Original Article

Adipocyte-Derived Serum Amyloid A3 and Hyaluronan Play a Role in Monocyte Recruitment and Adhesion

Chang Yeop Han,[1] Savitha Subramanian,[1] Christina K. Chan,[2] Mohamed Omer,[1] Tsuyoshi Chiba,[1] Thomas N. Wight,[2] and Alan Chait[1]

Obesity is characterized by adipocyte hypertrophy and macrophage accumulation in adipose tissue. Monocyte chemoattractant protein-1 (MCP-1) plays a role in macrophage recruitment into adipose tissue. However, other adipocyte-derived factors, e.g., hyaluronan and serum amyloid A (SAA), can facilitate monocyte adhesion and chemotaxis, respectively. The objective was to test the potential involvement of these factors in macrophage recruitment. Differentiated 3T3-L1 adipocytes made hypertrophic by growth in high glucose conditions were used to study SAA and hyaluronan regulation in vitro. Two mouse models of obesity were used to study their expression in vivo. Nuclear factor-κB was upregulated and peroxisome proliferator–activated receptor (PPAR)γ was downregulated in hypertrophic 3T3-L1 cells, with increased expression of SAA3 and increased hyaluronan production. Rosiglitazone, a PPARγ agonist, reversed these changes. Hypertrophic adipocytes demonstrated overexpression of SAA3 and hyaluronan synthase 2 in vitro and in vivo in diet-induced and genetic obesity. SAA and hyaluronan existed as part of a complex matrix that increased the adhesion and retention of monocytes. This complex, purified by binding to a biotinylated hyaluronan binding protein affinity column, also showed monocyte chemotactic activity, which was dependent on the presence of SAA3 and hyaluronan but independent of MCP-1. We hypothesize that adipocyte hypertrophy leads to increased production of SAA and hyaluronan, which act in concert to recruit and retain monocytes, thereby leading to local inflammation in adipose tissue. *Diabetes* 56:2260–2273, 2007

From the [1]Department of Medicine, University of Washington, Seattle, Washington; and the [2]Hope Heart Program, Benaroya Research Institute, Seattle, Washington.

Address correspondence and reprint requests to Alan Chait, MD, Division of Metabolism, Endocrinology, and Nutrition, Box 356426, University of Washington, Seattle, WA 98195-6426. E-mail: achait@u.washington.edu.

Received for publication 15 February 2007 and accepted in revised form 1 June 2007.

Published ahead of print at http://diabetes.diabetesjournals.org on 11 June 2007. DOI: 10.2337/db07-0218.

Additional information for this article can be found in an online appendix at http://dx.doi.org/10.2337/db07-0218.

CCR2, C-C motif chemokine receptor 2; CRP, C-reactive protein; ELISA, enzyme-linked immunosorbent assay; EMSA, electromobility shift assay; FITC, fluorescein isothiocyanate; FMLP, formyl-methionyl-leucyl-phenylalanine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HABP, hyaluronan binding protein; HAS, hyaluronan synthase; IL, interleukin; MALDI-TOF, matrix-assisted laser desorption ionization/time of flight; MCP-1, monocyte chemoattractant protein-1; NF-κB, nuclear factor-κB; PPAR, peroxisome proliferator–activated receptor; PPRE, PPAR response element; SAA, serum amyloid A; siRNA, small interfering RNA; TNF-α, tumor necrosis factor-α; WGA, wheat germ agglutinin.

Obesity is associated with chronic low-grade inflammation as evidenced by increased levels of inflammatory markers, such as C-reactive protein (CRP) and serum amyloid A (SAA) (1–5), and an increased risk of cardiovascular disease (6–9).

Two major features of obesity are adipocyte hypertrophy and macrophage infiltration into adipose tissue (10). Adipocyte expansion (genetic or diet-induced) promotes the accumulation of macrophages in adipose tissue in mice and in humans (11,12). Cross-talk between adipocytes and macrophage in adipose tissue may amplify the production of pro-inflammatory molecules by adipocytes and by macrophages, leading to local and systemic insulin resistance in obesity (10).

Monocyte chemoattractant protein-1 (MCP-1) plays an important role in the recruitment of macrophages to adipose tissue in obesity (13). In the absence of its receptor, C-C motif chemokine receptor 2 (CCR2), macrophage infiltration into adipose tissue was reduced and insulin resistance attenuated (14). However, substantial accumulation of macrophages was observed in adipose tissue from CCR2-deficient mice (14,15), suggesting that other molecules also are involved in macrophage recruitment. Molecules produced by adipocytes that have been implicated in monocyte recruitment and macrophage accumulation are SAA (16) and hyaluronan (17).

Recent studies suggest that SAA may play an important role in both local and systemic inflammation (2,18,19). SAA is able to recruit inflammatory cells, such as monocytes (20), and can activate endothelial cells, increasing their expression of adhesion molecules, such as intracellular adhesion molecule-1 and vascular adhesion molecule-1 (21). SAA is produced by the liver in response to inflammatory stimuli (22) and is secreted in lipoproteins, predominantly HDL (23). It also is produced by adipocytes and macrophages (16). In obesity, inducible forms of SAA are abundantly expressed in adipose tissue in humans and in mice (24). The isoform produced by extra-hepatic cells in mice, SAA3, is a different gene product from the hepatic forms of SAA (SAA1 and -2); little is known of its regulation or functional significance in adipocytes.

Hyaluronan, a nonsulfated glycosaminoglycan that lacks a core protein (25), binds monocytes via a number of receptors, predominantly CD44 (26). CD44-deficient atherosclerosis-prone mice have a marked reduction in the number of macrophages in their lesions plus a dramatic reduction in lesion size (27), suggesting that hyaluronan plays an important role in macrophage recruitment in

vascular tissue. Whether it plays a similar role in adipose tissue, where it is abundantly present, is unclear. Hyaluronan is secreted by 3T3-L1 cells during adipocyte differentiation (28,29). However, little is known regarding the regulation of its synthesis and secretion during adipocyte hypertrophy or its potential role in macrophage accumulation in adipose tissue.

In the current study, we induced adipocyte hypertrophy in vitro by growing 3T3-L1 cells in high glucose concentrations and in vivo either by feeding LDL receptor–deficient ($LDLR^{-/-}$) mice a high-fat, high-sucrose diet or by use of a genetically obese mouse model, the *ob/ob* mouse. We demonstrated that nuclear factor-κB (NF-κB) activity increased in hypertrophic adipocytes, indicative of a pro-inflammatory state, whereas activity of the anti-inflammatory transcription factor peroxisome proliferator–activated receptor (PPAR)γ decreased. SAA and hyaluronan expression increased during adipocyte hypertrophy. The PPARγ agonist, rosiglitazone, suppressed this effect. We also showed that SAA and hyaluronan existed as a complex that not only increased the adhesion and retention of monocytes by hypertrophic adipocytes but also had monocyte chemotactic activity. These findings indicate that adipocyte hypertrophy leads to increased production of SAA and hyaluronan and suggest that these molecules act in concert with MCP-1 to cause monocyte recruitment and adhesion, leading to adipose tissue inflammation.

RESEARCH DESIGN AND METHODS

Cell culture. 3T3-L1 murine preadipocytes were propagated and differentiated according to standard procedures (30), except that cells were differentiated and propagated in Dulbecco's modified Eagle's medium containing 5 or 25 mmol/l glucose or 5 mmol/l glucose plus 20 mmol/l mannitol as a control for osmolarity, and media were changed daily. To monitor glucose consumption, glucose concentrations were checked at the time of each medium change, because it is conceivable that exposure to excessively low glucose concentrations might limit 3T3-L1 differentiation. The U937 and THP-1 monocytic cell lines were cultured in RPMI 1640 for use in the monocyte adhesion and chemotaxis assays, respectively. Preliminary experiments indicated that similar effects on hypertrophy and SAA3, MCP-1, and hyaluronan synthase (HAS)2 gene expression were obtained if cells were differentiated in 25 mmol/l glucose and then switched to 5 or 25 mmol/l glucose–containing media (data not shown). Growth of cells in 5 mmol/l glucose plus 20 mmol/l mannitol had no effect on cellular hypertrophy or gene expression (Supplemental Fig. 1, which is detailed in the online appendix [available at http://dx.doi.org/10.2337/db07-0218).

In vitro SAA3 gene silencing. Small interfering RNA (siRNA) duplexes for SAA1, SAA3, negative control #1, and negative control #3 were synthesized and purified by Ambion. The sequences are as follows: SAA1, 5′GGACAUGAGGACACCAUUG3′; and SAA3, 5′GCUGGUCAAGGGUCUAGAG3′. Transfection of siRNA was performed using Hiperfect agent (Quiagene) 2 days after completion of the differentiation protocol.

Multiplex quantitative RT-PCR. Quantitative real-time RT-PCR was performed using the TaqMan Master kit (Applied Biosystems) in the Stratagene MX3000P system as described previously (31,32) (online appendix).

Western blot analysis. 3T3-L1 adipocytes were cultured for the indicated times and treated for 24 h with a cytokine mixture (interleukin [IL]-1β, IL-6, and tumor necrosis factor-α [TNF-α], all at 10 ng/ml) and/or rosiglitazone (100 nmol/l). After incubation, culture media and cell extracts were analyzed by Western blot using anti-SAA3 antibodies (generous gift from Dr. Philipp Scherer, University of Texas Southwestern, Dallas, TX) or an anti–MCP-1 antibody (R&D Systems).

Hyaluronan enzyme-linked immunosorbent assay. Hyaluronan content was measured in culture media and cell extracts from 3T3-L1 adipocytes or adipose tissue by enzyme-linked immunosorbent assay (ELISA) as described previously (33) (online appendix).

Electromobility shift assay. Nuclear extracts from adipocytes were isolated and analyzed by electromobility shift assay (EMSA) according to the manufacturer's protocol (Active Motif). The DNA-protein complexes were separated electrophoretically and autoradiographed at −80°C. To assess specificity, competition assays were performed with an excess of unlabeled NF-κB and PPAR response element (PPRE) oligonucleotide (unlabeled probe) or mutant NF-κB and PPRE oligonucleotide.

Monocyte adhesion assay. Monocyte adhesion to 3T3-L1 adipocytes was assessed using U937 cells as described previously (34) (online appendix).

Biotinylated hyaluronan binding protein affinity column. A hyaluronan binding protein (HABP) affinity column was used to purify the hyaluronan-containing complex from hypertrophic adipocytes. Briefly, streptavidin-agarose (Sigma) was mixed with biotinylated-HABP (Seikagaku) in PBS containing 50% glycerol and shaken overnight at 4°C. Hyaluronan was added in PBS to protect the HABP from cross-linking. Sulfo-EGS (Pierce) was added in PBS and incubated for 30 min at room temperature. Finally, HABP-conjugated streptavidin-agarose was poured into the column and rinsed with PBS. The column was washed with 4 mol/l guanidine, 0.5 mol/l sodium acetate, pH 5.8, to remove hyaluronan, equilibrated with PBS containing azide, and stored at 4°C. A control column with streptavidin-agarose column was prepared by the same method without biotinlyated HABP. Media and cell-associated samples from adipocytes cultured in either high or low glucose concentrations were applied to the control column before they were applied to the HABP affinity column. After washing seven times with PBS, the complex of hyaluronan and associated molecules was eluted with 4 mol/l guanidine and 0.5 mol/l sodium acetate, pH 5.8, dialyzed in PBS, and concentrated.

