

LOCALIZATION OF AMYLOID-RELATED SERUM PROTEIN  
SAA-LIKE MATERIAL TO  
INTERMEDIATE (10 nm) FILAMENTS OF CULTURED  
HUMAN EMBRYONAL FIBROBLASTS\*

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The amyloid-related serum protein SAA appears to be a precursor of protein AA (1, 2), the major constituent of amyloid fibrils deposited in secondary amyloidosis (3). We recently localized SAA-like material to fibrillar structures in fetal connective tissues by immunofluorescence microscopy (4). SAA was also detected in cultured embryonal fibroblasts: the fluorescence was cytoplasmic and formed a fine fibrillar staining pattern (4). It was clearly different from collagen and reticulin fibers, but showed certain similarities with components of elastic fibers (4). The aim of the present report was to study the intracellular relation of the SAA-like material in fibroblasts. We have shown that the SAA-like material is present in cytoskeletal 10-nm intermediate filaments of the cultured fibroblasts, but not associated with microfilaments or microtubules.

### Materials and Methods

*Cells in Tissue Culture.* Human embryonal fibroblasts (HEF) were prepared by trypsinization of small pieces of body wall: the dissociated cells were plated and maintained in secondary culture as described previously (4). Cells grown on small coverslips were fixed after 2 days in culture in 3.5% paraformaldehyde for 15 min and subsequently treated with acetone at  $-20^{\circ}\text{C}$  for 20 min. The fixed cells were washed in phosphate-buffered saline, pH 7.1 (PBS) for at least 30 min. Selected cell cultures were grown in the presence of 0.1  $\mu\text{g}/\text{ml}$  demecolcine (Colcemid, Ciba, Milan, Italy) for 12 h or with 10  $\mu\text{g}/\text{ml}$  vinblastine sulphate (Eli Lilly and Co., Indianapolis, Ind.) for 3 h (5).

*Amyloid-Related Serum Protein SAA.* SAA was prepared from patient sera with high SAA activity (1) by gel filtration in 10% formic acid (6).

*Antisera.* Antibodies against SAA were produced by immunization of rabbits with purified SAA in Freund's complete adjuvant (4). The specificity of these antibodies was assessed by immunodiffusion and indirect immunofluorescence (IFL) as reported previously (4, 6).

Anti-actin antibodies were from sera of patients with active chronic hepatitis producing smooth muscle antibodies (SMA) with anti-actin specificity (7). The antibodies were used as markers for cytoplasmic actin filaments (7, 8).

*Immunofluorescence and Immunoperoxidase Methods.* Binding of rabbit anti-human SAA to fixed normal demecolcine or vinblastine-treated fibroblasts was demonstrated by indirect immu-

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nofluorescence by using fluorescein-isothiocyanate-conjugated sheep anti-rabbit IgG (F/P molar ratio 2.0, 0.4 mg protein/ml) as previously described (4). The immunofluorescence was studied by using Zeiss Standard Microscope (Carl Zeiss, Inc., Oberkochen, West Germany) equipped with epi-illuminator IV FL and phase contrast optics.

The immunoperoxidase localization method was essentially that described by Sternberger and Cuculis (9). The cells were fixed in 3.5% paraformaldehyde for 15 min and subsequently treated with 0.05% nonionic detergent Nonidet P-40 (BDH) (Shell Chemical Co., New York) for 15 min,<sup>1</sup> incubated first with anti-SAA diluted in 1% goat serum, washed twice in PBS for 30 min followed by sheep anti-rabbit IgG. Then soluble complex of horseradish peroxidase and rabbit anti-horseradish peroxidase (PAP-complex, Dakopatts A.S., Copenhagen, Denmark) was added. After washing in PBS for 90 min the enzymatic reaction was developed with 3,3'-diaminobenzidine-tetrahydrochloride (DAB) (Fluka A.G., Buchs, Switzerland). This substrate for the enzyme reaction was made up as a 0.05% solution in 0.05 M Tris buffer pH 7.6, containing 0.003% H<sub>2</sub>O<sub>2</sub>. After 30 min incubation, the cells were washed in distilled water. For light microscopy the peroxidase-stained cells were mounted by using conventional methods. For electron microscopy the peroxidase-stained cells were postfixed in 1% aqueous osmium tetroxide, dehydrated, and embedded in Epon 812. Ultrathin sections were studied in a Jeol 100 B electron microscope (Jeol Ltd., Tokyo, Japan).

