Acute-phase response protein serum amyloid A stimulates renal tubule formation: studies in vitro and in vivo

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Kelly KJ, Kluve-Beckerman B, Dominguez JH. Acute-phase response protein serum amyloid A stimulates renal tubule formation: studies in vitro and in vivo. Am J Physiol Renal Physiol 296: F1355–F1363, 2009. First published March 25, 2009; doi:10.1152/ajprenal.90622.2008.—Serum amyloid A protein serum amyloid A (SAA) surges 1,000-fold in the blood of acute-phase animals, and yet its function during these acute events remains unknown. We report herein that SAA stimulates a developmental program in cultured NRK-52E cells that culminates in differentiated and functional tubules that feature a proximal tubule phenotype. We also found strong SAA expression in states of tubule formation (in utero stage) and regeneration (recovery from ischemia-reperfusion injury). These data lend support to a novel view of a more localized renal acute-phase reaction, where renal SAA may act as a paracrine or autocrine molecule that promotes tubule formation during development and repair.

THE ACUTE-PHASE RESPONSE IS a systemic reaction to inflammation from infection, physical trauma, or chemical injury, and is characterized by changes in the plasma levels of certain proteins (4). While few blood proteins decrease in the acute-phase response (4), the most dramatic change is the very rapid 1,000-fold surge in serum amyloid A protein (SAA) (26). The prevailing view is that the acute-phase response is generally beneficial, a concept largely based on relatively mild anti-inflammatory activity of C-reactive protein, another acute-phase protein that follows the acute phase would limit inflammatory tissue injury. However, this anti-inflammatory action is not shared by SAA, which does not have a known anti-inflammatory or a physiological target (4, 26). Hence it is not at all clear how the massive hepatic secretion of SAA protein that follows the acute phase would limit inflammatory tissue injury.

Contemporary investigation has revealed that while hepatic expression of SAA increases during inflammation, expression of apolipoprotein A-1 (apo A-1) decreases (6), resulting in near complete replacement of apo A-1 in high-density lipoprotein fraction 3 (HDL3) by SAA (10). Accordingly, some have suggested that the principal role of SAA is to modulate HDL function (1). However, this conclusion is seriously conflicted, in that investigators have reported that HDL enriched with SAA promotes cholesterol efflux (25), while others have described the opposite result (1). Furthermore, SAA may have different effects on cholesterol efflux with or without HDL (8). Therefore, assigning specific functions in lipid metabolism to acute-phase SAA is fraught with difficulty.

In adult animals, hepatic SAA is promptly secreted following the inflammatory stimulation that releases proinflammatory cytokines tumor necrosis factor-α (TNF-α) and interleukins 1 and 6 (4, 26). These cytokines activate SAA gene expression (4, 26). SAA is also expressed by extrahepatic tissues, including renal proximal tubules (20) and other epithelia (27). Regrettably, finding renal SAA expression during stress did not reveal its potential function. On the other hand, it was found that persistent hepatic secretion of SAA in chronic inflammation could lead to SAA deposition and formation of amyloid fibers at distant sites, a complication known as secondary amyloidosis (16). Our work was initially focused on amyloid formation by proximal tubular NRK-52E cells in vitro. However, we found instead that SAA was a powerful stimulant for renal tubule formation in vitro, and it was upregulated during states of renal tubulogenesis in vivo. Accordingly, we propose the hypothesis that SAA is a powerful stimulant of proximal tubule morphogenesis.

MATERIALS AND METHODS

Cell culture. NRK-52E cells, CRL-1571, were acquired from American Type Culture Collection (Manassas, VA). Cells (passage 2–5) were cultured on glass-bottom eight-well chambers. The culture medium was DMEM (Gibco Invitrogen, Carlsbad, CA) containing 1.5 g/l sodium bicarbonate and 5% bovine serum, in an atmosphere of 5% CO2–95% air at 37°C. We also studied S1 proximal tubule cells that were originally isolated from the earliest portion of the renal proximal tubule from a large T antigen transgenic mouse and were a gift of Dr. Glen T. Nagami (Veterans Affairs Greater Los Angeles Healthcare System at West Los Angeles) (11, 21). The S1 cells were cultured in DMEM-Ham’s F12 medium supplemented with 7% fetal bovine serum at 37°C in an atmosphere of 5% CO2–35% O2. When NRK-52E or S1 cells became confluent, recombinant murine SAA 1.1 (SAA, 5–100 μg/ml) or 60 mM urea vehicle (final concentration) were added to experimental or control cell groups. The culture media with SAA or urea were changed every 3 days. The cells were maintained in culture for 15 days and then were either fixed with 4% paraformaldehyde in preparation for immunohistochemistry (below) or subjected to fluorescence uptake studies (below).

