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Expression of apolipoprotein serum amyloid A mRNA in human atherosclerotic lesions and cultured vascular cells: Implications for serum amyloid A function

(endothelial cells/macrophages/smooth muscle cells/adipocytes/interleukin)

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ABSTRACT **Altered lipoprotein metabolism and vascular injury are considered to be major parts of the pathogenesis of atherosclerotic lesions. Serum amyloid A (SAA) is a family of acute-phase reactants found residing mainly on high density lipoproteins (HDL) in the circulation. Several functions for the SAAs have been proposed that could be important in atherosclerosis. These include involvement in cholesterol metabolism, participation in detoxification, depression of immune responses, and interference with platelet functions. Like other acute-phase reactants, the liver is a major site of SAA synthesis. However, studies in the mouse have revealed that several cell types including macrophages express SAA. Furthermore, we recently found that SAA mRNA expression can be induced in the human monocyte/macrophage cell line, THP-1. In the present study, human atherosclerotic lesions of coronary and carotid arteries were examined for expression of SAA mRNA by *in situ* hybridization. Surprisingly, SAA mRNA was found in most endothelial cells and some smooth muscle cells as well as macrophage-derived "foam cells," adventitial macrophages, and adipocytes. In addition, cultured smooth muscle cells expressed SAA1, SAA2, and SAA4 mRNAs when treated with interleukin 1 or 6 (IL-1 or IL-6) in the presence of dexamethasone. These findings give further credence to the notion that the SAAs are involved in lipid metabolism or transport at sites of injury and in atherosclerosis or may play a role in defending against viruses or other injurious agents such as oxidized lipids. Furthermore, expression of SAAs by endothelial cells is compatible with the evidence that SAA modulates platelet aggregation and function and possibly adhesion at the endothelial cell surface.**

Serum amyloid A (SAA), a multigene family of HDL [high density lipoprotein(s)] apolipoproteins (apo), is found in mammals and birds (1–72). They are major acute-phase reactants (8) whose plasma levels are elevated several hundred- to 1000-fold as part of a response to various injuries including trauma or infection (8, 9). While the functions of the apoSAAs are not known, they must have considerable physiological significance apart from the role as the precursor of amyloid A protein fibrils. Because the apoSAAs are acute-phase reactants, the liver has been considered to be the primary site of expression (8, 10), where they are induced by inflammatory cytokines interleukins 1 and 6 (IL-1 and IL-6) and tumor necrosis factor (TNF) (11–14). However, extrahepatic expression has been reported in mice and hamsters (15, 16). Macrophages and adipocytes are SAA-expressing cell types in the mouse (15, 17). The production of apoSAA by macrophages and other nonhepatic cells is an interesting feature in that these cells can provide a local source of apoSAA. As HDL apoproteins, the SAAs are poised to have a role in lipid transport and/or metabolism (18, 19). Evidence for several other actions of the SAAs includes detoxification (20, 21), depression of immune responses (22, 23), interference with platelet aggregation (24), induction of collagenase activity (25), and inhibition of neutrophil oxidative burst (26). We searched for SAA expression in atherosclerotic plaques because these lesions show evidence of altered lipid metabolism (27, 28), features of injury response (29–31) including expression of IL-1 and IL-6 (32), and the presence of microorganisms such as viruses and chlamydia (33, 34). Our observation of SAA mRNA expression by several cell types of the atherosclerotic lesions leads to the implication that the SAAs play a role in the vascular responses to injury.

MATERIALS AND METHODS

Vascular Tissues. Coronary arteries from six male heart transplant recipients 36–54 years of age, one carotid endarterectomy specimen from a 56-year-old female, and one segment of vein functioning as a coronary bypass from a 55-year-old male with atherosclerotic coronary disease were obtained with informed consent. The tissues were fixed in formalin, embedded in paraffin, and prepared for *in situ* hybridization as described elsewhere (35, 36).

