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Amyloid deposition is delayed in mice with targeted deletion of the serum amyloid P component gene

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The tissue amyloid deposits that characterize systemic amyloidosis, Alzheimer's disease and the transmissible spongiform encephalopathies always contain serum amyloid P component (SAP) bound to the amyloid fibrils. We have previously proposed that this normal plasma protein may contribute to amyloidogenesis by stabilizing the deposits. Here we show that the induction of reactive amyloidosis is retarded in mice with targeted deletion of the SAP gene. This first demonstration of the participation of SAP in pathogenesis of amyloidosis *in vivo* confirms that inhibition of SAP binding to amyloid fibrils is an attractive therapeutic target in a range of serious human diseases.

Amyloidosis is a disorder of protein metabolism in which normally soluble autologous proteins are deposited in the tissues as abnormal insoluble fibrils, which cause structural and functional disruption[1]. All the acquired and hereditary forms of systemic amyloidosis affecting the major viscera are usually fatal, and amyloid localized to the brain is associated with Alzheimer's disease and the transmissible spongiform encephalopathies (prion diseases). In each different type of amyloidosis the fibrils are derived from different proteins that share neither sequence homology nor similar native folds. However, the core structure of the fibrils is always the same and consists of a cross-β-sheet arrangement of polypeptide chains with their long axes perpendicular to the fiber's long axis[2–4]. This proteinase-resistant conformation is thought to underlie the persistence, and possibly also the pathogenicity, of amyloid fibrils *in vivo*, but another feature common to all types of amyloidosis is binding of the normal plasma protein, serum amyloid P component (SAP), to the fibrils[1,5]. The interaction is highly specific, and the abundance of SAP in amyloid deposits relative to its trace concentration in the plasma is remarkable.

It has lately been shown in several different types of systemic amyloidosis, that reduction in the supply of fibril precursor proteins arrests amyloid deposition and often leads to regression of established deposits, with corresponding clinical benefit[6]. Amyloid is thus in a state of dynamic turnover *in vivo*, but there is no specific treatment that itself promotes amyloid regression. We have previously proposed that the universal presence of SAP in amyloid, bound to the fibrils, may stabilize the deposits, and that inhibition of this binding could be a therapeutic option[7–10]. The evidence leading to this concept is, first, that the avid binding of SAP to amyloid fibrils[11] must be mutually stabilizing, purely on thermodynamic grounds. Second, the SAP in amyloid is intact and unaltered with respect to the circulating molecule[9], and this native structure may thereby mask the abnormal configuration of the fibrils. Third, SAP itself is highly resistant to proteolysis[12], especially when it forms complexes with its calcium-dependent ligands, and binding of SAP to amyloid fibrils *in vitro* protects them from proteolysis[10]. Fourth, SAP is catabolized *in vivo* only by hepatocytes[13], suggesting that other cells, especially the macrophages most likely to be responsible for amyloid fibril clearance, do not recognize or degrade it. Finally, although human plasma SAP levels are tightly controlled[14] and remain normal in patients with amyloidosis[15,16], in both mice and hamsters there is a strong positive correlation between circulating SAP concentrations and development of amyloidosis[17–19].

To investigate the role of SAP in amyloidogenesis *in vivo*, we have now generated SAP-deficient mice by targeted deletion of the SAP gene and have evaluated their development of reactive, systemic amyloid A (AA) amyloidosis in comparison with SAP-sufficient controls. Human AA amyloidosis is a serious and usually fatal complication of chronic inflammation; the amyloid fibrils are composed of amyloid A protein derived from the acute-phase reactant, serum amyloid A protein (SAA) (ref. 1). In mice, chronic inflammation elicited by repeated injections of casein[20] is associated with increased production of SAA (ref. 21), and AA amyloidosis is generally present in most animals after about 6 weeks. This is the most accessible experimental model of amyloidosis and closely resembles human AA amyloid disease.

