Chemicals and reagents

Human protein S was obtained from American Diagnostica Inc. (Stamford, CT). It was purified from fresh frozen human plasma via immunopurification and characterized of no protein S-C4BP complex, a single band at 69 kD on 10% SDS-PAGE and no reduction upon incubation with 2-mercaptoethanol. Phorbol myristate acetate (PMA) was purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in dimethyl sulfoxide (DMSO). For separate experiments, human protein S was also obtained from USBiological (Swampscott, MA) and Calbiochem (San Diego, CA). Unlabeled AcLDL was purchased from Medical technologies Inc. (Stoughton, MA). Alexa Fluor 488-AcLDL (fluorescent AcLDL) and Act-D were purchased from Invitrogen Co. (Carlsbad, CA). Goat anti-human SR-A antibody, rabbit anti-human protein S antibody, and mouse monoclonal anti-human protein S antibody were obtained from Biodesign (Saco, ME), Sigma, and US Biological Inc (Swampscott, MA), respectively. Goat antihuman Axl, Tyro3, Mer antibodies, recombinant human Gas6 and recombinant human Axl/Fc, Tyro3/Fc, Mer/Fc, and TrkB/Fc chimera, which contains the extracellular domain of the receptor fused to the Fc region of human immunoglobulin IgG1 heavy chain, were purchased from R&D Systems, Inc (Minneapolis, MN).

Cell culture

THP-1 and U937 was obtained from American Type Culture Collection (Manassas, VA) and maintained in the complete medium, which contains RPMI-1640 (GIBCO-BRL) medium supplemented with 10% FBS. THP-1 cells or U937 cells were differentiated into macrophages by PMA (50 ng/mL) for 48 h, and then treated with physiological concentrations of protein S (0, 5, 10 and 20 μg/mL) in the fresh complete medium for 24 h unless otherwise stated in the text. Fresh human blood samples were obtained from Gulf Coast Regional Blood Center (Houston, TX, USA). Human PBMC were isolated from fresh peripheral blood by Ficoll-Paque centrifugation (Sigma) and suspended in the RPMI 1640 medium with 10% human type AB serum (US Biological Inc, Swampscott, MA). PBMC were differentiated into macrophages by incubation in the RPMI 1640 medium with human type AB serum for 5 days, and then treated with protein S (0, 5, 10 and 20 μg/mL) for 24 h.

Primary human aorta smooth cells (AoSMC) obtained from Lonza Inc. (Allendale, NJ) and maintained according to the manufacture's instruction. AoSMC were treated with 20 μg /mL protein S or 20 ng/mL Gas6 in the fresh medium for 24 h.

AcLDL uptake and binding

For AcLDL uptake assay, protein S-treated human macrophages were incubated with Alexa Fluor 488-AcLDL (5 μg/mL) in the RPMI 1640 medium containing 2% lipoprotein-deficient human serum at 37°C for 3 h.[21] For the binding experiment, THP-1 macrophages were incubated at 4°C for 30 min because lowering the temperature can inhibit endocytosis of SR-A ligands. Unlabeled AcLDL in an excess amount (50-fold) was added together with the fluorescent AcLDL for competition assay. At the end of incubation, the cells were lysed with 0.1% SDS/0.1 N NaOH for direct measurement of fluorescence and protein concentrations. The fluorescence of each well was measured in triplicate by a fluorescence reader (BioTek Instruments Inc., VT). The excitation and emission wavelengths were set at 485 and 528 nm, respectively. Specific fluorescent intensity was determined by subtracting mean fluorescent intensity of unlabeled cells (autofluorescence) from that of Alexa Fluor 488-AcLDLincubated cells. Specific uptake (or

binding) was calculated by subtracting uptake (or binding) in the presence of an excess amount of unlabeled AcLDL from the total uptake (or binding) in the absence of unlabeled AcLDL.

Analysis of cellular cholesterol content

THP-1 macrophages were incubated with or without protein S for 24 h in the presence of 50 μg/mL AcLDL. Next, cellular lipids were extracted with isopropanol-hexane (3:2) and dissolved with isopropanol, and the cholesterol content was quantified with a cholesterol assay kit from Wako Chemicals (Richmond, GA). The amount of cholesterol ester was calculated by subtracting the free cholesterol from total cholesterol. After lipid extraction, the cellular protein was dissolved in sodium hydroxide, and the protein concentration was determined with the BCA kit (Pierce).