Production and purification of recombinant mouse SAA. SAA cDNA (corresponding to residues 1–103) was amplified from a cloned plasmid p8SAA2.63 kindly provided by K.-I. Yamamoto and S. Migita (Kanazawa University, Kanazawa, Japan) (30) and inserted into pET21a(+) (Novagen, Madison, WI) between NdeI and BamHI cloning sites. Recombinant pET-SAA plasmids were transformed into Escherichia coli strain B834(DE3)pLys (Novagen). Plasmid DNA isolated from large-scale culture was sequenced to verify the identity of SAA1.1. For SAA production, pET-SAA-containing E. coli were cultured in Luria-Bertani medium supplemented with carbenicillin (250 μg/ml) and chloramphenicol (34 μg/ml). Initially, 50-ml cultures were grown for 6 h at 37°C. E. coli were pelleted by centrifugation, resuspended in 1 liter of fresh medium, and incubated at 37°C until OD600 = 0.6. E. coli were again pelleted, resuspended in 1 liter of...
fresh medium containing 1 mM isopropylthio-β-D-galactoside to induce SAA expression, catalyzed by T7 RNA polymerase, and incubated at 30°C for 2 h. Rifampicin (200 µg/ml) was added after the first 30 min to inhibit E. coli RNA polymerase.

For SAA purification, E. coli cells were lysed by repeated freeze-thawing in 8 M guanidine-HCl, 0.05 M Tris-HCl, pH 8.2, and DNA was sheared by sonication. Lysates were cleared by centrifugation and subjected to molecular sieve chromatography using Sepharose CL-6B (Sigma-Aldrich, St. Louis, MO) in 4 M guanidine-HCl, 0.025 M Tris-HCl, pH 8.2. Peak fractions in the low-molecular-weight area were pooled, dialyzed against water, lyophilized, and dissolved in 6 M urea, 0.025 M Tris-HCl, pH 8.5, incubated at 37°C for 1 h, and centrifuged to remove insoluble material. The urea-soluble fraction was applied to a chromatofocusing column of Polybuffer exchanger PBE 94 (Sigma-Aldrich) equilibrated with 6 M urea, 0.025 M Tris-HCl, pH 8.5. Elution was accomplished using 7% Polybuffer 96, 3% Polybuffer 74, pH 5.5, in 6 M urea. SAA-containing fractions were identified by immunoreactivity with anti-(murine SAA) antiserum generated in this laboratory. SAA was precipitated with 80% saturated ammonium sulfate to remove nondialyzable Polybuffer components, dialyzed against water, and lyophilized. Purified preparations were dissolved at 10 mg/ml in 6 M urea, 0.025 M HEPES, pH 7.3, 0.15 M NaCl, and analyzed by Tris-tricine SDS-PAGE. Immediately before withdrawal of aliquots for addition to cell culture medium, SAA preparations were centrifuged at 13,000 g for 5 min.

Purified SAA preparations had endotoxin levels of <2.5 EU/mg (0.25 pg/g SAA) as measured by the Limulus amebocyte lysate assay (Cambrex, Walkersville, MD).

Fig. 1. Assessment of serum amyloid A (SAA) purity. Purified mouse SAA1.1 was analyzed by Tris-tricine SDS-PAGE as described in METHODS. Protein was transferred from gels onto membranes of polyvinylidene (PVDF) for NH₂-terminal sequencing (lanes 1 and 2) or nitrocellulose for immunodetection with anti-SAA antiserum (lanes 3 and 4). PVDF was stained with Coomassie blue R-250 before the indicated band for sequence analysis which confirmed identity with mouse SAA1.1 was cut out. Lanes 1 and 4, molecular weight markers; lanes 2 and 3, purified recombinant mouse SAA.