Probes and *in Situ* Hybridization. RNA probes were transcribed from pGEM-1 transcription plasmid (Promega) that contained a 110-bp sequence of mouse SAA1 cDNA (p125) (37). This nucleotide sequence encompasses a domain coding for amino residues 30–66 that is highly conserved among various species and is 81% homologous with human SAA1 and SAA2 mRNAs and is 71% homologous with human apoSAA4 mRNA (refs. 4 and 38–42; GenBank accession nos. M10906, X51445, and M81349). The probe has 100% identity with stretches of 42 and 43 nucleotides of SAA1 and SAA2 cDNAs, respectively. For apoSAA4, the longest regions of high identity are 24 of 25, 29 of 31, and 50 of 56 nucleotides. Fig. 1 shows the sense-strand sequence of the complementary RNA probe aligned with the corresponding sequences of human apoSAA1, apoSAA2, and apoSAA4. The probe should hybridize equally well with apoSAA1 and apoSAA2 mRNAs but may not hybridize efficiently with apoSAA4 mRNA. This mouse SAA probe hybridizes by Northern blot to apoSAA mRNA of human liver and human monocyte/macrophage THP-1 cells (unpublished observation).

The plasmid was linearized, and antisense or sense RNA was transcribed with T7 or SP6 RNA polymerase (Promega) in the presence of [^{3}H]UTP, [^{3}H]GTP, and [^{3}H]CTP (New England Nuclear). Probe was added (500,000 cpm) to 100 μl of hybridization buffer per slide, incubated overnight, and washed (35). Slides were coated with NTB-2 liquid emulsion

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Abbreviations: apo, apolipoprotein(s); apoSAA or SAA, serum amyloid A; HDL, high density lipoprotein(s); IL-1, interleukin 1; IL-6, interleukin 6; Dex, dexamethasone; SMC, smooth muscle cells.

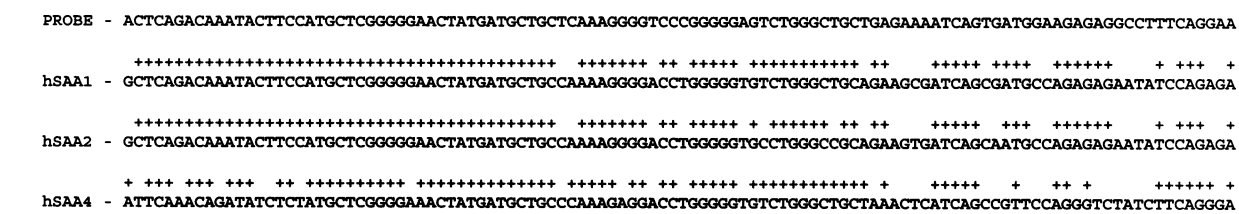

FIG. 1. Alignment of the apoSAA probe nucleotide sequence with the nucleotide sequence of the human apoSAAs. The sense-strand sequence of the probe, sp125, and corresponding sequences of human apoSAA1 (40), apoSAA2 (39), and apoSAA4 (4) mRNA are shown. The probe was subcloned from mouse apoSAA1 and begins in the codon for amino acid residue 30 at nucleotide 122. The human apoSAA sequences begin in the codon for amino acid residue 30 at nucleotide 146 numbered from the A of the ATG initiation codon. Matching nucleotides are indicated with a plus.

(Eastman Kodak), exposed in the dark for 12–14 days, developed in D-19, treated with Rapid Fix (Eastman Kodak), counterstained with hematoxolin and eosin, and mounted with Histomount (National Diagnostics). Sections of each tissue were hybridized in two or three separate experiments with similar results.

Immunohistochemistry. Sections from the same blocks used for *in situ* hybridization were immunostained with anti-α-actin antibody for smooth muscle cells (SMC) (Boehringer Mannheim); Ham-56 for macrophages, capillary endothelial cells, and monocytes (43); Leu-22 (Becton Dickinson) for lymphocytes; and CD-45 (Dakopatts, Glostrup, Denmark) for leukocytes. Antibodies were detected with biotinylated anti-rabbit IgG secondary antibody and visualized by treatment with avidin–biotin–peroxidase complex and 3,3′-diaminobenzidine (36).

