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Inhibition of Fibrocyte Differentiation by Serum Amyloid P[1]

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Wound healing and the dysregulated events leading to fibrosis both involve the proliferation and differentiation of fibroblasts and the deposition of extracellular matrix. Whether these fibroblasts are locally derived or from a circulating precursor population is unclear. Fibrocytes are a distinct population of fibroblast-like cells derived from peripheral blood monocytes that enter sites of tissue injury to promote angiogenesis and wound healing. We have found that CD14$^+$ peripheral blood monocytes cultured in the absence of serum or plasma differentiate into fibrocytes within 72 h. We purified the factor in serum and plasma that prevents the rapid appearance of fibrocytes, and identified it as serum amyloid P (SAP). Purified SAP inhibits fibrocyte differentiation at levels similar to those found in plasma, while depleting SAP reduces the ability of plasma to inhibit fibrocyte differentiation. Compared with sera from healthy individuals and patients with rheumatoid arthritis, sera from patients with scleroderma and mixed connective tissue disease, two systemic fibrotic diseases, were less able to inhibit fibrocyte differentiation in vitro and had correspondingly lower serum levels of SAP. These results suggest that low levels of SAP may thus augment pathological processes leading to fibrosis. These data also suggest mechanisms to inhibit fibrosis in chronic inflammatory conditions, or conversely to promote wound healing. ***The Journal of Immunology,*** **2003, 171: 5537–5546.**

Inflammation is the coordinated response to tissue injury or infection. The initiating events are mediated by local release of chemotactic factors, platelet activation, and initiation of the coagulation and complement pathways (1). These events stimulate the local endothelium, promoting the extravasation of neutrophils and monocytes. The second phase is characterized by the influx into the tissues of cells of the adaptive immune system, including lymphocytes (2). The subsequent resolution phase, when apoptosis of the excess leukocytes and engulfment by tissue macrophages take place, is also characterized by the repair of tissue damage by stromal cells, such as fibroblasts (3, 4). In chronic inflammation, the resolution of inflammatory lesions is disordered, with the maintenance of inflammatory cells, fibroblast hyperplasia, and eventually tissue destruction. The mechanisms that lead to this are complex, but include enhanced recruitment, survival and retention of leukocytes, and impaired emigration (4, 5).

The source of the fibroblasts responsible for the repair of wound lesions or the hyperplasia characteristic of chronic inflammation is controversial. The conventional hypothesis suggests that local quiescent fibroblasts migrate into the damaged area, produce extracellular matrix (ECM)[3] proteins, and promote wound contraction (1). An alternative hypothesis is that circulating fibroblast precursors (called fibrocytes) present within the blood migrate to sites of injury, where they differentiate and mediate tissue repair (6). Fibrocytes differentiate from a CD14$^+$ peripheral blood monocyte-precursor population (7). Fibrocytes express markers of both hemopoietic (CD45, MHC class II, CD34) and stromal cells (collagen I and III and fibronectin) (8, 9). Mature fibrocytes rapidly enter sites of tissue injury, where they secrete inflammatory cytokines and ECM proteins and promote angiogenesis and wound contraction (7, 10). Fibrocytes are also associated with the formation of fibrotic lesions after *Schistosoma japonicum* infection in mice and are implicated in fibrosis associated with autoimmune diseases (9, 11, 12).

The mechanisms that inhibit the differentiation of fibrocytes from CD14$^+$ peripheral blood monocytes are unknown. This is of profound importance, as unchecked fibrocyte differentiation would lead to enhanced and sustained fibrosis at sites of inflammation. Clearly, in healthy individuals this does not occur. In this study, we show that the differentiation of peripheral blood fibrocytes is inhibited by a factor in serum and plasma. Fractionation of human plasma identified the factor as serum amyloid P (SAP). SAP, a member of the pentraxin family of proteins that include C-reactive protein (CRP), circulates in the blood as stable pentamers (13–15). CRP and SAP bind to a variety of molecules including microbial polysaccharides, apoptotic cells, plasma proteins, and FcRs (16–18). The levels of SAP in serum from men are 32 $\pm$ 7 μg/ml (mean $\pm$ SD), while the levels in women are significantly lower, 24 $\pm$ 8 μg/ml (19). Intriguingly, ~80% of patients with fibrosing diseases, such as scleroderma, are women (20). We have found that sera from patients with scleroderma (systemic sclerosis) and mixed connective tissue disease (MCTD) are less able to inhibit fibrocyte differentiation and had lower levels of SAP protein. This suggests that circulating levels of SAP are important in regulating fibrocyte differentiation, and that relatively small changes in the circulating levels of SAP may have an important role in fibrosis.

Materials and Methods

Abs and proteins

Purified human CRP, SAP, protein S, and C4b were purchased from Calbiochem (San Diego, CA). mAbs to CD1a, CD3, CD10, CD11a, CD11b, CD11c, CD13, CD14, CD16, CD19, CD27, CD28, CD34, CD38, CD40,

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[3] Abbreviations used in this paper: ECM, extracellular matrix; C4BP, C4b-binding protein; CRP, C-reactive protein; MCTD, mixed connective tissue disease; RA, rheumatoid arthritis; SAP, serum amyloid P; SSc, systemic sclerosis/scleroderma.

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Pan CD45, CD49e, CD61, CD64, CD69, CD83, CD86, CD90, cutaneous lymphocyte-associated Ag, MHC class II, mouse IgM, mouse IgG1, and mouse IgG2a were from BD PharMingen (San Diego, CA). mAbs to CD18, CD49c, CD70, and CD154 were from DakoCytomation (Glostrup, Denmark). mAbs to CD29, CD49b, and CD49f were from Serotec (Oxford, U.K.). mAbs to CD49a, CD80, CD103, and CD162 were from Beckman Coulter (High Wycombe, U.K.). Chemokine receptor Abs were purchased from R&D Systems (Minneapolis, MN). mAbs to CD109, $\alpha_4\beta_7$, fibronectin, vimentin, and the rabbit anti-collagen I were from Chemicon International (Temecula, CA). Monoclonal C4b-binding protein (C4BP) was from Green Mountain Antibodies (Burlington, VT), and the sheep anti-human C4BP was from The Binding Site (Birmingham, U.K.). mAbs to CRP, SAP, and α smooth muscle actin and the polyclonal rabbit anti-protein S were from Sigma-Aldrich (St. Louis, MO). Polyclonal rabbit anti-SAP was from Biogenesis (Poole, Dorset, U.K.). The mAb to CD148 was from BioSource International (Camarillo, CA). The mAb to CD105 was obtained from the Developmental Studies Hybridoma Bank (NICHD, University of Iowa, Iowa City, IA). SeeBlue Plus2-prestained protein standards (Invitrogen, Carlsbad, CA) were used for native gel m.w. markers, and BenchMark protein ladder (Invitrogen) was used for reducing gel m.w. markers.

Patients

Patients with rheumatoid arthritis (RA) ($n = 7$) fulfilled the 1987 revised criteria of the American College of Rheumatology (ACR) (21); patients with systemic sclerosis ($n = 15$) fulfilled the 1980 diagnostic criteria of the ACR (formerly the American Rheumatism Association) (22); and all MCTD patients ($n = 10$) were positive for U1-ribonucleoprotein Abs. Sera from patients were stored at −80°C.

