

Research Article

Colocalization of Serum Amyloid A with Microtubules in Human Coronary Artery Endothelial Cells

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Serum amyloid A (SAA) acts as a major acute phase protein and represents a sensitive and accurate marker of inflammation. Besides its hepatic origin, as the main source of serum SAA, this protein is also produced extrahepatically. The mRNA levels of SAA become significantly elevated following proinflammatory stimuli, as well as, are induced through their own positive feedback in human primary coronary artery endothelial cells. However, the intracellular functions of SAA are so far unknown. Colocalization of SAA with cytoskeletal filaments has previously been proposed, so we analyzed the colocalization of SAA with all three cytoskeletal elements: actin filaments, vimentin filaments, and microtubules. Immunofluorescent double-labeling analyses confirmed by PLA method revealed a strict colocalization of SAA with microtubules and a very infrequent attachment to vimentin while the distribution of actin filaments appeared clearly separated from SAA staining. Also, no significant colocalization was found between SAA and endomembranes labeled with the fluorescent lipid stain DiO₆. However, SAA appears to be located also unbound in the cytosol, as well as inside the nucleus and within nanotubes extending from the cells or bridging neighboring cells. These different locations of SAA in endothelial cells strongly indicate multiple potential functions of this protein.

1. Introduction

The acute phase response represents an evolutionarily conserved mechanism of inflammatory events designed to rapidly react to infections, wounds, and injuries. It can lead to a dramatic increase (up to 1000 fold) in the levels of acute phase proteins (APPs) in the circulation and, ultimately, brings about resolution of the inflammatory reaction [1, 2]. Serum amyloid A (SAA) one of the major APPs in humans is mainly produced by the liver, although extrahepatic synthesis is also prevalent [3].

SAA originates from an evolutionarily conserved multi-gene family [4] ranging from invertebrates (with a wound-healing role in sea cucumbers [5]), vertebrates, to humans, where it represents an accurate and sensitive marker of inflammation [2]. Human SAA1 and SAA2 are the inducible isotypes (addressed jointly as SAA1/2, with over 95% sequence identity); SAA3, was thought in the past to be a pseudogene, not expressed in humans; however, recently there has been a

report of its mammary-associated expression found in milk [6]. SAA4 was found to be the constitutively expressed isotype [4]. There have been three acute phase SAA isotypes reported in the mouse SAA1, SAA2, and SAA3, with SAA3 being the primarily extrahepatic isoform [7]. SAA is a small protein (104 amino acids in length), 11.7 kDa in size, lipophilic, and poorly soluble in aqueous solution, originally described as a component of normal serum [8]. SAA fragments were found in amyloidosis, and the accumulation of these fibrils can lead to organ failure and ultimately death [9]. Murine and human SAA have been shown to form hexamers in solution, which can lead to the formation of membrane channels that could be involved in important pathological roles [10, 11].

SAA1/2 has a variety of multiple functions in humans, among them it acts as a cytokine and chemokine, induces matrix metalloproteinases, interferes with platelet functions, replaces Apo-A1 in high density lipoprotein particles in the circulation during inflammation, binds cholesterol and

influences its efflux, and plays a role in host defense [10, 12–18]. In physiological concentrations (~100–200 nM as measured in sera), SAA is thought to act in homeostasis of inflammatory events, while at prolonged higher pathological concentrations (above 1000 nM) SAA could act in a degradative manner causing injury to tissues and cells during chronic inflammatory conditions. The expression of SAA has also been shown in nonhepatic epithelial cells and can play a role in tumorigenesis [19]. Its ability to opsonize Gram-negative bacteria could contribute to the epithelial defense mechanism against these bacteria [15].

SAA is measured routinely in sera or plasma in the veterinary field (especially useful in equine [20], bovine, porcine, and also bird and fish pathologies) and has proven to be a relevant biomarker and predictor of many diseases in humans (among them cardiovascular diseases [21]). SAA was identified as an independent risk predictor of mortality following acute myocardial infarction [22] and indicated to play a causal role in atherogenesis [23, 24]. SAA mRNA expression has been localized to many different human tissues, including atherosclerotic plaques, where SAA protein is thought to play an active pathogenic role [7]. Some of the strongest signals in this study were observed in endothelial cells lining the lumen of coronary arteries and in macrophage-derived “foam cells.”