SAP knockout mice

Mice in which the SAP gene had been eliminated by homologous recombination ($SAP^{-/-}$) (Fig. 1) had no circulating SAP, but developed normally and were fertile. Histology of the $SAP^{-/-}$ animals at up to 6 months of age was normal, and no other obvious abnormality has been observed up to 11 months. When immunized with isolated mouse SAP they produced specific, high-titer, precipitating anti-mouse SAP antibodies, confirming absence of the autologous antigen. $SAP^{+/-}$ heterozygotes expressed SAP constitutively and as an acute-phase reactant, but at significantly lower levels than wild-type $SAP^{+/+}$ animals (Table 1).

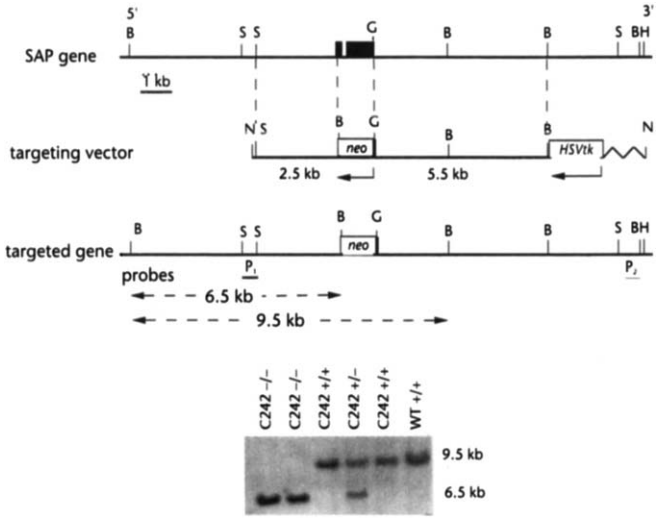

Fig. 1 Targeted disruption of the mouse SAP gene. *above*, The structure of the SAP gene, the targeting vector and the mutated allele. The coding regions are represented as filled boxes and the homologous fragments used in the targeting vector are delimited by dotted lines. Restriction enzymes used for cloning and Southern blot analysis were B, *Bam*HI; S, *Sph*I; G, *Bgl*II; H, *Hin*dIII; N, *Not*I. The double-headed arrows indicate sizes of fragments seen in Southern blot analysis of mutant and wild-type alleles after *Bam*HI digestion of genomic DNA hybridized to the 5′ probe, P1, outside the area of homologous recombination. *below*, Representative Southern blot of *Bam*HI-digested mouse tail DNA from offspring of a cross between animals that were heterozygotes for the disrupted SAP gene from clone 242. The wild-type SAP allele runs at 9.5 kb, and the disrupted allele at 6.5 kb, because of the *Bam*HI site in the neomycin resistance cassette.

Induction of AA amyloidosis with casein

Mice without the SAP gene ($SAP^{-/-}$) and control $SAP^{+/-}$ mice received daily injections of casein 5 days per week over a period of 66 days, and amyloid deposition was prospectively and serially monitored by quantitative scintigraphy with ^{123}I-labeled human SAP. This tracer binds specifically, but reversibly to amyloid fibrils *in vivo* and localizes on them in direct proportion to their quantity, in contrast to its prompt elimination in mice without amyloid deposits[22,23]. More than 1000 human clinical studies, and autopsy correlations in humans and mice, have validated serial use of this method to quantify amyloid *in vivo* in all phases of deposition and regression as well as in the steady state[23–25]. Serial *in vivo* monitoring of live mice, which has not been done before during the induction of amyloidosis, confirmed the expected pattern in the $SAP^{+/-}$ controls, with amyloid first appearing in some animals at day 37 and then progressively accumulating in all except one. However, deposition of amyloid was significantly delayed and reduced in the $SAP^{-/-}$ mice (Fig. 2).