RA patients were all female with a mean age of 67 ± 12 years (mean ± SD), had a duration of disease of 13 ± 11 years (mean ± SD), and were rheumatoid factor positive. Ten scleroderma patients were female and five male, with a mean age of 53 ± 12 years and a mean duration of disease of 5 ± 4.5 years, and all but three had high titers of anti-nuclear Abs. Nine MCTD patients were female and one male, with a mean age of 40 ± 18 years.

Cell separation

PBMC were isolated from buffy coats (Gulf Coast Regional Blood Center, Houston, TX) by Ficoll-Paque Plus (Amersham Biosciences, Piscataway, NJ) following the manufacturer's protocol. For the experiment to determine which subset of PBMC differentiates into fibrocytes, we depleted specified leukocyte subsets using negative selection with magnetic Dynabeads (Dynal Biotech, Lake Success, NY), as described previously (23–25). Briefly, PBMC were incubated for 30 min at 4°C with primary Abs against either CD3 (to deplete T cells), CD14 (to deplete monocytes), CD19 (to deplete B cells), or MHC class II (to deplete all APCs). Cells were then washed and incubated with Dynabeads coated with goat anti-mouse IgG for 30 min, before removal of Ab-coated cells by magnetic selection. This process was repeated twice. The negatively selected cells were routinely in excess of 98% pure, as determined by mAb labeling.

Cell culture and fibrocyte differentiation assay

Cells were incubated in serum-free medium, which consisted of RPMI (Invitrogen) supplemented with 10 mM HEPES (Invitrogen), 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.2% BSA (Sigma-Aldrich), 5 μg/ml insulin (Sigma-Aldrich), 5 μg/ml iron-saturated transferrin (Sigma-Aldrich), and 5 ng/ml sodium selenite (Sigma-Aldrich). Normal human serum (Sigma-Aldrich or Gulf Coast Regional Blood Center), normal human plasma (Gulf Coast Regional Blood Center), FCS (Sigma-Aldrich), column fractions, and sera from patients or purified proteins were added at the stated concentrations. PBMC were cultured in 24- or 96-well tissue culture plates (BD Biosciences, Franklin Lakes, NJ) in 2 ml or 200 μl vol, respectively, at 2.5×10^5 cells/ml in a humidified incubator containing 5% CO_2 at 37°C for the indicated times. Fibrocytes were also cultured on tissue culture plates (BD Biosciences, or Corning Costar, Acton, MA) or glass slides coated with BSA, bovine plasma fibronectin, human cellular or plasma fibronectin, or human vitronectin (Sigma-Aldrich). Fibrocytes were identified by morphology in viable cultures as adherent cells with an elongated spindle-shaped morphology as distinct from small lymphocytes or adherent monocytes. Enumeration of fibrocytes was performed on cells cultured for 6 days in flat-bottom 96-well plates, with 2.5×10^4 cells/well. Cells were air dried, fixed in methanol, and stained with H&E (Hema 3 Stain; VWR, Houston, TX). Fibrocytes were counted in five different 900-μm-diameter fields, using the above criteria of an elongated spindle shape and the presence of an oval nucleus. In addition, fibrocyte identity was confirmed by immunoperoxidase staining (see below). The fibrocyte-inhibitory activity of a sample was defined as the reciprocal of the dilution at which it inhibited fibrocyte differentiation by 50%, when added to serum-free medium.

Purification and characterization of serum and plasma proteins

A total of 250 ml of frozen human serum or fresh-frozen plasma was thawed rapidly at 37°C, and 1× Complete protease inhibitor (Roche, Indianapolis, IN), 1 mM benzamidine HCl (Sigma-Aldrich), and 1 mM Pefabloc (4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride; Roche) were added. All subsequent steps were performed on ice or at 4°C. Barium citrate adsorption of plasma was performed, as described previously (26, 27). The precipitate was collected by centrifugation at 10,000 × g for 15 min, resuspended in 20 ml of 100 mM $BaCl_2$ plus inhibitors, and recentrifuged. After two rounds of washing, the pellet was resuspended to 20 ml in 10 mM sodium phosphate buffer, pH 7.4, containing 5 mM EDTA and 1 mM benzamidine HCl, and dialyzed for 24 h against three changes of 4 L of the same buffer. Chromatography was performed using an Econo system (Bio-Rad, Hercules, CA) collecting 1-ml samples with a flow rate of 1 ml/min. The dialyzed barium citrate precipitate was loaded onto a 5-ml Hi-Trap Heparin column (Amersham Biosciences), and the column was washed extensively in 10 mM sodium phosphate buffer, pH 7.4, until the absorbance at 280 nm returned to baseline. Bound material was eluted with a stepped gradient of 15 ml each of 100, 200, 300, and 500 mM NaCl in 10 mM sodium phosphate buffer, pH 7.4. The fractions that inhibited monocyte to fibrocyte differentiation eluted at 200 mM NaCl. These were pooled (2 ml), diluted to 10 ml in sodium phosphate buffer, and loaded onto a 5-ml Econo-Pak High Q column. After washing the column in 10 mM phosphate buffer, the bound material was eluted with the stepped gradient, as above, with the active fraction eluting at 300 mM NaCl.

Active fractions from the High Q chromatography were concentrated to 200 μl using Aquacide II (Calbiochem) and then loaded onto a 4–20% native polyacrylamide gel (Cambrex Bio Science, Rockland, ME), as described previously (28). After electrophoresis, gel lanes were cut into 5-mm slices, mixed with 200 μl 20 mM sodium phosphate, 150 mM NaCl, 5 mM EDTA (pH 7.4), containing 1 mM benzamidine HCl, crushed with a small pestle in an Eppendorf tube, and placed on an end-over-end mixer at 4°C for 3 days. Proteins that eluted from the gel were analyzed for activity. To obtain amino acid sequences, proteins eluted from the gel slices were loaded onto a 4–20% reducing SDS-polyacrylamide gel with 100 μM thioglycolic acid (Sigma-Aldrich) in the upper chamber. After electrophoresis, the gel was rapidly stained with Coomassie Brilliant Blue, destained, and washed in water, and the bands were excised out of the gel. Amino acid sequencing was performed by R. Cook (Protein Sequencing Facility, Department of Immunology, Baylor College of Medicine, Houston, TX).

Immunohistochemistry

Cells cultured on 8-well glass microscope slides (Lab-Tek, Nalge Nunc International, Naperville, IL) were air dried before fixation in acetone for 15 min. Endogenous peroxidase was quenched for 15 min with 0.03% H_2O_2, and then nonspecific binding was blocked by incubation in 2% BSA in PBS for 60 min. Slides were incubated with primary Abs in PBS containing 2% BSA for 60 min. Isotype-matched irrelevant Abs were used as controls. Slides were then washed in three changes of PBS over 15 min and incubated for 60 min with biotinylated goat $F(ab')_2$ anti-mouse Ig (Southern Biotechnology, Birmingham, AL). After washing, the biotinylated Abs were detected by ExtrAvidin peroxidase (Sigma-Aldrich). Staining was developed with diaminobenzidine (Sigma-Aldrich) for 3 min and counterstained for 30 s with Mayer's hemalum (Sigma-Aldrich). All procedures were at room temperature.

