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The Role of Fibronectin in the Development of Experimental Amyloidosis

Evidence of Immunohistochemical Codistribution and Binding Property with Serum Amyloid Protein A

Ei Kawahara, Masahiro Shiroo,* Isao Nakanishi, and Shunsuke Migita*

From the Department of Pathology, School of Medicine, and the Department of Molecular Immunology, Cancer Research Institute, Kanazawa University, Kanazawa, Japan*

Azocasein-induced amyloid A (AA) amyloidosis in CBA/K1Jms mice was investigated to elucidate a preference of serum amyloid A (SAA) deposition in the spleen. By indirect immunofluorescence using anti-SAA/AA antibodies the initial deposition of SAA/AA was recognized in the marginal zone of spleen at 20 days after azocasein injection. Indirect immunofluorescence using anti-fibronectin antibodies also showed meshwork positivity in the corresponding area more intensely than that in controls. Immunoelectron microscopy using anti-SAA/AA revealed the presence of positively stained flocculent materials on cell surfaces of macrophages in the marginal area in addition to amyloid fibril. The tissue fibronectin rapidly increased in the spleen and maintained 10 times more than that of controls until the 20th day. Binding assay of SAA on frozen sections revealed the presence of SAA-binding substances in the perifollicular area. Affinity chromatographic assay showed fibronectin have a binding capacity to SAA1 and SAA2. By binding assay on the microtiter plate, SAA had more affinity to fibronectin than those of heparan sulfate, collagen type I, or serum amyloid P component. These results indicate that fibronectin plays an important role in the development of amyloidosis by working as a linking protein between SAA and the cell surface of macrophages. (Am J Pathol 1989, 134:1305–1314)

It is well known that in systemic amyloidosis there are preferential sites of amyloid deposits during the early development. Also, in experimental amyloidosis of mice, amyloid fibrils are always detected in specific locations,[1,2] such as in Disse's space of the liver, in mesangial area of glomeruli in the kidney, and in the marginal zone of the splenic follicle. Why does amyloid protein deposit in such areas? There may be a specific binding protein to amyloid precursor that is interposed in the tissue during early development of amyloidosis.

Serum amyloid A protein (SAA) synthesized in the liver is a precursor of amyloid A (AA) fibril in the tissue.[3,4] It has been reported that several serine proteinases are capable of degrading SAA,[5] and in *in vitro* experiments AA is formed as a result of partial degradation of SAA on the cell surface of macrophages, where the activity of such proteinases could be abolished by proteinase inhibitors.[6,7] In a previous report[7] we clarified that degradation of SAA occurs not in the serum but in the tissue, probably around the cell surfaces of tissue macrophages. The mechanism of SAA deposition in the tissue, however, remains unclear. Hypothetically, SAA binds to one of extracellular matrix components in the specific sites where SAA is partially degraded. Fibronectin seems to be a candidate for such substances specifically bound to SAA because it has various specific binding domains to several proteins[8,9] and it increases particularly in the acute phase of tissue reactions.[10–12] Furthermore, immunohistochemical studies,[12,13] show that fibronectin is accumulated in the tissues in which SAA is usually entrapped. Thus, the close topographic relation between fibronectin and SAA led us to study the role of fibronectin in amyloidogenesis.

This study reveals codistribution of fibronectin and SAA in the early stage of experimental amyloidosis and a strong binding capacity of fibronectin to SAA is demonstrated.

Materials and Methods

Materials

CBA/K1Jms mice (provided by Medical Research Institute, Tokyo University) aged 2 months were bred under

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Address reprint requests to Ei Kawahara, MD, Department of Pathology, School of Medicine, Kanazawa University, 13-1 Takaramachi, Kanazawa, Ishikawa 920, Japan.

conventional conditions, and fed Sankyo FI chow and water *ad libitum*. AA amyloidosis was induced by daily subcutaneous injection of 0.5 ml of 10% azocasein.[7] The spleen tissues were examined during the experimental period at the 4, 8, 12, 16, 20, and 90th day of azocasein injection.

Preparation of Fibronectin and Its Antibody

Rat, human, and mouse plasma fibronectin was purified by affinity chromatography.[9,14] The affinity gels used were gelatin-sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) and heparin-sepharose 4B. Porcine heparin (Sigma Chemical Co., St. Louis, MO) was coupled to CNBr-activated sepharose 4B according to manufacture's instruction (Pharmacia). Pooled plasma anticoagulated by citrate was applied to the gelatin-sepharose column. The bound fibronectin was then eluted with 4 M urea in 0.02 M phosphate-buffered saline (PBS) at pH 7.4. The pooled protein fractions were applied to a heparin-sepharose column that had been preequilibrated with the 4 M urea solution, and then the fibronectin was eluted with 0.5 M NaCl in 0.02 M phosphate buffer (pH 7.4). The protein peak was determined by absorbance at 280 nm. The peak fractions were pooled and dialyzed against PBS.

Antibodies against the purified rat plasma fibronectin, which were raised in a rabbit, were obtained after being purified by affinity chromatography consisting of CNBr-activated sepharose 4B coupled with purified rat plasma fibronectin. Unbound materials to the gels were removed with PBS and bound antibodies were eluted with 0.2 M glycine-HCl buffer (pH 2.8). Purified antibodies were immediately dialyzed against PBS. Specificity of affinity-purified antibodies was checked by Western blotting.

Monoclonal Antibody to SAA/AA

Rat anti-murine SAA/AA monoclonal antibodies used in this study had been described and characterized previously.[7] MSA 4-26 antibodies show high affinity to SAA and AA of the mouse.

Localization of SAA and Fibronectin

Indirect immunofluorescence was used to assess immunolocalization of fibronectin and SAA/AA. Frozen sections of the spleens were fixed with 95% ethanol for 10 minutes. Sections were treated with the primary antibodies diluted in PBS for 90 minutes and subsequently with fluorescein isothiocyanate (FITC)-conjugated secondary antibodies for 90 minutes with a rinse in PBS. For the primary antibodies, rabbit anti-rat fibronectin affinity-purified antibody and rat anti-murine SAA/AA monoclonal antibody were used. As the secondary antibodies, FITC-conjugated goat anti-rat IgG (Cappel, Malvern, PA) and FITC-conjugated goat anti-rabbit IgG (Cappel) were used. FITC-conjugated anti-rat IgG was used after the following treatment: FITC-conjugated goat anti-rat IgG purchased from Cappel was mixed with 1 to 10 volumes of normal murine serum, incubated overnight at 4 C, and centrifuged at 10,000*g* to avoid cross reactivity to murine IgG.[15]

For double immunofluorescence for both fibronectin and SAA/AA, rat anti-murine SAA/AA, FITC-conjugated goat anti-rat IgG, rabbit anti-rat fibronectin, and rhodamine isothiocyanate (RITC)-conjugated goat anti-rabbit IgG (Cappel) were incubated on a single frozen section in this order. Immunohistochemical controls consisted of rat anti-murine SAA/AA, FITC-conjugated goat anti-rat IgG, and RITC-conjugated goat anti-rabbit IgG, and another FITC-conjugated goat anti-rat IgG, rabbit anti-rat fibronectin, and RITC-conjugated goat anti-rabbit IgG.