The specificity controls were essentially as described previously (4). Rabbit anti-human SAA antiserum was substituted with normal rabbit serum and anti-SAA activity was neutralized both with purified human SAA and with patient sera containing SAA. In the immunoenzymatic method an additional control included leaving out the PAP-complex to detect endogenous peroxidase activity. This control was essentially negative.

*Electron Microscopy.* For electron microscopy, the cells were fixed in 3.5% glutaraldehyde, postfixed in 1% osmium tetroxide, dehydrated, and embedded in Epon 812. The ultrathin sections were stained with lead citrate and uranyl acetate.

## Results

*Immunofluorescent Staining with Anti-SAA and Anti-Actin.* After staining with anti-SAA a fine fibrillar fluorescence was seen in the cytoplasm of embryonal fibroblasts (Fig. 1 A). This staining pattern was clearly different from the filamentous staining produced by anti-actin antibodies. Thus the SAA-like material detected by anti-SAA did not correspond to the cytoplasmic microfilaments.

*Effects of Drugs Altering Intracellular Microtubules.* Further studies to localize SAA-like material intracellularly were performed with drugs, which alter intracellular microtubules. The localization of anti-SAA was markedly altered in cells treated with demecolcine or vinblastine sulphate, as compared to untreated cells. In treated cells the immunofluorescence staining was usually seen in pronounced perinuclear bundles of varying diameter (Fig. 1 B). It sometimes formed a perinuclear ring, in other cells a branching cytoplasmic network was seen. The fluorescent fibers and bundles were often localized in one segment of the cytoplasm. The most intensely stained bundles were closely associated with the nucleus. The localization of SAA in vinblastine-treated cells did not differ from that of demecolcine-treated cells. Vinblastine-induced paracrystals were seen in phase contrast microscopy (Fig. 1 C). They were not stained by anti-SAA in immunofluorescence (Fig. 1 D).

The fibrillar immunofluorescence obtained with anti-SAA both in treated and in untreated cells was completely inhibited when the anti-SAA antiserum was

<sup>1</sup> P. Laurila, I. Virtanen, J. Wartiovaara, and S. Stenman. Fluorescent antibodies and lectins stain intracellular structures in fixed cells treated with nonionic detergent. Manuscript in preparation.

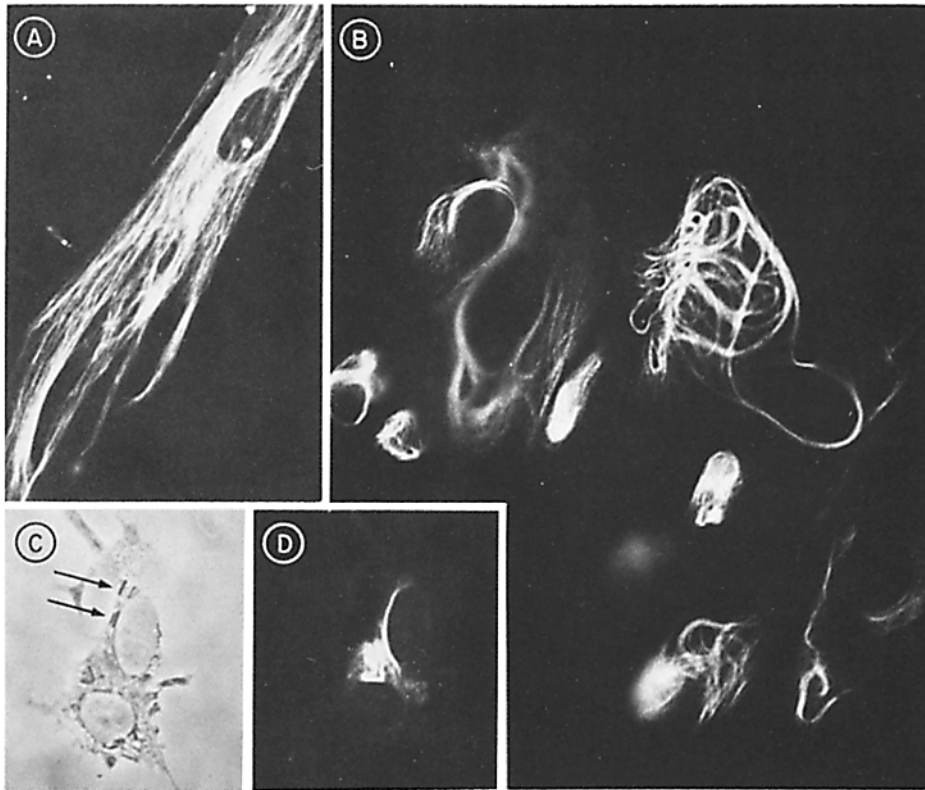


FIG. 1. Localization of SAA-like material in cultured human embryonal fibroblasts. A. Fine fibrillar IFL in the cytoplasm with anti-SAA. B. Perinuclear immunofluorescent bundles with anti-SAA in cells cultured in the presence of demecolcine. C. Cytoplasmic paracrystals (arrows) in cells cultured in the presence of vinblastine sulphate. Phase contrast micrograph. D. IFL staining with anti-SAA of the same cells as in Fig. 1 C  $\times$  2,000.

preincubated with purified SAA protein in a concentration of  $10 \mu\text{g/ml}$  or with SAA-containing patient sera in dilutions up to 400. The fluorescence was not affected when absorbed with SAA-negative sera.