Depletion of SAP from plasma

Plasma was diluted to 20% in 10 mM Tris, pH 8, 150 mM NaCl, and 5 mM $CaCl_2$ buffer, and mixed with an equal volume of Bio-Gel A-1.5m agarose beads (Bio-Rad, Hercules, CA) for 2 h at 4°C. Beads were then removed by centrifugation, and the process was repeated twice. Control plasma was diluted to 20% in 10 mM Tris, pH 8, 150 mM NaCl, 5 mM $CaCl_2$ buffer. To assess the specificity of Bio-Gel A depletion, beads were washed in 10 mM Tris, pH 8, 150 mM NaCl, and 5 mM $CaCl_2$, and the bound material was eluted with 10 mM Tris, pH 8, 150 mM NaCl, and 5 mM EDTA. Fractions were run on 4–20% polyacrylamide gels and stained with Coomassie blue for the presence of total protein. SAP was detected by Western blotting. Ab depletion of plasma was performed using protein G-coated beads (Roche, Indianapolis, IN). A total of 500 μl of protein G beads was coated with a mixture of 20 μg monoclonal (Chemicon) and 300 μl of 1/50 rabbit polyclonal antisera (BioGenesis) anti-SAP Abs, according to the manufacturer's instructions. A total of 500 μl of plasma, pretreated at 56°C for 30 min, was diluted to 20% in 20 mM sodium phosphate buffer and

precleared with 100 μl of unlabeled protein G beads for 60 min at 4°C. Beads were then removed by centrifugation. SAP was then depleted from plasma by overnight incubation at 4°C with 100 μl anti-SAP beads. Beads were removed by centrifugation before cell culture.

Western blotting

For Western blotting, plasma and serum samples were diluted 1/500 in 20 mM sodium phosphate, pH 7.4. Fractions from heparin and High Q columns and gel-purified material were not diluted. Samples were mixed with Laemmli's sample buffer, containing 20 mM DTT, and heated to 100°C for 5 min. Samples were loaded onto 4–20% Tris/glycine polyacrylamide gels (Cambrex). Samples for native gels were analyzed in the absence of DTT or SDS. Proteins were transferred to polyvinylidene difluoride (Immobilon P; Millipore, Bedford, MA) membranes in Tris/glycine/SDS buffer containing 20% methanol. Filters were blocked overnight at 4°C with TBS, pH 7.4, containing 5% BSA, 5% nonfat milk protein, and 0.1% Tween 20. Primary rabbit polyclonal anti-SAP Ab was preincubated with human IgG-coated agarose beads (Jackson ImmunoResearch Laboratories, West Grove, PA) to reduce the known reactivity of commercial anti-SAP Abs to Ig (29). Membranes were incubated with rabbit anti-SAP (1/1000 dilution) diluted in TBS, pH 7.4, containing 5% BSA, 5% nonfat milk protein, and 0.1% Tween 20 for 1 h at room temperature. Blots were washed in TBS containing 0.1% Tween 20 and further incubated for 1 h at room temperature with 100 pg/ml biotinylated goat anti-rabbit Ab (Southern Biotechnology) in TBS containing 5% BSA, 5% nonfat milk protein, and 0.1% Tween 20. ExtrAvidin-peroxidase (Sigma-Aldrich) diluted in TBS, pH 7.4, containing 5% BSA and 0.1% Tween 20 was used to identify biotinylated Ab, and chemiluminescence (ECL; Amersham Biosciences) was used to visualize the result, using Kodak BioMax MR film (Eastman Kodak, Rochester, NY).

Detection of SAP by Western blotting

Serum was diluted to 2% in 20 mM sodium phosphate buffer, pH 7.4, mixed with Laemmli's sample buffer containing 20 mM DTT, and heated to 100°C for 5 min. Samples were loaded onto 4–20% Tris/glycine polyacrylamide gels (Cambrex). After transfer to polyvinylidene difluoride membranes, samples were assessed by Western blotting, as described above. Commercial SAP preparations (Calbiochem) were used as standards on each membrane, with a range of 2–0.125 μg/ml.

Detection of SAP by ELISA

Maxisorb 96-well plates (Nalge Nunc International) were coated overnight at 4°C with anti-SAP mAb (SAP-5; Sigma-Aldrich) in 50 mM sodium carbonate buffer, pH 9.5. Plates were then incubated in TBS, pH 7.4 (TBS), containing 4% BSA (TBS-4% BSA) to inhibit nonspecific binding. Serum and purified proteins were diluted to 1/1000 in TBS-4% BSA, to prevent SAP from aggregating (15), and incubated for 60 min at 37°C. Plates were then washed in TBS containing 0.05% Tween 20. Polyclonal rabbit anti-SAP Ab (BioGenesis) diluted 1/5000 in TBS-4% BSA was used as the detecting Ab. After washing, 100 pg/ml biotinylated goat $(Fab')_2$ anti-rabbit (Southern Biotechnology) diluted in TBS-4% BSA was added for 60 min. Biotinylated Abs were detected by ExtrAvidin peroxidase (Sigma-Aldrich). Undiluted peroxidase substrate 3,3,5,5-tetramethylbenzidine (Sigma-Aldrich) was incubated for 5 min at room temperature before the reaction was stopped by 1 N HCl and read at 450 nm (BioTek Instruments, Winooska, VT). The assay was sensitive to 200 pg/ml.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software (GraphPad Software, San Diego, CA). Differences between two groups were assessed by Mann-Whitney or between multiple groups by ANOVA (Kruskal-Wallis) using Dunn's posttest. Significance was defined as $p < 0.05$.

Results

Serum and plasma inhibit the rapid differentiation of monocytes into fibroblast-like cells

While examining the possible role of cell density in the survival of peripheral blood T cells (30–32), we observed that in serum-free medium PBMC gave rise to a population of fibroblast-like cells. These cells were adherent and had a spindle-shaped morphology (Fig. 1*a*). Approximately 0.5–1% of PBMC differentiated into fibroblast-like cells in serum-free medium, and this occurred whether cultures were performed in tissue culture-treated plastic-

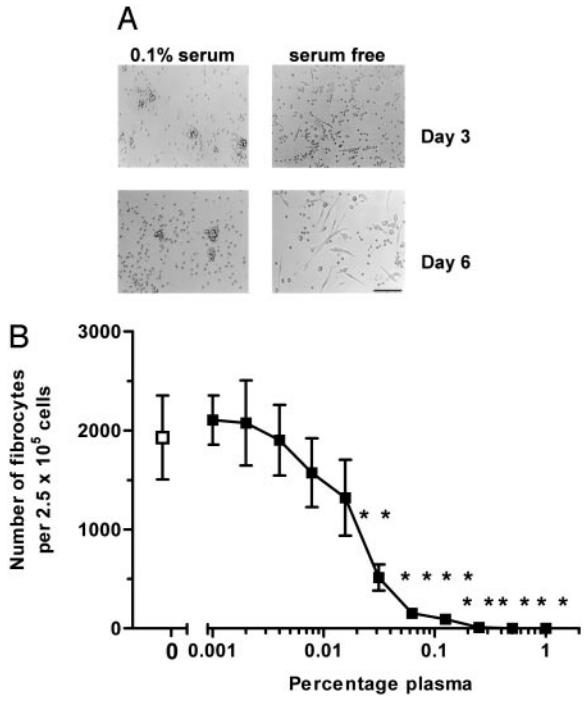

FIGURE 1. Serum and plasma inhibit the rapid differentiation of fibroblast-like cells. *A*, PBMC at 2.5×10^5/ml were cultured in serum-free medium for 3 or 6 days in the presence or absence of 0.1% human serum, and then examined by microscopy for the appearance of fibroblast-like cells. Bar is 100 μm. *B*, PBMC at 2.5×10^5/ml were cultured in flat-bottom 96-well plates in serum-free medium for 6 days in dilutions of human plasma. Cells were then air dried, fixed, and stained, and fibrocytes were enumerated by morphology. Results are expressed as mean ± SD of the number of fibrocytes per 2.5×10^5 PBMCs ($n = 7$ separate experiments). Plasma significantly inhibited fibrocyte differentiation above 0.031% ($p < 0.005$), as determined by a Mann-Whitney test.