Immunoelectron Microscopy

Indirect immunoperoxidase methods were performed to investigate ultrastructural localization of SAA/AA as described previously.[15] Frozen sections fixed with periodate-lysine-paraformaldehyde were reacted with anti-SAA/AA antibodies followed by peroxidase-conjugated anti-rat IgG (Cappel). The sections were osmicated with 2% osmium tetroxide, dehydrated in graded ethanol, and embedded in Epon 812. Ultrathin sections were not counterstained.

Quantitation of Tissue Fibronectin

Tissue fibronectin was extracted by using the modified method of Isemura et al.[16] Spleen tissues were homogenized at 4 C in 4 volumes of PBS containing protease inhibitors (5 mM ethylene-diamine-tetraacetic acid, 1 mM phenylmethylsulfonyl fluoride, and 20 mM ε-aminocaproic acid). Homogenized tissues were centrifuged at 3000 rpm, and the precipitates were suspended in 4 volumes of the buffer described above. After these procedures were repeated four times, the precipitates were suspended in an equal volume of 8 M urea in PBS containing 1 M NaCl, 2% sodium dodecyl sulfate (SDS), and protease inhibitors. The suspension was stirred for 1 hour and the supernatant was centrifuged at 3000 rpm and used in this experiment.

Tissue fibronectin extracted from these supernatants with 4 M urea was measured by a modified Vuento's method[17] of competitive enzyme-linked immunosorbent assay (competitive ELISA). Polyvinyl microtiter plates (200 μl/well Costar, Cambridge, MA) were coated by adding

100 μl of 5 μg/ml purified rat fibronectin in 0.1 M sodium carbonate-bicarbonate buffer (pH 9.8) to the wells. After incubation for 1 hour at 37 C, the wells were washed three times with the washing solution (PBS/0.05% Tween 20). Residual binding sites were blocked by incubation for 1 hour with 1% bovine serum albumin. After washing three times, 50 μl of standard fibronectin or unknown samples diluted serially in 4 M urea in PBS were mixed with 50 μl of anti-fibronectin antibodies in PBS/Tween 20 in the wells. After 2 hours incubation at 37 C, and three washings, 100 μl of alkaline phosphatase-labeled anti-rabbit IgG (Cappel) diluted at 1:1000 was applied in the wells to detect fibronectin. This was done by incubating the plates at 37 C with 100 μl of 0.1% paranitrophenyl phosphate in 10% diethanolamine. An absorbance at 405 nm was measured.

Preparation of Samples Containing SAA

Sera or high-density lipoprotein (HDL) rich in soluble SAA were used in the following experiment because purified SAA is insoluble in neutral buffer. The sera rich in SAA (SAA-serum) were obtained from CBA/K1Jms mice 4 days after subcutaneous injections of azocasein. HDL_3 fraction was obtained by sequential ultracentrifugation from the sera.[3] SAA1 and SAA2 in those samples were separated by 13% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) containing 6 M urea,[18] and they were detected by Coomasie blue staining and by Western blotting using anti SAA/AA antibodies. Western blotting was performed using an avidin-biotin complex method described previously.[15] These samples contained SAA1 and SAA2 almost equally by Western blotting.

Assay for Binding Material to SAA on Frozen Section

To clarify the presence of SAA-binding material, frozen sections of splenic tissues were fixed with 95% ethanol for 10 minutes, and HDL-SAA was applied *in vitro* on them. After extensive washings with PBS, SAA bound on the sections was detected by indirect immunofluorescence using anti-SAA antibody.

Affinity Chromatographic Assay for Binding of SAA to Fibronectin

One hundred microliters of SAA-serum was mixed with 1 ml of fibronectin-sepharose (3 mg of rat plasma fibronectin coupled to 4 ml of CNBr-activated sepharose 4B) pre-equilibrated in PBS, and stirred overnight at 4 C. After washing with 100 ml of PBS in the column, bound proteins were eluted with 6 M urea in PBS. The protein fractions of eluate were detected by SDS-PAGE followed by Coomasie blue staining, and Western blotting using anti-SAA/AA antibodies.

Assay for Binding of SAA on the Microtiter Plate

Three assay systems using ELISA[19,20] were employed to determine the binding capacity of SAA to several proteins. In the first, microtiter plates were coated with 100 μl (10 μg/ml) of human plasma fibronectin, bovine heparan sulfate (Sigma), or soluble collagen type I purified from human placenta[21] in PBS and incubated at 37 C for 90 minutes. The wells were washed with PBS/Tween 20 three times, and then blocked with 1% BSA for 90 minutes at 37 C. HDL-SAA serially diluted in PBS was allowed to react with each substance bound to the solid phase at 20 C for 90 minutes, and after washing, bound SAA was detected by a monoclonal antibody against SAA incubated at 20 C for 90 minutes and subsequent alkaline-phosphatase-conjugated goat anti-rat IgG (Cappel) antibody reacted at 37 C for 20 minutes. After washing, final quantitation was made using paranitrophenyl phosphate. The second and third assay systems were applied for murine plasma fibronectin, murine serum amyloid P component (SAP), and murine serum albumin[22] with same procedures as in the first system, and calcium ion was added in the third as follows: 1 mM $CaCl_2$ in 0.02 M TRIS-buffered saline (pH 7.4) was used instead of PBS for dilution of coating substances, BSA, HDL-SAA, primary and secondary antibodies and in washings, because SAP was expected to have calcium-dependent ligand binding properties.[23] SAP used in the present study was extracted from pooled sera of lipopolysaccharide-injected mice by affinity chromatography with agar gel particle,[24] and further purified by anti-SAA antibody-bound Sepharose 4B to remove a small amount of contaminated SAA.