Cell Culture and RNA Blot Analysis. Human adult SMC were derived from a nondiseased thoracic aorta of a 51-year-old male. The aortic media was enzymatically digested (44), and cells were grown in Waymouth's medium containing 20% (vol/vol) fetal bovine serum (HyClone), penicillin (100 units/ml), and streptomycin (100 μg/ml). Confluent monolayers of SMC were treated in serum-free Waymouth medium as follows: with and without 1 μM dexamethasone (Dex); with IL-1α and IL-1β, each at 10 ng/ml with and without Dex; IL-6 at 500 units/ml with and without Dex. Twenty hours after additions, the medium was removed, and total RNA was isolated, electrophoresed, transferred to nylon membrane, and hybridized with a ^{32}P-labeled human SAA1 cDNA (40, 69) and a 28S rRNA probe. The blot was subjected to PhosphorImager analysis (Molecular Dynamics) in the Markey Molecular Medicine Center at the University of Washington. The quantity of SAA mRNA in each sample was normalized to the level of 28S rRNA.

PCR. The contiguous sequence of human apoSAA1 was derived from two partial but overlapping sequences: GenBank accession nos. M10906 and X51439. The sequences of SAA2 and SAA4 are from accession nos. X51445 and M81349, respectively. PCR primers for the three human SAA genes were synthesized (Operon Technologies, Alameda, CA). We have demonstrated by complete sequence analysis that these PCR primers amplify the specific human SAA1, SAA2, or SAA4 products from cDNA synthesized from human liver and the human monocyte/macrophage cell line THP-1 (69). For PCR amplification, SAA1-, SAA2-, and SAA4-specific primers were used to amplify cDNA reverse-transcribed from SMC RNA. Amplification reactions were carried out as recommended (GeneAmp, Perkin–Elmer/Cetus) in a Coy TempCycler (Coy Laboratory Products, Ann Arbor, MI) described elsewhere (69).

RESULTS

SAA mRNA Is Expressed by Various Cells of Atherosclerotic Lesions. Tissue sections were subjected to *in situ* hybridization with either antisense or sense RNA. The antisense RNA probe hybridized to several cell types, whereas hybridization with the sense probe was always negative. Fig. 2 shows parallel sections hybridized with sense (Fig. 2*A*) and antisense (Fig. 2*B*) SAA RNA. The endothelial cells (arrows) overlay the plaque cap (p. cap), and their flattened nuclei can be seen at the border of the lumen (Lu). Silver grains are clearly associated with the endothelial cells (arrows) and some cells within the lesion cap of sections hybridized with the antisense probe (Fig. 2*B*) but not the sense probe (Fig. 2*A*). Furthermore, this observation also shows that the antisense hybridization to lumenal endothelial cells is not a result of an artifact produced at the tissue edge. Some of the strongest signals in endothelial cells were observed in those lining the lumen of atherosclerotic coronary arteries (Fig. 2), in new-plaque vessels, in vaso vasorum (Fig. 2*G*), and in adventitial vessels (not shown).

Focal areas of tissue rich in macrophage "foam cells," as determined histologically and by HAM-56 immunoreactivity, exhibited very strong hybridization signals for SAA mRNA (Fig. 2 *C* and *G*). Hybridization was also observed in macrophages of the intima and extravasated blood in the adventitia (Fig. 2*D*).

Many adventitial adipocytes were strongly positive for SAA mRNA (Fig. 2*E*), with silver grains overlaying the thin layer of cell cytoplasm surrounding each intracellular vacuole.

Some SMC, identified by their location, morphology, and comparison with sections of the same tissue immunolabeled with anti-smooth-muscle α-actin antibody, expressed SAA mRNA. Fig. 2*F* shows labeled and unlabeled SMC.

Fig. 2*G* is a dark-field photomicrograph (×125) showing a portion of an atherosclerotic plaque in an occluded vessel. A focus of foamy macrophages (M) and endothelial cells of new vessels (V) in the plaque shows high levels of hybridization as indicated by the bright refractile silver grains. Several smaller but highly labeled vessels are also present in this field as well as numerous labeled macrophages and SMC.