ware, borosilicate, or on standard glass slides. Differentiation was also observed on both glass and plastic coated with BSA, fibronectin, or vitronectin. The rapid appearance of these cells, within 3 days of culture, was inhibited by human serum or plasma. To examine this process in more detail, we cultured PBMC in serum-free medium containing increasing concentrations of human plasma for 6 days. When plasma was present at concentrations between 10 and 0.1%, there was a significant reduction in the number of the fibroblast-like cells ($p < 0.005$) (Fig. 1*b*). However, at or below 0.01% plasma, fibroblast-like cells rapidly developed. The activity in the serum or plasma that inhibited fibrocyte formation was retained by a 30-kDa cutoff spin filter (data not shown). If serum was heated to 56°C for 30 min, the efficacy was reduced ~10-fold, and heating to 95°C abolished the inhibitory activity (data not shown). These data suggested that the inhibitory factor might be a protein. The inhibitory function appeared to be mediated by an evolutionary conserved factor, as bovine, caprine, equine, and rat sera were also able to inhibit the appearance of these fibroblast-like cells from human peripheral blood precursors (data not shown). Human and rat sera also inhibited the differentiation of fibroblast-like cells from rat PBMC (data not shown).

Characterization of fibroblast-like cells

The differentiation of fibroblast-like cells from peripheral blood leukocytes suggested that they may be the same as previously described peripheral blood fibrocytes (7, 8, 11). Fibrocytes are a population derived from peripheral blood monocytes that differentiate in vitro and in vivo into fibroblast-like cells. Their phenotype in-

Table I. *Expression of surface markers on fibrocytes*

Marker	Alternative Name[a]	Fibrocyte Expression	Marker	Alternative Name	Fibrocyte Expression
CD11a	LFA-1	Positive	CD1a		Negative
CD11b	Mac-1	Positive	CD3		Negative
CD11c		Positive	CD10		Negative
CD13		Positive	CD14		Negative
CD18	β_2 integrin	Positive	CD19		Negative
CD29	β_1 integrin	Positive	CD25		Negative
CD34		Positive	CD27		Negative
CD40		Weak positive	CD28		Negative
CD45	LCA	Positive	CD38		Negative
CD49a	α_1 integrin	Weak positive	CD49c	α_3 integrin	Negative
CD49b	α_2 integrin	Positive	CD49d	α_4 integrin	Negative
CD49e	α_5 integrin	Positive	CD49f	α_6 integrin	Negative
CD61	β_3 integrin	Positive	CD69		Negative
CD80	B7-1	Weak positive	CD70	CD27-L	Negative
CD86	B7-2	Positive	CD83		Negative
CD105	Endoglin	Positive	CD90		Negative
CD148		Positive	CD103	α_E integrin	Negative
MHC class II		Positive	CD109		Negative
CD162	PSGL-1	Positive	CD154	CD40-L	Negative
CCR1		Weak positive	$\alpha_4\beta_7$		Negative
CCR3		Weak positive	CLA		Negative
CCR4		Weak positive	CCR2		Negative
CCR5		Weak positive	CCR6		Negative
CCR7		Weak positive	CXCR2		Negative
CCR9		Weak positive	CXCR5		Negative
CXCR1		Positive	CXCR6		Negative
CXCR3		Positive			
CXCR4		Weak positive			
Collagen I		Positive			
Fibronectin		Positive			
α smooth muscle actin		Positive			
Vimentin		Positive			

[a] LCA, Leukocyte common Ag; PSGL, P-selectin glycoprotein ligand.

cludes hemopoietic markers such as CD45 and MHC class II and also stromal markers, such as collagen I and fibronectin. However, to delineate these cells in previous reports, PBMC were generally cultured for 1–2 wk in medium containing serum (7, 8, 11). To characterize whether the cells observed in our system were fibrocytes, PBMC were depleted of T cells with anti-CD3, B cells with anti-CD19, monocytes with anti-CD14, or all APCs with anti-HLA class II, and then cultured in serum-free conditions for 6 days. Depletion of PBMC with anti-CD3 or anti-CD19 did not eliminate fibroblast-like cells from PBMC when cultured in serum-free cultures (data not shown). However, depletion of APCs with anti-HLA class II or monocytes with anti-CD14 Ab did prevent the appearance of fibroblast-like cells, indicating that the fibroblast-like cells were derived from monocytes.

To further characterize the fibroblast-like cells, we cultured PBMC in serum-free medium for 5 days on glass slides. Cells were then air dried, fixed in acetone, and labeled with a variety of Abs (Table I and Fig. 2). Fibrocytes are known to express CD11a;

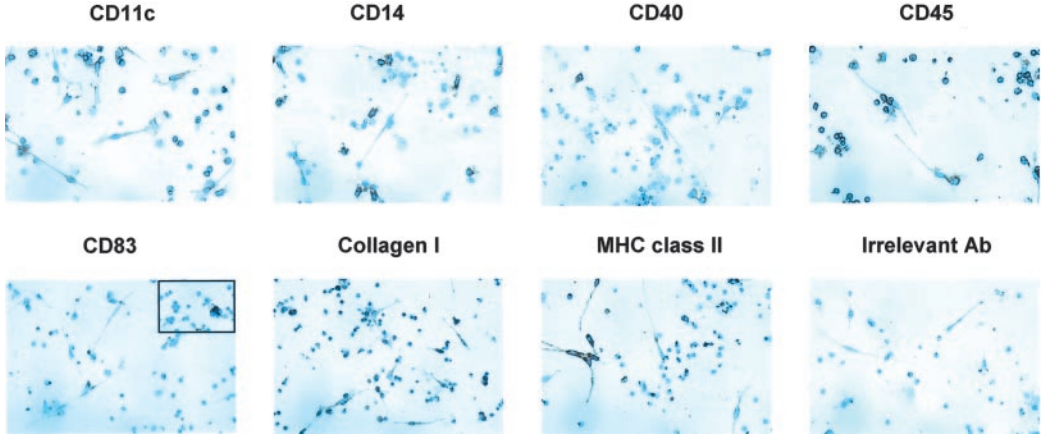

FIGURE 2. Expression of surface molecules on fibroblast-like cells. PBMC were cultured on glass slides in serum-free medium for 6 days. Cells were air dried and analyzed by immunohistochemistry. mAbs used are as indicated, and identified by biotin-conjugated goat anti-mouse Ig, followed by ExtrAvidin peroxidase. Cells were counterstained with Mayer's hematoxylin to identify nuclei. Positive staining was identified by brown staining; nuclei are counterstained blue. Insert for CD83 shows positive staining on a dendritic cell.