Ligand blot analysis. Ligand blotting was performed to examine the interaction between hyaluronan and SAA3, as described previously (35) (online appendix).

Monocyte chemotaxis assay. The chemotactic activity of conditioned media and the purified complex (using the HABP affinity column) from 3T3-L1 adipocytes grown in 5 or 25 mmol/l glucose was studied in a 96-well microchamber (ChemoTx; Neuro Probe) as described previously (36) (online appendix).

Animals, diet, and tissue collection. Eight-week-old male $LDLR^{-/-}$ mice bred onto a C57BL/6 background were fed a high-carbohydrate, high-fat diet (BioServ No.F1480, containing 35.5% fat [primarily lard] and 36.6% carbohydrate [primarily sucrose]) for 24 weeks. *ob/ob* mice and C57BL/6 littermate controls were fed a chow diet for 24 weeks.

Immunohistochemistry. Immunohistochemistry was performed as described previously (37,38) (online appendix).

Statistical analysis. Statistical significance was determined by Student's *t* tests. All data are shown as means ± SD of three independent experiments performed in triplicate. $P < 0.05$ was considered significant.

RESULTS

Achieving adipocyte hypertrophy by exposure of 3T3-L1 cells to high glucose concentrations. To induce cellular hypertrophy, we exposed 3T3-L1 cells to high glucose concentrations during differentiation from the preadipocyte to the adipocyte-like state and for 1, 7, and 14 days after completion of differentiation. 3T3-L1 adipocytes grown in 25 mmol/l glucose were hypertrophic by 7 days, with the appearance of large cells containing considerable numbers of lipid droplets compared with cells maintained in 5 mmol/l glucose (Fig. 1*A*). Because undifferentiated adipocytes do not express adiponectin (30), we ascertained that adiponectin mRNA expression levels did not differ after differentiation in high and low glucose concentrations (data not shown). This indicated that cells grown under low glucose conditions can fully differentiate into 3T3-L1 adipocytes. To investigate the effect of PPARγ agonists in cellular hypertrophy, the PPARγ agonist rosiglitazone (100 nmol/l) was added to cells exposed to both high and low glucose concentrations with each medium change. Rosiglitazone prevented the effect of high glucose on adipocyte hypertrophy (Fig. 1*A*).

Hypertrophy induced by high glucose conditions induces SAA3 and MCP-1 expression in 3T3-L1 adipocytes. The effect of adipocyte hypertrophy on SAA3 expression levels was measured using real-time RT-PCR at the indicated times after completion of differentiation. SAA3 mRNA level increased in cells grown in high glucose medium in a time-dependent manner but did not change in cells grown in low glucose medium (Fig. 1*B*). Although

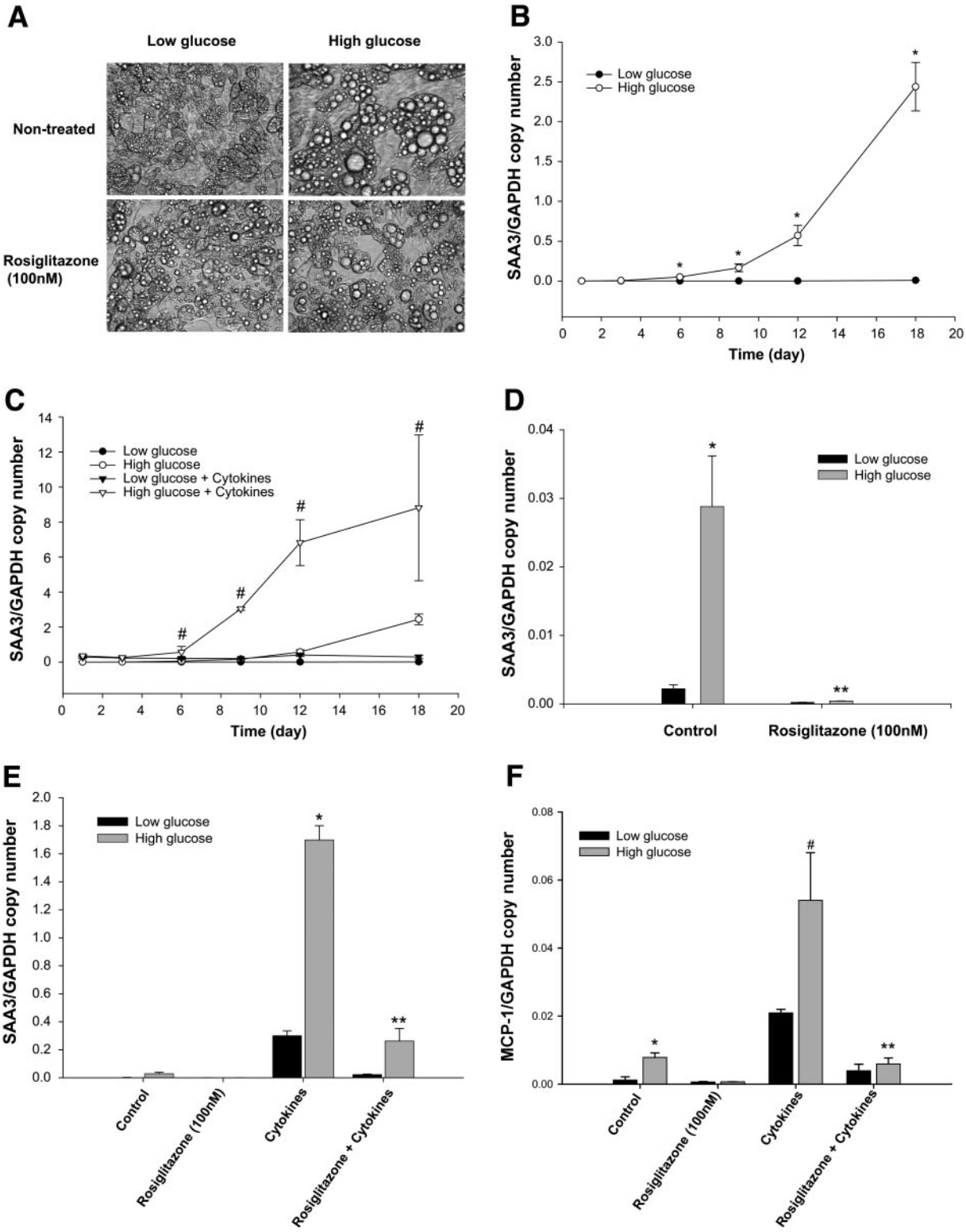

FIG. 1. Expression of SAA3 and MCP-1 mRNA is induced in hypertrophic adipocytes and suppressed by rosiglitazone. 3T3-L1 preadipocytes were differentiated into adipocytes and cultured in low (5 mmol/l) or high (25 mmol/l) glucose media with or without rosiglitazone (100 nmol/l). *A*: Photomicrographs were performed 7 days after completion of the differentiation protocol. 3T3-L1 adipocytes were differentiated in low (5 mmol/l) or high (25 mmol/l) glucose and then cultured in the same medium for the indicated times (*B* and *C*) or 7 days (*D*, *E*, and *F*) with or without a cytokine mixture (10 ng/ml IL-1β, IL-6, and TNF-α) or added rosiglitazone (100 nmol/l). After differentiation, rosiglitazone was replenished daily, whereas the cytokine mixture was added 24 h before harvesting. Total RNA was isolated and analyzed by multiplex real-time RT-PCR using SAA3-specific primers and probes (*B*, *C*, *D*, and *E*) or MCP-1 specific primers and probes (*F*), and normalized to GAPDH. **P* < 0.001 vs. low glucose, ***P* < 0.001 vs. cytokine mixture, #*P* < 0.001 vs. high glucose.

SAA1 and -2 mRNA levels also increased, their expression levels were very low compared with SAA3 (data not shown). Treatment with a mixture of pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) amplified SAA3 expression (Fig. 1*C*). Rosiglitazone dramatically inhibited SAA3 expression in cells exposed to high glucose concentrations (Fig. 1*D*) and blocked the amplifying effect of cytokines on SAA3 mRNA expression (Fig. 1*E*).

Preliminary experiments demonstrated that each isoform of SAA contains a unique peptide that ionizes with high efficiency. Peptide mapping therefore represents a highly sensitive and specific method that is able to identify the various isoforms of SAA. To determine whether hypertrophic 3T3-L1 adipocytes secrete SAA3 protein, medium isolated from the cells was fractionated by SDS-PAGE and immunoblotted with an antibody specific for SAA. The single immunoreactive band of material (which exhibited an apparent molecular weight consistent with that of SAA) was cut out, digested with trypsin, and analyzed by matrix-assisted laser desorption ionization/time of flight (MALDI-TOF) mass spectrometry. A peptide unique to SAA3, but not SAA1 and SAA2, was detectable in the medium from 3T3-L1 adipocytes cultured in high glucose media (data not shown).

Another inflammatory molecule believed to have an important role in monocyte recruitment into adipose tissue is MCP-1 (10,15). Therefore, MCP-1 expression levels were measured using real-time RT-PCR. As with SAA3, MCP-1 mRNA levels increased in cells that had become hypertrophic by exposure to high glucose. Treatment with cytokines amplified the increased MCP-1 expression induced by growth in high glucose concentrations. Rosiglitazone inhibited the induction of MCP-1 by growth in high glucose and by inflammatory cytokines (Fig. 1*F*). These results indicate that hypertrophic adipocytes have the potential ability to make two proteins known to be capable of recruiting monocytes, i.e., SAA and MCP-1.

NF-κB DNA binding activity is increased and PPARγ DNA binding activity decreased in differentiated 3T3-L1 adipocytes, effects that are reversed by rosiglitazone. NF-κB DNA activation regulates transcription of a wide range of inflammatory mediators in pro-inflammatory states, whereas PPARγ activation has anti-inflammatory properties. Therefore, NF-κB and PPARγ DNA binding activity was examined in differentiated 3T3-L1 cells exposed to high or low glucose concentrations, with or without the addition of cytokines and rosiglitazone (Fig. 2). Hypertrophic adipocytes showed evidence of increased NF-κB DNA binding activity without any change of NF-κB mRNA expression levels (Fig. 2*A* and *B*). Conversely, PPARγ DNA binding activity decreased in hypertrophic adipocytes without any change in PPARγ mRNA levels (Fig. 2*C* and *D*). Rosiglitazone treatment restored these effects, resulting in increased PPARγ and decreased NF-κB DNA binding activity in hypertrophic adipocytes. These results imply that the pro-inflammatory state associated with adipocyte hypertrophy is reciprocally regulated by NF-κB and PPARγ.

