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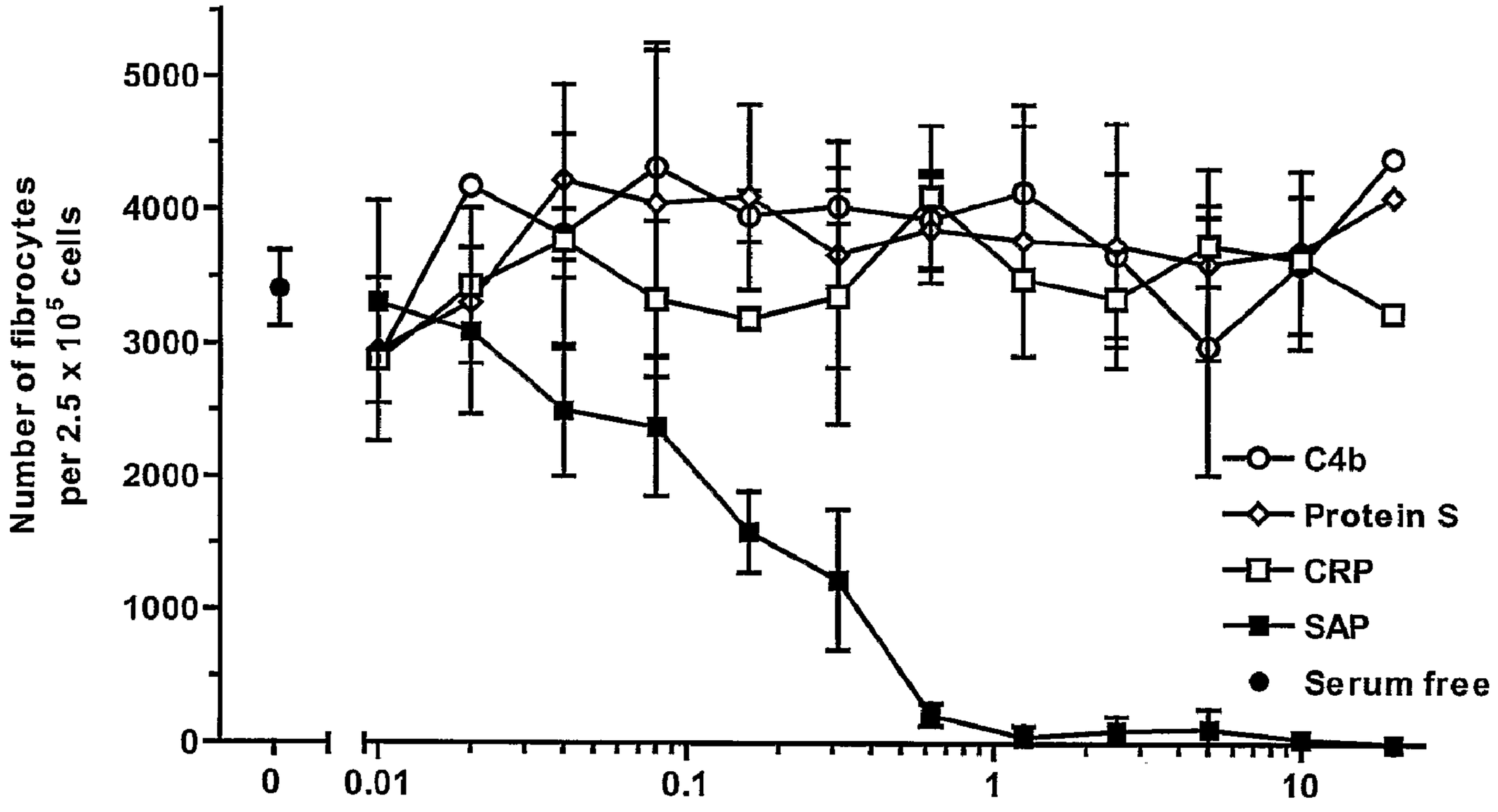
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(54) Titre : UTILISATION D'UN COMPOSE AMYLOIDE P SERIQUE ET DE COMPOSITIONS CONNEXES DANS LA SUPPRESSION DE MALADIES FIBREUSES