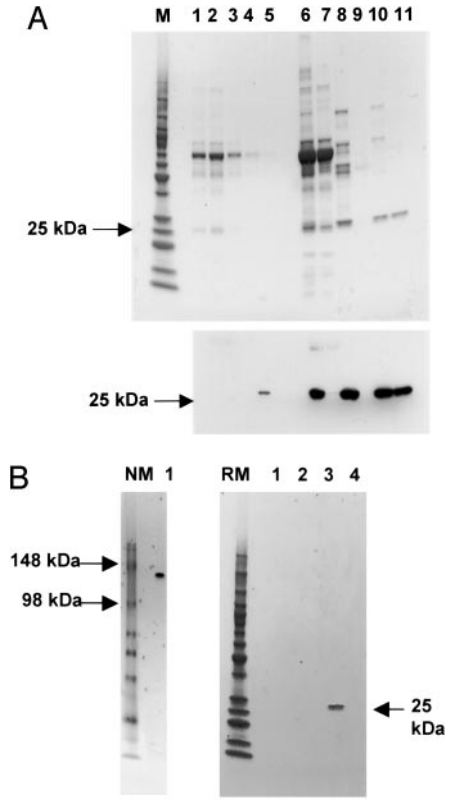

FIGURE 3. Characterization of the inhibitory molecule present in plasma that inhibits fibrocyte differentiation. Citrated plasma was treated with $BaCl_2$, and the precipitated material was collected by centrifugation and dialyzed against 20 mM sodium phosphate containing 10 mM EDTA and protease inhibitors. This material was then fractionated by heparin and ion exchange chromatography. *A*, Fractions were analyzed by PAGE on a 4–20% reducing gel. M, m.w. markers. *Lane 1*, plasma; *lane 2*, $BaCl_2$ supernatant; *lane 3*, wash 1; *lane 4*, wash 2; *lane 5*, $BaCl_2$ precipitate (*lanes 1–5*, diluted 1/500 in sodium phosphate buffer; *lanes 6–11*, undiluted); *lane 6*, $BaCl_2$ precipitate; *lane 7*, heparin flow through; *lane 8*, heparin fraction; *lane 9*, High Q flow through; *lane 10*, High Q fraction; *lane 11*, gel-purified fraction. *Upper panel* is a gel stained with Coomassie blue; *lower panel* is a duplicate gel assessed by Western blotting, using a rabbit anti-SAP Ab. *B*, Active fractions from the High Q ion exchange column and eluted from gel slices were analyzed by 4–20% PAGE on native or reducing gels. NM, native gel markers; *lane 1*, active fraction. RM, reduced gel markers; *lanes 1–3*, control gel slice samples, *lane 4*, active fraction.

CD11b; CD45; CD80; CD86; MHC class II; collagen I; fibronectin; the chemokine receptors CCR3, CCR5, CCR7, and CXCR4; and α-smooth muscle actin (7–11). Our fibroblast-like cells expressed all these markers. Fibrocytes are negative for CD1a, CD3, CD19, and CD38, as were our fibroblast-like cells. Based on these data, we concluded that the fibroblast-like cells observed in these experiments were fibrocytes. We were also able to extend this phenotype. Fibrocytes expressed several β_1 integrins, including α_1 (CD49a), α_2 (CD49b), α_5 (CD49e), β_1 (CD29), and β_3 (CD61), along with high levels of β_2 (CD18), but were negative for α_3, α_4, α_6, $\alpha_4\beta_7$, α_E, and cutaneous lymphocyte-associated Ag (Fig. 2 and Table I).

Characterization of the fibrocyte-inhibitory factor

Initial characterization of the serum factor that inhibits fibrocyte differentiation indicated that it was a heparin-binding molecule that eluted from an ion exchange column (High Q) as one of four proteins. By sequencing tryptic fragments of protein in a band cut from a native gel, we were able to identify one of these proteins as C4BP. C4BP is a 570-kDa protein, composed of seven α-chains (70 kDa) and usually a single β-chain (40 kDa), which is involved in regulating the decay of the C4b and C2a components of the complement system (33). C4BP also interacts with the vitamin K-dependent anticoagulant protein S and SAP (27, 34). The C4BP/protein S complex can be purified from serum or plasma using $BaCl_2$ precipitation (27).

To assess whether C4BP, or an associated protein, was the factor responsible for inhibiting fibrocyte differentiation, citrated plasma was treated with $BaCl_2$. The inhibitory factor was present in the $BaCl_2$ precipitate (Fig. 3*a* and Table II). This fraction was applied to a heparin column, and the fractions, eluted by increasing concentrations of NaCl, were assessed for their ability to inhibit monocyte to fibrocyte differentiation in serum-free medium. The active factor was eluted from the heparin column in a peak at 200 mM NaCl (Fig. 3 and Table II). The fractions from the 200 mM peak were pooled and further fractionated by High Q ion exchange chromatography. A small peak eluting at 300 mM NaCl contained activity that inhibited fibrocyte differentiation. Analysis of the proteins present in this fraction showed a major band at 27 kDa and minor bands at 80 and 130 kDa (Fig. 3*a*). The high Q fraction was concentrated, fractionated by electrophoresis on a nondenaturing polyacrylamide gel, followed by elution of the material in the gel slices. A single band that migrated at ~140 kDa was able to inhibit differentiation (Fig. 3*b*). This band had a molecular mass of 27 kDa on a reducing polyacrylamide gel, suggesting that the native conformation of the protein was a pentamer (Fig. 3*b*). This band was excised from the gel, digested with trypsin, and analyzed by matrix-assisted laser desorption ionization mass spectrometry. Three major and two minor peptides were identified: VFVFPR, VGEYSLYIGR, AYSLFSYNTQGR, QGYFVEAQPK, and IVLGQEQDSYGGK. These sequences exactly matched amino acid sequences 8–13, 68–77, 46–57, 121–130, and 131–143 of SAP.

To confirm that the active fractions contained SAP, a Western blot of fractions collected from column chromatography was analyzed with anti-SAP Abs (Fig. 3*a*, *lower panel*). The presence of SAP at 27 kDa was detected in all fractions that inhibited fibrocyte differentiation (Fig. 3*a*, *lanes 5*, *6*, *8*, *10*, and *11*). A considerable

Table II. *Recovery of protein and fibrocyte-inhibitory activity from fractionated human plasma*

		Protein			Activity			
	Volume (ml)	Protein (mg/ml)	Total protein (mg)	Yield (%)	Activity (U/ml)	Total activity (U)	Yield (%)	Specific activity (U/mg)
Plasma	250	70	17,500	100	10,000	2.5×10^6	100	143
$BaCl_2$ supernatant	240	60	14,400	82.3	6,666	1.6×10^6	64	111
$BaCl_2$ precipitate	31	1	31	0.18	1,666	5.1×10^4	2	1,600
Heparin fraction	4.3	0.25	1.075	0.006	500	2,150	0.086	2,000
High Q fraction	1.96	0.05	0.098	0.00056	400	720	0.029	7,300
Gel slice	0.075	0.025	0.0018	0.00001	2,000	150	0.006	80,000