The expression of SAA mRNA within cultured human primary coronary artery endothelial cells (HCAEC) was first reported in 2007 [25] and these cells were also shown to be highly responsive to human recombinant SAA1/2, as an inflammatory stimulus, indicating a strong positive feedback onto its own synthesis. However, very little is known about the localization of SAA within these cells, whether it is released and could act locally at the extracellular level or SAA remains in HCAEC and could influence endothelial function in an intracellular manner.

So, our objective was to determine the localization of intracellular SAA in HCAEC and to elucidate possible associations of SAA with particular cytoskeletal elements.

2. Materials and Methods

2.1. Materials. Rabbit anti-SAA polyclonal antibodies were a gift from Professor Ernst Malle (Institute of Molecular Biology and Biochemistry, Medical University of Graz, Austria). The anti-SAA antibodies used for the immunofluorescent staining were raised against synthetic peptides of SAA ranging from the indicated amino acid sequences: 27–44 (pAb 3), 59–72 (pAb 5), and 68–84 (pAb 6). For the immunoblot analysis, eight separate anti-SAA polyclonal antibodies were used, ranging in consecutive order, as raised against synthetic SAA peptides from pAb 1 to pAb 8. (SAA 1–17, SAA 14–30, SAA 27–44, SAA 40–63, SAA 59–72, SAA 68–84, SAA 79–94, SAA 89–104, resp.).

2.2. Cell Culture. HCAEC were purchased from Lonza (Walkersville, USA). Cells were cultured in EGM-2M medium (Lonza, Walkersville, USA) containing 5% fetal bovine serum at 37°C in a humidified atmosphere and 5% CO₂. For immunofluorescent staining, HCAEC were used through passage 5 of subconfluent (80–85% confluency) cell cultures.

2.3. Fluorescent Labeling of Cells. Cells were fixed in 4% paraformaldehyde for 30 min at 37°C, then washed in PBS.

For actin staining, cells were labelled with phalloidin-fluorescein isothiocyanate (FITC) (2 μM in 20% methanol) (Sigma, Germany).

For SAA, α-tubulin, and vimentin, cells were incubated with rabbit antibodies against SAA and/or mouse antibody against vimentin (Dako, USA) or α-tubulin (Sigma, Germany) and with the appropriate secondary antibodies conjugated with FITC or TRITC (Sigma, Germany). The Von Willebrand factor was determined with immunofluorescence microscopy (Abcam, Cambridge, Great Britain).

For labelling of endomembranes, a lipophilic stain 3,3'-dihexyloxycarbocyanin iodide (DiO₆) was used. It mainly labels endoplasmic reticulum and mitochondria (Molecular Probes manual). A stock solution of membrane marker vibrant DiO₆ (0.5 mg DiO₆/mL ethanol) (Invitrogen, Molecular Probes, Leiden, The Netherlands) was freshly prepared, and a 0.5% dilution of DiO₆ in culture medium was added to cells for 3 min. After washing in PBS, the coverslips with cells were embedded with antibleaching medium Vectashield containing the nuclear stain diamidinophenylindole dilactate (DAPI) (Vector Laboratories, Burlingame, Calif, USA) and observed with a fluorescence microscopes (Nikon eclipse TE 300 and AxioImager Z1, Carl Zeiss) supplemented with Apo Tome device for generation of optical sections.

2.4. In Situ Proximity Ligation Assay (PLA). In order to determine the colocalization between SAA and MT, SAA and VIM, the PLA assay (Olink Bioscience, Uppsala, Sweden) was performed according to the manufacturer's protocol. In brief, fixation, permeabilization, blockade, and incubation with primary antibodies were performed as described in the Section 2.3. Subsequently, the cells were incubated with the secondary mouse PLUS and rabbit MINUS antibodies. After hybridization and ligation of the oligonucleotides, the DNA was amplified by addition of an amplification mixture. A detection mixture detected the amplicons resulting in red fluorescence signals. In the final step, the nuclei were counterstained with DAPI and the cells were mounted with mounting medium (Vector Laboratories, Burlingame, Calif, USA). As a control, the cells were incubated without primary antibodies. Cells were analyzed with a 63x objective on a fluorescent microscope AxioImager Z1 (Carl Zeiss) supplemented with ApoTome device for optical sections generation.