(54) Title: USE OF SERUM AMYLOID P (SAP) AND RELATED COMPOSITIONS FOR THE SUPPRESSION OF FIBROSIS-RELATED DISEASES




(57) Abrégé/Abstract:
The present invention relates to the ability of Serum Amyloid P (SAP) protein to suppress the differentiation of monocytes into fibrocytes. It also relates to the ability of IL-12, laminin-1, crosslinked IgG and IgG aggregates to suppress the differentiation of monocytes into fibrocytes. Methods and compositions for suppressing differentiation of monocytes into fibrocytes using these

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proteins are provided. These methods are useful in a variety of applications including treatment and prevention of fibrosing diseases such as scleroderma and asthma. Finally, the invention includes assays for detecting the ability of various agents to modulate monocyte differentiation into fibrocytes and to detect monocyte defects. Such assays may also be used to diagnose scleroderma or other fibrosing diseases.

ABSTRACT

The present invention relates to the ability of Serum Amyloid P (SAP) protein to suppress the differentiation of monocytes into fibrocytes. It also relates to the ability of IL-12, laminin-1, cross-linked IgG and IgG aggregates to suppress the differentiation of monocytes into fibrocytes. Methods and compositions for suppressing differentiation of monocytes into fibrocytes using these proteins are provided. These methods are useful in a variety of applications including treatment and prevention of fibrosing diseases such as scleroderma and asthma. Finally, the invention includes assays for detecting the ability of various agents to modulate monocyte differentiation into fibrocytes and to detect monocyte defects. Such assays may also be used to diagnose scleroderma or other fibrosing diseases.

# USE OF SERUM AMYLOID P (SAP) AND RELATED COMPOSITIONS FOR THE SUPPRESSION OF FIBROSIS-RELATED DISEASES

## FIELD OF THE INVENTION

The present invention relates to the ability of SAP to suppress differentiation of monocytes into fibrocytes. Accordingly, it includes compositions and methods for suppressing such differentiation. These compositions and methods may be useful in a variety of applications in which decreased fibrocyte formation is beneficial, such as treatment of fibrosing diseases and asthma. The invention additionally includes methods for detecting problems in the ability of monocytes to differentiate into fibrocytes or for SAP to inhibit this differentiation. These problems may be correlated with a disease or may be drug-induced.

## BACKGROUND

*Fibrocytes*

Inflammation is the coordinated response to tissue injury or infection. The initiating events are mediated by local release of chemotactic factors, platelet activation, and initiations of the coagulation and complement pathways. These events stimulate the local endothelium, promoting the extravasation of neutrophils and monocytes. The second phase of inflammation is characterized by the influx into the tissue of cells of the adaptive immune system, including lymphocytes. The subsequent resolution phase, when apoptosis of the excess leukocytes and engulfment by tissue macrophages takes

place, is also characterized by repair of tissue damage by stromal cells, such as fibroblasts.

In chronic inflammation, the resolution of inflammatory lesions is disordered, with the maintenance of inflammatory cells, fibroblast hyperplasia, and eventual tissue destruction. The mechanisms that lead to these events are complex, but include enhanced recruitment, survival and retention of calls and impaired emigration.

The source of fibroblasts responsible for repair of wound lesions or in other fibrotic responses is controversial. The conventional hypothesis suggests that local quiescent fibroblasts migrate into the affected area, produce extracellular matrix proteins, and promote wound contraction or fibrosis. An alternative hypothesis is that circulating fibroblast precursors (called fibrocytes) present within the blood migrate to the sites of injury or fibrosis, where they differentiate and mediate tissue repair and other fibrotic responses.

Fibrocytes are known to differentiate from a CD14+ peripheral blood monocyte precursor population. Fibrocytes express markers of both hematopoietic cells (CD45, MHC class II, CD34) and stromal cells (collagen types I and III and fibronectin). Mature fibrocytes rapidly enter sites of tissue injury where they secrete inflammatory cytokines. Fibrocytes are also capable of secreting extracellular matrix proteins, other cytokines and pro-angiogenic molecules, which may result in fibrosis.