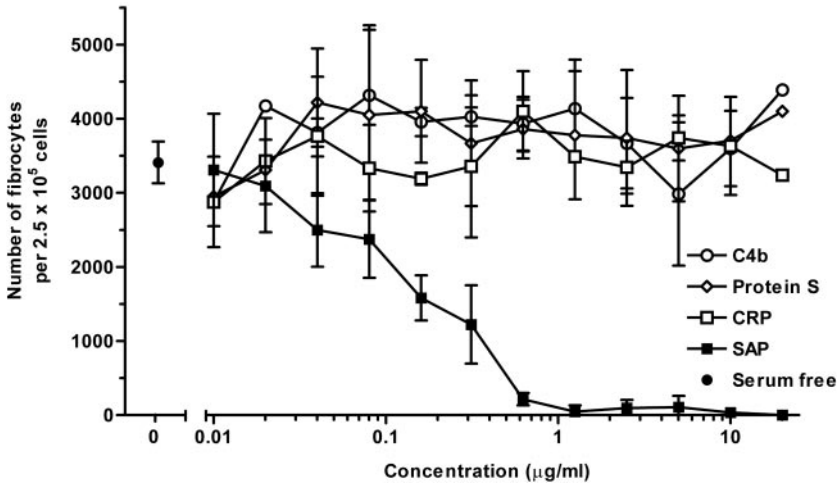

FIGURE 4. Inhibition of fibrocyte differentiation by SAP, but not CRP or other plasma proteins. PBMC at 2.5×10^5/ml were cultured in serum-free medium for 6 days in the presence of commercially available purified SAP, CRP, protein S, or C4b, and then examined for the appearance of fibroblast-like cells. Cells were then air dried, fixed, and stained, and fibrocytes were enumerated by morphology. Results are mean ± SD of fibrocytes per 2.5×10^5 PBMC ($n = 3$ separate experiments). Compared with the other proteins, SAP at concentrations greater than 1 μg/ml significantly inhibited fibrocyte differentiation ($p < 0.001$), as determined by ANOVA.

amount of fibrocyte-inhibitory activity was present in the supernatant from the $BaCl_2$ precipitation step, indicating that this procedure was inefficient, with the recovery of only ~2% of the fibrocyte-inhibitory activity in the $BaCl_2$ pellet (Table II). Fractions were also analyzed for the presence of CRP, C4BP, and protein S. Western blotting indicated that C4BP and protein S were present in plasma and the barium precipitation, but were absent from the active fractions collected from heparin chromatography (data not shown). There was no reactivity with CRP, as noninflammatory plasma was used.

Specificity of SAP

SAP is a constitutive plasma protein and is closely related to CRP, the major acute-phase protein in humans. To assess whether other plasma proteins could also inhibit the differentiation of fibrocytes, we cultured PBMC in serum-free medium in the presence of commercially available purified SAP, CRP, C4b, or protein S. The commercially obtained SAP was purified using calcium-dependent affinity chromatography (35). Of the proteins tested, only SAP was able to inhibit fibrocyte differentiation, with essentially complete inhibitory activity at 1 μg/ml ($p < 0.001$) (Fig. 4). A dilution curve indicated that the commercially available SAP has a fibrocyte-inhibitory activity of ~6.6×10^3 U/mg (Fig. 4).

To determine whether removal of SAP from plasma and serum would lead to increased fibrocyte differentiation, SAP was depleted from plasma using agarose beads (Bio-Gel A) or protein G beads coated with anti-SAP Abs. Compared with untreated plasma, the agarose bead-treated plasma was significantly less able to inhibit fibrocyte differentiation at intermediate concentrations of plasma ($p < 0.05$) (Fig. 5*a*). To confirm that the Bio-Gel A beads depleted SAP, beads were washed and the material was eluted off with 10 mM Tris, pH 8, 150 mM NaCl, and 5 mM EDTA. Protein gels indicated that a 27-kDa band was the major protein binding to the beads, and Western blotting indicated that this band corresponded to SAP (data not shown). Plasma was also depleted of SAP using protein G beads coated with anti-SAP Abs. Removal of SAP led to a significant reduction in the ability of plasma to inhibit fibrocyte differentiation compared with plasma, or plasma treated with beads coated with control Abs ($p < 0.05$) (Fig. 5*b*). The beads coated with control Abs did remove some of the fibrocyte-inhibitory activity from plasma, but this was not significantly different from cells cultured with plasma. This probably reflects SAP binding to the agarose in the protein G beads. These data, together with the ability of purified SAP to inhibit fibrocyte differentiation,

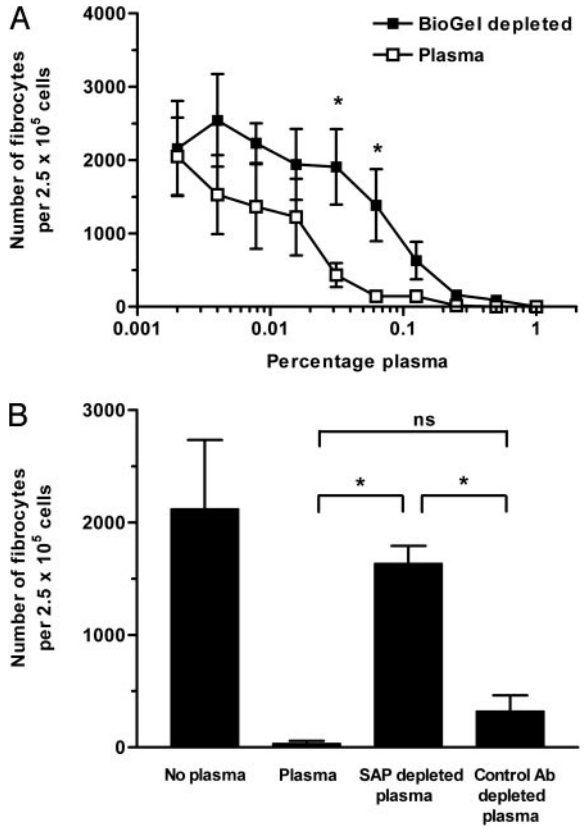

FIGURE 5. Depletion of SAP from plasma reduces the inhibition of fibrocyte differentiation. *A*, SAP was depleted from plasma using Bio-Gel A agarose beads. Control plasma was diluted in 10 mM Tris, pH 8, 150 mM NaCl, and 5 mM $CaCl_2$ buffer alone. PBMC at 2.5×10^5/ml were cultured in serum-free medium for 6 days in the presence of dilutions of plasma. Cells were then air dried, fixed, and stained, and fibrocytes were enumerated by morphology. Results are mean ± SD of fibrocytes per 2.5×10^5 PBMC ($n = 3$ separate experiments). At two dilutions of plasma, 0.3 and 0.6%, the depletion had a significant effect on fibrocyte differentiation ($p < 0.05$). *B*, Anti-SAP Ab-coated protein G beads were used to deplete plasma. Twenty percent plasma in sodium phosphate buffer was incubated with protein G coupled to anti-SAP or rabbit IgG control Ab. After depletion, plasma was diluted to 0.25% and cultured with PBMC at 2.5×10^5/ml for 6 days. Fibrocytes were enumerated by morphology. Results are mean ± SD of fibrocytes per 2.5×10^5 PBMC ($n = 4$ separate experiments). Depletion of SAP from plasma significantly impaired the inhibition of fibrocyte differentiation ($p < 0.05$), as determined by Mann-Whitney test. ns, Indicates a statistically insignificant difference.