Results

Localization of SAA/AA

There were no discernible changes in the splenic tissue until 16 days after azocasein injection in this experimental run of 2-month-old CBA/K1Jms mice. Amorphous eosinophilic deposits were first recognized in the marginal zone of the follicle at the 20th day by conventional histologic examinations (Figure 1A). Scattered macrophages were found associated with the amorphous materials. Congo red staining on the corresponding sections exhibited fine fibrillary green birefringence in the amorphous materials when viewed with polarized light (Figure 1B).

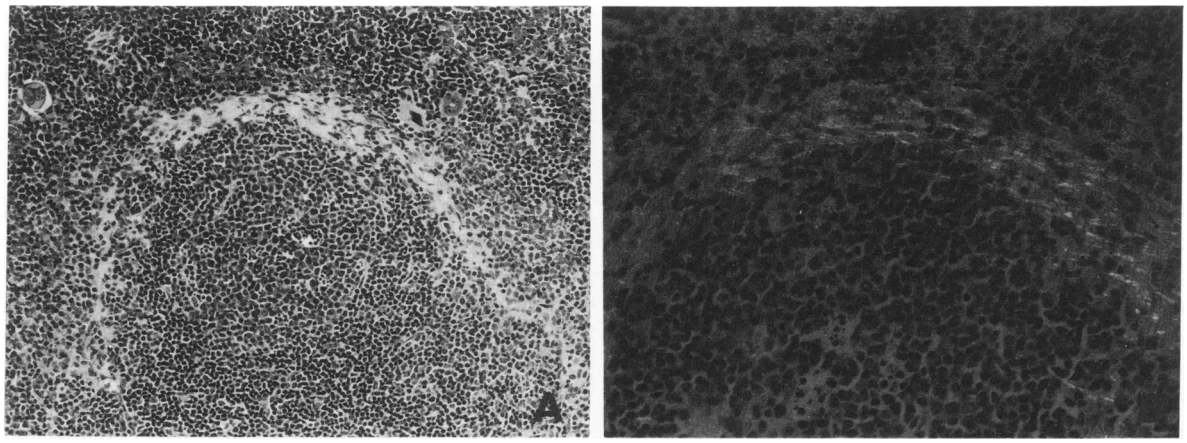


Figure 1. *Histology of the splenic tissue at the 20th day of azocasein injection.* **A:** *A hematoxylin and eosin-stained (H & E) section showing eosinophilic amorphous materials that appear initially in the current study in the marginal zone of the follicle (×130).* **B:** *A Congo red-stained section showing faint fibrillary birefringence in the amorphous materials (×260).*

An immunohistochemical examination using anti-SAA/AA antibodies also showed no immunofluorescence detectable in the splenic tissue before the 20th day, when the crescent shaped immunoreaction was detected in the marginal zone of the follicles (Figure 2A). On the 90th day, SAA/AA deposits became extensive and crystalline-like in configuration (Figure 3A).

Ultrastructural immunolocalization of SAA/AA was investigated on the tissue section at the 20th day after azocasein injection. The immunoreactive products detected were largely associated with two structures in the intercellular space between macrophages. The first was the small aggregates of microfibrils about 10 nm in diameter, which were compatible with usual amyloid fibrils (Figure 4A). The second reactive structure was the flocculent material lying on the outer cell membrane of macrophages in the marginal zone in which cytoplasmic processes of macrophages were interdigitated with each other (Figure 4B). Thus, the flocculent materials may represent extracellular deposits of SAA or SAA-intermediates before AA protein resulting in amyloid fibrils.

Localization of Fibronectin

Tissue fibronectin in the normal spleen was observed faintly in the perifollicular area by an immunofluorescent examination. At the fourth experimental day, the immunofluorescence of fibronectin became increased intensely in the perifollicular marginal area and it extended in the intercellular tissue spaces along pericellular or perivascular spaces (Figure 5A). The fluorescent positivity showed a meshwork pattern, probably due to the deposits of fibronectin chiefly around the macrophages (Figure 5B). However, the fluorescence was sparse in germinal centers. In the perivascular area strong positivity also was noted. The positivity and localization of fibronectin were unchanged until the 16th day. At the 20th day, when amyloid deposits were initially detected, focal densities were accentuated in the marginal zone of the follicles (Figure 2B). The distribution pattern was identical with that of immunofluorescence of SAA/AA (Figure 2A, B). On the 90th day immunoreactivities for fibronectin and SAA/AA

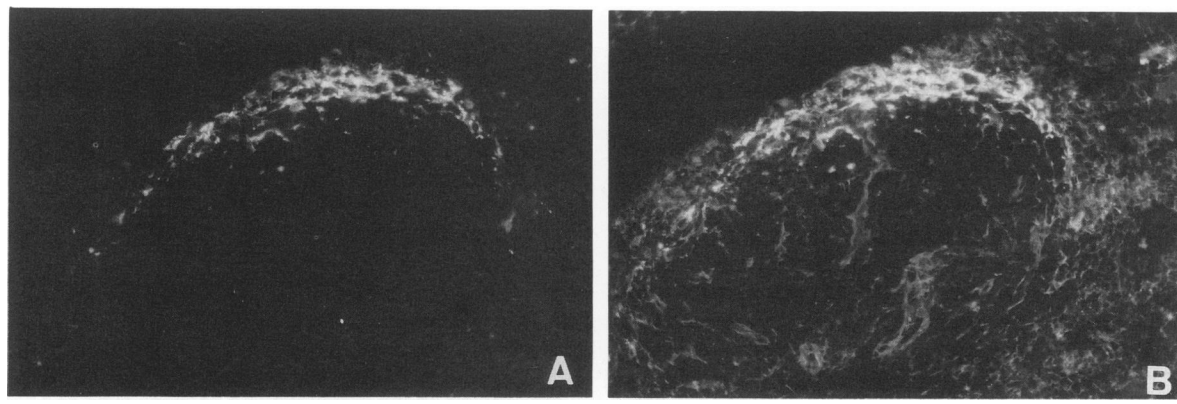


Figure 2. *Double immunofluorescence for SAA/AA and fibronectin on a frozen section of the 20th day.* **A:** *SAA/AA appearing in the marginal zone.* **B:** *Fibronectin detected in meshwork pattern in the marginal zone and perivascular area of a follicle. Note a focal density of marginal zone coincides with that of SAA (A and B, ×130).*