Parallel cohorts of $SAP^{-/-}$ mice and $SAP^{+/-}$ controls received casein in an identical protocol, but were not monitored before day 53 in order to avoid introduction of extraneous SAP. The difference in whole-body amyloid load between the groups at this time point (Fig. 3*a*) was confirmed by quantification of amyloid in the viscera by using both ^{123}I-labeled SAP retention and histochemical staining with Congo red (Fig. 3, *b* and *c*). Eight of 11 $SAP^{+/-}$ mice had amyloid, 7 with massive deposits, compared with only 2 of 9 $SAP^{-/-}$ mice with just trace amounts ($P = 0.02$). However, the serum concentrations of SAA, the actual precursor of the AA amyloid fibril protein, measured at bleed-out, were the same in the $SAP^{-/-}$ and the $SAP^{+/-}$ mice: (mean ± s.d.) 17 ± 3 and 17 ± 8 mg/l, respectively.

Induction of AA amyloidosis with AEF and silver nitrate

In the casein model there is a long initial lag phase followed by rapid accumulation of AA amyloid in most animals and, even if inflammatory stimulation is halted before any amyloid appears, the mice remain primed for explosive amyloid deposition in the future. Very similar phenomena occur in human AA amyloidosis[1]. The lag phase reflects the generation of a potent activity known as amyloid-enhancing factor (AEF), which is present in all amyloidotic tissues, but has not yet been biochemically characterized[26]. Parenteral injection in mice of a minute quantity of amyloid tissue extract, together with the powerful inflammatory stimulus provided by a single injection of silver nitrate, induces AA amyloid deposition within 24–48 hours and rapid accumulation thereafter. When groups of ten $SAP^{-/-}$ and control $SAP^{+/-}$ mice received AEF and silver nitrate, they had similar amyloid deposits when killed on day 3. However, all AEF preparations contain SAP, and we therefore sought to determine whether this was required for the AEF activity. First, we investigated in $SAP^{+/+}$ wild-type mice whether amyloid fibrils formed *in vitro* from pure synthetic peptides had AEF activity, but we found no amyloid in the recipients, albeit after just a single silver nitrate injection, in contrast to a recent report[27], in which AEF activity was seen after several such injections. These synthetic fibrils were therefore not tested in $SAP^{-/-}$ mice. Second, we used immobilized sheep anti-mouse SAP antibodies to remove all the SAP from a standard AEF preparation, but this SAP-depleted material was fully active in $SAP^{-/-}$ mice stimulated with silver nitrate. SAP is thus not required for AEF activity.

Induction of AA amyloidosis with AEF and casein

Although AEF dramatically accelerates amyloid deposition, the difference between $SAP^{-/-}$ and control $SAP^{+/-}$ or $SAP^{+/+}$ animals was still seen when the mice received casein rather than silver nitrate after injection of SAP-depleted AEF (Fig. 4). This was not due to differences in SAA production between SAP-deficient and SAP-sufficient mice; their SAA levels at bleed-out were not significantly different: day 4, $SAP^{-/-}$ (mean ± s.d.) 112 ± 91 mg/l, median (range) 98 (19–281); $SAP^{+/+}$ 76 mg/l (38), 74 (15–145); day 16, $SAP^{-/-}$ 258 mg/l (182), 172 (64–529); $SAP^{+/-}$ 235 mg/l (158), 176 (78–515). However, prolonged casein stimulation with uninterrupted daily injections eventually induced comparable amounts of amyloid in $SAP^{-/-}$ and $SAP^{+/-}$ mice, even without administration of AEF, showing, as expected, that SAP is not essential for amyloidogenesis in this system.