Fig. 2. Tubule formation in cultured NRK-52E cells. Congo red stain of a monolayer of NRK-52E cells exposed to SAA (100 µg/ml) for 15 days is shown. A: large convoluted tubule-like structure (black arrow). B and C: phase-contrast microscopy of NRK-52E cells exposed to 60 mM urea (B) or SAA (100 µg/ml; C). D–F: immunohistochemistry of NRK-52E cells exposed to SAA (100 µg/ml) for 15 days. Antibody to sodium/hydrogen exchanger 3 (NHE3) labeled mainly tubule-like structures, with a branch (open arrow) showing a luminal deposition (inset, arrowhead; D). Antibody to organic anion transporter 1 (OAT1) labeled mainly tubule-like structures (E). F: summary chart of samples labeled for NHE3 and OAT1 showed increase in tubule-like structures as a function of SAA concentration (means ± SE; n = 16).
SDS-PAGE, immunodetection, and NH₂-terminal sequence analysis. Tris-tricine SDS-PAGE was carried out as described by Schagger and von Jagow (23). Separating, spacing, and stacking layers contained 16.5, 10, and 4% polyacrylamide, respectively. Proteins were stained with Coomassie blue R-250 or transferred to nitrocellulose for immunodetection of SAA, which was accomplished using rabbit anti-mouse recombinant SAA antiserum generated in this laboratory (1:2,000) followed by goat anti-rabbit immunoglobulin conjugated to alkaline phosphatase (1:1,000, Biosource International, Camarillo, CA). Color was developed with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (Bio-Rad Laboratories, Richmond, CA). SAA in a companion gel was transferred to a polyvinylidene fluoride membrane (ProBlott, Applied Biosystems, Foster City, CA) for NH₂-terminal sequence analysis, which was performed on an Applied Biosystems Procise model 491 cLC protein sequencer and confirmed identity to mouse SAA.

Induction of acute-phase response in mice. All animal experimentation was conducted in conformity with the Guiding Principles for Research Involving Animals and Human Beings. The investigative protocols were approved by the Institutional Animal Care and Use Committee at Indiana University. C57BL mice were injected subcutaneously with casein [5 ml of 10% (wt/vol)] (C-5890, Sigma-Aldrich) dissolved in 10 mM NaOH. Mice were anesthetized 24 h later; blood was collected, and then death was ensured by cervical dislocation. Sera collected from control (untreated) and casein-treated mice were tested for SAA by immunodiffusion using the anti-mouse SAA antiserum described above. The liver and kidneys were also harvested and stained for SAA by immunofluorescence (below).

Acute kidney injury. Adult C57BL mice were anesthetized with pentobarbital sodium (40 –70 mg/kg ip), sterilely prepped, shaved, and placed on a homeothermic table to maintain core body temperature at 37°C. The left renal pedicle was occluded with a nontraumatic microvascular clip via a midline incision for 30 min. After ensuring color change of the kidney consistent with reperfusion, the incision was closed and the animal recovered. The contralateral kidney was used as a control. After surgery, at 24 and 48 h mice were again anesthetized with pentobarbital sodium (40–70 mg/kg ip), steriley prepped, shaved, and placed on a homeothermic table to maintain core body temperature at 37°C. The left renal pedicle was occluded with a nontraumatic microvascular clip via a midline incision for 30 min. After ensuring color change of the kidney consistent with reperfusion, the incision was closed and the animal recovered. The contralateral kidney was used as a control. After surgery, at 24 and 48 h mice were again anesthetized with pentobarbital sodium (80 –140 mg/kg), kidneys were surgically exposed, perfusion fixed with 3.8% paraformaldehyde, and removed (13). In preparation for Western blot analysis, three additional mice were also subjected to unilateral renal ischemia as described. At termination, blood was collected and the serum was separated. The abdominal organs, including liver and kidneys, were then manually perfused for 3 min with...
30 ml of ice-chilled PBS infused into the left ventricle. The effluent was collected from the left iliac vein. We also collected the last milliliter of perfusate, the perfused liver, and both kidneys. These samples were analyzed for SAA content by Western blotting (below).

**Congo red staining.** Congo red staining was performed according to the method of Westermark and colleagues (29). Briefly, cells cultured on eight-well chamber slides were rinsed in PBS, fixed in 10% neutral-buffered formalin for 10 min, rinsed in water, immersed in Gill’s no. 3 hematoxylin for 90 s, rinsed in water, immersed for 20 min in alkaline solution A (1% NaCl in 80% ethanol made alkaline by adding 1 ml of 0.1% NaOH/100 ml), immersed for 45 min in alkaline solution B (0.2% Congo red in solution A), and rinsed twice for 5 s in 100% ethanol.