The mechanisms that inhibit fibrocyte differentiation from CD14+ monocytes were largely unknown prior to the present invention. However, control of

fibrocyte differentiation is likely to be important in the control of many diseases and processes. Fibrocytes are associated with a variety of processes and diseases including scleroderma, keloid scarring, rheumatoid arthritis, lupus, nephrogenic fibrosing dermopathy, and idiopathic pulmonary fibrosis. They play a role in the formation of fibrotic lesions after *Schistosoma japonicum* infection in mice and are also implicated in fibrosis associated with autoimmune diseases. Fibrocytes have also been implicated in pathogenic fibrosis associated with radiation damage, Lyme disease and pulmonary fibrosis. CD34+ fibrocytes have also been associated with stromal remodeling in pancreatitis and stromal fibrosis, whereas lack of such fibrocytes is associated with pancreatic tumors and adenocarcinomas. Fibrosis additionally occurs in asthma patients and possibly other pulmonary diseases such as chronic obstructive pulmonary disease when fibrocytes undergo further differentiation into myofibroblasts.

In accordance with one aspect of the present invention there is provided use of a Serum Amyloid P (SAP) protein in an amount sufficient to suppress fibrosis in a mammal having a fibrosing disease.

In accordance with another aspect of the present invention there is provided use of a Serum Amyloid P (SAP) protein, in an amount sufficient to suppress differentiation of monocytes into fibrocytes in a patient having a fibrosis disease or condition.

In accordance with yet another aspect of the present invention there is provided use of a Serum Amyloid P (SAP) protein in an amount sufficient to suppress fibrosis in the patient after surgery.

In accordance with still yet another aspect of the present invention there is provided use of Serum Amyloid P (SAP) protein, or a portion thereof, said portion

comprising one or more sequences selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:3, to suppress pulmonary fibrosis in a mammal suffering from pulmonary fibrosis, wherein the SAP or portion thereof binds to Fcγ receptors and inhibits fibrocyte differentiation from monocytes.

In accordance with still yet another aspect of the present invention there is provided use of Serum Amyloid P (SAP) protein or a portion thereof, said portion comprising one or more sequences selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:3, in an amount and for a time sufficient to suppress differentiation of monocytes into fibrocytes in the kidney thereby to treat a fibrotic or fibroproliferative disorder of the kidney in a patient.

*Serum Amyloid P*

Given the wide range of processes and diseases in which fibrocytes play a role, the mechanisms that regulate their differentiation from peripheral blood monocytes are of great interest. Aspects of the present invention are based upon the discovery that differentiation of peripheral blood fibrocytes from monocytes is suppressed by a factor in serum and plasma. Fractionation of human plasma identified this factor as serum amyloid p (SAP). SAP, a member of the pentraxin family of proteins that include C-reactive protein (CRP), is secreted by the liver and circulates in the blood as

stable pentamers. The exact role of SAP is still unclear, although it appears to play a role in both the initiation and resolution phases of the immune response. SAP binds to sugar residues on the surface of bacteria leading to their opsonisation and engulfment. SAP also binds to free DNA and chromatin generated by apoptotic cells at the resolution of an immune response, thus preventing a secondary inflammatory response. Molecules bound by SAP are removed from extracellular areas due to the ability of SAP to bind to all three classical Fcγ receptors (FcγR), with a preference for FcγRI (CD64) and FcγRII (CD32). After receptor binding, SAP and any attached molecule are likely engulfed by the cell.

FcγR are necessary for the binding of IgG to a wide variety of hematopoietic cells. Peripheral blood monocytes express both CD64 and CD32 (a subpopulation of monocytes express CD16), whereas tissue macrophages express all three classical FcγR. Clustering of FcγR on monocytes by IgG, either bound to pathogens or as part of an immune complex, initiates a wide variety of biochemical events. The initial events following receptor aggregation include the activation of a series of src kinase proteins. In monocytes, these include lyn, hck and fgr, which phosphorylate tyrosine residues on the ITAM motif of the FcR-γ chain associated with FcγRI and FcγRIII, or the ITAM motif with the cytoplasmic domain of FcγRII. Phosphorylated ITAMs lead to the binding of a second set of src kinases, including syk. Syk has been shown to be vital for phagocytosis of IgG-coated particles. However, the wide distribution of syk in non-hematopoietic cells and the evidence that syk is involved in both integrin and G-protein coupled receptor

signaling, indicates that this molecule has many functions.