*Ultrastructural Studies.* In thin sections of demecolcine-treated fibroblasts, bundles of filaments were seen at the perinuclear regions in electron microscopy (Fig. 2). They consisted of intermediate filaments with an average diameter of 10 nm. By using immunoperoxidase labeling technique, the relationship between these 10-nm intermediate fibrils and the SAA-like material detected by anti-SAA was further investigated. Light microscopic analysis of peroxidase-stained fibroblasts demonstrated patterns analogous to those seen in immunofluorescence microscopy with anti-SAA antiserum. (Fig. 3, insert). In addition, when the demecolcine-treated cells were studied by immunoelectron microscopy with anti-SAA (Fig. 3), the peroxidase reaction product was localized to the perinuclear bundles of 10-nm filaments similar to that identified by electron microscopy in Fig. 2. The specificity of the staining reaction was proved by blocking experiments where isolated SAA and SAA-positive, but not SAA-negative sera, totally inhibited the reaction. Thus the SAA-like material as

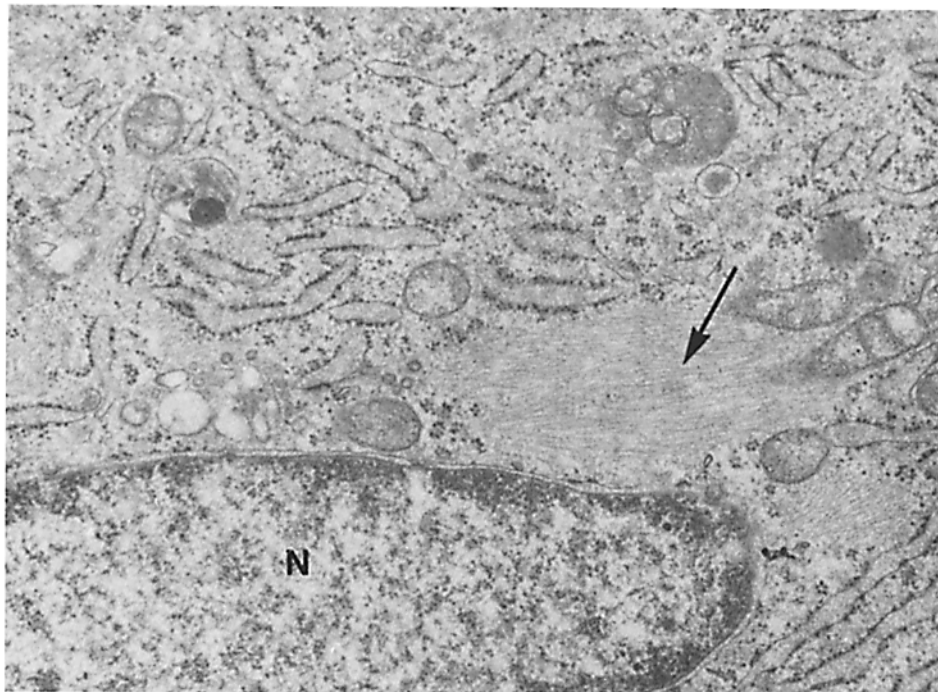


FIG. 2. Electron micrograph from a human embryonal fibroblast treated with demecolcine. Bundles of intermediate filaments (arrows), about 10 nm in diameter, are seen perinuclearly. N, nucleus.  $\times 35,000$ .

detected by specific anti-SAA antisera corresponded to the intracellular 10-nm filaments of the fibroblasts.

### Discussion

In the present study we have localized SAA-like material to cytoplasmic intermediate (10 nm) filaments in fibroblasts by IFL and immunoelectron microscopy. The intermediate filaments constitute part of the cellular cytoskeleton (10, 11). In this study we differentiated these filaments from other fibrillar cytoplasmic structures, microfilaments and microtubules, on the basis of the unique property of the intermediate filaments to form bundles when treated with demecolcine and vinblastine (8, 12).