**Immunohistochemistry for organic anion transporter 1 and sodium/hydrogen exchanger 3.** Cells on eight-well chamber slides were rinsed in PBS, fixed in 10% neutral-formalin for 15 min and rinsed again in PBS. Antigen retrieval was performed by incubation in 10 mM sodium citrate, pH 6.0, for 15 min at 95°C and then for 20 min at room temperature. After slides were rinsed in PBS, endogenous peroxidase was quenched by incubation in 0.3% hydrogen peroxide in 100% methanol for 20 min. Blocking was performed for 30 min in 1.5% normal goat serum in PBS. Primary and secondary antibodies were diluted in blocking solution. Slides were incubated sequentially in primary antiserum for 1 h, secondary antiserum for 30 min, and horseradish peroxidase ABC (Vector Laboratories, Burlingame, CA) for 45 min. Color was developed using Sigma FAST diaminobenzidine and urea tablets (Sigma-Aldrich) as a substrate. Primary antibodies were rabbit polyclonal anti-rat organic anion transporter 1 (OAT1; affinity-purified IgG) used at a concentration of 6.5 μg/ml (catalog no. OAT11-A, Alpha Diagnostic International, San Antonio, TX) and rabbit anti-rat sodium/hydrogen exchanger 3 (NHE3; affinity-purified...
IgG) used at a concentration of 20 μg/ml (catalog no. NHE31-A, Alpha Diagnostic International). Secondary antibodies, biotinylated goat anti-rabbit IgG (diluted 1:200), were from Vector Laboratories.

Immunofluorescence. Pregnant CD1 mice of timed gestational ages were purchased from Charles River (Wilmington, MA). After maternal death, the embryos were extracted immediately by midline abdominal and uterine incisions. The mouse embryos, aged embryonic days 12.5-18.5 (E12.5–E18.5), were fixed in 3.8% paraformaldehyde overnight, rinsed in PBS, and then cryoprotected in 30% sucrose for 3 more days. The fixed tissue was rinsed in PBS, frozen in OCT embedding medium (Sakura Finetek, Torrance, CA) and cryosectioned into 30 μM tissue slices. Sagittal embryo sections (~0.7 mm from midline in E14.5) containing the developing kidney were identified via light microscopy for immunostaining.

Paraformaldehyde-fixed sections were then incubated with anti-SAA (1:1,000, above) overnight, rinsed in PBS, followed by a FITC-conjugated goat anti-rabbit IgG (1:200, Jackson ImmunoResearch, West Grove, PA) and rhodamine-peanut agglutinin (PNA; 1:200, Vector Laboratories) for 1 h and the nuclear dye 4,6-diamidino-2-phenylindole (DAPI; Molecular Probes, Eugene, OR) for 20 min. The sections were rinsed with PBS and imaged with a Zeiss LSM 510 confocal microscope equipped with UV, argon, and helium lasers (12).

Positive controls consisted of acute-phase livers, harvested from casein-injected mice, incubated with anti-SAA, secondary antibody (FITC-conjugated goat anti-rabbit IgG) and DAPI. Negative controls consisted of acute-phase livers incubated with secondary antibody and DAPI, control kidneys, and embryo sections incubated with secondary antibody, PNA, and DAPI. Control mice kidneys were incubated with anti-SAA, secondary antibody, PNA, and DAPI.

Living NRK-52E cells were imaged after the addition to the culture media of fluorescein (2 μM) overnight without and with probenecid (2 mM). After rinsing of the cells in PBS five times, Hoescht 33342 was added (0.1 mg/ml, Molecular Probes) for 10 min, cells were again rinsed in PBS and imaged as above.

SAA by Western blot analysis. Tissues were homogenized in 25 mM Tris, pH 7.6, 150 mM NaCl, 1% deoxycholate, 1% Norisil P-40, 0.1% SDS, and 2× Halt Protease Inhibitor Cocktail (Thermo Scientific, Rockford, IL) and adjusted to a protein concentration of 2 mg/ml. Samples, including homogenates (20 μg) and purified recombinant mouse SAA1.1 (50 ng), were fractionated by electrophoresis through 16.5% polyacrylamide Tris-tricine gels. After electrophoresis, proteins were transferred to a nitrocellulose filter. Blocking was carried out in 1% casein, 1× PBS for 1 h. Incubation with primary antibodies diluted in 1× PBS was for 1 h; primary antibodies were rabbit anti-mouse SAA (1:2,000) generated in this laboratory and mouse anti-actin (1:1,000, clone AC-40, Sigma-Aldrich). The filter was then washed in 1× PBS and incubated with secondary antibodies diluted in 1× PBS for 1 h. Secondary antibodies were IRDye 800 goat anti-rabbit IgG (1:15,000, i-Cor Biosciences, Lincoln, NB) and IRDye 800 CW goat anti-mouse IgG (1:20,000, i-Cor Biosciences). After washing in 1× PBS, the filter was scanned using an Odyssey Infrared Imaging System (Li-Cor Biosciences) for visualization of immunoreactive proteins. All steps were carried out at room temperature.