Adipocyte hypertrophy induced by high glucose conditions is associated with changes in hyaluronan. Extracellular matrix molecules secreted by adipocytes may play an important role in local inflammation because they can anchor lipoproteins, cells, and pro-inflammatory molecules. Hyaluronan secretion was investigated because hyaluronan increases during the differentiation of 3T3-L1 preadipocytes into adipocytes (28,29) and because hyaluronan is involved in monocyte attachment (26,39). Hyaluronan secreted into the media and bound to differentiated 3T3-L1 cells were measured by ELISA. In adipocytes in which hypertrophy was induced by high glucose conditions, media hyaluronan increased by ~40% (Fig. 3*A*). However, cell-associated hyaluronan increased by ~400% (Fig. 3*B*). The expression of the HAS1, -2, and -3 genes, which are responsible for hyaluronan production, was measured using real-time RT-PCR. HAS2 mRNA expression levels were increased in hypertrophic adipocytes (Fig. 3*C*), whereas HAS1 and -3 mRNA were not detected. Rosiglitazone inhibited the increase of hyaluronan in both the media and cell-associated compartments. Interestingly, pro-inflammatory cytokines did not stimulate the production of cell-associated hyaluronan (Fig. 3).

To explore the role of NF-κB on SAA3, MCP-1, and HAS2 mRNA expression levels, the effect of a specific NF-κB inhibitor, SN50 (100 μg/ml; Calbiochem), was investigated. SN50 treatment effectively inhibited the increase of SAA3, MCP-1, and HAS2 in cells exposed to high glucose concentrations (Supplemental Fig. 2, online appendix).

SAA3 and hyaluronan are coordinately induced by high glucose concentrations and associate with each other in a complex. We used three independent approaches to determine whether SAA3 and hyaluronan released by hypertrophic adipocytes exist in a free form or are present in a complex. First, we used hyaluronidase to determine whether hyaluronan degradation could release cell-associated SAA. 3T3-L1 adipocytes cultured in 5 or 25 mmol/l glucose for 7 days after differentiation were treated with cytokines or/and rosiglitazone. After an additional 24-h incubation, media were collected and replaced with new media with or without hyaluronidase for 30 min before re-harvesting (Fig. 4*A*, *a*). Hyaluronidase released cell-associated SAA3 into the media from hypertrophic adipocytes grown in 25 mmol/l glucose (Fig. 4*A*, *b*, compare lanes 1 and 3 with lanes 6 and 8). However, SAA3 was not released without hyaluronidase treatment (Fig. 4*A*, *b*, lane 10). Second, we immunostained cells with an SAA antibody and identified hyaluronan with HABP. To determine whether the complex was at the cell surface or intracellular sites, cells were stained with Alexa Fluor 594–conjugated wheat germ agglutinin (WGA; Molecular Probes), which selectively labels glycoproteins on plasma membranes. SAA and hyaluronan (green fluorescence, Fig. 4*B*, *c* and *h*) and Alexa Fluro 594–conjugated WGA (red fluorescence, Fig. 4*B*, *d* and *i*) were colocalized (compare with merge; Fig. 4*B*, *e* and *j*, yellow). SAA (green fluorescence, Fig. 4*B*, *m*) and hyaluronan (red fluorescence, Fig. 4*B*, *n*) also colocalized in hypertrophic adipocytes (yellow, Fig. 4*B*, *o*). Hyaluronidase treatment simultaneously removed hyaluronan and SAA staining (Fig. 4*B*, *r*, *w*, β, and γ) but not plasma membrane staining (Fig. 4*B*, *s* and *x*), suggesting that hyaluronan and SAA associate in a complex matrix present at the cell surface. Finally, we used a HABP column to isolate the hyaluronan-containing matrix. Media from hypertrophic adipocytes were loaded onto the HABP column, after which hyaluronan-containing complexes were eluted with 4 mol/l guanidine. SAA protein was detected in the eluate but not in the pre-elution washes (Fig. 4*C*, *a*). SAA protein was not detected in the eluate when media were pretreated with hyaluronidase before loading onto the HABP column (Fig. 4*C*, *b*). To assess the relative abundance and purity of the complex containing hyaluronan and SAA3, the complexes were run on SDS-PAGE, immunoblotted with an anti-SAA antibody,

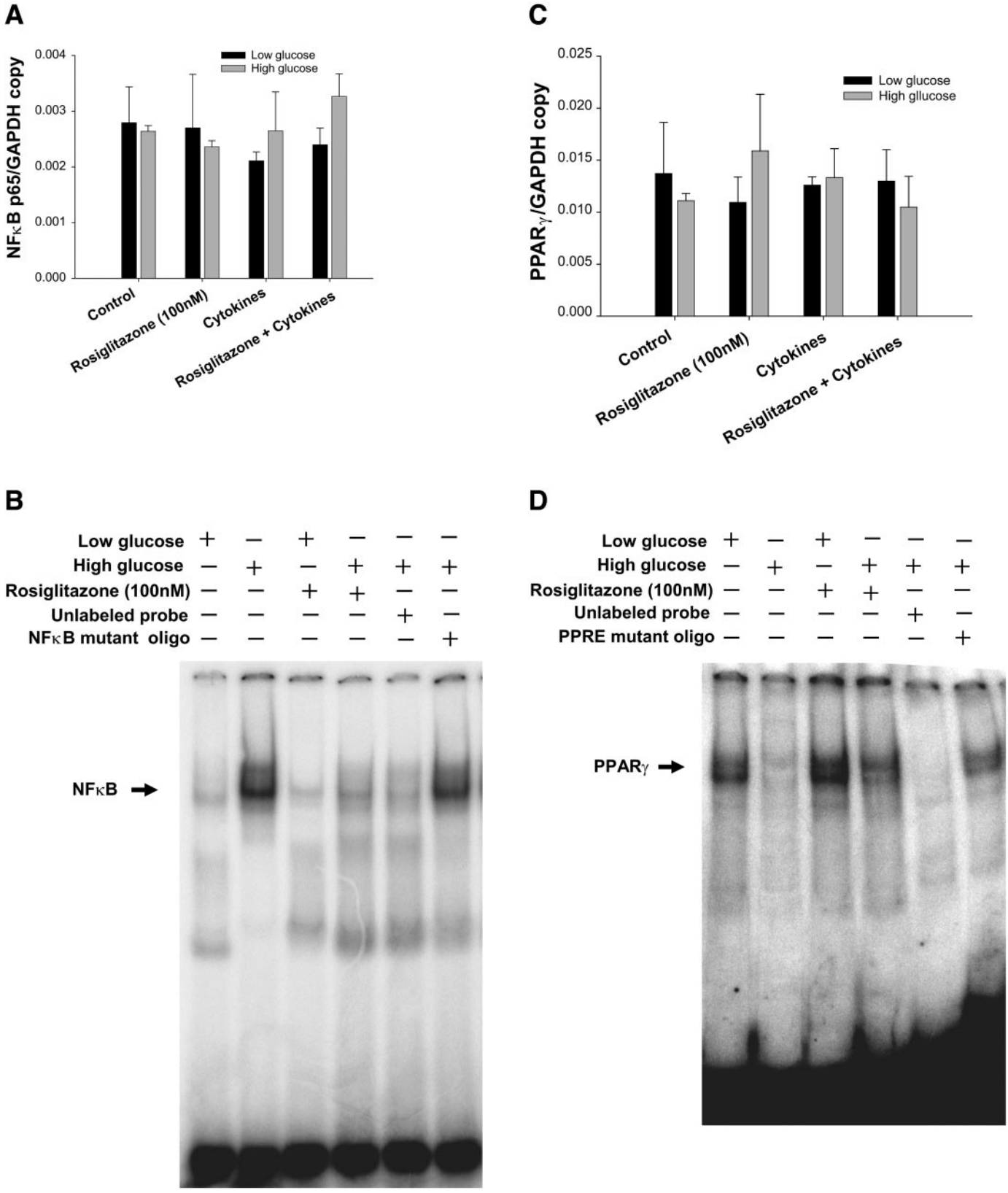

FIG. 2. Reciprocal regulation of NF-κB and PPARγ DNA binding activity during hypertrophy induced by high glucose concentrations in differentiated 3T3-L1 adipocytes. 3T3-L1 adipocytes were differentiated in low (5 mmol/l) or high (25 mmol/l) glucose and cultured for 7 days in the same medium with or without a mixture of cytokines or rosiglitazone (100 nmol/l). Total RNA was isolated and analyzed by multiplex real-time RT-PCR using NF-κB– and PPARγ–specific primers and probes (*A* and *C*) and normalized to GAPDH. Nuclear extracts were analyzed by EMSA using radiolabeled NF-κB (*B*) and PPRE (*D*) oligonucleotides. Specificity was determined by the addition of unlabeled NF-κB and PPRE (unlabeled probes) or mutant NF-κB and PPRE oligonucleotides before addition of the labeled probe.