Discussion

All known amyloid fibril proteins can be converted from the soluble to the fibrillar form *in vitro* in the absence of any other pro-

Table 1 Acute-phase SAP response in $SAP^{-/-}$, $SAP^{+/-}$ and $SAP^{+/+}$ mice

	Serum SAP concentration (mg/l) after 4 casein injections in 4 days			Serum SAP concentration (mg/l) after 8 casein injections in 10 days		
	Mean ± s.d.	Range	(*n*)	Mean ± s.d.	Range	(*n*)
$SAP^{-/-}$	0		(14)	0		(26)
$SAP^{+/-}$	66 ± 37	15–160	(24)	48 ± 39	4–140	(13)
$SAP^{+/+}$	165 ± 31	120–210	(13)	194 ± 104	40–460	(21)

Differences between $SAP^{+/-}$ and $SAP^{+/+}$ mice on day 4 and on day 10, $P < 0.0001$.

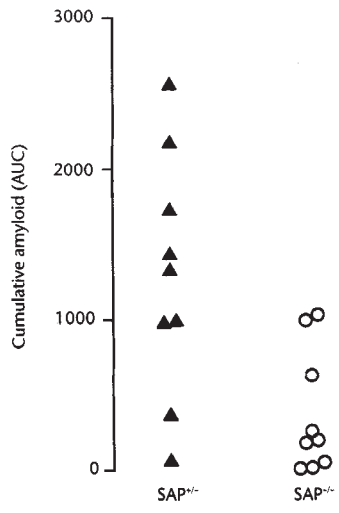

Fig. 2 Induction of AA amyloidosis in female SAP$^{-/-}$ and SAP$^{+/-}$ mice by repeated casein injection. Daily subcutaneous injections were given for 5 days per week from day 1 to 66. Amyloid deposition was quantified by whole-body scintigraphy 24 h after intravenous injection of tracer doses of ^{123}I-labeled human SAP on days 8, 22, 29, 36, 45, 50, 57 and 66. Retention of tracer above the background (<10%) in untreated mice without amyloid deposits was first detected on day 37. The cumulative amyloid load for each mouse from day 37 to day 67 inclusive was estimated from the area under the curve of tracer retention (corrected for background) plotted against time. The means of the two groups were significantly different, $P = 0.008$.

tein[28], and there are no clinical or experimental results suggesting that this process should require SAP *in vivo*. However, as indicated above, there is much circumstantial evidence that SAP contributes to the pathogenesis of amyloid deposition *in vivo*, and the present results strongly support this concept. Although the precise mechanisms involved are not yet clear, the appearance of AA amyloid deposits was delayed and their quantity reduced in SAP$^{-/-}$ mice compared with SAP$^{+/-}$ and SAP$^{+/+}$ animals, despite equal production of SAA.

It is notable that human SAP binds more avidly to amyloid fibrils *in vivo* than does mouse SAP (ref. 23), and for a given quantity of AA amyloid, there is up to 30 times more human SAP than mouse SAP in the respective deposits (unpublished observations) despite the lower plasma concentration of human SAP. The mouse AA amyloid model, in which more than 90% of controls develop deposits, reflects extreme stimulation compared with the clinical situation, where AA amyloidosis rarely occurs in more than 10% of those at risk[1]. Also when overproduction of SAA ceases, there is relatively much more rapid regression of murine than human AA amyloid[29,30]. The contribution of SAP to amyloid pathology may thus be even greater in humans than in the mouse.

The present findings therefore validate our strategy of seeking inhibitors of SAP binding for use as drugs[7–10] and, if they were effective, such agents would be applicable in all disorders in which amyloid is involved. SAP is present in amyloid deposits of all types, including those in Alzheimer's disease[31–33] and the prion diseases[34], and presumably contributes to their pathogenesis, as we have demonstrated here that it does in AA amyloidosis. If *in vivo* inhibition of SAP binding to amyloid fibrils retarded amyloid deposition, as observed here in the SAP$^{-/-}$ mice, and/or accelerated amyloid regression, it would clearly be of clinical benefit.