Cultured SMC Express mRNAs of Three SAA Genes. Human adult aortic SMC grown in culture were exposed to IL-1 or IL-6 in serum-free medium with or without Dex. SAA mRNA was not detected unless Dex was included in the medium. The addition of Dex alone permitted a low level of SAA mRNA expression (Fig. 3, control), and IL-1 and IL-6 were very effective inducers of SAA mRNA expression but only in the presence of Dex, stimulating 20- and 50-fold increases compared with Dex alone (Fig. 3). To determine what *SAA* genes are expressed in SMC, total RNA was reverse-transcribed and PCR-amplified with specific primers designed to yield a different-size PCR product from SAA1, SAA2, and SAA4 mRNAs (Fig. 4). PCR products for all three *SAA* genes were amplified for the SMC treated with Dex (not shown), Dex and IL-1, or IL-6. The *SAA1*, *SAA2*, and *SAA4* products are respectively 303, 328, and 397 nucleotides long (Fig. 4).

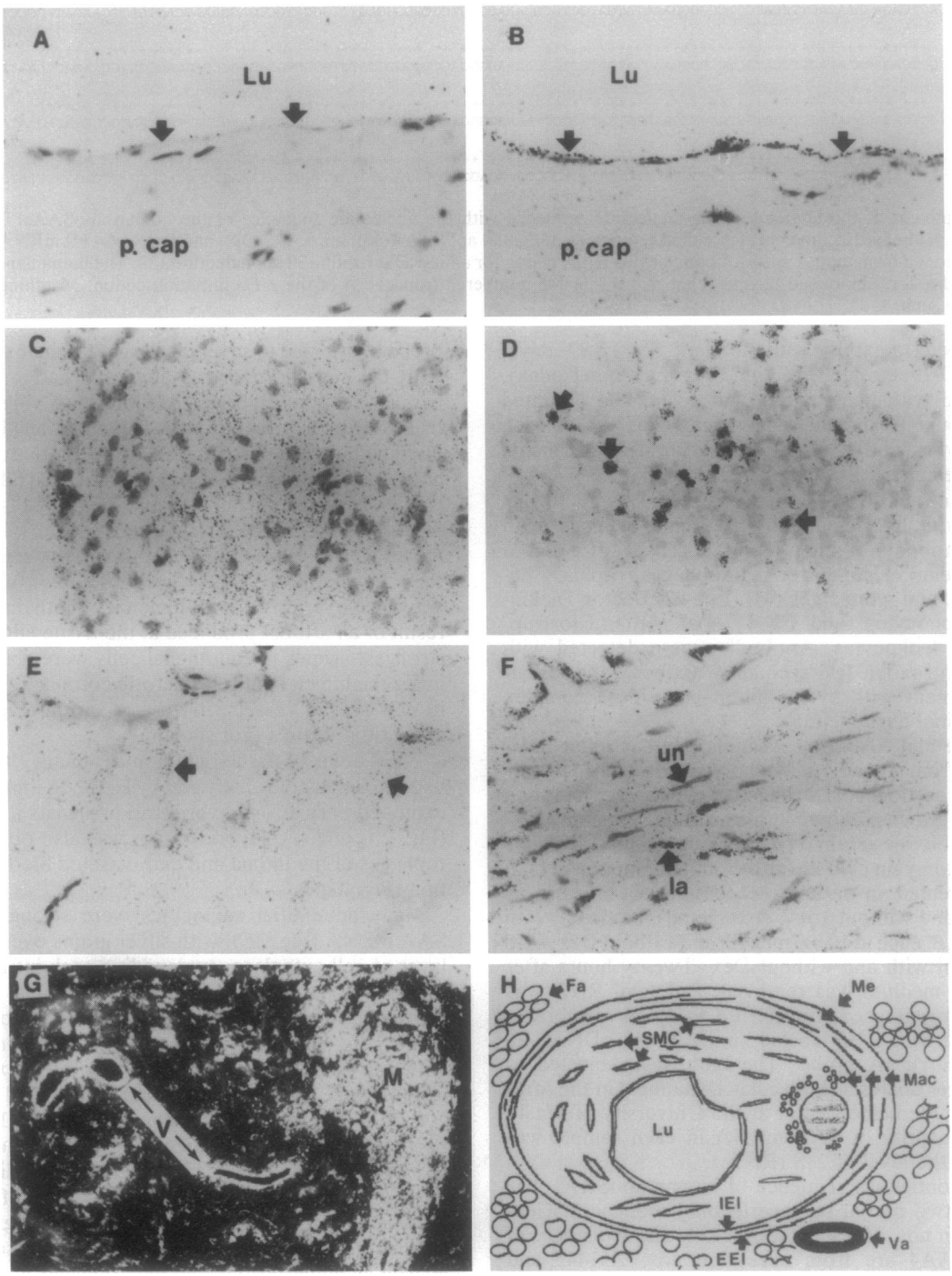

FIG. 2. Photomicrographs of *in situ* hybridized atherosclerotic lesions. (*A* and *B*) Parallel sections of thickened intima and lumen hybridized with sense (*A*) and antisense (*B*) SAA RNA. Note the specific hybridization to endothelial cells (arrows) and to some intimal cells with the antisense (*B*) but not the sense (*A*) SAA RNA. The lumen (Lu) and plaque cap (p. cap) are indicated. (*C–G*) Hybridization with the antisense RNA probe to foam cells (*C*); macrophages of blood located in adventitia, where the arrow indicates several cells hybridized with the SAA RNA probe (*D*); adventitial fat adipocytes, with the silver grains overlaying the thin cytoplasm (*E*); a section of thickened medium, showing labeled (la) as well as unlabeled (un) SMC (*F*); and a lower power dark-field photomicrograph of an atherosclerotic lesion (*G*). M, macrophages; V, new vessels. (*H*) A representative schematic of an arterial atherosclerotic lesion. Lu, lumen; Me, medium; Va, vasa vasorum; Fa, adventitial fat; IEI, internal elastica; EEI, external elastica; Mac, macrophage-rich region. (*A* and *B*, ×500; *C–F*, ×250; and *G*, ×125.)