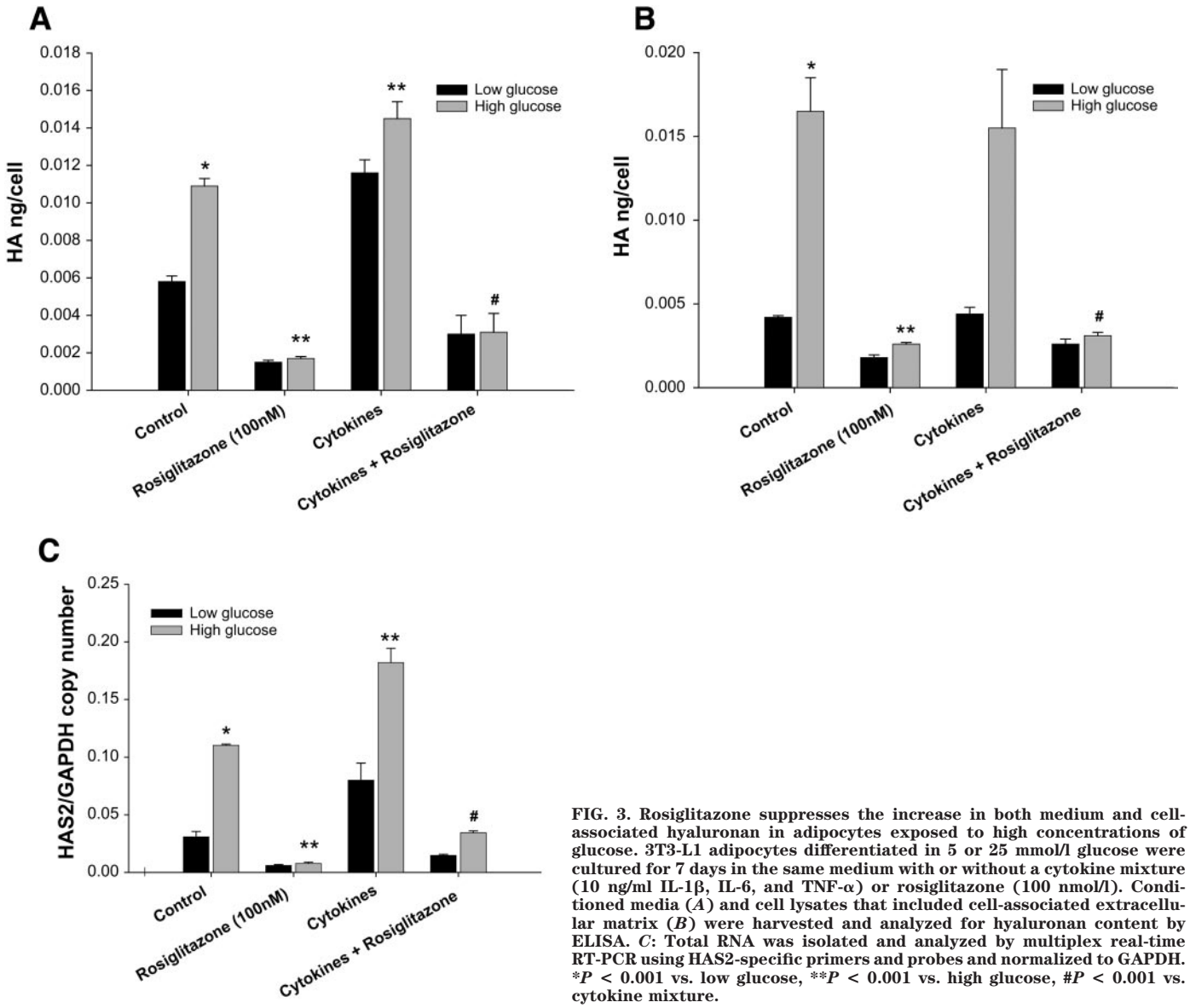

FIG. 3. Rosiglitazone suppresses the increase in both medium and cell-associated hyaluronan in adipocytes exposed to high concentrations of glucose. 3T3-L1 adipocytes differentiated in 5 or 25 mmol/l glucose were cultured for 7 days in the same medium with or without a cytokine mixture (10 ng/ml IL-1β, IL-6, and TNF-α) or rosiglitazone (100 nmol/l). Conditioned media (*A*) and cell lysates that included cell-associated extracellular matrix (*B*) were harvested and analyzed for hyaluronan content by ELISA. *C*: Total RNA was isolated and analyzed by multiplex real-time RT-PCR using HAS2-specific primers and probes and normalized to GAPDH. **P* < 0.001 vs. low glucose, ***P* < 0.001 vs. high glucose, #*P* < 0.001 vs. cytokine mixture.

and ligand blotted using biotinylated hyaluronan. A band of ~15 kDa (Fig. 4*C*, *c*), which was confirmed as SAA3 by immunoblot analysis (Fig. 4*C*, *d*) and MALDI-TOF mass spectrometry, was the major protein detected in the complex. Moreover, hyaluronan selectively detected the SAA3 band on the ligand blot assay (Fig. 4*C*, *e*). These results indicate that SAA3 binds to hyaluronan and suggest that hyaluronan plays a role in anchoring SAA3 in the extracellular matrix of hypertrophic adipocytes.

The complex containing SAA3 and hyaluronan increases monocyte adhesion. To investigate the potential of the complex containing SAA3 and hyaluronan to recruit monocytes into adipose tissue, monocyte adhesion assays were performed with 3T3-L1 adipocytes grown in high or low glucose concentrations. Adhesion of monocytes to hypertrophic adipocytes was increased compared with cells grown in low glucose. Rosiglitazone, a PPARγ agonist that decreased both SAA and hyaluronan production in response to hypertrophy, inhibited monocyte adhesion. Pretreatment of the cell cultures with hyaluronidase, which removes hyaluronan from the extracellular matrix of the cells, also inhibited the monocyte adhesion (Fig. 5*A*).

To examine the role of SAA3 present in the complex, SAA3 expression was silenced by transfecting a specific SAA3 siRNA into hypertrophic 3T3-L1 adipocytes. siRNA transfection decreased SAA3 mRNA and protein levels compared with transfection of control siRNA constructs and untreated cells (Fig. 5*B* and *C*). This SAA3 siRNA was specific for the SAA3 gene and did not silence other genes, such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and SAA1 (data not shown). By using this siRNA silencing technique, SAA3 was largely removed from the complex extracellular matrix (see below). Silencing of SAA3 partially inhibited the adhesion of monocytes (Fig. 5*D*). These data imply that both the hyaluronan and SAA3 in the complex extracellular matrix produced by hypertrophic adipocytes play an important role in monocyte adhesion to adipose tissue.

The complex of SAA3 and hyaluronan induces chemotaxis of monocytes, an effect that is inhibited by SAA3 silencing. To further characterize biological properties of the complex of SAA3 and hyaluronan that might recruit monocytes into adipose tissue, the chemotactic activity of this complex was determined using a Boyden chamber assay. Addition of the complex, purified from

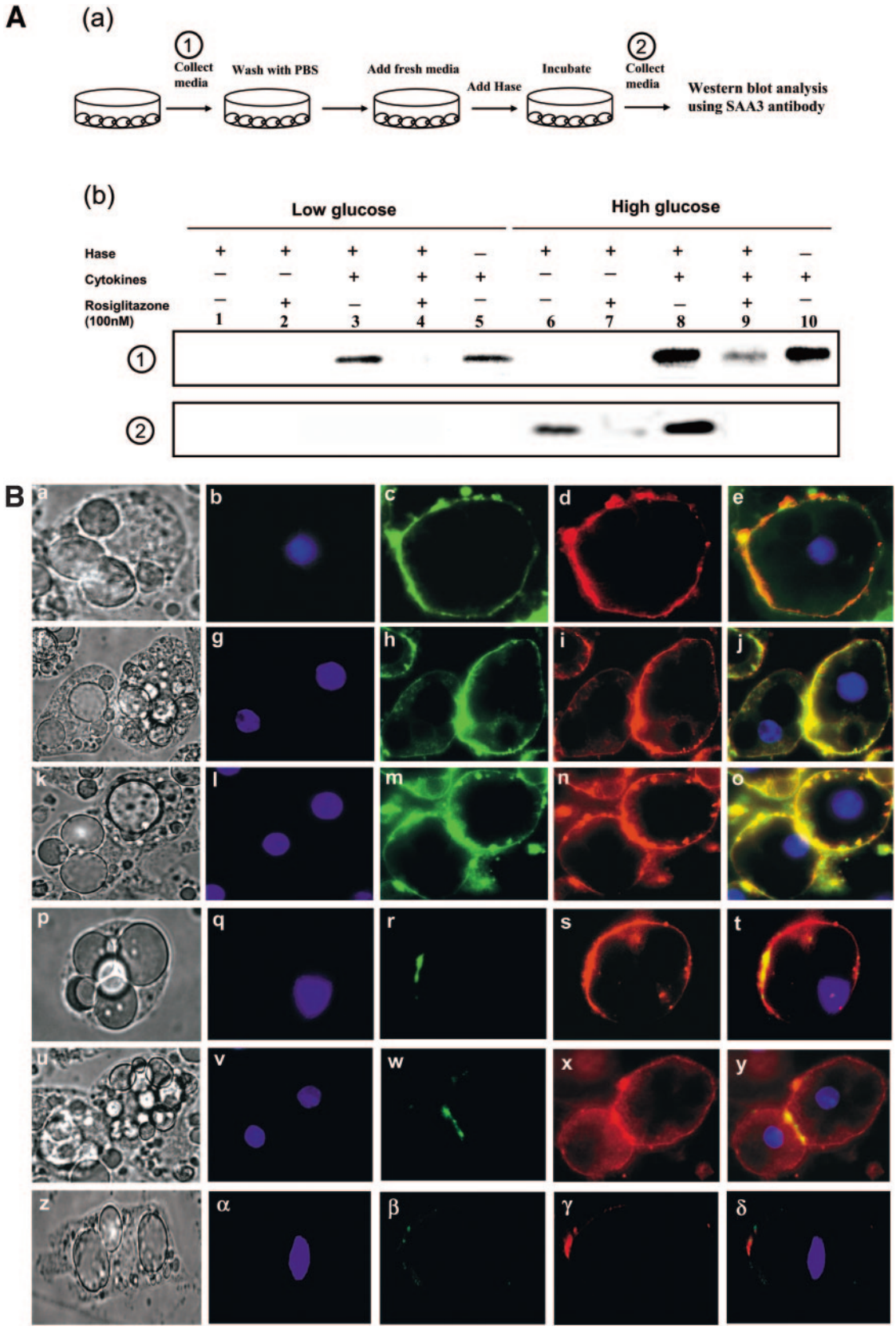

FIG. 4—Continues on the following page.

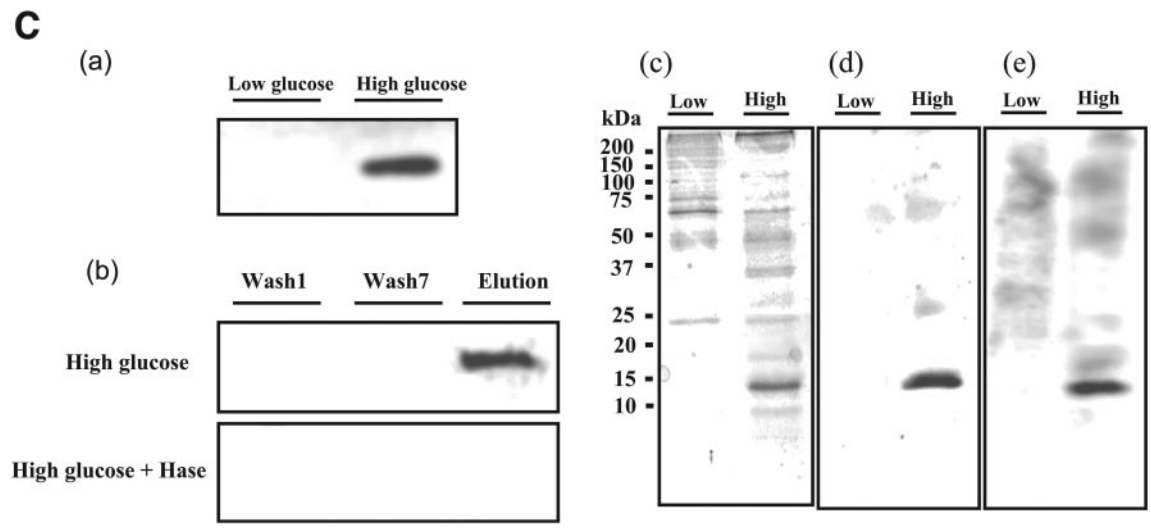

FIG. 4. SAA3 and hyaluronan are bound in a complex. *A*: 3T3-L1 adipocytes differentiated in 5 or 25 mmol/l glucose were cultured for 7 days with a cytokine mixture (10 ng/ml IL-1β, IL-6, and TNF-α) or rosiglitazone (100 nmol/l). *a*: After 24 h of incubation, conditioned media were collected (the first collection was designated as 1), replaced by new media with or without hyaluronidase (200 mU/ml) for 30 min and collected again (the second collection was designated as 2). *b*: These two collections were analyzed by immunoblot using anti-SAA3 antibody. Hyaluronidase released cell-associated SAA3 into the medium of adipocytes exposed to high glucose. *B*: 3T3-L1 adipocytes differentiated in high glucose medium on 22-mm^2 coverslips were cultured in the same medium for 7 days. *p–δ*: For controls, cells were treated with hyaluronidase (200 mU/ml) for 30 min before fixation. Cells were stained with a SAA3 (green) antibody following fluorescein isothiocyanate (FITC)-secondary antibody (*c*, *m*, *r*, and *β*) and HABP following FITC-conjugated (green) (*h* and *w*) or Cy5-conjugated (red) (*n* and *g*) streptavidin. *d*, *i*, *s*, and *x*: To distinguish the outside of cells from intracellular sites, plasma membranes were stained with Alexa Fluor 594–conjugated WGA (red). *b*, *g*, *l*, *q*, *v*, and *α*: Cell nuclei were counter-stained by Hoechst 33342 (blue), and cells were photographed using fluorescence microscopy. *e*, *j*, *o*, *t*, *y*, and *δ*: Merged fluorescence is shown in yellow (original magnification, ×1,000). *a*, *f*, *k*, *p*, *u*, and *z*: Cell morphology was shown by phase-contrast photography. *o*: Thus, hyaluronan and SAA3 colocalize in hypertrophic adipocytes. *C*: Conditioned media from adipocytes exposed to high or low glucose conditions were loaded onto a HABP column to purify the hyaluronan complex, washed extensively, and eluted with 4 mol/l guanidine. *a*: Each washing fraction and the fraction eluted with guanidine were analyzed by immunoblotting using a SAA3 antibody. Only cells grown in high glucose conditions have SAA complexed with hyaluronan. *b*: To test for the specificity of the HABP column, some media were treated with hyaluronidase before loading onto the column. SAA3 was lost by pretreatment with hyaluronidase. To examine the abundance and purity of the complex, the isolated complexes (20 μg/ml) were loaded on 10–20% gradient SDS-PAGE, visualized by Coomassie staining (*c*), by immunoblot using an anti-SAA antibody (*d*), or by ligand blot using biotinylated-HA (*e*). Thus, hyaluronan exists in a complex with SAA3 in media from adipocytes induced to become hypertrophic by growth in high glucose conditions.