2.5. Immunoblots. Human recombinant SAA (5.3 μg/cm gel) (PeproTech Ec Ltd., London, UK) was loaded onto SDS-PAGE. After transfer to nitrocellulose membranes (100 V, 250 mA, 35 min), blocking was performed in TBS with 5% milk. As primary antibodies, polyclonal rabbit anti-SAA antibodies were used (gift of prof. Malle) in a 1:1000 dilution for 2 hrs. Following washing, secondary incubation was done with antirabbit HRP conjugate (Biorad, Munich, Germany) at 1:200 dilution with subsequent Western blot luminol reagent (Santa Cruz, Calif, USA) and chemiluminescent detection performed following manufacturer's instructions.

3. Results

In order to determine the distribution of SAA within HCAEC and its colocalization with cytoskeletal elements, endomembranes, and the nucleus, fluorescent labeling of these components was performed in spread nonconfluent cells. To confirm the endothelial nature of these cells, the Von Willebrand factor was determined (Figure 1(a)).

3.1. Cytoskeleton. Labeling of SAA together with phalloidin confirmed that the majority of actin filaments were at the cell periphery and clearly separated from the location of SAA which is mainly concentrated in the central part of the cells (Figure 1(b)). Vimentin was organized as a network spreading from the nuclear region toward the cell periphery and was generally distributed in the same cell area as SAA. However, double labeling of vimentin and SAA revealed a rare colocalization of the two signals and thus gave a very low probability for their codistribution (Figure 1(d)) with antivimentin. The labeling of SAA in many locations, especially in the cell periphery of HCAEC cells, largely fits in with the linear pattern of microtubular labeling providing a high probability for the actual colocalization with this cytoskeletal component (Figure 1(c)). The attachment of SAA to microtubules is limited to small spots of SAA labeling while larger clumps of SAA (could be possible multimerization of SAA) appear to be nonattached to any of the labeled structures (Figure 1(e)).

To evaluate the data showing colocalization by double labeling of SAA with microtubules and vimentin filaments, we performed the PLA method demonstrating close proximity of labeled proteins (maximum distance between proteins that still enables reaction is 30 nm). The strong reaction product confirmed the colocalization of SAA and tubulin (Figure 1(g)) while a weak reaction product in the case of SAA and vimentin (Figure 1(h)) makes specific colocalization very improbable. The negative control by omitting primary antibodies gave a completely negative result (data not shown).

3.2. Nucleus. Often the strongest SAA staining was observed in the nucleus (Figures 2(a) and 2(a')) and in the area closely surrounding the nucleus where the majority of endomembranes are concentrated (Figure 2(c)). To find out if SAA colocalizes with endomembranes, especially endoplasmic reticulum, Golgi apparatus, or transport vesicles, endomembranes were labeled with a lipophilic stain DiO₆ that preferentially labels membranes of the endomembrane system and the mitochondrial membranes (Figures 2(b) and 2(c)). The double labeling of DiO₆ with SAA did not show any specific colocalization of the two labels making functional connection of SAA to endomembranes doubtful.

3.3. Communication. Surprisingly, intense labeling of SAA was found in tubular protrusions extending from the cells (Figure 2(d)). SAA was found at the tips of shorter extensions, probably filopodia, and also in longer nanotubes bridging neighboring cells. Occasionally, SAA labeling was

detected in dilatations of nanotubes (white arrows) described as gondolas in our previous work [26] and in extracellular membrane vesicles of cells that were attached to the bottom of the tissue culture dishes, labeled with DiO₆ (white arrowheads).

3.4. Multimerization. In order to determine potential multimerization of SAA, polyclonal anti-SAA antibodies against specific human synthetically derived SAA peptides were tested on immunoblots. Anti-SAA antibodies bound to different multimers of SAA following immunoblotting (Figure 3), including dimers, trimers, and higher multimers.

Anti-SAA antibodies against SAA peptides 1, 4, and 7 gave a similar pattern on the immunoblots, with a strong signal around 20 kD, whereas anti-SAA antibodies against SAA peptides 3, 5, 6, and 8 gave the strongest monomer band signal, with higher bands appearing also at multimers and potentially indicating conformational epitopes. The antibody directed against SAA peptide 2 gave only one high multimer signal.

4. Discussion

The cellular localization and potential functions of SAA are largely unknown in extrahepatic tissues.