Real-time quantitative RT-PCR

Total cellular RNA was extracted using the RNAqueous-4PCR kit (Ambion, TX). One μg of RNA was reverse-transcribed into cDNA. Real-time quantitative RT-PCR primers were selected from the Harvard primer bank (Table S1). The cDNA was subjected to real-time quantitative PCR using the SYBR green supermix and iCycler iQ Real-time PCR detection system (Bio-Rad). All results were normalized to the amount of GAPDH or 18S rRNA. To assess the half life (t1/2) of SR-A mRNA, THP1 cells pretreated with 50 ng/mL PMA for 48 h were washed twice with PBS, and 5 μg/mL Act-D was added to the culture in the presence or absence of protein S (20 μg/mL). Total cellular RNA was isolated at multiple time points (0, 5, 10, 14 and 16 h) and analyzed for mRNA levels by real-time PCR using SR-A and 18S rRNA primers. Thermal cycle condition used for RT was as follows: 5 min at 25ºC, 30 min at 42ºC, and 5 min at 85ºC. The condition used for real-time PCR was as follows: 3 min at 95ºC, 40 repeats of 20 sec at 95ºC, and 1 min at 60ºC. Controls were performed with no RT (mRNA sample only) or no mRNA (water only) to demonstrate the specificity of the primers and the lack of DNA contamination in samples. Sample cycle threshold (Ct) values were determined from plots of relative fluorescence units (RFU) versus PCR cycle number during exponential amplification so that sample measurement comparisons were possible. The gene expression in each sample was calculated as $2^{(40-Ct)}$ and further normalized to GAPDH or 18S rRNA expression as $[2^{(Ct[\text{GAPDH or 18S rRNA}]-Ct[\text{gene of interest}])}]$.

Western blot and immunodepletion of protein S

Western blot analysis and immunodepletion were performed as previously described.[17] The cell lysates (40 μg protein/lane) were applied to a SDS-polyacrylamide gel electrophoresis (PAGE). The electrophoresed proteins were transblotted onto a nitrocellulose membrane. After blocking, the membrane was incubated with an antibody against human SR-A (Biodesign), Axl, Tyro3, and Mer, respectively. The membrane was then incubated with an appropriate HRP-conjugated second antibody and developed with ECL western-blot detection reagents (Amersham Biosciences, Piscataway, NJ).

The blot was reblotted with a β-actin antibody (Chemicon, Temecula, CA) for a loading control. For the protein S immunodepletion experiment, protein S was incubated with rabbit anti-human protein S, normal rabbit IgG, mouse monoclonal anti-human protein S, or normal mouse IgG for 2 h at 4°C. Protein G sepharose was then added, the suspension was mixed for 1 h at 4°C and then subjected to centrifugation (12,000 × g) for 2 min, the resultant supernatant fluid was used

for the AcLDL uptake assay in THP-1 macrophages and the degree of protein S depletion was monitored by immunoblotting using the same anti-protein S antibody.

SR-A promoter activity assay

SR-A promoter fragment was kindly provided by Dr. Christopher K. Glass (University of California, San Diego).[22,23] The promoter fragment (from −630 to +48 bp) was subcloned into the pGL3-Basic vector carrying firefly luciferase. SR-A promoter mutants were created by site-directed mutagenesis of the positive transcriptional elements corresponding to the PU.1 site at −198 to −185 bp, and AP-1 element at −67 to −50 bp, as previously described.[23] THP-1 cells were co-transfected with pGL3-BasicSR-A constructs and pRL-SV40 vector carrying Renilla luciferase (as an internal control reporter) by electroporation (nucleofector program V-01/V-001) in cell line nucleofector solution V (Amaxa Inc., Gaithersburg, MD). After transfection, the cells were differentiated with 50 ng/mL PMA for 24 h. THP-1 macrophages were then treated with 20 μg/mL protein S for 24 h. Luciferase activity was measured using a dual luciferase assay kit (Promega) and a luminometer. Luciferase activities were normalized by the ratio of firefly and Renilla luciferase activities. All experiments were carried out in triplicate.