hypertrophic adipocytes on a HABP column, showed the typical dose response of migration of monocytes compared with the complex from adipocytes grown in low glucose conditions (Fig. 6*A*). The optimal concentration of the complex containing SAA3 and hyaluronan for induction of monocyte migration was 100 ng/ml. To address the role of each component of the complex on monocyte chemotaxis, the complex was pretreated with either pronase or hyaluronidase. Pretreatment with pronase completely blocked monocyte migration, whereas hyaluronidase reduced migration by ~20% (Fig. 6*B*).

Because MCP-1 expression was increased in hypertrophic adipocytes, we needed to establish whether MCP-1 was present in the complex because its presence could have a major effect on monocyte chemotaxis. Therefore, the complex was pretreated with a neutralizing antibody against MCP-1. Pretreatment with an anti–MCP-1 antibody had no effect on the chemotactic activity of complex, whereas the chemotactic activity of purified MCP-1 added alone was completely blocked by pretreatment with the anti-MCP antibody (Fig. 6*C*). Moreover, MCP-1 was not detected by immunoblot on the complex containing SAA3 and hyaluronan (Fig. 6*D*). These data indicate that the monocyte chemotactic activity of the complex containing SAA3 and hyaluronan is not dependent on MCP-1.

Because SAA is known to be a chemoattractant (20), the chemotactic activity of SAA3 in the complex was evaluated by transfection of cells with SAA3 siRNA. SAA3 protein was not detected in the complex purified from SAA3 siRNA-transfected hypertrophic adipocytes, compared with matrix from cells transfected with a control siRNA construct and from untreated cells (Fig. 7*A*). Also, the concentration of hyaluronan did not change with transfection of the SAA3-specific siRNA (Fig. 7*B*). SAA3 deficiency inhibited the chemotactic activity of the complex (Fig. 7*C*). These results indicate that SAA3 plays an important role in the chemotactic activity of the complex of SAA3 and hyaluronan, which might work in concert with MCP-1 to attract macrophages to adipose tissue or become an alternative chemoattractant to MCP-1 in CCR2 knockout mice.

We next compared the chemotactic potency of MCP-1 and the complex containing SAA3 and hyaluronan, both of which are secreted into the medium by hypertrophic adipocytes. Conditioned medium from hypertrophic adipocyte increased monocyte chemotaxis. Pretreatment with an anti–MCP-1 antibody reduced migration by ~38%, whereas medium from SAA3 siRNA-transfected hypertrophic adipocyte reduced monocyte chemotaxis by ~32% (Fig. 7*D*). Pretreatment of conditioned medium from SAA3 siRNA-transfected hypertrophic adipocyte with an anti–MCP-1 antibody showed an additive effect of SAA3 and MCP-1 on monocyte chemotaxis (Fig. 7*D*). These data indicate that not only MCP-1 but also SAA3 plays a major role on monocytes chemotaxis to hypertrophic adipocytes.

SAA3 and hyaluronan increase during the development of obesity in mouse models. To extend our in vitro findings in 3T3-L1 adipocytes to in vivo models, we used male LDLR$^{-/-}$ mice fed a high-fat, high-sucrose diet, which previously has been shown to result in obesity, insulin resistance, and atherosclerosis (40). In addition,

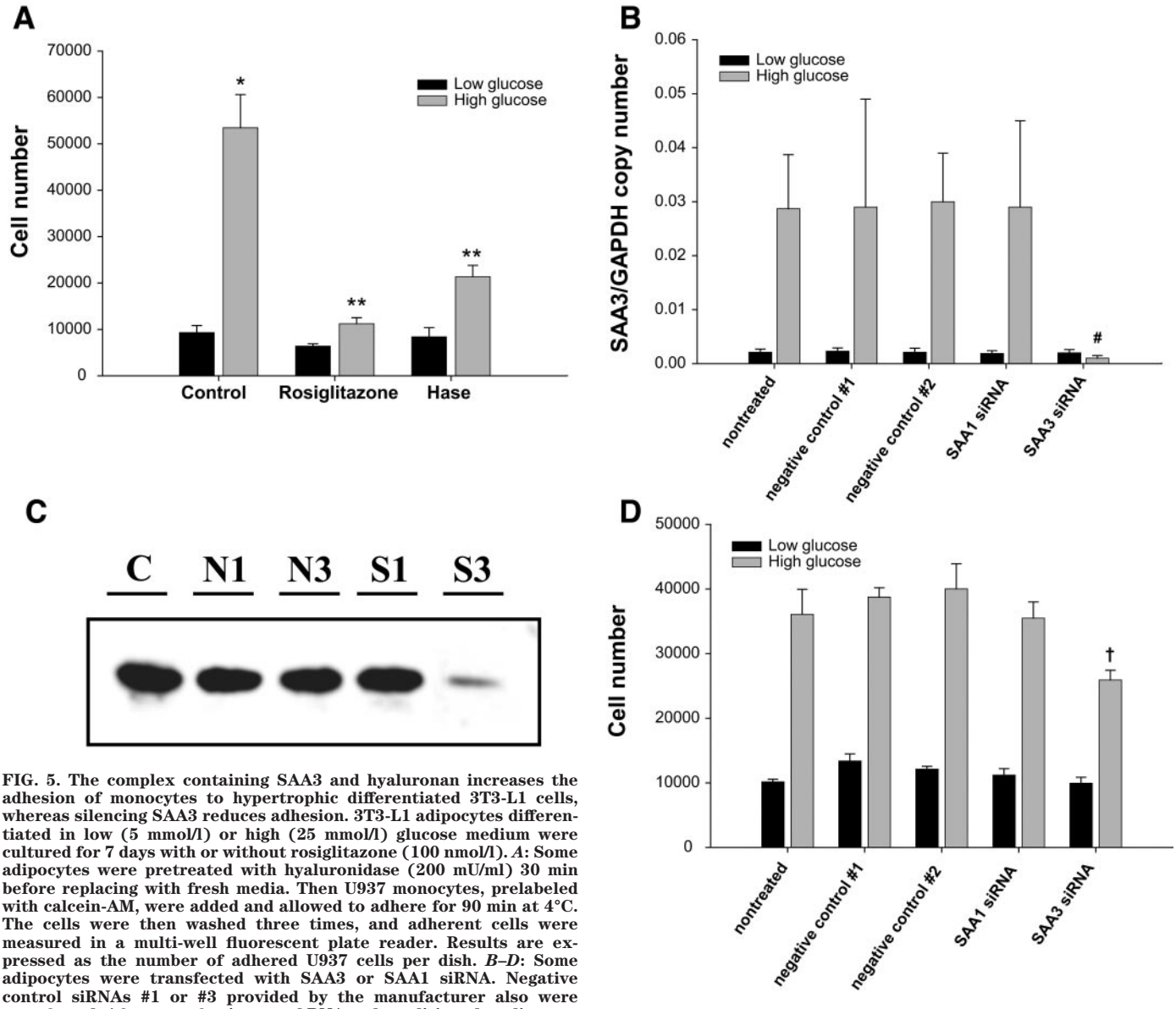

FIG. 5. The complex containing SAA3 and hyaluronan increases the adhesion of monocytes to hypertrophic differentiated 3T3-L1 cells, whereas silencing SAA3 reduces adhesion. 3T3-L1 adipocytes differentiated in low (5 mmol/l) or high (25 mmol/l) glucose medium were cultured for 7 days with or without rosiglitazone (100 nmol/l). *A*: Some adipocytes were pretreated with hyaluronidase (200 mU/ml) 30 min before replacing with fresh media. Then U937 monocytes, prelabeled with calcein-AM, were added and allowed to adhere for 90 min at 4°C. The cells were then washed three times, and adherent cells were measured in a multi-well fluorescent plate reader. Results are expressed as the number of adhered U937 cells per dish. *B–D*: Some adipocytes were transfected with SAA3 or SAA1 siRNA. Negative control siRNAs #1 or #3 provided by the manufacturer also were transfected. After transfection, total RNA and conditioned media were collected for analysis of SAA3 and SAA1 mRNA expression by real-time RT-PCR using SAA3 and SAA1 specific primers and probes and normalized to GAPDH (*B*) or by immunoblot using a SAA3 antibody (*C*). *D*: Two days after finishing the transfection, adipocytes were subjected to the monocyte adhesion assay described in *A*. **P* < 0.001 vs. low glucose; ***P* < 0.001 vs. high glucose; #*P* < 0.001 vs. negative control siRNA 3; †*P* < 0.01 vs. negative control siRNA 3.

ob/ob mice, which lack leptin and develop massive obesity (41), were investigated. Both mouse models had systemic inflammation as evidenced by increased circulating levels of SAA (data not shown). SAA3 mRNA and protein increased dramatically in adipose tissue of $LDLR^{-/-}$ mice in response to consumption of the high-fat, high-sucrose diet (Fig. 8*A* and *B*). *ob/ob* mice also exhibited a marked increase in SAA3 mRNA levels in adipose tissue (Fig. 8*D*). In addition, hyaluronan concentration and HAS2 mRNA increased dramatically in both diet-induced and genetically obese mice (Fig. 8*C* and *E*). Immunohistochemical staining showed an increase in SAA (using an antibody that recognizes all isoforms of SAA), hyaluronan (detected with HABP), and macrophages (detected with Mac-2), in visceral adipose tissue from $LDLR^{-/-}$ mice fed the high-fat, high-sucrose diet (Fig. 8*F*). Adipose tissue from these mice also showed evidence of adipocyte hypertrophy and macrophage accumulation. Moreover, hyaluronan and SAA were detected in the same regions where macrophages infiltrated (Fig. 8*F*). These findings imply that the complex of SAA and hyaluronan might have an important role in the accumulation and recruitment of macrophages into adipose tissue in vivo.