To determine the location of SAA in endothelial cells, HCAEC were double labeled for codistribution of this protein with cytoskeletal elements, endomembranes, and nucleus. There was no apparent colocalization of SAA found with either actin filaments and very rare with vimentin (Figures 1(b) and 1(d)). However, this paper is the first, to our knowledge, indicating colocalization of SAA with microtubules in HCAEC (Figures 1(c) and 1(e)). The colocalization was further confirmed with the PLA method indicating the distance of less than 30 nm for the two proteins (as evaluated and confirmed by the manufacturer) between SAA and tubulin. The PLA method has recently been determined as comparable to FRET (fluorescence resonance energy transfer) method in case of semiquantitative and qualitative analysis [27]. The positive reaction with PLA makes it rather unlikely for SAA to be located inside any transport vesicles attached to microtubules by motor proteins, because in this case, the distance should largely exceed the maximum value enabling the reaction and even more the vesicle membrane would probably prevent the ligation process required for the reaction product to form. In conclusion, SAA seems to be directly attached to microtubules giving this protein a new potential role as a cytosolic protein distinct from the already known secretory version of this protein, which is mainly found in the serum.

Few studies to date have addressed the intracellular localization of SAA. In the 1970s, SAA-like protein had been detected using anti-SAA antibodies in human placenta. SAA localized to the cytoplasm of cells within the mesenchymal stroma (thought to be fibroblasts), to fetal stem vessel endothelium, and to perivascular tissue. SAA had been shown to be localized to 10 nm intermediate filaments which form characteristic perinuclear bundles following treatment with