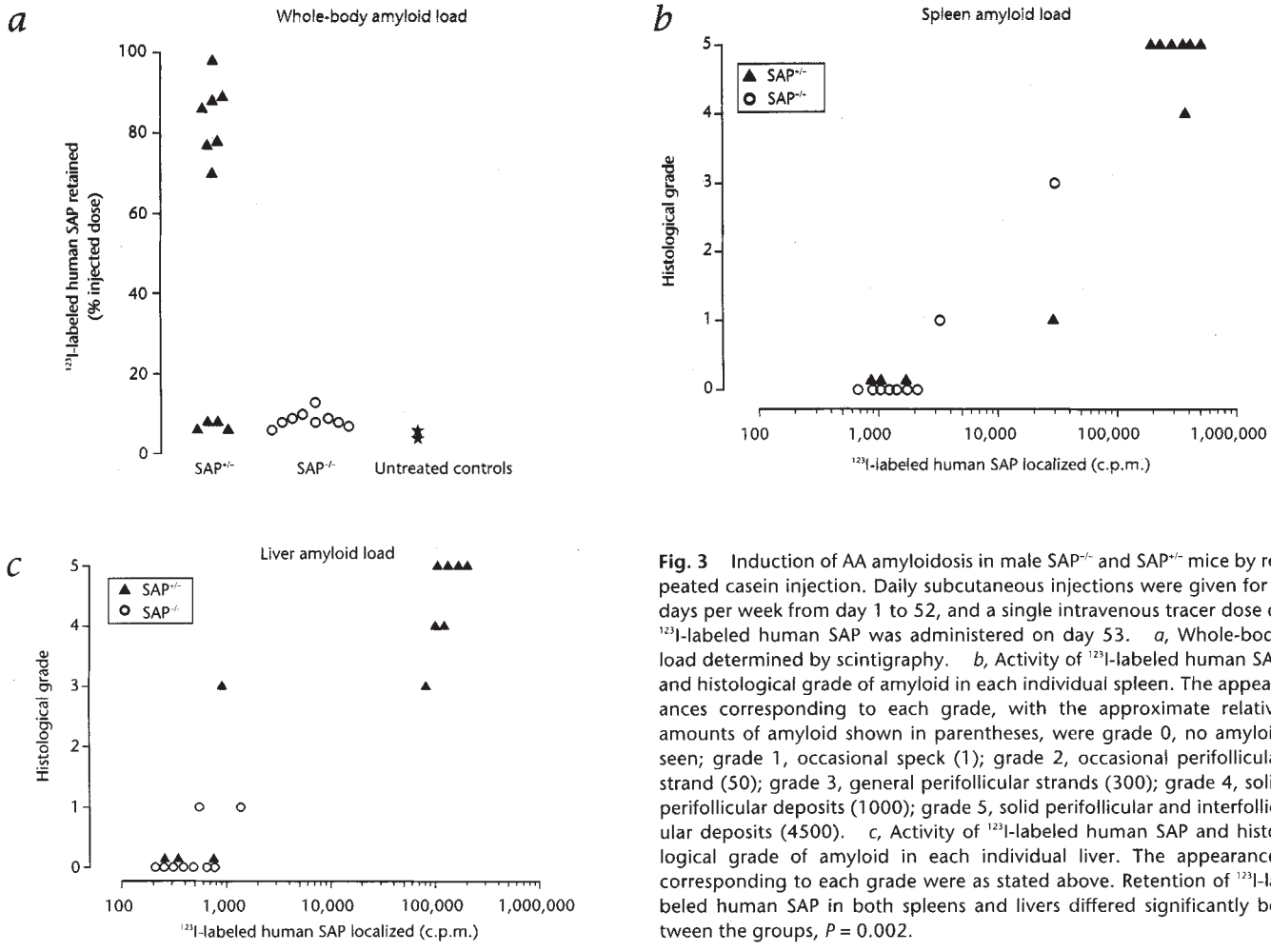

Fig. 3 Induction of AA amyloidosis in male SAP$^{-/-}$ and SAP$^{+/-}$ mice by repeated casein injection. Daily subcutaneous injections were given for 5 days per week from day 1 to 52, and a single intravenous tracer dose of ^{123}I-labeled human SAP was administered on day 53. *a*, Whole-body load determined by scintigraphy. *b*, Activity of ^{123}I-labeled human SAP and histological grade of amyloid in each individual spleen. The appearances corresponding to each grade, with the approximate relative amounts of amyloid shown in parentheses, were grade 0, no amyloid seen; grade 1, occasional speck (1); grade 2, occasional perifollicular strand (50); grade 3, general perifollicular strands (300); grade 4, solid perifollicular deposits (1000); grade 5, solid perifollicular and interfollicular deposits (4500). *c*, Activity of ^{123}I-labeled human SAP and histological grade of amyloid in each individual liver. The appearances corresponding to each grade were as stated above. Retention of ^{123}I-labeled human SAP in both spleens and livers differed significantly between the groups, $P = 0.002$.

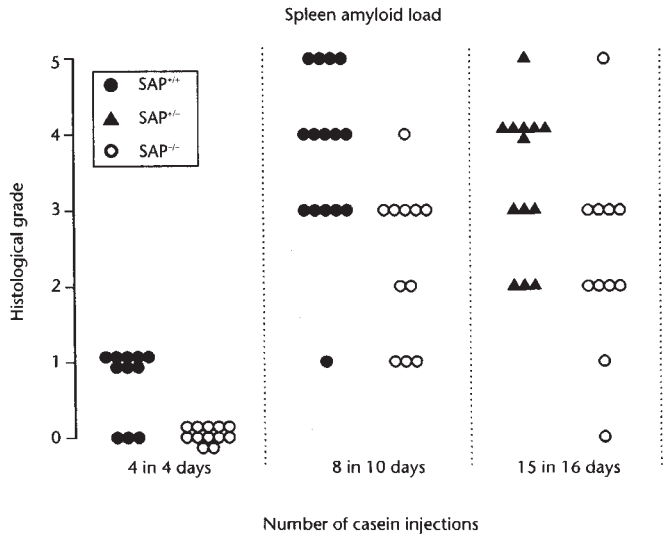

Fig. 4 Induction of AA amyloidosis in SAP$^{-/-}$, SAP$^{+/-}$ and SAP$^{+/+}$ mice with SAP-depleted AEF followed by subcutaneous casein injections. Groups of mice were killed at the times shown, and amyloid deposition in the spleens was graded histologically as indicated in Fig. 4. There was significantly less amyloid in the SAP$^{-/-}$ mice at each time point: day 4, $P = 0.037$; days 10 and 16, $P < 0.001$. On day 16 there was also a significant difference, $P < 0.001$, between the liver amyloid scores in the two groups (data not shown); before then only some mice had occasional traces.

For example, preservation of organ function in the systemic amyloidoses would be invaluable, while delaying the onset of clinical Alzheimer's disease by even a few years would have a major impact on the incidence of this condition[35]. It will therefore be of interest to investigate transgenic Alzheimer's disease models[36,37] and prion infections[38] in the SAP$^{-/-}$ mice, and such studies have lately been funded by the Medical Research Council and UK Department of Health. Finally, the absence, so far, of a deleterious phenotype in the SAP$^{-/-}$ mice suggests in an encouraging way that, despite the evolutionary conservation and presumably significant physiological function of SAP, blocking SAP binding *in vivo* may not have major adverse effects.

