distribution and size of thioflavin S-stained plaques were also very similar in hAPP^{+/+} mice with or without SR expression (Figures 2 and 4). The plaque load was greatest in the hippocampus, and most callosal and neocortical plaques were found immediately overlying the hippocampus. No plaques were observed in the thalamus, cerebellum, or brainstem (data not shown).

Lack of the SR Does Not Affect Extent of Neurodegeneration in hAPP Mice

One of the best neuropathological correlates of cognitive deficits in AD is the loss of synaptophysin-immunoreactive presynaptic terminals in specific brain regions.²⁸⁻³¹ Increased expression of A β in PDGF-hAPP mice is also associated with a significant decrease in synaptophysin-immunoreactive presynaptic terminals in the outer molecular layer of the hippocampus and with more subtle decreases in MAP-2-immunoreactive neuronal dendrites.^{3,7} We therefore used confocal microscopy of immunolabeled brain sections and computer-aided image analysis to assess the integrity of presynaptic terminals and neuronal dendrites in the different groups of mice. This semiquantitative assessment of neurodegenerative alterations (see Materials and Methods for details) has been used successfully in diverse experimental models^{3,24,32} and in diseased human brains.^{22,33,34} It has also been validated previously by comparisons with quantitative immunoblots,³⁵ quantitations of synaptic proteins by enzyme-linked immunosorbent assay,^{32,36} and modifications of the stereological "disector" approach.³⁷

Compared with hAPP^{-/-}-SR^{+/+} controls, hAPP^{-/-}-SR^{-/-} mice had normal levels of synaptophysin-immunoreactive presynaptic terminals (Figure 5), suggesting that lack of the SR does not by itself result in abnormal central

Figure 2. Similarity of amyloid plaques in hAPP mice that are wild type or knockout for the SR. Sagittal hippocampal sections from 12-month-old mice were stained either with thioflavin S (**a**, **c**, **e**, and **g**) or with an anti- $A\beta$ antibody (3D6) (**b**, **d**, **f**, and **h**) and imaged by confocal microscopy as described in Materials and Methods. hAPP^{-/-} mice (**a–d**) had no amyloid plaques. The bright signal in the left lower portion of **d** represents a staining artifact. Comparable plaque deposition was found in SR^{+/+} and SR^{-/-} mice expressing hAPP/ $A\beta$ (**e–h**). Thioflavin S stains primarily β -pleated sheets characteristic of more mature amyloid deposits, whereas 3D6 also labels more diffuse $A\beta$ deposits, which accounts for the difference in staining patterns obtained with these two markers. Scale bar, 180 μ m.

nervous system (CNS) development or neurodegenerative alterations.

In contrast, hAPP^{+/-} mice with or without SR expression had significant losses of synaptophysin-immunoreactive presynaptic terminals in the hippocampal outer molecular layer, but the presence or absence of the SR in

these mice did not significantly affect the extent of neurodegeneration (Figure 5). The density of MAP-2-immunoreactive neuronal dendrites in the outer molecular layer of the hippocampus was also reduced in hAPP^{+/-} mice compared to hAPP^{-/-} controls ($P < 0.05$ by Tukey-Kramer post hoc test), with similar levels observed in

Figure 3. Comparable age-dependent accumulation of amyloid plaques in hAPP mice that are wild type or knockout for the SR. Thioflavin S-positive plaques were visualized by fluorescence microscopy and counted at 6 (**a**) and 12 (**b**) months of age as described in Materials and Methods. Each data point represents results from a different mouse. No statistically significant effect of SR genotype was identified on plaque number in hAPP mice at 6 or 12 months of age. At 12 months, the average number of plaques per section was 8.8 ± 3.0 (mean) and 8.3 (median) in hAPP^{+/+}SR^{-/-} mice compared with 11.3 ± 4.5 (mean) and 6.6 (median) in hAPP^{+/+}SR^{+/+} mice. **c:** The average area of hippocampus occupied by amyloid plaques at 12 months of age was assessed by staining three sections per animal with an anti-A β antibody (3D6). The hippocampal areas occupied by A β -positive deposits in hAPP^{+/+}SR^{+/+} mice (mean, $2.2 \pm 1.3\%$; median, 0.9%) and hAPP^{+/+}SR^{-/-} mice (mean, $1.6 \pm 0.6\%$; median, 0.9%) were not significantly different.

mice with or without SR expression (hAPP^{-/-}SR^{+/+} 37.5 ± 1.3 , hAPP^{-/-}SR^{-/-} 37.1 ± 1.3 , hAPP^{+/+}SR^{+/+} 31.8 ± 1.9 , hAPP^{+/+}SR^{-/-} 32.3 ± 1.6 ; values represent percent of image area occupied).