The fibrillar fluorescence in intermediate filaments was clearly distinct from the pattern produced by anti-actin antibodies which react with cytoplasmic microfilaments (5, 8). On the other hand, SAA-like material seem to be unrelated to microtubules as well; vinblastine treatment causes fragmentation of microtubules and formation of tubulin-containing paracrystals. Such microtubules-derived material did not stain in IFL with anti-SAA.

Intermediate filaments seem to be ubiquitous occurring in e.g. smooth muscle cells (11), vascular endothelium (12), and neural cells (13), but the problem whether SAA is present in 10-nm filaments of different cellular origin remains to be solved. However, the fluorescence seen in liver sinusoids (4) and fetal vessel endothelium of placenta after staining with anti-SAA (14) indicates that

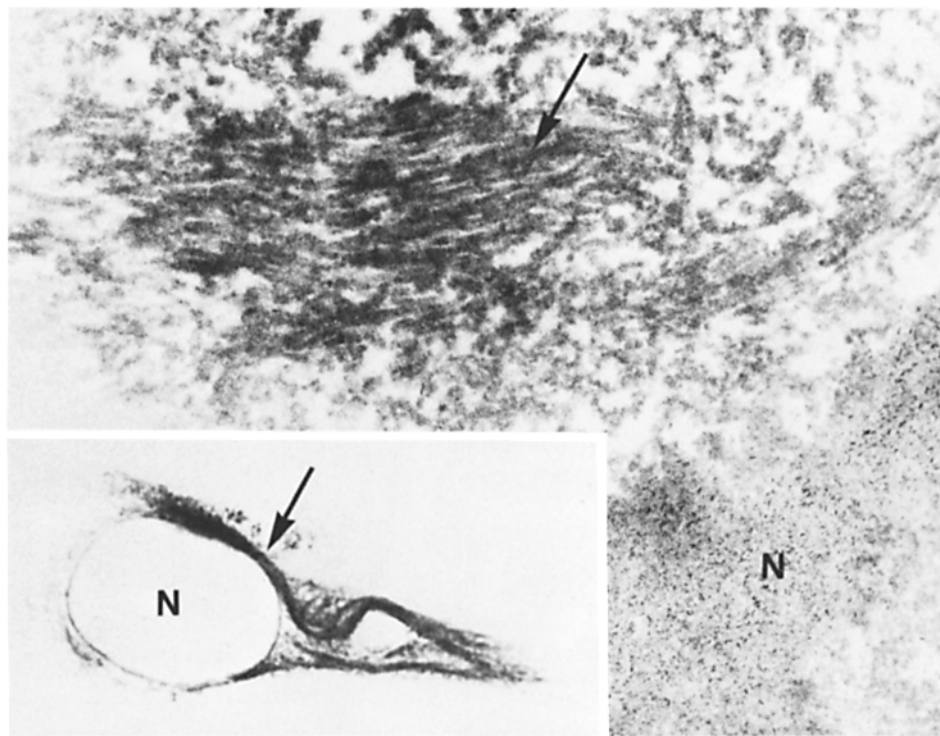


FIG. 3. Immunoperoxidase localization with anti-SAA of SAA-like material in cells treated with demecolcine. In light microscopy perinuclear bundles are seen similar to those seen in IFL (insert). In electron microscopy the peroxidase reaction product is localized to perinuclear intermediate filament bundles (arrow). N, nucleus.  $\times 42,000$ .

SAA-like material might be present also in 10-nm filaments of other cell types than fibroblasts. Previous immunofluorescence studies suggested that SAA might be related to the microfibrillar component of elastic fibers (14). This component has a size of about 10–11 nm (100–110 Å) (15) and this corresponds to the size of the intracellular filaments which react with anti-SAA. Studies on the composition of 10-nm filaments have shown one major component, desmin, with a mol wt of about 55,000 (12). Further studies are presently being performed to see whether these morphological and antigenic similarities reflect a common biochemical composition.

### Summary

Further studies are presented on the intracellular localization of the amyloid-related serum protein SAA previously shown to be produced by embryonal fibroblasts. In cultured embryonal fibroblasts, the fine fibrillar cytoplasmic immunofluorescence obtained by anti-SAA was distinguished from that of microfilaments and microtubules. By using electron microscopy and cells treated with drugs known to specifically alter intracellular fibrils, SAA was localized to 10-nm intermediate size filaments. These filaments form characteristic perinuclear bundles upon treatment with drugs such as demecolcine or vinblastine

which disrupt microtubules. The results indicate that SAA is a constituent of the intracellular cytoskeleton.

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