DISCUSSION

We have demonstrated increased SAA3 and hyaluronan expression in 3T3-L1 adipocytes induced to become hypertrophic by culture in high glucose concentrations in vitro and in diet-induced and genetic obesity in vivo. These changes are coordinately and reciprocally regulated by DNA binding activity of NF-κB and PPARγ. Moreover, SAA3 and hyaluronan produced by hypertrophic adipocytes associate with each other in a complex. This SAA3- and hyaluronan-containing complex facilitates monocyte adhesion and increases monocyte chemotaxis. Thus, mol-

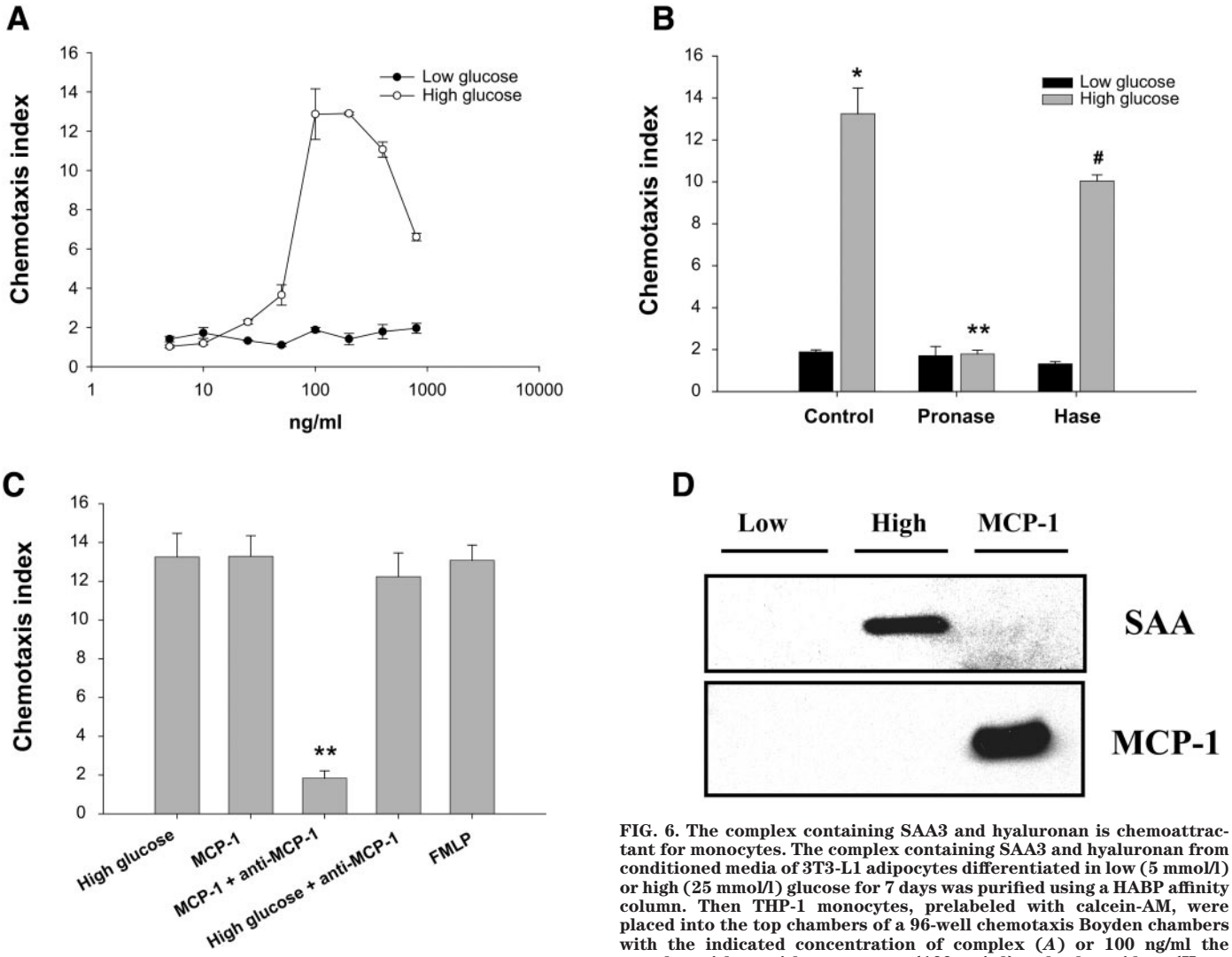

FIG. 6. The complex containing SAA3 and hyaluronan is chemoattractant for monocytes. The complex containing SAA3 and hyaluronan from conditioned media of 3T3-L1 adipocytes differentiated in low (5 mmol/l) or high (25 mmol/l) glucose for 7 days was purified using a HABP affinity column. Then THP-1 monocytes, prelabeled with calcein-AM, were placed into the top chambers of a 96-well chemotaxis Boyden chambers with the indicated concentration of complex (*A*) or 100 ng/ml the complex with or without pronase (100 μg/ml) or hyaluronidase (Hase; 200 mU/ml) (*B*) in the bottom chambers. The complexes (100 ng/ml) purified from conditioned media of 3T3-L1 adipocytes cultured in high glucose were pretreated with neutralizing anti–MCP-1 antibody (5 μg/ml). Then, the complexes or MCP-1 (100 ng/ml), with or without added anti–MCP-1 antibody, were analyzed with the chemotaxis assay. *C*: The chemotactic peptide, formyl-methionyl-leucyl-phenylalanine (FMLP) (10 nmol/l, Sigma), was used as a control. *D*: Finally, the complex containing SAA3 and hyaluronan (20 μg/ml) and MCP-1 (10 ng/ml) as control was loaded on SDS-PAGE and subjected to immunoblot analysis using an anti-SAA (*top*) or anti–MCP-1 antibody (*bottom*). *$P < 0.001$ vs. low glucose; **$P < 0.001$ vs. high glucose; #$P < 0.01$ vs. high glucose.

ecules present in this complex could facilitate the recruitment and retention of monocytes in adipose tissue in obesity.

An important feature of obesity is that adipocytes become hypertrophic due to the delivery of an excess amount of energy in the form of glucose or fatty acids. To mimic obesity, a major feature of which is adipocyte hypertrophy, we used high glucose concentrations to make 3T3-L1 adipocytes hypertrophic in vitro. Previous studies have shown that hyperglycemia per se can rapidly increase the secretion of SAA by adipocytes in vitro and by adipose tissues from streptozotocin-treated mice without adipocyte hypertrophy (30,42). However, we were unable to show increased expression of SAA3 in the absence of adipocyte hypertrophy, possibly because of the different conditions used in the previous study (30).

Coordinate and reciprocal regulation of NF-κB and PPAR transactivation is an important determinant of the inflammatory state. NF-κB DNA transactivation leads to the expression of pro-inflammatory molecules (34,43–45), whereas PPARs ameliorate inflammation by regulating the expression of anti-inflammatory molecules (46,47). In the present study, we have shown that hypertrophic adipocytes are in a pro-inflammatory state in which NF-κB activity is increased, PPARγ activity is decreased, and expression of SAA and hyaluronan is increased. Moreover, these inflammatory changes were reversed by rosiglitazone, which decreased NF-κB DNA binding activity, increased PPARγ DNA binding activity, and reduced SAA and MCP-1 expression.

Our data show that adipocyte hypertrophy is specifically associated with an increase in SAA3 mRNA and protein expression both in vitro and in vivo. SAA3 is the isoform produced by extra-hepatic cells, such as adipocytes, in mice (16). Although adipocyte hypertrophy was also associated with an increase in the expression of SAA1 and -2, isoforms produced primarily by the liver, their transcripts levels were considerably lower than for SAA3.

Our data also indicate that hyaluronan is increased during adipocyte hypertrophy because of HAS2 induction in vitro, changes that are also reversed by rosiglitazone. Similar observations were made in vivo, because steady-

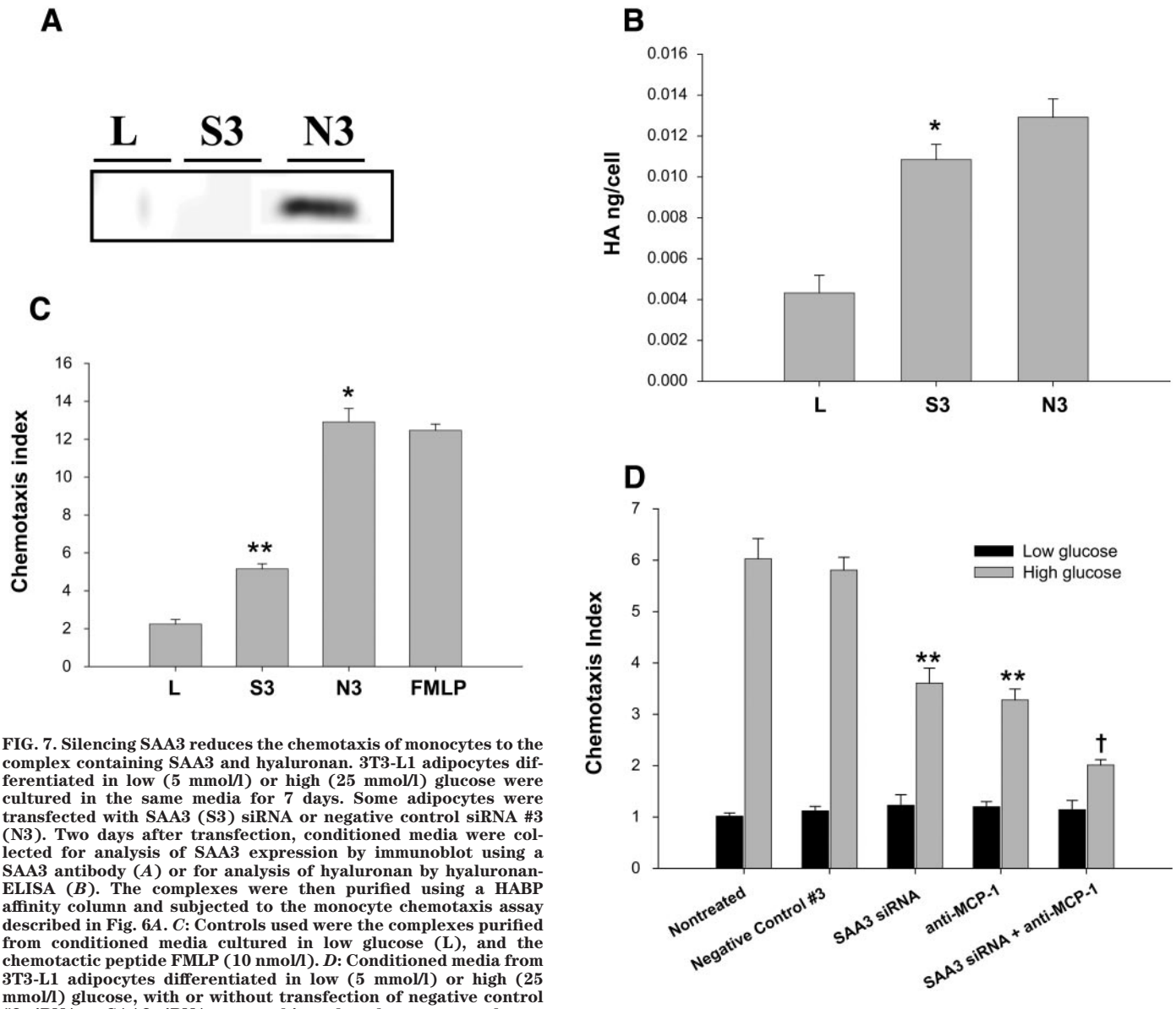

FIG. 7. Silencing SAA3 reduces the chemotaxis of monocytes to the complex containing SAA3 and hyaluronan. 3T3-L1 adipocytes differentiated in low (5 mmol/l) or high (25 mmol/l) glucose were cultured in the same media for 7 days. Some adipocytes were transfected with SAA3 (S3) siRNA or negative control siRNA #3 (N3). Two days after transfection, conditioned media were collected for analysis of SAA3 expression by immunoblot using a SAA3 antibody (*A*) or for analysis of hyaluronan by hyaluronan-ELISA (*B*). The complexes were then purified using a HABP affinity column and subjected to the monocyte chemotaxis assay described in Fig. 6*A*. *C*: Controls used were the complexes purified from conditioned media cultured in low glucose (L), and the chemotactic peptide FMLP (10 nmol/l). *D*: Conditioned media from 3T3-L1 adipocytes differentiated in low (5 mmol/l) or high (25 mmol/l) glucose, with or without transfection of negative control #3 siRNA or SAA3 siRNA, were subjected to the monocyte chemotaxis assay described in Fig. 6. *P < 0.001 vs. low glucose, **P < 0.001 vs. negative control #3 siRNA, †P < 0.01 vs. SAA3 siRNA.