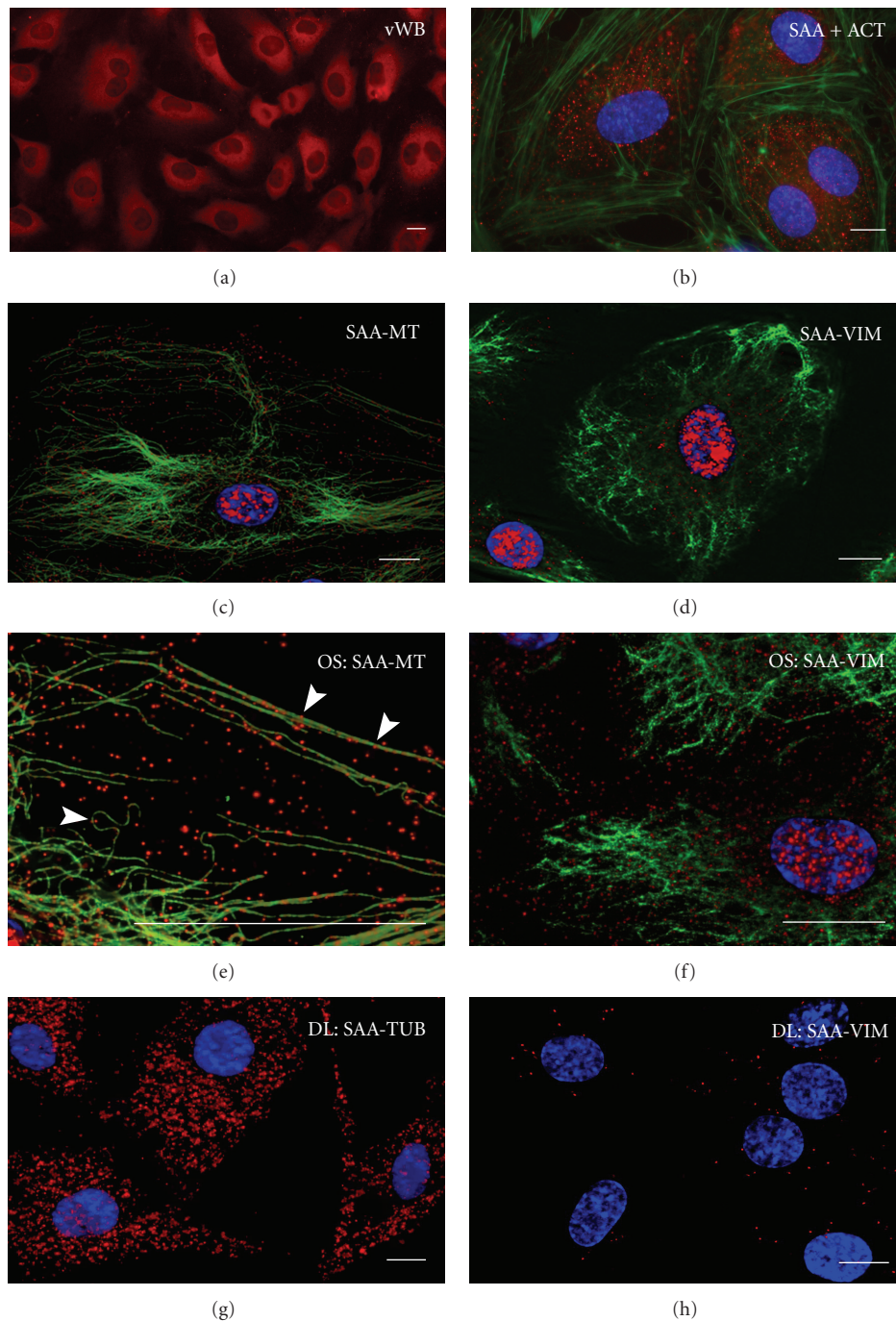


FIGURE 1: SAA localizes with microtubules in untreated HCAEC, but not with actin filaments or vimentin. In HCAEC Von Willebrand factor (vWB) is demonstrated (red) in all cells (a). In (b) labeling of actin filaments, SAA, and nuclei is presented. In (c) (stack of optical sections) and (e) (larger magnification) SAA labeling can be detected attached to microtubules MT (arrowheads). Vimentin labeling together with SAA does not show any specific colocalization ((d)—stack of optical sections and (f) larger magnification). In (b, c, d, e and f) the labeling of SAA is red, of cytoskeletal elements is green, nuclei is blue. With PLA method colocalization of SAA with tubulin ((g)-TUB) or with vimentin ((h)-VIM) resulted in red reaction product strongly expressed only for tubulin and SAA. Anti-SAA polyclonal antibodies 3, 5, and 6, which target SAA peptides with amino acid sequences 27–44, 59–72, and 68–84, respectively, were used in the designated SAA panels. Bar = 10 μm .