EMSA

Cellular nuclear extracts were prepared using the nuclear extraction kit (Invitrogen). After the cell debris was removed by centrifugation, the supernatant was collected. The concentration of nuclear proteins was determined by the Bio-Rad protein assay kit. Biotin end-labeled double-stranded oligonucleotides containing consensus sequences of activator protein AP-1 (5′-biotin-CGCTTGATGACTCAGCCGGAA), Ets (5′-biotin GGAGGAGGGCTGCTTGAGGAAGTATAAGAAT) and PU.1 (5′-biotin-GCCTCCTACTTCTCCTTTTCT) (Panomics, Fremont, CA) were used for this experiment. The binding reaction mixture contained 5 μg of nuclear extract proteins, 2 μl binding buffer, 1 μg of poly d(I-C), and 10 ng of biotin-labeled DNA, and was incubated at 18°C for 30 min. The competition reaction was performed by adding 60-fold excess unlabeled double-stranded consensus oligonucleotide to the reaction mixture. After the reaction, the DNA-protein complexes were subjected to a 6% native PAGE and were transferred to a Pall biodyne B nylon membrane at 120 V for 1 h in 0.5 × TBE buffer. Then the membrane was baked at 80°C for 1.5 h. The biotin-labeled DNA was detected using streptavidin-linked horseradish peroxidase and EMSA kit (Panomics).

Analysis of protein S by native PAGE and western blot

Human protein S (25 ng) or human plasma (1.5 μl) was subjected to native PAGE, transferred to a nitrocellulose membrane, and detected by rabbit anti-protein S antibody as described above. In addition, protein S (2.5 μg) was heated at 80°C for different time points and loaded in 4–12% non-SDS polyacrylamide gel using Tris-Glycine buffer system. After electrophoresis, the gel was stained with Coomassie blue for 45 min.

Tyrosine phosphorylation assay

THP-1 macrophages were treated with human protein S (20 μg/mL) at 37°C for 30 min. The cells were lysed in EBC buffer. The lysates were immunoprecipitated with goat anti-Axl, anti-Tyro 3 or anti-Mer antibody, and immunoblotted with a phosphotyrosine-specific monoclonal antibody (4G10, Chemicon), anti-Axl, anti-Tyro 3 or anti-Mer antibody, respectively. Following

incubation with an appropriate HRP-conjugated second antibody, bands were visualized with ECL western-blot detection reagents (Amersham Biosciences, Piscataway, NJ).

Binding assay of human protein S to Mer/Fc fusion protein

Purified human protein S (10 nM) was mixed with 10 nM Mer-Fc or TrkB-Fc at 4 °C for 2 h. Protein A agarose beads were added and complexes were incubated for additional 1 h. After centrifugation, the precipitates were washed four times with cold PBS containing 0.1% Triton X-100. Bound proteins were subjected to SDS-PAGE and immunobloted with anti-protein S antibody.

Immunohistochemical analysis

Full-thickness arterial wall specimens of aorta, carotid and coronary arteries were obtained from 5 patients with or without atherosclerosis undergoing autopsy (National Disease Research Interchange, Philadelphia, PA). All samples were fixed in formalin and embedded in paraffin. Immunohistochemistry was done with anti-human protein S antibody (1:200) (sigma, St. Louis, MO), anti-human SR-A antibody (1:100) (Cosmo Bio USA, Inc., Carlsbad, CA), biotinylated secondary antibody, and avidin-biotin reaction using peroxidase enzyme (ABC kit; Vector Laboratories, Burlingham, CA). For colocalization of protein S and SR-A, we used serial slides in immunostaining. Non-immune rabbit IgG (sigma, St. Louis, MO) were used as negative controls. In addition, protein S antibody was pre-incubated with soluble protein S before adding to the slides. The protocol of use of human tissues obtained from NDRI was approved by the Institutional Review Board (IRB) at the Baylor College of Medicine. The investigation conformed to the principles outlined in the Declaration of Helsinki.

ELISA assay for human Gas6

Human Gas6 concentration was determined by using human Gas6 ELISA kit (R&D Systems Inc, Minneapolis, MN). An assay volume of 100 μl was used throughout. After the plate was coated with capture antibody and blocked with PBS-1% BSA, human Gas6 standards or 100 μg protein S samples were added and incubated for 2 h. The plate was then sequentially incubated with detection antibody, streptavidin-HRP, and ABTS substrate solution. The absorbance was measured at 405 nm in a microplate reader.

Statistical analysis

Results were expressed as the mean ± SD absolute values or as the percentage of control. Statistical comparison between two groups was performed using the Student's *t* test. One-way analysis of variance (ANOVA) was used to compare the means of multiple groups. A probability (*P*) value <0.05 was considered statistically significant.