Methods

Gene targeting. The homologous recombination vector was constructed by replacing the whole mouse SAP gene with a neomycin resistance cassette (pMC1Neo-Poly(A)$^+$, Stratagene, La Jolla, CA) flanked by approximately 2.5- and 5.5-kb homologous 5′ and 3′ arms. The fragment was subcloned into a vector containing the herpes simplex virus thymidine kinase gene (*HSV-tk*) for positive and negative selection with G418 and ganciclovir. Transfection of the linearized plasmid into embryonic stem cells resulted in 1% homologous recombinants, as shown by Southern blot analysis of individual clones using two external probes (Fig. 1, P1 and P2) and the neomycin resistance gene. Four independent clones, karyotyped and microinjected into C57BL/6 blastocysts, generated chimeric mice that transmitted the targeted allele through the germ line. Homozygotes (SAP$^{-/-}$) for the deleted SAP gene, heterozygotes (SAP$^{+/+}$) and wild-type (SAP$^{+/+}$) mice were initially bred as (C57BL/6 × 129/sv)F_1 and the experiments shown used (C57BL/6 × 129/sv)F_2 mice.

Induction of amyloidosis. Mice received 0.5 ml of 10% (wt/vol) vitamin-free casein (ICN Pharmaceuticals, Cleveland, OH) in 0.1 M $NaHCO_3$ by subcutaneous injection daily for 5 or 7 days per week. For accelerated induction mice received a single, intravenous injection of glycerol-extracted AEF (ref. 39) on day 0 immediately followed by a single, subcutaneous injection of 0.5 ml of 2% (wt/vol) silver nitrate in water. Some of this AEF preparation, which contained 20 mg/l of SAP, was subjected to two separate batch absorptions each with a large excess of sheep anti-mouse SAP antibody immobilized on CNBr-Sepharose, though no SAP was detectable (assay sensitivity 1 mg/l) even after the first absorption. Injection of 0.1 ml of a 1:100 dilution of the SAP-depleted AEF, containing much less than 1 ng of SAP, retained full activity when used with silver nitrate in either SAP$^{+/-}$ or SAP$^{-/-}$ mice, and this dose was used in the limited casein injection experiments (Fig. 4). The amyloid induction results reported here in SAP$^{-/-}$ and control mice are representative of all our experiments, involving a total of 582 animals so far. There was no difference between mice of different ages or genders or those derived from any of the four separate clones of embryonic stem cells in which the SAP gene was deleted.

Quantification of amyloidosis. Tracer studies with ^{123}I-labeled human SAP and histochemical staining for amyloid were performed as previously described[23]. Histological grading of amyloid was performed "blind" by at least two independent expert observers in each experiment. Fig. 3 shows the close correlation between ^{123}I-labeled SAP retention and histological grade, but neither method provides a linear estimate. The tracer dose is limited, and once there is sufficient amyloid to bind more than 90% of the injected activity, neither whole-body retention nor organ counting can quantify more extensive deposits. Similarly the histological grading gives only a semiquantitative estimate, because the continuous focusing and polarization adjustments involved in scanning sections for Congo red-stained amyloid preclude quantitative image analysis. Nevertheless, grading is consistent within and between observers despite the very wide range in approximate relative amounts between trace or minor amyloid and massive deposits (Figs. 3 and 4).

Serum protein immunoassays. SAP was measured by electroimmunoassay with sheep anti-mouse SAP antiserum[40,41], and SAA by dot-blot immunoassay standardized with purified acute-phase mouse high-density lipoprotein and using rabbit anti-mouse SAA antibody (kindly provided by E. Benditt) that was extensively absorbed before use with normal mouse serum containing <3 mg/l of SAA, followed by affinity-purified peroxidase-labeled goat anti-rabbit IgG antibody absorbed before use with normal mouse serum.

Statistics. Two-tailed Student's *t*-tests were used to analyze cumulative amyloid load estimates (Fig. 2) (untransformed data) and organ localization of ^{123}I-labeled SAP (Fig. 3, *b* and *c*) (log-transformed data). Incidence of amyloid in different groups (Fig. 2, Fig. 4, day 4) was compared by chi-square test. The histological amyloid grades described in Fig. 3 were assigned consecutive integer values from 1 (no amyloid) to 6 (heaviest deposits) and were compared by Mann-Whitney U-test (Fig. 4, days 10 and 16).

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