DISCUSSION

Our finding that SAA mRNA is expressed by several cell types in atherosclerotic lesions raises a number of possibilities for SAA functions and their role in the pathogenesis or regression of atherosclerotic plaques (46). The association of apoSAAs with HDL and to a lesser extent with other lipoprotein fractions is compatible with the idea that apoSAA function(s) is related to lipid metabolism. The fact that thus far the apoSAAs or mRNAs are expressed at high levels only during inflammation or following injury suggests that SAA function(s) is part of a mechanism of recovery from injury. ApoSAA in lesions could modify cellular lipid content by facilitating lipid removal or contribute to the accumulation of lipid and further lesion development. This process may be important in preventing or reversing atherosclerosis. Several other observations of SAA behavior suggest possible roles in lipid metabolism. (*i*) Since apoSAA replaces apoA-1 (47, 48),

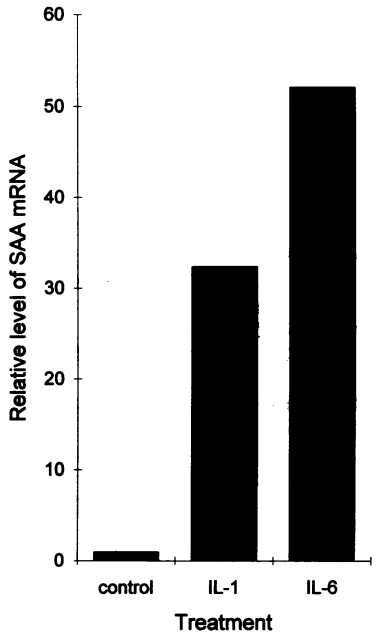

FIG. 3. SAA mRNA is expressed by cultured human adult aortic SMC. At confluence, monolayers of SMC were incubated in serum-free Waymouth's medium with 1 μM Dex for 20 hr with no additions (control), IL-1α and IL-1β (10 ng/ml of each), and IL-6 (500 units/ml). RNA was isolated and electrophoresed through a denaturing 1.2% agarose gel, transferred to nylon membrane, and hybridized with ^{32}P-labeled human SAA1 cDNA. The washed RNA blot was quantitated by PhosphorImager analysis and the data are presented as fold stimulation compared with Dex-alone treatment.