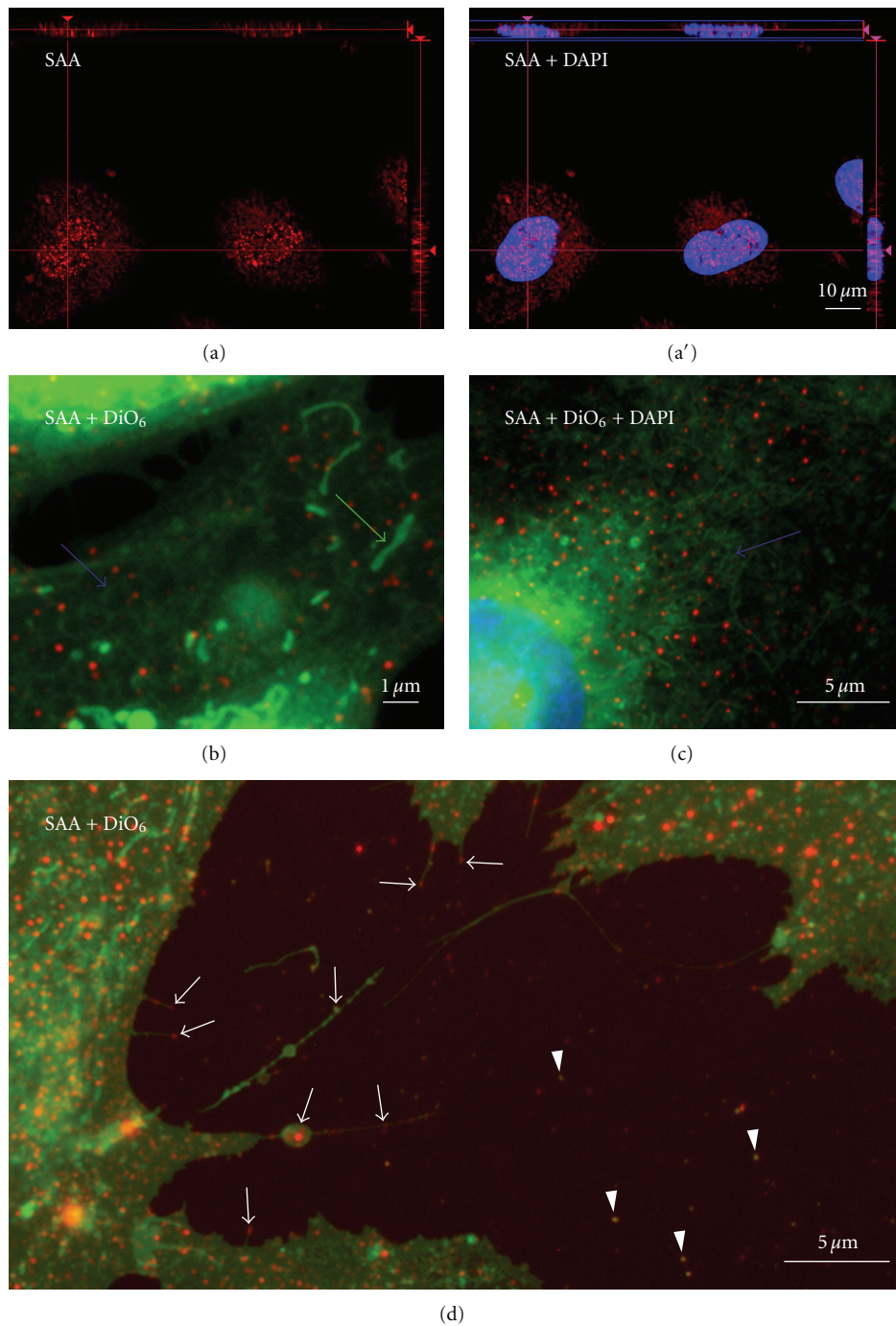


FIGURE 2: Staining for SAA is found in the nucleus, nanotubes, and budding vesicles; however, its association with endomembranes remains unclear. SAA in HCAEC shows a predominantly nuclear localization, with a strong signal also concentrated in the juxta-nuclear cytoplasm (a'). In (a) and (a') the cut views above and on the right of each panel represents the SAA mainly inside nuclei and in the perinuclear space. In HCAEC nuclei were stained with DAPI (blue), endomembranes were stained with 3,3'-dihexyloxycarbocyanin iodide (DiO₆) (green) and SAA was labeled with anti-SAA antibodies (red) (c). Merged labeling indicates SAA staining distinct from the endomembranes (b) and (c). Endoplasmic reticulum is shown by the purple arrows, mitochondrion with the green arrow. HCAEC were stained with DiO₆ and show colocalization of SAA with nanotubes (d). SAA was clearly detected at the tips of the filopodial protrusions (white arrows), and in vesicles dispersed outside the cells (white arrowheads). The association of SAA with endomembranes remains unclear. Anti-SAA polyclonal antibodies 3, 5, and 6, which target SAA peptides with amino acid sequences 27–44, 59–72, and 68–84, respectively, were used in the designated SAA panels.

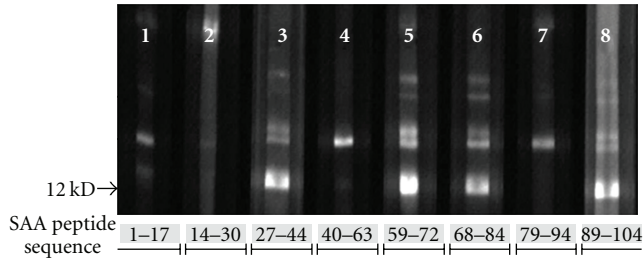


FIGURE 3: Multimerization of SAA. Human recombinant SAA was blotted onto nitrocellulose membranes and overlaid with polyclonal antibodies against synthetic SAA human peptides ranging from the indicated amino acid sequences: lane 1: SAA1–17, lane 2: SAA14–30, lane 3: SAA27–44, lane 4: SAA40–63, lane 5: SAA59–72, lane 6: SAA68–84, lane 7: SAA79–94, and lane 8: SAA 89–104. HRP-labeled secondary goat antirabbit antibody was used for secondary incubation followed by luminol and chemiluminescent detection. The SAA monomer at around 12 kD is indicated.

vinblastine, which disrupts microtubules in cultured embryonic fibroblasts [28]. SAA-like material has also been shown previously to be present intracellularly in cultured human endothelial cells, specifically in HUVEC [29]. In that study, SAA was found to be localized in an irregular filament-like staining pattern in the perinuclear regions of HUVEC. Our results showing colocalization of SAA with microtubules (Figure 1, antitubulin panels) explain the perinuclear concentration of SAA because of the well-known and, in our figures, well-documented concentration of microtubules close to the nucleus. We have previously shown differential mRNA expression of SAA in HCAEC versus HUVEC [25], indicating endothelial cell specificity.