Both SAP and CRP augment phagocytosis and bind to Fcγ receptors on a variety of cells. CRP binds with a high affinity to FcγRII (CD32), a lower affinity to FcγRI (CD64), but does not bind FcγRIII (CD16). SAP binds to all three classical Fcγ receptors, with a preference for FcγRI and FcγRII, particularly FCγRI. Although there are conflicting observations on the binding of CRP to FcγR, both SAP and CRP have been shown to bind to Fc receptors and initiate intracellular signaling events consistent with FcγR ligation.

In human blood serum, males normally have approximately 32 μg/ml +/- 7 μg/ml of SAP, with a range of 12-50 μg/ml being normal. Human females generally have approximately 24 μg/ml +/- 8 μg/ml of SAP in blood serum, with a range of 8-55 μg/ml being normal. In human cerebral spinal fluid there is normally approximately 12.8 ng/ml SAP in human males and approximately 8.5 ng/ml in females. Combining male and female data, the normal SAP level in human serum is 26 μg/ml +/- 8 μg/ml with a range of 12-55 μg/ml being normal. (The above serum levels are expressed as mean +/- standard deviation.)

*IL-12*

Other aspects of the present invention relate to the newly discovered ability of IL-12 to suppress fibrocyte formation by monocytes. IL-12 has been previously implicated in fibrosis and fibrosing diseases, but most studies have focused on the role of IL-12 in promoting the Th1 immune response or by triggering the production

of interferon-γ. The direct effects of IL-12 on fibrocyte formation do not appear to have been previously recognized.

*Laminin-1*

Still other aspects of the present invention relate to the newly discovered ability of laminin-1 to inhibit monocyte differentiation into fibrocytes. Laminins are extracellular matrix proteins involved in movement of monocytes from the circulation into tissues.

In order for leukocytes to enter tissues, they must cross through endothelial cells and the surrounding basement membrane of blood vessel wall. This process involves the tethering, rolling and stopping of the leukocytes on the endothelial cells. Following adhesion to the endothelial cells, leukocytes then cross between the endothelial cells, through the blood vessel wall and into the tissues. The process of extravasation of cells through blood vessel walls alters their phenotype and function.

These events are controlled by a series of cell surface adhesion receptors, including integrins. Integrins bind to a wide variety of ligands, including extracellular matrix proteins (ECM), such as fibronectin, vitronectin, collagen and laminin. Matrix proteins are present within the basement of the blood vessel wall, including laminins. Laminin are a large family of glycoproteins, with a heterotrimeric structure of α, β and γ chains. The use of different α, β and γ chains leads to the expression of at least 12 different laminin isoforms. Different laminins are expressed at different

stages of development and at different sites within the body.

*Scleroderma*

In investigations described herein, it was found that sera from patients with scleroderma (systemic sclerosis) were less able to inhibit fibrocyte differentiation from monocytes that that of normal patients. Further scleroderma patients were found to have lower levels of circulating SAP.

Scleroderma is a non-inherited, noninfectious disease that has a range of symptoms. It involves the formation of scar tissue containing fibroblasts in the skin and internal organs. The origin of the fibroblasts is unknown. In mild or early cases of scleroderma, there is a hardening of the skin, fatigue, aches and sensitivity to cold. In more sever and later stages, there is high blood pressure, skin ulcers, difficulty moving joints, and death from lung scarring or kidney failure. Approximately 300,000 people in the U.S. have scleroderma. The disease has similarities to lupus and rheumatoid arthritis. There is no cure or significant treatment for scleroderma and even diagnosis is difficult because there is no clinical test.