Image analysis/statistics. Images were analyzed and fluorescent intensity representing immunoreactive SAA was quantified using Zeiss LSM and MetaMorph (Molecular Imaging, Downingtown, PA) software (12). Data are expressed as means ± SE. Analysis of variance was used to determine whether differences among mean values reached statistical significance. Student’s t-test was used for comparisons between groups. Tukey’s test was used to correct for multiple comparisons. The null hypothesis was rejected at P < 0.05.

RESULTS

The initial goal was to determine whether soluble murine SAA added to cultured NRK-52E renal tubular cells would be processed as SAA amyloid in vitro. This approach was similar to previous work from our laboratory performed using other cells (15). NRK-52E kidney cells obtained from American Type Culture Collection were cultured for 15 days in the presence of recombinant mouse SAA, 25–100 μg/ml, or with only 60 mM urea used as the vehicle for SAA (Fig. 1). Whereas we failed to show amyloid formation by Congo red staining or polarized microscopy, we found that organized complex structures resembling renal tubules grew on the two-dimensional culture dish in the presence of SAA (Fig. 2A). We then verified the formation of tubule-like structures under phase-contrast microscopy of live cells (Fig. 2B, C and D). Urea by itself did not affect cell morphology or viability after 15 days in culture (Fig. 2B), but tubule-like structures were readily seen in the presence of SAA (100 μg/ml). In the next series of experiments, we examined the possibility that the tubule-like structures expressed markers of renal proximal tubule differentiation. Confluent NRK-52E cells were cultured for 15 days with urea vehicle alone or with several concentrations of SAA. In the presence of SAA, cells that organized into tubule-like structures also expressed OAT1 (Fig. 2F) and NHE3 (Fig. 2D) proteins. These two proximal tubule transporters were far less distinct in cells that remained on the flat monolayer. The average number of tubule-like structures expressing proximal tubule differentiation markers as a function of SAA concentrations is shown in Fig. 2F.

The specific expression of OAT1 in the tubule-like structures implied their potential for weak acid transport. Accordingly, fluorescein transport was evaluated in NRK-52E cells
that had organized into tubule-like structures in the presence of SAA (50 μg/ml) for 15 days. The renal tubules were exposed overnight to fluorescein (2 μM) with or without probenecid (2 mM). The following day, the tubule-like structures were washed five times with fresh media and fluorescein uptake was measured under confocal microscopy (Fig. 3). Cells treated with SAA for 15 days again organized into tubule-like structures (Fig. 3A), while control cells exposed to only urea remained as a monolayer (Fig. 3F). Fluorescein entered and was retained in the luminal space of the tubule-like structures (Fig. 3, A–C), a transport process drastically reduced by the competitive antagonist probenecid (Fig. 3E).

Stimulation of tubule formation by SAA was also studied in cultured S1 proximal tubule cells (11, 21). The S1 cells were

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Fig. 8. Expression of SAA in recovery from acute kidney injury. Immunoreactive SAA (green) is found in renal tubules 24 h after renal ischemia (F, I, and L), but not in the contralateral kidney (C). Higher-power images (J–L,×63) show expression of SAA (arrows, L) in areas of apoptosis (as indicated by fragmented nuclei in DAPI staining; arrows, J). Tubular SAA expression decreased by 48 h postischemia (M–O). Nuclei were stained with DAPI (blue; A, D, G, J, M). Peanut agglutinin staining is shown in the middle panels (red).
cultured until they became confluent and were then exposed to SAA (100 μg/ml) for an additional 2 wk or to 60 mM urea control vehicle. Control S1 cells remained as a monolayer during the entire period of observation (Fig. 4, A, F, and J). In contrast, S1 cells exposed to SAA organized into tubules (Fig. 4, B–E). The mouse S1 cell tubules developed patent lumens, which also retained transported fluorescein after overnight fluorescein exposure (Fig. 4, G–I). The formed and weak acid-transporting mouse S1 cell tubules also expressed the transporter OAT1 (Fig. 4K).