In our paper (Figures 2(a) and 2(a')), there is a persistent immunofluorescent labeling of SAA also observed in the nucleus of HCAEC that varied considerably among cells, which could point to an additional role of SAA in the regulation of gene expression. A similar nuclear distribution of SAA was found in macrophages where an implication for SAA-dependent gene regulation was proposed. In this study, SAA was shown to be uptaken along with HDL in murine macrophages. SAA (or its fragments) was detected proceeding the perinuclear region and further the nucleus [30]. Within 60 min, SAA was found in the cytoplasm with subsequent localization at the plasma membrane and further extrusion from the cell. This study was the first to follow the path of SAA through a particular cell type. This paper gave the indication that SAA itself might regulate macrophage gene regulation. Both murine acute phase isoforms of SAA1.1 and SAA2.1 bind well to the plasma membrane, with only SAA2.1 taken up by the cell at 37°C and later detected in the nucleus. This suggests that the two isoforms have differential localizations and distinct physiological roles in cells [30]. This might very well be the case also in humans, and a more detailed analysis of the SAA isotypes and their locations within cells would be prudent in the future.

In addition to its putative intracellular functions, SAA seems to be involved in intercellular communication based on its location in nanotubes (Figure 2(d)). Intercellular

nanotubes (ICNs) are recently described thin protrusions that can connect cells which are several cell diameters apart [31]. Nanotubes are very dynamic structures, involving motor proteins in molecular cargo transport [32]. The discovery of SAA localized in nanotubes and their dilated regions (Figure 2, bottom micrograph) previously described as gondolas [26] indicate a possible exchange of SAA among cells and the role of this molecule in intercellular communication. Numerous extracellular vesicles (microparticles or exosomes) containing SAA (possibly arising from the budding of the plasma membrane) also point to the proposed role in intercellular communication.

Since SAA has been implicated in host defense, as having beneficial functions in the protection against microbes [12, 15, 33], we speculate that SAA localization could be an important additional prerequisite for this process to occur, especially in epithelial cells. The microtubular association of SAA in correlation to the recently found microtubular involvement in bacterial internalization into epithelial cells [34] points to a possible contribution of microtubules bearing SAA in the epithelial resistance to bacterial infection. In 2002, two groups almost simultaneously reported on acute phase SAA being able to form channels in planar lipid bilayer membranes at physiological concentrations and, thus, play a role in host defense, by placing a severe metabolic strain on bacterial cells [10, 11]. Electron microscopy by Wang et al. revealed that these channels were formed by SAA subunits arranged in hexamers, and circular-dichroism spectrum deconvolution and secondary structure prediction suggested that SAA contains ~50% residues in an α -helical conformation and below 10% in β structure [11]. Later studies indicated that these hexamers could be totally dissociated into monomers by urea treatment, with a concerted loss of its α -helical structure [35]. We have confirmed that, upon addition of 6M urea to human recombinant SAA, there is an apparent lack of self-multimerization and only monomers are recognized by anti-SAA antibodies from human sera on an immunoblot [36]. Since the studies by Wang et al. [35] were performed on murine SAA and little information is available in this regard concerning human SAA, the immunoblots we performed (Figure 3 and [36]) indicated that similar multimerization was occurring for human SAA. No X-ray crystallographic data is currently available for SAA, but it appears that the structure and function of this important acute phase protein is modulated *in vivo* upon binding to apolipoproteins and/or other factors into complexes in the circulation. Intracellular associations of SAA multimers within eukaryotic cells have been largely unaddressed; however, they could provide important clues to how SAA functions within these cells and whether its locations are influenced by these interactions.

5. Conclusion

SAA localizes with microtubules in untreated HCAEC, but not with actin filaments or vimentin. These data indicate that SAA can be attached to microtubules and can possibly be transported between neighboring cells by means of

nanotubes or budding vesicles. Staining for SAA is also found in the nucleus which indicates that SAA might have nuclear-specific functions.

Intra- and intercellular SAA could play different roles in the physiological state, depending on the environment where it is located. On the basis of the distribution in endothelial cells, SAA can be predicted to contribute also to the antibacterial barrier function of this epithelium.

Conflict of Interests

The authors declare that there is no conflict of interests.

Acknowledgments

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