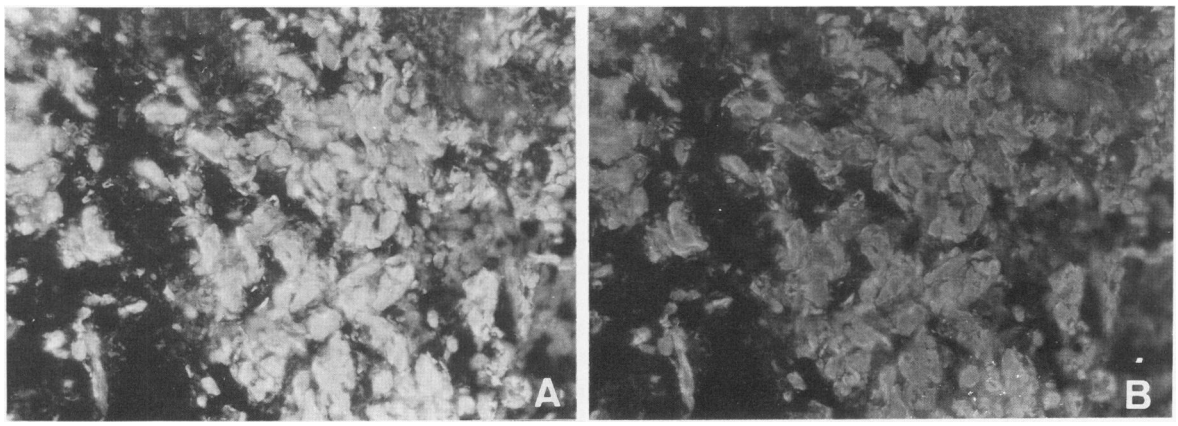
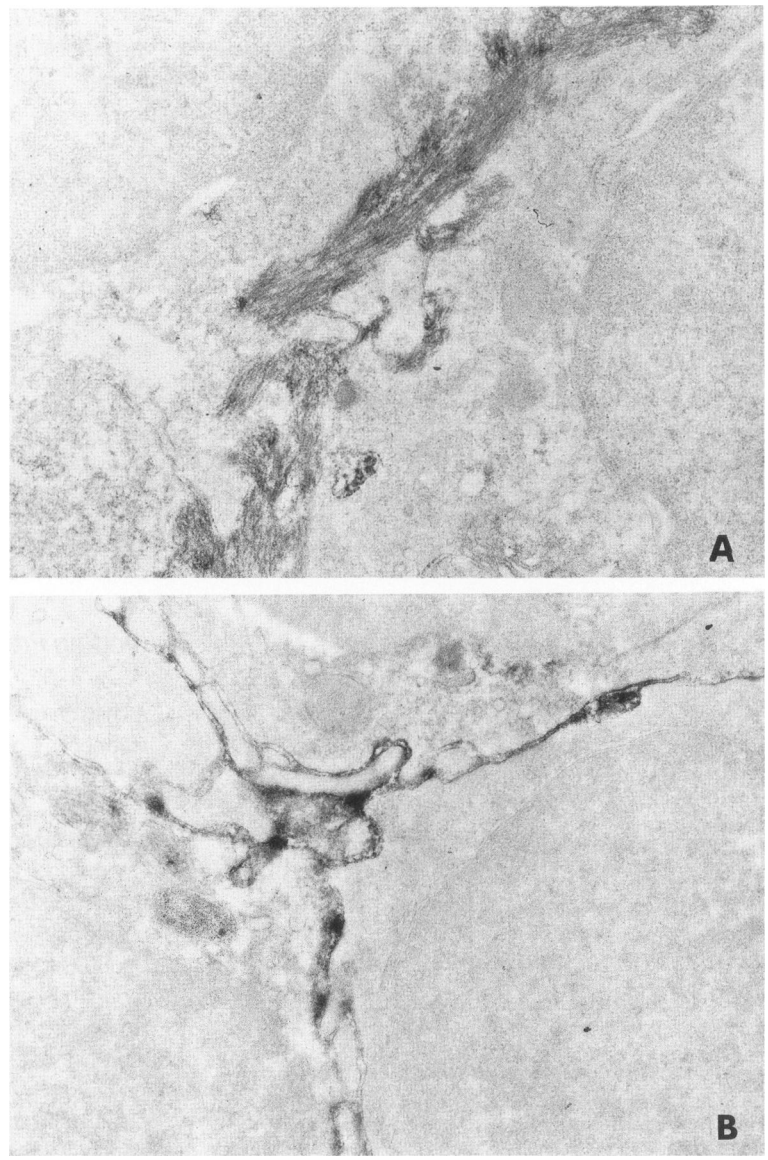


Figure 3. *Double immunofluorescence for SAA/AA and fibronectin on a frozen section of the 90th day.* **A:** *SAA/AA distributing throughout the spleen in crystallinelike deposition.* **B:** *Staining pattern of fibronectin almost coinciding with that of SAA with slight intensity at the periphery of crystallinelike deposits (A and B, ×130).*

Figure 4. *Immunoelectron peroxidase using anti-SAA/AA antibodies at the 20th day.* **A:** *Small aggregates of microfibrils about 10 nm in diameter compatible with amyloid fibrils in the intercellular space between macrophages.* **B:** *Flocculent immunoreactive products identifiable in the narrow intercellular spaces surrounded by intertwined foot processes of macrophages (A and B, no counter staining, ×20,000).*



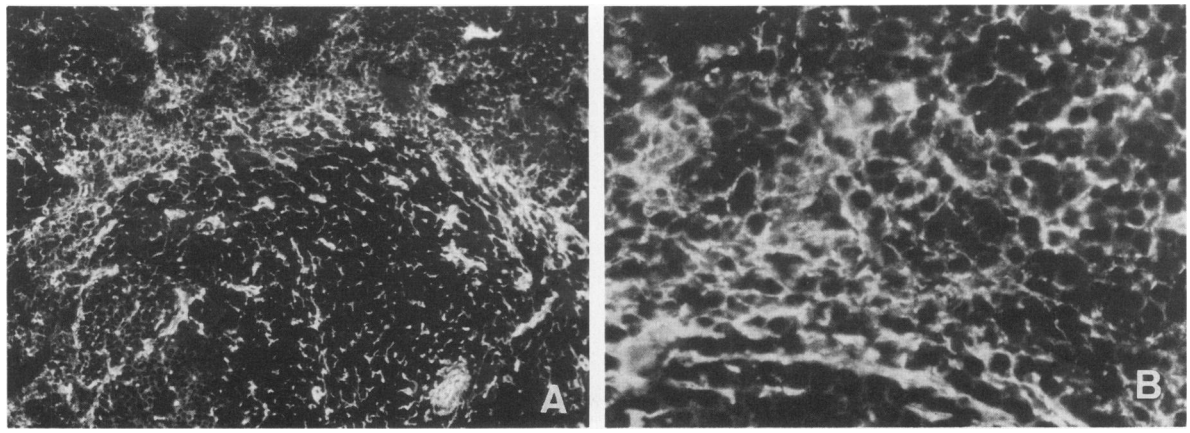


Figure 5. *Indirect immunofluorescence for fibronectin on a frozen section of the fourth day.* **A:** *Immunofluorescence of fibronectin locating chiefly in the marginal zone (×65).* **B:** *Higher magnification showing meshwork pattern delineating tissue macrophages (×300).*

showed similar patterns with slight condensation at the periphery of the bundles of amyloid fibrils (Figure 3A, B).