Discussion

Cell culture studies have implicated the SR in A β clearance and A β -induced neurotoxicity.^{11,15} Because both processes could play important roles in AD pathogenesis, pharmacological manipulation of the SR pathway

Figure 4. Regional distribution of amyloid plaques in hAPP mice is unaffected by the SR. Thioflavin S-positive plaques were counted in different brain regions of 12-month-old mice ($n = 8$ per genotype) as described in Materials and Methods. Data represent the average number of plaques per section. The majority of plaques were found in the hippocampus, and there were no statistically significant differences in plaque numbers between hAPP^{+/+}SR^{+/+} and hAPP^{+/+}SR^{-/-} mice for any of the brain regions analyzed.

might be considered for the development of better AD treatments. However, the present study demonstrates that ablation of the SR in transgenic mice expressing FAD-mutant forms of hAPP does not affect amyloid plaque formation or neurodegeneration *in vivo*.

Elimination of the SR did not significantly alter the type, extent, or distribution of amyloid plaques in the brains of hAPP transgenic mice at 6 or 12 months of age. These findings suggest that amyloid plaque formation, at least in these models, is not critically influenced by the SR. We cannot exclude the possibility that the high A β levels in the brain of hAPP mice saturate SR-mediated A β clearance processes, resulting in A β deposition regardless of whether the SR has been eliminated or not. However, the genetic manipulation of other molecules such as apoE³⁸ and transforming growth factor β ¹³⁹ in a similar hAPP model can drastically alter amyloid deposition. Taken together, these studies indicate that the SR plays either no or only a minor role in plaque formation in these models.

To our knowledge, this is the first study to examine the cerebral cytoarchitecture of SR^{-/-} mice. No differences between brains of SR^{-/-} and SR^{+/+} mice at 6 or 12 months of age were detected by inspection of hematoxylin/eosin-stained sections (data not shown) or by the detailed confocal microscopic analysis described above. Thus the lack of the SR does not appear to affect CNS development or result in the significant accumulation of neurotoxic endogenous ligands. Although the measurements of amyloid deposition and of immunolabeled neuronal processes used in this study are sensitive, we cannot exclude the possibility that other aspects of CNS integrity or pathology might be affected by the absence of the SR.

Eliminating SR expression did not significantly alter the extent of neurodegenerative changes in hAPP mice. This suggests that microglial SR expression is not essential to the development of A β -induced neurodegenerative alterations *in vivo*, at least not in these experimental models. It

Figure 5. Lack of SR expression does not prevent decrease in synaptophysin-immunoreactive presynaptic terminals in hippocampi of 12-month-old hAPP mice. **a:** Computer-aided image analysis was used to determine the density of synaptophysin-immunoreactive (SYN-IR) presynaptic terminals as described in Materials and Methods (seven or eight mice per genotype). SR^{-/-} mice without hAPP/A β expression had a normal density of immunolabeled presynaptic terminals compared with wild-type controls. In contrast, hAPP^{+/+} mice showed significant decreases in immunolabeled presynaptic terminals (** $P < 0.01$ versus nontransgenic mice by Dunnett's post hoc test (n.s.)). The difference between hAPP^{+/+} SR^{+/+} and hAPP^{+/+} SR^{-/-} mice was not statistically significant. **b:** Sections from hAPP^{+/+} mice with prominent decreases in SYN-IR presynaptic terminals are shown for illustration. Scale bar, 16 μ m.

is possible that A β injures neurons directly, rather than indirectly via microglial activation, an interpretation that is supported by the finding that fibrillar A β is toxic to neurons in cell culture, even in the absence of microglia.^{40,41} Another potential explanation is that A β induces neurotoxicity via alternative receptors.

Binding of A β to the receptor for advanced glycation end products (RAGEs) activates cultured microglia and triggers them to release neurotoxins,⁴² and neuronal expression of the p75 neurotrophin receptor potentiates A β -induced apoptosis in cultured neurons.⁴³ The LDL receptor-related protein (LRP), like the SR, is a lipoprotein receptor with broad ligand specificity. It binds apoli-

poprotein E-enriched lipoproteins and functions as a receptor for the proteinase inhibitors Nexin II (α -secreted hAPP751) and α_2 -macroglobulin.^{9,44} α_2 -Macroglobulin avidly binds the A β peptide^{45,46} and through interaction with LRP can mediate A β uptake.⁴⁷ The extent to which these and related pathways contribute to detrimental effects of A β in AD or to the physiological clearance of A β *in vivo* remains to be determined.

Acknowledgments

We thank Mr. S. Ordway and Dr. G. Howard for editorial assistance, Mr. J. Carroll for help with graphics, Ms. G.-Q. Yu and Ms. H. Ordanza for expert technical assistance, Dr. E. Masliah for advice on the histological analysis of amyloid plaques and neurodegeneration, and Ms. D. Murray for help in the preparation of the manuscript.

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