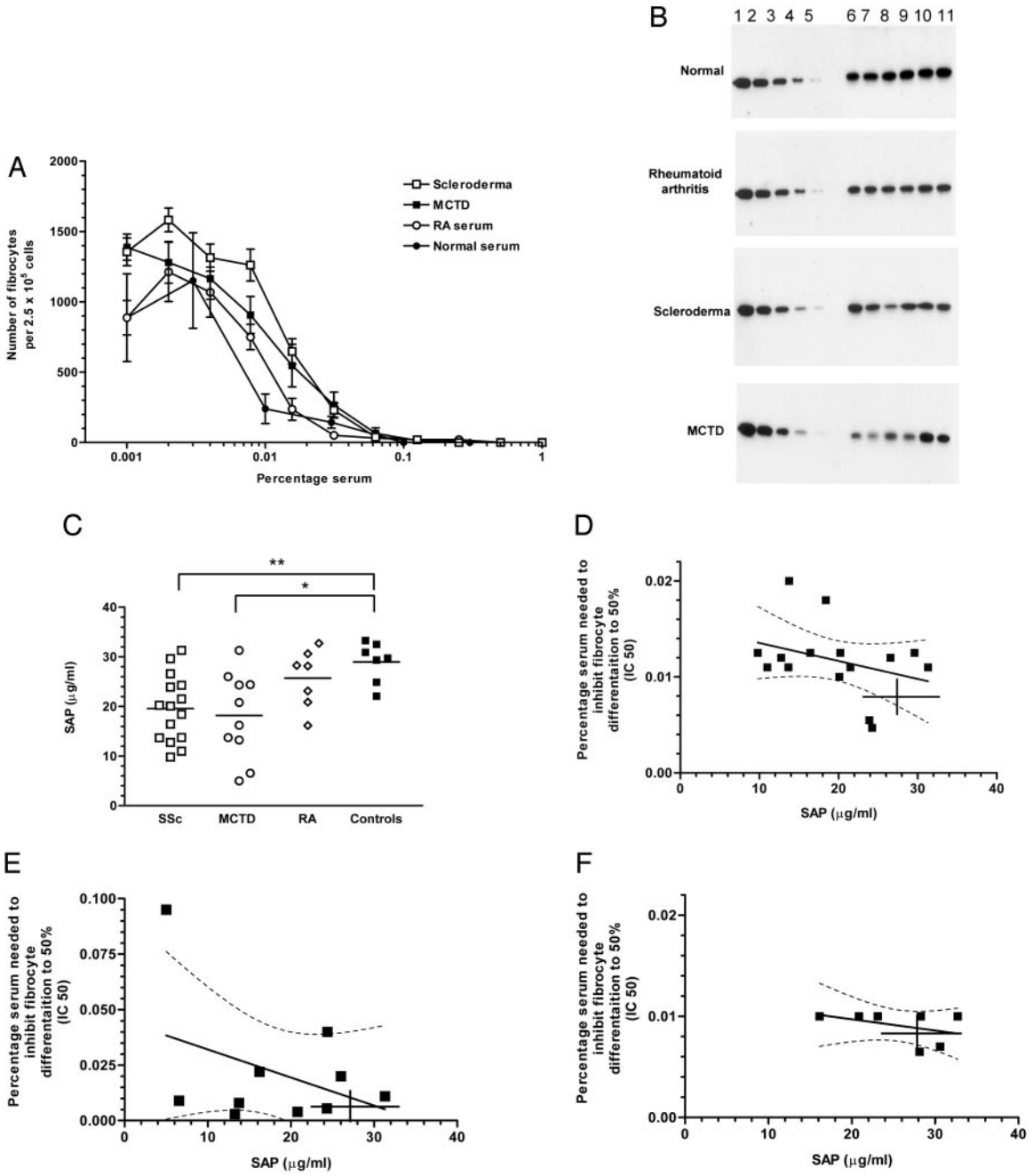

FIGURE 6. Sera from scleroderma and MCTD patients have a reduced ability to inhibit fibrocyte differentiation and a corresponding low level of SAP. *A*, PBMC at 2.5×10^5/ml were cultured in serum-free medium for 6 days in the presence of dilutions of sera from scleroderma, MCTD, RA patients, and normal controls. Cells were then air dried, fixed, and stained, and fibrocytes were enumerated by morphology. Results are mean ± SD of fibrocytes per 2.5×10^5 PBMC. Sera were from patients with RA ($n = 7$ patients), systemic sclerosis ($n = 15$ patients), MCTD ($n = 10$ patients), or from normal controls ($n = 7$). *B*, Commercial SAP preparations were used as standards at 2, 1, 0.5, 0.25, and 0.125 μg/ml. SAP standards (*lanes 1–5*) and six randomly selected normal or patient serum samples (*lanes 6–11*) were analyzed by PAGE on a 4–20% reducing gel. Western blots of the gels were then stained with anti-SAP Abs. *C*, Concentration of SAP in serum samples was assessed by ELISA. Bar indicates the mean value. There were significantly lower levels of SAP in patients with SSc ($p < 0.01$) and MCTD ($p < 0.05$) compared with normal sera, as determined by ANOVA. There was no significant difference in the SAP levels between RA patients and normal sera. *D*, Correlation of SAP concentration and IC_{50} inhibitory activity in sera from SSc, MCTD, and RA patients, respectively. The solid lines in *D*, *E* and *F* represent the regression line with the dotted line the 95% confidence limits. Crosshairs represent the range (maximum and minimum) of SAP and IC_{50} values observed in the controls.

strongly suggest that SAP is the active factor in serum and plasma that inhibits fibrocyte differentiation.

Correlation of SAP levels in patient sera with the ability to inhibit fibrocyte differentiation

Although blood levels of SAP are stable in humans, there are small variations in SAP levels in disease states (36). These levels are likely to be important, as small changes in the concentration of SAP can have a large effect on fibrocyte differentiation (Fig. 4). To test the hypothesis that the circulating levels of SAP may inversely correlate with the presence of fibrocytes, or fibrosis in disease states, we tested the ability of sera from patients with scleroderma/systemic sclerosis (SSc), MCTD, and RA, to inhibit fibrocyte differentiation (Fig. 6*a*). The ability of sera from RA patients to in-

hibit fibrocyte differentiation was similar to healthy controls, with both sera inhibiting fibrocyte differentiation to a dilution of 0.03%. The average half-maximal activity of normal sera was at 0.0075%, whereas the average half-maximal activity of rheumatoid sera was at 0.012%. SSc sera were ~2.7-fold less active than normal sera, with an average half-maximal activity at 0.021%. The fibrocyte-inhibitory activity of normal sera was thus ~13,000 U/ml, while the activity of scleroderma sera was ~4,800 U/ml. Sera from MCTD patients also had a poor ability to inhibit fibrocyte differentiation, with an average half-maximal activity at 0.02%. Together, these data suggest that sera from patients with different fibrosing diseases have a reduced capacity to prevent fibrocyte differentiation.

To determine whether the differences observed in the inhibition of fibrocyte differentiation were due to alterations in the size or composition of SAP, serum was analyzed by staining Western blots with anti-SAP Abs (Fig. 6*b*). All the serum samples showed a band at the same m.w. as the commercially purified SAP, indicating that the observed differences in the ability of the patients' sera to inhibit fibrocyte differentiation was not due to large changes in the molecular mass of the SAP protein. Some of the sera from the SSc and MCTD patients appeared to have lower levels of SAP. We next measured the concentration of SAP in patient and normal sera by ELISA, to test whether the differences observed in the bioassays were due to variations in the circulating levels of SAP. The normal (mean ± SD, 28.95 ± 4.08 μg/ml) and RA (25.68 ± 5.87 μg/ml) sera all had approximately the same concentration of SAP (Fig. 6*c*). Sera from patients with SSc (19.54 ± 6.69 μg/ml) or MCTD (18.15 ± 8.65 μg/ml) had significantly lower levels of SAP. Together, these data suggest that SSc and MCTD patients as a group have lower levels of fibrocyte-inhibitory activity and SAP in their sera. We next assessed whether the levels of SAP in individuals correlated with the ability to inhibit fibrocyte differentiation. Sera with a high concentration of SAP tended to have a corresponding low IC_{50}, indicating an increased ability to inhibit fibrocyte differentiation (Fig. 6, *d–f*). These findings suggest that the circulating concentration of SAP may well contribute to the bodies' ability to regulate fibrocyte differentiation.