Increase of Tissue Fibronectin Deposit

The sequence of fibronectin deposits in the tissues evaluated by competitive enzyme immunoassay in the 4 M urea extract of spleen homogenate was demonstrated in Figure 6. The fibronectin increased at the fourth day of azocasein injection and the amount was 10 times more than that of the normal spleen. The amounts at the 8, 12, 16, and 20th days remained unchanged. This implies that the increase of fibronectin in the tissue precedes the deposits of SAA/AA in the marginal zone. At the 90th day the amount reached the levels of the 4 to 20th day only twice.

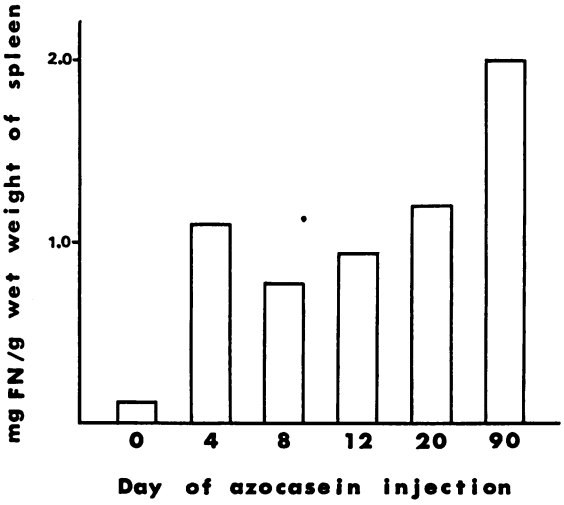


Figure 6. *Quantitation of tissue fibronectin in 4 M urea extracts from precipitates of spleen homogenates. At the fourth day fibronectin increases 10 times more than a control. Until the 20th day the amounts were unchanged and at the 90th day it increases twice more.*

Binding Site of SAA in the Tissue

HDL-SAA was applied *in vitro* on the frozen sections, and bound SAA was detected by immunofluorescence. The fluorescence appeared positive in the perifollicular area of the spleen 4 days after azocasein injection (Figure 7A), and this was the same in a section of the eighth day. On a control section of normal mouse spleen, however, binding SAA was not detected by immunofluorescence (Figure 7B). This indicates that there are substances that are able to bind with SAA that increase or accumulate in the marginal zone of the spleen before SAA/AA deposition.

Binding Property of SAA to Fibronectin

Affinity chromatographic isolation of bound SAA from the fibronectin-bound column was detected by Western blotting. The Western blotting revealed two bands of SAA1 and SAA2. Several faint bands of high molecular weight were detected in a polyacrylamide gel electrophoresis by Coomasie blue staining (Figure 8A). This faint banding pattern also was obtained from those of fibronectin-bound sepharose as well as from those of fibronectin-unbound Sepharose as a control. These faint bands were thought to represent nonspecific binding proteins to Sepharose 4B. SAA was not detected by Coomasie blue staining, but, using Western blotting (Figure 8B), two bands of SAA1 and SAA2 were clearly detected in the eluate from the fibronectin column, not in that from the control column. Thus, it is presumed that these proteins have specific binding properties to fibronectin.

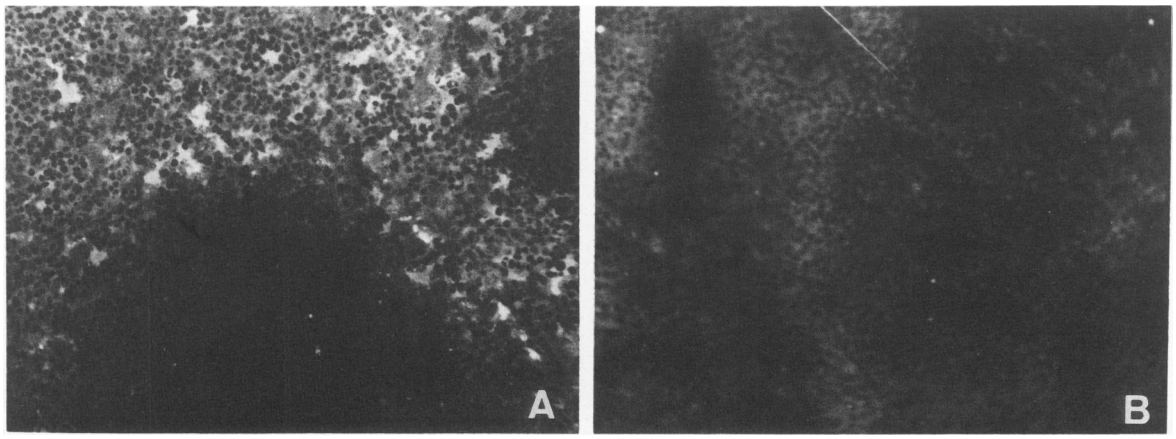


Figure 7. *Binding assay of SAA on frozen sections of the fourth day* (A) *and a control* (B). *A frozen section of the fourth day shows the presence of binding substance to SAA in the perifollicular area (A), although in a frozen section of the normal spleen this is ambiguous (B) (A and B, immunofluorescence, ×130).*

Comparison of Binding Affinity of SAA to Fibronectin, Collagen, Heparan Sulfate, SAP, and Albumin

The binding capacity of SAA to fibronectin, collagen type I, and heparan sulfate, which are major components of the extracellular matrix, was evaluated by optical density using ELISA and is shown in Figure 9. Figure 10 shows the binding capacity of SAA to fibronectin in comparison with SAP and albumin in the supplementation of calcium ion. In calcium-free conditions the result was the same as that shown in Figure 10. SAA showed strong affinity to fibronectin compared with heparan sulfate, collagen type I, SAP, and albumin. A gradual increase in affinity curves of heparan sulfate, collagen type I, SAP, and albumin may show background.