state levels of SAA3, HAS2 mRNA, and hyaluronan concentrations were increased in adipose tissue from two different mouse models of obesity.

Recent studies show that macrophages are present, albeit in reduced numbers, in adipose tissue of obese CCR2-deficient mice (14). These observations suggest that molecules other than MCP-1 also must be involved in the recruitment and retention of macrophages into adipose tissue. SAA is known to be a chemoattractant (20), and hyaluronan can bind macrophages via an interaction with cell surface receptors, especially CD44 (26,48), events that could potentially lead to monocyte recruitment and macrophage retention in adipose tissue. Because both SAA and hyaluronan increase during adipocyte hypertrophy, we postulate that SAA and hyaluronan have a synergistic effect on macrophage recruitment. Several lines of evidence indicate that SAA3 and hyaluronan coexist in a complex surrounding the cells. First, hyaluronidase treatment of the cell-associated matrix led to the release of SAA3. Second, SAA and hyaluronan colocalized on adipocytes, and treatment with hyaluronidase removed both SAA and hyaluronan immunostaining. Third, SAA bound to a HABP affinity column, indicating that it existed in a complex with hyaluronan. And finally, SAA and hyaluronan were colocalized in adipose tissue from mice with diet-induced obesity.

We have demonstrated that the complex of SAA and hyaluronan that surrounds hypertrophic adipocytes increases both monocyte adhesion and retention. Both hyaluronan and SAA appear to facilitate monocyte adhesion, because pretreatment of the cells with hyaluronidase and silencing of SAA3 production both partially reduced monocyte adhesion. We also showed that the SAA and hyaluronan-containing complex, which is free of cells, increased monocyte chemotaxis. Degradation of hyaluronan with hyaluronidase and inhibition of SAA3 production by the use of a SAA3-specific siRNA both partially inhibited monocyte chemotaxis to the hyaluronan-containing complex. Moreover, this effect was independent of the chemotactic factor MCP-1, which was absent from the matrix

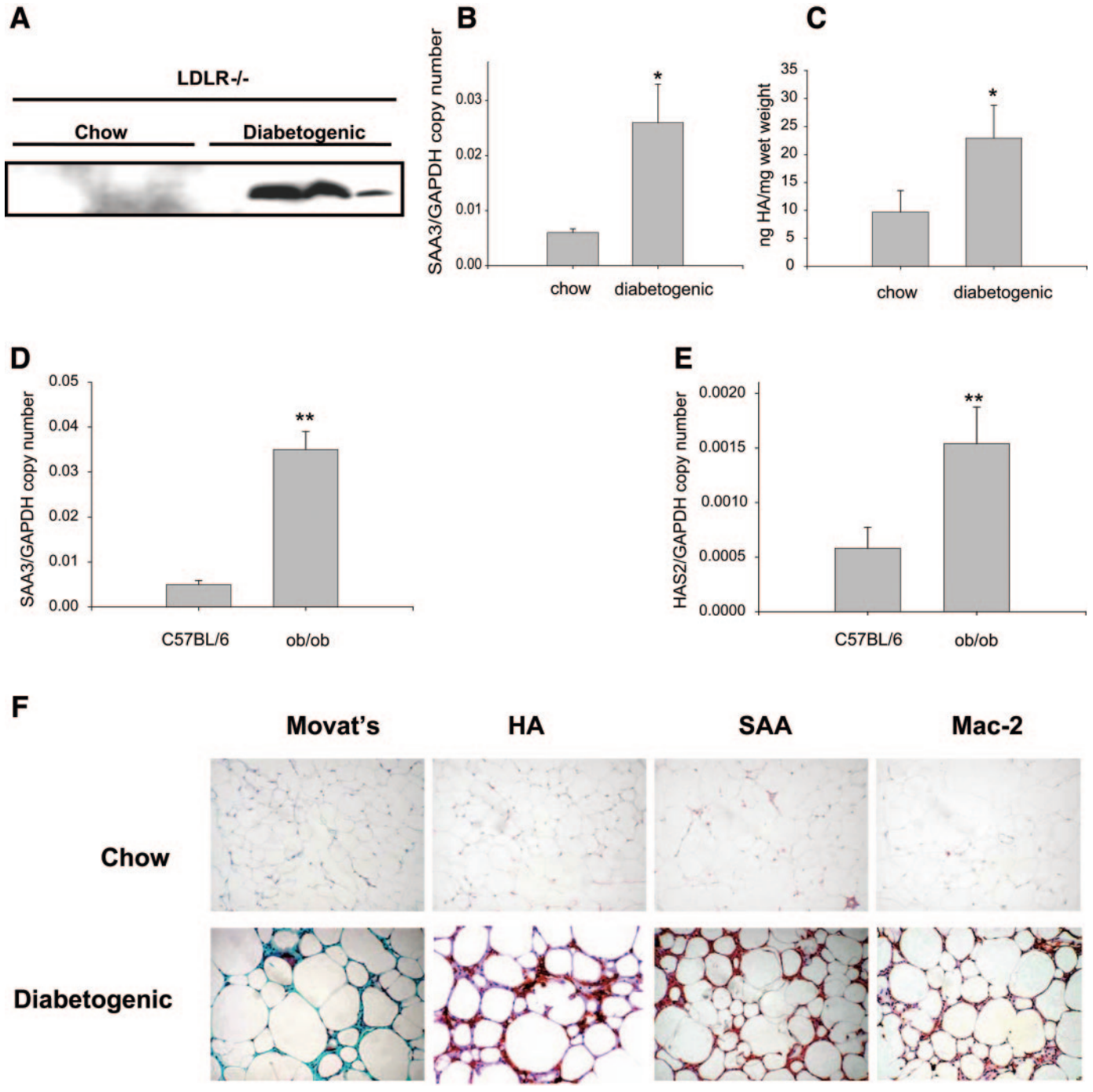

FIG. 8. SAA3 and hyaluronan are increased and colocalized in adipose tissue from diet-induced and genetically obese mice. *A*, *B*, *C*, and *F*: LDLR$^{-/-}$ mice were fed a chow or high-fat, high-sucrose diet for 16 weeks. Epididymal fat was isolated and analyzed by immunoblot using a SAA3 antibody (*A*; $n = 4$) and by real-time RT-PCR using a SAA3-specific primer and probes (*B*; $n = 4$), and hyaluronan content was measured by ELISA (*C*; $n = 5$). *D* and *E*: *ob/ob* mice and C57BL/6 littermates were fed a chow diet for 24 weeks. Epididymal fat was isolated and analyzed by real-time RT-PCR using SAA3-specific (*D*; $n = 5$) and HAS2-specific (*E*; $n = 5$) primers and probes and normalized to GAPDH. *$P < 0.01$ vs. chow, **$P < 0.001$ vs. C57BL/6 mice. *F*: Epididymal fat was isolated and analyzed by immunohistochemistry using a SAA3 antibody, HABP, and a Mac-2 antibody, which detects SAA3, hyaluronan, and murine macrophages, respectively. Tissues were photographed using microscopy (original magnification ×100).

although induced by adipocyte hypertrophy. Furthermore, chemotaxis induced by the isolated complex was not reduced by the use of an antibody against MCP-1. However, use of media from the hypertrophic adipocytes indicated that MCP-1 and SAA3 each accounted for about 35% of the chemotaxis, with the remainder presumably due to some as-yet-unidentified chemotactic factor(s). It is unclear why SAA3 should play a role in the adhesion of monocytes to the matrix. It is conceivable that SAA3 present in the deeper layers of the complex might induce monocyte chemotaxis, thereby facilitating the retention of monocytes by the matrix surrounding the adipocytes. Hyaluronan might thus facilitate monocyte retention via CD44 and other receptors and also by anchoring SAA3, thereby creating a gradient of this chemotactic factor, which might facilitate monocyte adhesion.

Plasma SAA is derived mainly from the liver and circulates predominantly in association with HDL (16). It is not

present in a lipoprotein-free form in plasma (37). Because adipocytes do not produce structural apolipoproteins, such as apo A-I or apo B, non–lipoprotein-associated SAA produced by adipocytes might have a local function, such as monocyte recruitment. Although hyaluronan is involved in monocyte adhesion, it might also work to retain immune cells, such as macrophages, by anchoring proinflammatory molecules, such as SAA.

We propose a model whereby the extracellular matrix surrounding adipocytes acts as an anchor that traps lipoprotein-free SAA3 and that these molecules act in concert to facilitate the recruitment, adhesion, and retention of monocyte-macrophages in adipose tissue, playing a role in the local inflammation that is characteristic of adipose tissue in obesity.

ACKNOWLEDGMENTS

C.Y.H. received a postdoctoral fellowship from the American Heart Association. This work received grants from the National Institutes of Health (HL-30086, DK-02456, HL-18645, HL-079117, DK-17047, and DK-35816).

Mass spectrometry experiments were performed by the Mass Spectrometry Resource, Department of Medicine, and the DERC Mass Spectrometry Core, University of Washington.