a major ligand for HDL–cell interactions and cholesterol efflux (49, 50), SAA could influence HDL clearance of excess cellular cholesterol. (*ii*) Evidence that mouse macrophages possess a receptor for SAA (51) suggests that SAA-rich HDL may be redirected to cells that possess an SAA receptor. (*iii*) SAA could modulate LCAT (lecithin-cholesterol acyltransferase) activity (52, 53), thereby effecting net cholesterol accumulation by HDL. (*iv* and *v*) Other proposed SAA functions could be active in the vascular wall lesion; thus, SAA (*iv*) could induce secretion of collagenase in SMC (54, 55) as it does in rabbit synovial fibroblasts (25, 56) and thereby play a role in remodeling of the plaque or the vessel wall, or (*v*) could modulate platelet aggregation (24) or

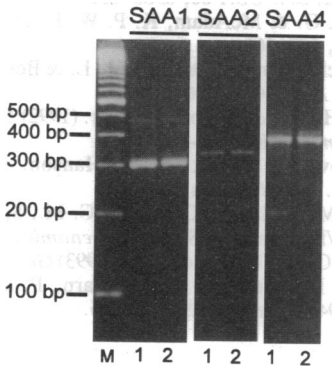

FIG. 4. SAA1, SAA2, and SAA4 mRNAs are expressed by cultured human adult aortic SMC. Total RNA from SMC treated with Dex and IL-1 (lanes 1) or IL-6 (lanes 2) was reverse-transcribed and PCR-amplified in separate reactions with specific oligonucleotides for the three SAA mRNAs. PCR fragments were separated on a 2.5% low-melting agarose gel, stained with ethidium bromide, and photographed. Markers of a DNA ladder (100 bp steps) are shown in Lane M.

adhesion at the endothelial cell surface and effect thrombus formation (an important process in development of vascular occlusion) and thereby may be a means of controlling platelet activity at vascular injury sites. Finally, it should be noted that the differences in the amino acid sequence of the three expressed apoSAAs suggest that each may be adapted to a particular function, as observed with products of other polygene families of ancient origin (57).

Only some SMC were SAA-mRNA-positive in lesions, and the fraction of labeled SMC varied among the different lesions examined. The lack of a hybridization signal by some cells may reflect the absence of apoSAA mRNA or a level of mRNA below the threshold of detection. Since SMC are known to be a heterogeneous population (58, 59), SAA expression may be another example of SMC heterogeneity.

Cells of human atherosclerotic lesions (32, 60) and cultured endothelial and SMC can express IL-1, IL-6, and TNF (61, 62). These cytokines also induce SAA expression in human primary hepatocytes and hepatoma-derived transformed cell lines (11, 12, 14, 63). Our finding that IL-1 and IL-6 induce SAA mRNA expression in cultured SMC (Figs. 3 and 4) suggests that SMC, endothelial cells, and macrophage SAA expression is induced by these cytokines *in vivo*. Furthermore, because vascular cells and macrophages can express these cytokines in response to various injurious agents like oxidized LDL (64) or viruses (65), SAA expression in the atherosclerotic lesion may be induced by paracrine or autocrine mechanisms in response to events that stimulate cytokine production by these cells.

The human apoSAA gene family is known to be composed of four genes (1). Human apoSAA1, apoSAA2, and apoSAA4 are transcribed and translated (4, 39–42, 66, 70); but human apoSAA3 is a nonexpressed gene (67, 71). Another gene locus originally identified as *SAA4* (68) was misidentified but may represent a fifth SAA gene, "*SAA5*" (71). In the mouse most extrahepatic SAA expression is of the *SAA3* gene (15, 17, 45). Whether a select set of apoSAA mRNAs is expressed in cells of atherosclerotic plaques has not yet been determined. But expression of SAA1, SAA2, and SAA4 mRNAs in cultured SMC (Fig. 4) and in the human monocyte/macrophage cell line THP-1 (69) indicates that expression of all three *SAA* genes is likely.

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