Discussion

Serum amyloid A protein is a serum precursor of AA amyloid fibril. Shiroo et al[7] clarified using SDS-PAGE and Western blotting that SAA and SAA-intermediates, as well as AA, are contained in amyloid-laden tissues. They presumed that SAA *per se* is deposited in the intercellular space and subsequently degrades into AA protein, finally

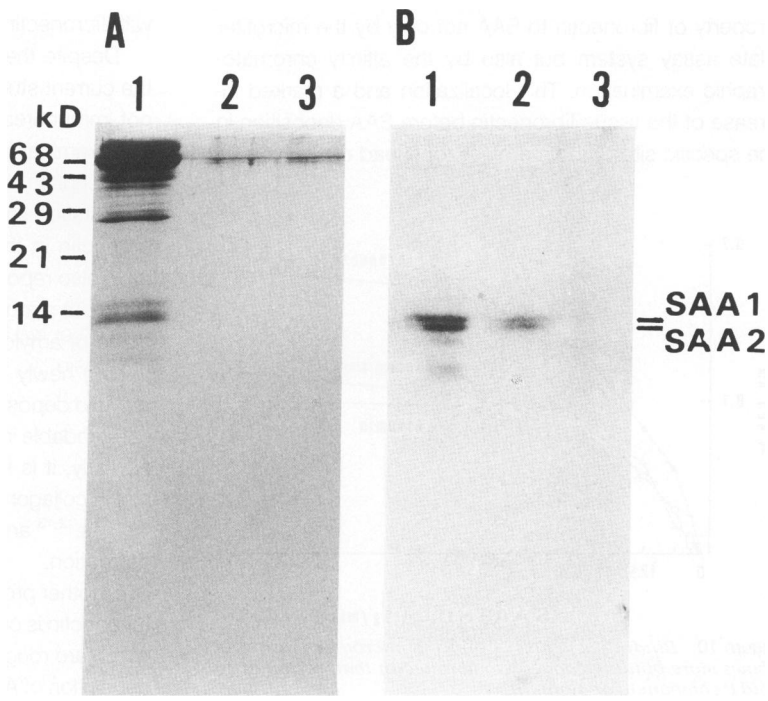


Figure 8. *Affinity chromatographic assay for binding SAA to fibronectin.* A: *Coomasie blue staining after 6 M urea-13% SDS-PAGE. Faint bands of proteins bound to sepharose 4B are shown in lane 2 and 3, but SAA is not detected by this staining.* B: *Western blotting using anti-SAA/AA antibodies. Two adjacent bands of SAA1 and SAA2 are detected by Western blotting in lane 1 (SAA-rich serum) and lane 2 (6 M urea eluate from fibronectin-Sepharose 4B), whereas in lane 3 (6 M urea eluate from fibronectin-unbound control) no bands are detected.*

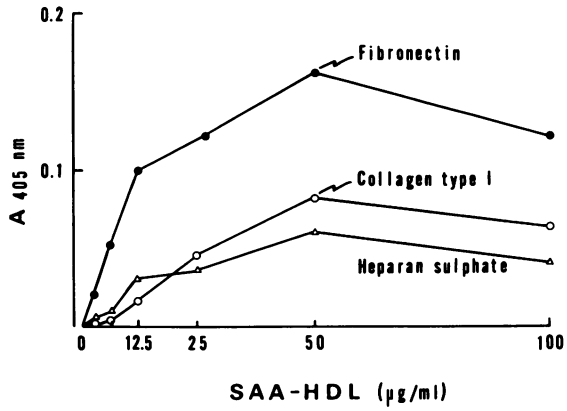


Figure 9. *Binding assay of SAA on the microtiter plate. SAA shows more binding capacity to fibronectin than collagen type I or heparan sulfate.*

forming amyloid fibrils. In terms of enzymatic breakdown of SAA, SAA-degrading proteinases actively work on the surface of tissue macrophages and are presumably inactivated by proteinase inhibitors that accomplish the partial proteolysis to AA.[6] Thus, the initial event of amyloidosis appears to occur on the outer surface of macrophages. In the present study, we identified flocculent materials immunoreactive to SAA/AA on the surface of macrophages exclusively in the perifollicular area of the spleen at the early stage. These materials seem to correspond to SAA or SAA-intermediates. This may be one of the reasons why SAA/AA is always deposited in a specific location, that is, in the marginal zone of the follicle in which tissue macrophages are abundant.[25,26]

This study clarified colocalization of SAA/AA and fibronectin in the perifollicular area, and a specific binding property of fibronectin to SAA not only by the microtiter plate assay system but also by the affinity chromatographic examination. This localization and a marked increase of the tissue fibronectin before SAA deposition in the specific site of the marginal zone lead us to suspect

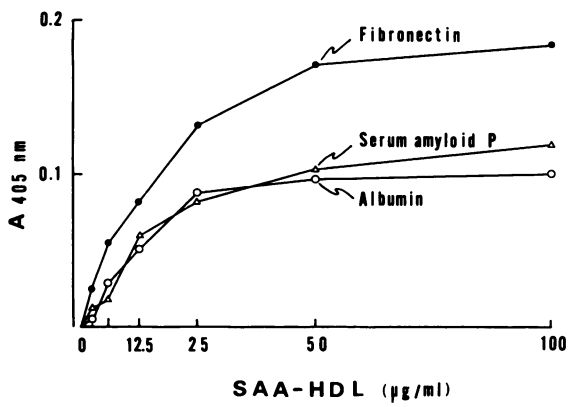


Figure 10. *Binding assay of SAA on the microtiter plate. SAA shows more binding capacity to fibronectin than serum amyloid P component or albumin.*

that fibronectin is involved in the development of amyloidosis, particularly during a preamyloidotic period in the two-phase theory of amyloidogenesis postulated by Teilum.[27]

It is known that fibronectin promotes spreading, moving, differentiation of the mesenchymal cells, and has different binding domains to cells or various extracellular matrix components, and promotes opsonization of macrophages.[9,28,29] Macrophages are noted to be one of the cell populations to which fibronectin binds.[28,30,31] As shown in the present study, SAA binds more strongly to fibronectin than to heparan sulfate or collagen type I. Therefore, if the fibronectin molecule has different binding domains to macrophages and SAA, tissue fibronectin, which increases during the preamyloidotic phase, plays an important role in the binding of SAA to the cell surface of marginal zone macrophages, where amyloid fibrils are formed as a result of partial degradation of SAA. In other words, fibronectin works as a linking protein between SAA and macrophages during the early stage.