REFERENCES

1. Maachi M, Pieroni L, Bruckert E, Jardel C, Fellahi S, Hainque B, Capeau J, Bastard JP: Systemic low-grade inflammation is related to both circulating and adipose tissue TNFalpha, leptin and IL-6 levels in obese women. *Int J Obes Relat Metab Disord* 28:993–997, 2004
2. Poitou C, Viguerie N, Cancello R, De Matteis R, Cinti S, Stich V, Coussieu C, Gauthier E, Courtine M, Zucker JD, Barsh GS, Saris W, Bruneval P, Basdevant A, Langin D, Clement K: Serum amyloid A: production by human white adipocyte and regulation by obesity and nutrition. *Diabetologia* 48:519–528, 2005
3. Ouchi N, Kihara S, Funahashi T, Nakamura T, Nishida M, Kumada M, Okamoto Y, Ohashi K, Nagaretani H, Kishida K, Nishizawa H, Maeda N, Kobayashi H, Hiraoka H, Matsuzawa Y: Reciprocal association of C-reactive protein with adiponectin in blood stream and adipose tissue. *Circulation* 107:671–674, 2003
4. Cancello R, Clement K: Is obesity an inflammatory illness? Role of low-grade inflammation and macrophage infiltration in human white adipose tissue. *BJOG* 113:1141–1147, 2006
5. Fried SK, Bunkin DA, Greenberg AS: Omental and subcutaneous adipose tissues of obese subjects release interleukin-6: depot difference and regulation by glucocorticoid. *J Clin Endocrinol Metab* 83:847–850, 1998
6. Sowers JR: Obesity as a cardiovascular risk factor. *Am J Med* 115 (Suppl. 8A):37S–41S, 2003
7. Bray GA: Risks of obesity. *Endocrinol Metab Clin North Am* 32:787–804, viii, 2003
8. Wang CC, Goalstone ML, Draznin B: Molecular mechanisms of insulin resistance that impact cardiovascular biology. *Diabetes* 53:2735–2740, 2004
9. Lebovitz HE: Insulin resistance: a common link between type 2 diabetes and cardiovascular disease. *Diabetes Obes Metab* 8:237–249, 2006
10. Wellen KE, Hotamisligil GS: Obesity-induced inflammatory changes in adipose tissue. *J Clin Invest* 112:1785–1788, 2003
11. Xu H, Barnes GT, Yang Q, Tan G, Yang D, Chou CJ, Sole J, Nichols A, Ross JS, Tartaglia LA, Chen H: Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *J Clin Invest* 112:1821–1830, 2003
12. Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW Jr: Obesity is associated with macrophage accumulation in adipose tissue. *J Clin Invest* 112:1796–1808, 2003
13. Neels JG, Olefsky JM: Inflamed fat: what starts the fire? *J Clin Invest* 116:33–35, 2006
14. Weisberg SP, Hunter D, Huber R, Lemieux J, Slaymaker S, Vaddi K, Charo I, Leibel RL, Ferrante AW Jr: CCR2 modulates inflammatory and metabolic effects of high-fat feeding. *J Clin Invest* 116:115–124, 2006
15. Kanda H, Tateya S, Tamori Y, Kotani K, Hiasa K, Kitazawa R, Kitazawa S, Miyachi H, Maeda S, Egashira K, Kasuga M: MCP-1 contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in obesity. *J Clin Invest* 116:1494–1505, 2006
16. Uhlar CM, Whitehead AS: Serum amyloid A, the major vertebrate acute-phase reactant. *Eur J Biochem* 265:501–523, 1999
17. Toole BP, Wight TN, Tammi MI: Hyaluronan-cell interactions in cancer and vascular disease. *J Biol Chem* 277:4593–4596, 2002
18. Poitou C, Coussieu C, Rouault C, Coupaye M, Cancello R, Bedel JF, Gouillon M, Bouillot JL, Oppert JM, Basdevant A, Clement K: Serum amyloid A: a marker of adiposity-induced low-grade inflammation but not of metabolic status. *Obesity* (*Silver Spring*) 14:309–318, 2006
19. O'Brien KD, Chait A: Serum amyloid A: the "other" inflammatory protein. *Curr Atheroscler Rep* 8:62–68, 2006
20. Badolato R, Wang JM, Murphy WJ, Lloyd AR, Michiel DF, Bausserman LL, Kelvin DJ, Oppenheim JJ: Serum amyloid A is a chemoattractant: induction of migration, adhesion, and tissue infiltration of monocytes and polymorphonuclear leukocytes. *J Exp Med* 180:203–209, 1994
21. Mullan RH, Bresnihan B, Golden-Mason L, Markham T, O'Hara R, FitzGerald O, Veale DJ, Fearon U: Acute-phase serum amyloid A stimulation of angiogenesis, leukocyte recruitment, and matrix degradation in rheumatoid arthritis through an NF-kappaB-dependent signal transduction pathway. *Arthritis Rheum* 54:105–114, 2006
22. Lindhorst E, Young D, Bagshaw W, Hyland M, Kisilevsky R: Acute inflammation, acute phase serum amyloid A and cholesterol metabolism in the mouse. *Biochim Biophys Acta* 1339:143–154, 1997
23. Eriksen N, Benditt EP: Isolation and characterization of the amyloid-related apoprotein (SAA) from human high density lipoprotein. *Proc Natl Acad Sci U S A* 77:6860–6864, 1980
24. Yang RZ, Lee MJ, Hu H, Pollin TI, Ryan AS, Nicklas BJ, Snitker S, Horenstein RB, Hull K, Goldberg NH, Goldberg AP, Shuldiner AR, Fried SK, Gong DW: Acute-phase serum amyloid A: an inflammatory adipokine and potential link between obesity and its metabolic complications. *PLoS Med* 3: e287, 2006
25. Kolodgie FD, Burke AP, Farb A, Weber DK, Kutys R, Wight TN, Virmani R: Differential accumulation of proteoglycans and hyaluronan in culprit lesions: insights into plaque erosion. *Arterioscler Thromb Vasc Biol* 22:1642–1648, 2002
26. de La Motte CA, Hascall VC, Calabro A, Yen-Lieberman B, Strong SA: Mononuclear leukocytes preferentially bind via CD44 to hyaluronan on human intestinal mucosal smooth muscle cells after virus infection or treatment with poly(I.C). *J Biol Chem* 274:30747–30755, 1999
27. Cuff CA, Kothapalli D, Azonobi I, Chun S, Zhang Y, Belkin R, Yeh C, Secreto A, Assoian RK, Rader DJ, Pure E: The adhesion receptor CD44 promotes atherosclerosis by mediating inflammatory cell recruitment and vascular cell activation. *J Clin Invest* 108:1031–1040, 2001
28. Allingham PG, Brownlee GR, Harper GS, Pho M, Nilsson SK, Brown TJ: Gene expression, synthesis and degradation of hyaluronan during differentiation of 3T3–L1 adipocytes. *Arch Biochem Biophys* 452:83–91, 2006
29. Calvo JC, Gandjbakhche AH, Nossal R, Hascall VC, Yanagishita M: Rheological effects of the presence of hyaluronic acid in the extracellular media of differentiated 3T3–L1 preadipocyte cultures. *Arch Biochem Biophys* 302:468–475, 1993
30. Lin Y, Berg AH, Iyengar P, Lam TK, Giacca A, Combs TP, Rajala MW, Du X, Rollman B, Li W, Hawkins M, Barzilai N, Rhodes CJ, Fantus IG, Brownlee M, Scherer PE: The hyperglycemia-induced inflammatory response in adipocytes: the role of reactive oxygen species. *J Biol Chem* 280:4617–4626, 2005
31. Han CY, Chiba T, Campbell JS, Fausto N, Chaisson M, Orasanu G, Plutzky J, Chait A: Reciprocal and coordinate regulation of serum amyloid A versus apolipoprotein A-I and paraoxonase-1 by inflammation in murine hepatocytes. *Arterioscler Thromb Vasc Biol* 26:1806–1813, 2006
32. Goueffic Y, Potter-Perigo S, Chan CK, Johnson PY, Braun K, Evanko SP, Wight TN: Sirolimus blocks the accumulation of hyaluronan (HA) by arterial smooth muscle cells and reduces monocyte adhesion to the ECM. *Atherosclerosis*. In press
33. Wilkinson TS, Potter-Perigo S, Tsoi C, Altman LC, Wight TN: Pro- and anti-inflammatory factors cooperate to control hyaluronan synthesis in lung fibroblasts. *Am J Respir Cell Mol Biol* 31:92–99, 2004
34. Barnes PJ, Karin M: Nuclear factor-kappaB: a pivotal transcription factor in chronic inflammatory diseases. *N Engl J Med* 336:1066–1071, 1997
35. Yannariello-Brown J, Zhou B, Ritchie D, Oka JA, Weigel PH: A novel ligand blot assay detects different hyaluronan-binding proteins in rat liver hepatocytes and sinusoidal endothelial cells. *Biochem Biophys Res Commun* 218:314–319, 1996
36. Frevert CW, Wong VA, Goodman RB, Goodwin R, Martin TR: Rapid fluorescence-based measurement of neutrophil migration in vitro. *J Immunol Methods* 213:41–52, 1998
37. Lewis KE, Kirk EA, McDonald TO, Wang S, Wight TN, O'Brien KD, Chait A:

Increase in serum amyloid a evoked by dietary cholesterol is associated with increased atherosclerosis in mice. *Circulation* 110:540–545, 2004
38. Underhill CB, Nguyen HA, Shizari M, Culty M: CD44 positive macrophages take up hyaluronan during lung development. *Dev Biol* 155:324–336, 1993
39. Wilkinson TS, Bressler SL, Evanko SP, Braun KR, Wight TN: Overexpression of hyaluronan synthases alters vascular smooth muscle cell phenotype and promotes monocyte adhesion. *J Cell Physiol* 206:378–385, 2006
40. Schreyer SA, Vick C, Lystig TC, Mystkowski P, LeBoeuf RC: LDL receptor but not apolipoprotein E deficiency increases diet-induced obesity and diabetes in mice. *Am J Physiol Endocrinol Metab* 282: E207–E214, 2002
41. Pelleymounter MA, Cullen MJ, Baker MB, Hecht R, Winters D, Boone T, Collins F: Effects of the obese gene product on body weight regulation in ob/ob mice. *Science* 269:540–543, 1995
42. Lin Y, Rajala MW, Berger JP, Moller DE, Barzilai N, Scherer PE: Hyperglycemia-induced production of acute phase reactants in adipose tissue. *J Biol Chem* 276:42077–42083, 2001
43. Ruan H, Hacohen N, Golub TR, Van Parijs L, Lodish HF: Tumor necrosis factor-α suppresses adipocyte-specific genes and activates expression of preadipocyte genes in 3T3–L1 adipocytes: nuclear factor-κB activation by TNF-α is obligatory. *Diabetes* 51:1319–1336, 2002
44. Ajuwon KM, Spurlock ME: Palmitate activates the NF-kappaB transcription factor and induces IL-6 and TNFalpha expression in 3T3–L1 adipocytes. *J Nutr* 135:1841–1846, 2005
45. Bokarewa M, Nagaev I, Dahlberg L, Smith U, Tarkowski A: Resistin, an adipokine with potent proinflammatory properties. *J Immunol* 174:5789–5795, 2005
46. Chawla A, Schwarz EJ, Dimaculangan DD, Lazar MA: Peroxisome proliferator-activated receptor (PPAR) gamma: adipose-predominant expression and induction early in adipocyte differentiation. *Endocrinology* 135: 798–800, 1994
47. Lehrke M, Lazar MA: The many faces of PPARgamma. *Cell* 123:993–999, 2005
48. Konttinen YT, Li TF, Mandelin J, Ainola M, Lassus J, Virtanen I, Santavirta S, Tammi M, Tammi R: Hyaluronan synthases, hyaluronan, and its CD44 receptor in tissue around loosened total hip prostheses. *J Pathol* 194:384–390, 2001