The finding that SAA stimulated the formation of functional renal tubules from rat and mouse cells in vitro was followed by studies of SAA expression in situations of ongoing renal tubulogenesis in vivo. Accordingly, we examined the expression of SAA in mouse embryos before, during, and after E14.5 (Fig. 5), a critical stage for renal tubule formation in the embryonic metanephric mesenchyme (7, 27). The results of these experiments are summarized in Fig. 6. Nuclear staining of embryonic kidneys revealed pronounced cellular enrichment in the embryonic renal cortex. Very early SAA expression was barely detectable at E12.5 in organizing renal vesicles (Fig. 6, A–C). At E14.5, the branches of the ureteric bud were visualized with PNA, which outlined their surfaces (Fig. 6, E, H, and K). The ureteric bud prototubules were also labeled with anti-SAA antibody that distinctly revealed SAA in the same prototubules positive for PNA (Fig. 6, C, F, I, and L). Embryonic renal SAA expression was also detected in the metanephric mesenchyme and in emerging nephrons (Fig. 6, E, F, and I).

Subsequently, renal SAA expression was gradually restricted, such that from E16.5 to E18.5 the SAA label was limited to luminal surfaces of ureteric bud prototubules (Fig. 6, M, O, and Q) and was progressively lost in the more mature nephron segments (Fig. 6, N, P, and R).

Figure 7 is a composite of negative and positive immunofluorescence controls, including sections of mouse acute-phase liver (Fig. 7, A and D), normal adult kidney (Fig. 7, B and E), and E14.5 kidney (Fig. 7C). All five sections were incubated with DAPI and FITC-conjugated secondary anti-rabbit IgG. One acute-phase liver section (Fig. 7D) and one normal kidney section (Fig. 7E) were preincubated with primary anti-SAA antibody. The adult and embryonic kidney sections (Fig. 7, B, E, and C, respectively) were also incubated with rhodamine-conjugated PNA. Figure 7D illustrates SAA labeling in the positive control acute-phase liver, whereas all other negative controls lacked nonspecific FITC labeling.

Tubular repair following ischemia-reperfusion (I/R) injury is dependent on the proliferation of renal epithelial cells, ultimately leading to the restitution of tubular structures (9). Hence, the tubular renovation that follows I/R injury could be another example of SAA expression, this time in the adult kidney. Therefore, we searched for SAA tubular expression in mouse renal sections obtained 24 and 48 h following ischemia for 30 min (Ref. 12 and Methods in this study). In this experiment, one of the two mouse kidneys was clipped for 30 min, renal blood flow was restored, and the kidneys were studied 24 and 48 h later (Fig. 8). The proximal tubules of unaffected kidneys had normal nuclei, and while PNA label was visible, the SAA label was absent (Fig. 8, A–C). In contrast, some nuclei in proximal tubules from postischemic kidneys (Fig. 8, D–L) had condensed chromatin consistent with apoptosis. The injured tubules were significantly disrupted, but their PNA label confirmed their epithelial phenotype, which colocalized with strong SAA expression at 24 h postischemia (Fig. 8, F, I, and L). Kidneys imaged 48 h postischemia (Fig. 8, M–O) had significantly fainter expression of SAA (Fig. 8O).

In preparation for Western blot analysis, three additional mice were subjected to unilateral ischemia and killed 24 h later. To minimize the role of surgically induced acute-phase circulating SAA, the kidneys and liver were arterially perfused with 30 ml of ice-chilled PBS. The homogenates from surgically stimulated acute-phase liver, postischemic and contralateral kidneys, unrelated normal kidney, perfusate effluent, recombinant SAA, and original serum were then probed with the anti-SAA antibody by Western blotting (Fig. 9). We used an anti-actin antibody to normalize for protein loading. SAA was markedly positive in the liver of ischemic mouse, which is the expected acute-phase reactivity to surgery. SAA was also strongly positive in the three postischemic kidneys, whereas it was undetectable in the corresponding three contralateral kidneys. SAA was also absent in the unrelated normal adult kidney. The effluent contained a small amount of SAA and even actin, the latter likely derived from flushed cellular debris. Recombinant SAA was the positive control, and it comigrated with tissue-derived SAA. The original serum was strongly SAA positive, consistent with the postoperative hepatic acute-phase reaction.