There are many other amyloid-related proteins, such as glycosaminoglycan,[1,2] SAP component,[23,32] and vitronectin.[33] These proteins are closely correlated with amyloid deposition, but their precise role remains unclear. We examined the binding property of heparan sulfate to SAA, which is thought to represent one of the most closely amyloid-correlating species of glycosaminoglycan.[34] Our results, however, failed to show a specific binding capacity of heparan sulfate or SAP to SAA *in vitro*. Although we did not check all amyloid-related proteins, it is equivocal that they bind directly with SAA. Because SAP,[32] heparin,[35] or heparan sulfate[20] could bind to fibronectin, they may be concerned with the amyloidogenesis by the interaction with fibronectin.

Despite the important role of fibronectin indicated in the current study, it is reported that amyloid deposits are not immunoreactive with anti-fibronectin.[36,37] In the present examination, fibronectin was detected especially at the periphery of amyloid deposits in the late stage. The quantitation study revealed only a twofold increase of fibronectin in the tissue of amyloid spleen. Dixon et al[36] have also reported that fibronectin is negative on amyloid fibrils but strongly positive around them. Thus, in the late stage of amyloidosis, fibronectin may play a role only in adding newly formed amyloid fibrils to the periphery of amyloid deposit. Thus, the negative staining would be understandable in the late and inactive stage of amyloidosis. Similarly, it is known that fibronectin plays an important role in collagen deposition during the early reparative processes,[12,13] and it becomes scanty during the late collagenization.

Another problem in amyloidogenesis is whether or not fibronectin is one of the amyloid-enhancing factors (AEF), which are roughly defined as something promoting rapid deposition of AA amyloid in the mouse.[38,39] AEF is at least

a substance that could be extracted from amyloidotic or pre-amyloidotic tissues to a greater extent than from normal tissues. In this sense, as shown in the present experiment, fibronectin would be a AEF. Baltz et al[39] reported that AEF is a glycoprotein that has an apparent molecular weight of 440,000, comparable with that of plasma fibronectin dimer. Reportedly AEF also is extractable from the culture medium of fibroblasts,[39] is sensitive to trypsin treatment,[39] has a low isoelectric point,[40] and exhibits a tendency to form aggregate when thawed.[41] These properties are similar to those of fibronectin. A recent study[41] on the biochemical nature of AEF have shown that AEF activity is abolished by serine protease inhibitors and detected in the lower molecular weight fractions between 43,000 and 66,000. However, such a biochemical nature is inconsistent with the report of Baltz et al[39] that AEF is not affected by serine protease inhibitors. Low molecular weight fractions might include fragments of fibronectin because fibronectin is easily degraded through extraction procedures. These differences in the biochemical properties of AEF lead us to consider the heterogeneity of AEFs. Cytokines that promote synthesis of SAA,[42] proteases that degrade SAA to AA, and protease inhibitors that contribute to the partial degradation of SAA, would all be AEF. Fibronectin that is assumed to be a linking protein between SAA and macrophages would also be an AEF.

There are two major SAA isotypes in the mouse: SAA1 and SAA2.[18,43] Only SAA2 seems to be a precursor of AA.[4,7,44] In this study, we could not demonstrate why SAA2, not SAA1, evolved in amyloid fibrils, because both of them have equal binding properties to fibronectin. The reason why only SAA2 but not SAA1 forms amyloid fibril may due to a proteolytic process that starts after the localization of both SAA1 and SAA2 on the marginal area. Shirasawa et al[45] reported *in vitro* cleavage of SAA2 by cyanogen bromide and fibril formation in the mixture of SAA1 and SAA2 because SAA2 has ^{76}Met instead of ^{76}Ile in SAA1.

References

1. Snow AD, Kisilevsky R: Temporal relationship between glycosaminoglycan accumulation and amyloid deposition during experimental amyloidosis: A histochemical study. Lab Invest 1985, 53:37–44
2. Linker A, Carney HC: Presence and role of glycosaminoglycans in amyloidosis. Lab Invest 1987, 57:297–305
3. Benditt EP, Eriksen N, Hanson RH: Amyloid protein SAA is an apolipoproteins of mouse plasma high density lipoprotein. Proc Natl Acad Sci USA 1979, 76:4092–4096
4. Yamamoto K, Shiroo M, Migita S: Diverse gene expression for isotypes of murine serum amyloid A protein during acute phase reaction. Science 1986, 232:227–229
5. Skogen B, Natvig JB: Degradation of amyloid proteins by different serine proteases. Scand J Immunol 1981, 14:389–396
6. Zucker-Franklin D, Fuks A: The role of the macrophage phagocytic system (MPS) in the development of secondary amyloidosis, Amyloidosis. Edited by GG Glenner, EF Osserman, EP Bendit, E Calkins, AS Cohen, and D Zucker-Franklin. New York, Plenum Press, 1986, pp 329–340
7. Shiroo M, Kawahara E, Nakanishi I, Migita S: Specific deposition of serum amyloid A protein 2 in the mouse. Scand J Immunol 1987, 26:709–716
8. Vaheri A, Mosher DF: High molecular weight, cell surface-associated glycoprotein (fibronectin) lost in malignant transformation. Biochim Biophys Acta 1978, 516:1–25
9. Yamada KM: Isolation of fibronectin from plasma and cells, Immunochemistry of the Extracellular Matrix, Vol I. Edited by H Furthmayr. Florida, CRC Press, 1982, pp 111–123
10. Dyck RF, Roger SL: Fibronectin is an acute phase reactant in mice. Clin Invest Med 1985, 8:148–151
11. Kurkinen M, Vaheri A, Roberts PJ, Stenmans S: Sequential appearance of fibronectin and collagen in experimental granulation tissue. Lab Invest 1980, 43:47–51
12. Martinez-Hernandez A: The hepatic extracellular matrix: II. Electron immunohistochemical studies in rats with CCl_4-induced cirrhosis. Lab Invest 1985, 53:166–186
13. Stenman S, Vaheri A: Distribution of a major connective tissue protein, fibronectin, in normal human tissues. J Exp Med 1978, 147:1054–1064
14. Engvall E, Ruoslahti E: Binding of soluble form of fibroblast surface protein, fibronectin, to collagen. Int J Cancer 1977, 20:1–5
15. Kawahara E, Oda Y, Ooi A, Katsuda S, Nakanishi I, Umeda S: Expression of glial fibrillary acidic protein (GFAP) in peripheral nerve sheath tumors. Am J Surg Pathol 1988, 12:115–120
16. Isemura M, Yamaguchi Y, Munakata H, Aikawa J, Kan M, Yamane I, Yosizawa Z: Isolation and characterization of human placenta fibronectin. J Biochem 1984, 96:163–169
17. Vuento M, Salonen E, Pasanen M, Stenman U-H: Competitive enzyme immunoassay for human plasma fibronectin. J Immunol Methods 1981, 40:101–108
18. Hoffman JS, Benditt EP: Changes in high density lipoprotein content following endotoxin administration in the mouse. J Biol Chem 1982, 257:10510–10517
19. Schmidt G, Robenek H, Haraach B, Glossl J, Nolte V, Hormann H, Richter H, Kresse H: Interaction of small dermatan sulfate proteoglycan from fibroblasts with fibronectin. J Cell Biol 1987, 104:1683–1691
20. Isemura M, Saito N, Yamaguchi Y, Aikawa J, Munakata H, Hayashi N, Yosizawa Z: Isolation and characterization of fibronectin-binding proteoglycan carrying both heparan sulfate and dermatan sulfate chains from human placenta. J Biol Chem 1987, 262:8926–8933
21. Minamoto T, Ooi A, Okada Y, Mai M, Nagai Y, Nakanishi I: Desmoplastic reaction of gastric carcinoma: A light and electron-microscopic immunohistochemical analysis using collagen type-specific antibodies. Human Pathol 1988, 19: 812–821
22. Migita S, Matsusita N: A new large scale preparative agarose gel electrophoresis. Meth Immunol Exp 1976, 5:1401–1409