Discussion

During the resolution phase of an immune response, repair of tissue damage and replacement of the ECM involve fibroblasts. Whether these are locally derived fibroblasts or are from a circulating precursor population is still controversial (1, 6). Fibrocytes are a peripheral blood monocyte-derived cell population that appear at wound sites within 3 days (7, 8). Fibrocytes have a variety of distinct functions. They are capable of presenting Ag to T cells, secreting angiogenic factor to promote new blood vessel growth, and expressing myofibroblast proteins important in contracting collagen for wound closure (7, 10, 11). These functions suggest that fibrocytes may be present early in the inflammatory response to present Ag and yet are retained at the same site for the resolution of an inflammatory lesion.

Fibrocytes also differentiate in vitro after 1–2 wk in culture in the presence of serum (7, 11). We have found that in serum-free culture conditions, fibrocyte differentiation occurred within 3 days. The addition of serum or plasma inhibited this process, although fibrocytes did eventually appear after ~10–14 days. We were able to purify and identify the protein responsible for the inhibition of fibrocyte differentiation as SAP. We hypothesize that SAP present in the circulation and in the peripheral tissues regulates the differentiation of fibrocytes, and that relatively small changes in the circulating levels of SAP are likely to have an important role in fibrosis.

The commercial preparation of SAP had 6.6×10^3 U/mg of fibrocyte-inhibiting activity, while our preparation had 8.0×10^4 U/mg of activity. This suggests that our preparation was either more pure or that the commercial preparation contained some amount of inactive SAP. The dilution curves of purified SAP indicate that a small reduction in the concentration of SAP leads to a significant change in the number of differentiating fibrocytes. A reduction of SAP from 0.6 to 0.156 μg/ml SAP leads to a 10-fold increase in fibrocyte numbers. This suggests that SAP regulates fibrocyte differentiation with a threshold cutoff that is very narrow. Therefore, even small reductions in SAP levels in the body may have profound affects on the inhibition of fibrocyte development and may be necessary to regulate differentiation in peripheral tissues.

SAP is a member of the pentraxin family of proteins, which also contains the classic acute-phase protein CRP (13, 14). SAP and CRP are secreted by the liver and circulate in the plasma as pentamers. SAP and CRP are 51% identical and 66% similar at the protein level. The exact role of these molecules is still unclear, although they appear to play a role in the initiation of immune responses and clearance of apoptotic cells (16, 17, 37). CRP binds preferentially to small nuclear ribonucleoprotein particles, whereas SAP binds free DNA and chromatin (38, 39). They both augment phagocytosis and bind to FcγRs on a variety of cells. CRP binds with a high affinity to FcγRII, a lower affinity to FcγRI, but does not bind FcγRIII (40, 41). SAP binds to all three classical FcγRs, with a preference for FcγRI and FcγRII (42). CRP is the archetypal acute-phase response protein in most mammals, except mice, with increases of up to 1000-fold after infection. However, SAP levels remain relatively constant, with only small changes in disease states (36). The subtle differences in the biology of SAP and CRP suggest that they perform discrete functions in vivo, with likely candidates for their differences being the preference of FcγRs and the differences in specificity for apoptotic material. Our observation that, in contrast to SAP, CRP was incapable of inhibiting fibrocyte differentiation, suggests that this process may be mediated through differential FcγR signaling or receptor expression on a subset of fibrocyte precursors.

The ability of SAP to inhibit fibrocyte differentiation suggests that its presence in peripheral tissues would be a means of regulating monocyte to fibrocyte differentiation. In peripheral tissues, SAP is associated with several ECM proteins, such as elastin in the skin and collagen and laminin in the glomerular basement membrane of the kidney (43–48). The observation that fibrocytes are evident in experimental wound chambers within days of induction suggests that fibrocytes differentiate quickly after wounding (7, 8). This suggests that in a wound SAP is likely to be either absent or rendered incapable of inhibiting monocyte-fibrocyte differentiation. Following skin remodeling, the underlying ECM protein composition is altered, which is also likely to affect SAP localization.

Dysregulation of fibrocyte biology has also been postulated to be a contributing factor in fibrosing diseases, such as scleroderma and RA (9, 12). Our observation that the sera of MCTD and scleroderma patients are less able to inhibit monocyte to fibrocyte differentiation adds weight to this argument. In RA patients, the serum levels of SAP were similar to the levels in healthy individuals, as has been described previously (49), suggesting that a simple primary defect of SAP secretion is unlikely to be the initiating event in this disease. In RA, the fibrotic process is restricted to the synovial compartment and peripheral lesions such as rheumatoid nodules (50). There are lower levels of SAP in the tissues compared with the circulation, which are consistent with a role for SAP in local inhibition of fibrocyte differentiation (51, 52). This suggests that the combination of reduced SAP levels in the blood of scleroderma patients along with the limited diffusion of SAP from

blood to tissue could lead to a tissue SAP concentration that is below the threshold level to prevent fibrocyte differentiation and thus result in the formation of fibroses. The elevated female to male ratio of patients in many fibrosing diseases also correlates with the reduced level of SAP found in women compared with men. This suggests the possibility that SAP may represent a useful therapy for some patients with fibrosing diseases. Conversely, if localized fibrocyte differentiation is impaired in wound healing, an interesting possibility is that a localized depletion or inactivation of SAP from a wound site might enhance the local differentiation of fibrocytes and thus enhance wound healing.

Chronic inflammation is associated with two major problems, fibrosis and amyloidosis. Fibrosis associated with keloid scarring, synovitis in RA, and collagen deposition and excessive fibroblast proliferation in scleroderma is a major clinical issue. In certain individuals, chronic inflammation also leads to amyloidosis with the deposition of amyloid at organs and tissues that may subsequently lead to organ failure (53). Data presented in this work suggest that these two conditions may be opposing and mutually exclusive conditions, associated with chronic inflammation. In environments in which amyloidosis is promoted, such as occurs after Ig deposition, protein aggregation, and amyloid fiber formation, the appearance of fibrocytes will be inhibited due to the excess amounts of SAP. This may then prevent the appearance of cells capable of secreting proteases necessary for ECM protein degradation and remodeling. Alternatively, in situations in which SAP levels are low, biologically inactive, or absent, such as in the rheumatoid joint or in scar tissue, uncontrolled differentiation of fibrocytes and the unregulated deposition of ECM proteins will occur. This is observed clinically in RA, in which amyloidosis is particularly a renal, but not synovial complication (53).

We have previously proposed that stromal cells are active participants in immune responses, secreting factors such as IFN-β, TGF-β, and stromal cell-derived factor-1 to promote T cell survival and retention at sites of inflammation (4, 54, 55). We can extend this hypothesis to include the regulation of fibrosis by SAP, a systemic protein involved in the elimination of bacteria and apoptotic cells. At the resolution of an inflammatory response, the local effective levels of SAP may be reduced due to the binding to DNA and chromatin from dead or damaged cells. This would then allow peripheral blood monocytes to differentiate into fibrocytes that would aid tissue regeneration. A positive feedback loop may develop in which fibrocytes would provide survival and retention signals for lymphocytes, which in turn would further stimulate the fibrocytes. Clearly, mechanisms that could inhibit this process would offer profound clinical advantages for the treatment of chronic inflammation and fibrosis, and the activation of this pathway may aid the resolution of ulcers and other nonhealing wounds.

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