**DISCUSSION**

SAA is a protein that to this day posits a complex interpretative problem in adult life. In natural conditions, it is nearly undetectable in blood, but SAA can burst to levels exceeding 1 g/l when stimulated by inflammation (26), a level 10 times higher than the highest stimulatory concentration used in our in vitro experiments. SAA is also very lipophilic with affinity for HDL, and at some point it was thought to be an acute-phase lipoprotein (1). Our observations in vitro and in vivo are consistent with a different viewpoint, in that we discovered that SAA stimulated the organization of rat and mouse renal epithelial cells into complex tubule-like structures with specific markers of proximal tubular differentiation. Moreover, the tubules depicted vectorial transport of organic acid molecules in vitro, also consistent with a renal proximal tubule phenotype.
(17). Hence, SAA is a powerful mediator of tubulogenesis in vitro, which occurred on a flat surface without the benefit of added colloidal matrices.

Our findings in rat NRK-52E and mouse S1 cells are reminiscent of branching morphogenesis observed in NRK-52E cells when exposed to unrelated heparin-binding epidermal growth factor-like growth factor (24). However, the earlier report on NRK-52E cells lacked evidence of specific tubular differentiation, and we cannot really equate the two observations. Madin-Darby canine kidney (MDCK) cells also branch into tubules when cultured in collagen matrices and exposed to hepatocyte growth factor (HGF; 22). However, limited evidence for further MDCK cell differentiation into collecting duct cells (2), prevents us from reaching conclusions as to whether the two phenomena are related. It is possible that SAA and HGF signaling systems activate independent events. Favoring this idea are the differing time lines and originating sites of tubular embryonic morphogenesis. The earlier forming ureteric bud, a precursor of MDCK-like collecting ducts (2), and subsequent tubular formation in the metanephric mesenchyme, a precursor for NRK-52E-like proximal tubules, appear in uniquely different tissues and at distinctly separate temporal and spatial dimensions (3). We also wish to point out that these systems are very different in vitro models. MDCK tubulogenesis, with minor variations, requires that cells first form cysts in matrix gels followed by exposure to HGF (22). In contrast, NRK-52E tubulogenesis arises from a flat cell monolayer in the presence of SAA. Moreover, the differentiating features induced by SAA in the NRK-52E and S1 cells systems include expression and function of specific proximal tubular transporters. These inducible differentiating properties have not been reported in the MDCK system to our knowledge. However, we also recognize that we do not know which signaling system is responsible for SAA-induced tubular differentiation, some aspects of which could be shared by both systems of tubulogenesis.

The finding of SAA expression in embryonic kidney was remarkable. First, its association with rapidly developing renal epithelia is consistent with a putative role of SAA as a promoter of tubulogenesis. Second, the unexpected association between two disparate systems, acute-phase response and development of renal tubules, reveals a novel convergence of signaling networks that interact and seem to share components, a situation reminiscent of the role of the complement system in cell differentiation and proliferation (18). Furthermore, the association of SAA expression with the recovery stage of renal I/R injury is also noteworthy because it implies that inflammatory reactions in renal recovery are not all noxious (13), but rather, some elements of the inflammatory response could potentially aid in the recovery process. Hence, expression of the SAA activators TNF-α and interleukins (13) and of SAA isoforms in I/R injury (5) may aid tubule recovery in part by activating tubular SAA. We close by emphasizing the remarkable timing in the acute response of renal SAA. Other investigators have previously reported this property (19). Specifically, renal SAA mRNA levels are undetectable in normal mice kidneys, or from mice injected with casein. However, high renal SAA mRNA levels are detected within hours of lipopolysaccharide (LPS) injections (19). In our hands, SAA protein was also undetectable in kidneys from control or from casein-injected mice, but renal SAA surged to high levels within 24 h of ischemia, and nearly faded by 48 h postischemia. This characteristic rapid response was also observed in embryonic murine kidneys, where critical steps in tubule formation must occur within hours (3). Hence, the rapid in vivo response inherent to SAA gene expression fits in with the rapid requirements for tubular formation during renal injury and in renal morphogenesis of fetal life. Accordingly, our initial results lend support to an emerging and novel view of a more localized renal acute-phase reaction, where renal SAA may act as a paracrine or autocrine molecule that promotes tubule formation during development and repair.

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