23. Pepys MB, Dyck RF, De Beer FC, Skinner M, Cohen AS: Binding of serum amyloid P-component (SAP) by amyloid fibrils. Clin Exp Immunol 1979, 38:284–293
24. Pepys MB, Dash AC, Munn EA, Feinstein A, Skinner M, Cohen AS, Gewurz H, Osmand AP, Painter RH: Isolation of amyloid P-component from normal serum as a calcium-dependent binding protein. Lancet 1977, 1:1029–1031
25. Buckley PJ, Smith M, Braverman MF, Dickson SA: Human spleen contains phenotypic subsets of macrophages and dendritic cells that occupy discrete microanatomic locations. Am J Pathol 1987, 128:505–520
26. Humphrey JH, Grennan D: Different macrophage populations distinguished by means of fluorescent polysaccharides. Recognition and properties of marginal-zone macrophages. Eur J Immunol 1981, 11:221–228
27. Teilum G: Pathogenesis of amyloidosis. Acta Pathol Microbiol Scand 1964, 61:21–45
28. Gudewicz PW, Molnar J, Lai MZ, Beezhold DW, Siefring GE, Credo RB, Lorand L: Fibronectin-mediated uptake of gelatin-coated latex particles by peritoneal macrophages. J Cell Biol 1980, 87:427–433
29. Hayashi M, Yamada KM: Domain structure of the carboxyl-terminal half of human plasma fibronectin. J Biol Chem 1983, 258:3332–3340
30. Bevilacqua MP, Amrani D, Mosesson MW, Bianco C: Receptors for cold-insoluble globulin (plasma fibronectin) on human monocytes. J Exp Med 1981, 153:42–60
31. Rovin B, Molnar J, Chevalier D, Ng P: Interaction of plasma fibronectin (pFN) with membranous constituents of peritoneal exudate cells and pulmonary macrophages. J Leuk Biol 1984, 36:601–620
32. De Beer FC, Baltz ML, Holford S, Pepys MB: Fibronectin and C4-binding protein are selectively bound by aggregated amyloid P component. J Exp Med 1981, 154:1134–1149
33. Dahlbäck K, Löfberg H, Dahlbäck B: Immunohistochemical demonstration of vitronectin in association with elastin and amyloid deposits in human kidney. Histochemistry 1987, 87: 511–515
34. Snow AD, Kisilevsky R, Stephens C, Anastassiades T: Characterization of tissue and plasma glycosaminoglycans during experimental AA amyloidosis and acute inflammation: Qualitative and quantitative analysis. Lab Invest 1987, 56: 665–675
35. Ruoslahti E, Engvall E: Complexing of fibronectin glycosaminoglycans and collagen. Biochim Biophys Acta 1980, 631: 350–358
36. Dixon AJ, Burns J, Dunnill MS, McGee JO'D: Distribution of fibronectin in normal and diseased human kidneys. J Clin Pathol 1980, 33:1021–1028
37. Breathnach SM, Bhogal B, De Beer FC, Black MM, Pepys MB: Primary localized cutaneous amyloidosis: Dermal amyloid deposits do not bind antibodies to amyloid A protein, prealbumin or fibronectin. Br J Dermatol 1982, 107:453–459
38. Axelrad MA, Kisilevsky R, William J, Chen SJ, Skinner M: Further characterization of amyloid-enhancing factor. Lab Invest 1982, 47:139–146
39. Baltz ML, Caspi P, Hind CRK, Feinstein A, Pepys MB: Isolation and characterization of amyloid enhancing factor (AEF), Amyloidosis. Edited by GG Glenner, EF Osserman, EP Bendit, E Calkins, AS Cohen, and D Zucker-Franklin. New York, Plenum Press, 1986, pp 115–121
40. Kisilevsky R, Snow AD, Subrahmanyan L, Bondreau L, Tan R: What factors are necessary for the induction of AA amyloidsis (AA amyloid, glycosaminoglycans, amyloid enhancing factor, apo-SAA), Amyloidosis. Edited by J Marrink and MH van Rijswijk. Marpinus, Nijhoss publishers. 1987, pp 301–310
41. Abankwa GV, Ali-Khan Z: Alveolar hydatid cyst induced amyloid enhancing factor (AEF): Physiochemical properties and abolition of AEF activity by serine protease inhibitors. Br J Exp Pathol 1988, 69:133–148
42. Selinger MJ, McAdam KPWJ, Kaplan MM, Sipe JD, Vogel SN, Rosenstreich DL: Monokine-induced synthesis of serum amyloid A protein by hepatocytes. Nature 1980, 285:498–500
43. Yamamoto K, Migita S: Complete primary structures of two major murine serum amyloid A proteins deduced from cDNA sequences. Proc Natl Acad Sci USA 1985, 82:2915–2919
44. Hoffman JS, Ericsson LH, Eriksen N, Walsh KA, Benditt EP: Murine tissue amyloid protein A, NH_2-terminal sequence identity with only one of two serum amyloid protein (apo SAA) gene products. J Exp Med 1984, 159:641–646
45. Baba S, Miura K, Fujie M, Shirasawa H: In vitro formation of amyloid fibrils from serum amyloid A (apo SAA). 5th International Symposium on Amyloidosis Program. 1987, p 148