*Nephrogenic fibrosing dermopathy*

Nephrogenic fibrosing dermopathy (NFD) is a newly recognized scleroderma-like fibrosing skin condition. It develops in patients with renal insufficiency. Yellow scleral plaques and circulating antiphospholipid antibodies have been proposed as markers of NFD. Dual

immunohistochemical staining for CD34 and pro-collagen in the spindle cells of NFD suggest that the dermal cells of NFD may represent circulating fibrocytes recruited to the dermis. Therefore, inhibition of fibrocyte formation may alleviate symptoms of this disease.

*Asthma*

Asthma affects more than 100 million people worldwide, and its prevalence is increasing. Asthma appears to be cause by chronic airway inflammation. One of the most destructive aspects of asthma is remodeling of the airways in response the chronic inflammation. This remodeling involves thickening of the lamina reticularis (the subepithelial reticular basement membrane surrounding airways) due to fibrosis. The airway passages then become constricted due to the thickened airway walls.

The thickened lamina reticularis in asthma patients contains abnormally high levels of extracellular matrix proteins such as collagen I, collagen III, collagen V, fibronectin and tenascin. The source of these proteins appears to be a specialized type of fibroblast called myofibroblasts.

In asthma patients, CD34+/collagen I+ fibrocytes accumulate near the basement membrane of the bronchial mucosa within 4 hours of allergen exposure. 24 hours after allergen exposure, labeled monocytes/fibrocytes have been observed to express α-smooth muscle actin, a marker for myofibroblasts. These observations suggest that in asthma patients allergen exposure causes fibrocytes from the blood to enter the bronchial mucosa, differentiate into myofibroblasts, and then cause airway

wall thickening and obstruct the airways. Further, there is a correlation between having a mutation in the regulatory regions of the genes encoding monocyte chemoattractant protein 1 or TGFβ-1 and the severity of asthma. This also suggests that recruitment of monocytes and appearance of myofibroblasts lead to complications of asthma.

Thickening of the lamina reticularis distinguishes asthma from chronic bronchitis or chronic obstructive pulmonary disease and is found even when asthma is controlled with conventional medications. An increased extent of airway wall thickening is associated with severe asthma. No medications or treatments have been found to reduce thickening of the lamina reticularis. However, it appear likely that reducing the number of myofibroblasts found in the airway walls may reduce thickening or help prevent further thickening.

*Idiopathic pulmonary fibrosis*

Idiopathic pulmonary fibrosis (IPF) is a unique type of chronic fibrosing lung disease of unknown etiology. The sequence of the pathogenic mechanisms is unknown, but the disease is characterized by epithelial injury and activation, the formation of distinctive subepithelial fibroblast/myofibroblast foci, and excessive extracellular matrix accumulation. These pathological processes usually lead to progressive and irreversible changes in the lung architecture, resulting in progressive respiratory insufficiency and an almost universally terminal outcome in a relatively short period of time. While research has largely focused on inflammatory mechanisms for initiating the fibrotic

response, recent evidence strongly suggests that disruption of the alveolar epithelium is an underlying pathogenic event. Given the role played by fibrocytes in wound healing and their known role in airway wall thickening in asthma, it appears likely that overproduction of fibrocytes may be implicated in IPF.

## SUMMARY OF THE INVENTION

The present invention includes compositions and methods for suppressing fibrocyte differentiation from monocytes. In selected embodiments, fibrocyte differentiation in a target location may be suppressed by providing SAP, IL-12, Laminin-1, IgG aggregates, cofactors of any of the above, and any combination thereof. (Designations for "SAP", "IL-12" and "Laminin-1" as used herein also refer to functional fragments of these proteins unless it is clear that such fragments are excluded from the usage in a given context.) The target location may be located *in vitro* or *in vivo*. *In vivo* the target location may include an entire organism or a portion thereof and the composition may be administered systemically or it may be confined to a particular area, such as an organ or tissue. The compositions may include those supplied directly or produced in target location or the same organism as the target location, for instance through expression of a transgene. These compositions may be given in amount sufficient to increase concentrations above normal levels or to bring their concentrations up to normal levels or restore their normal activity levels. Concentrations or activity of certain of these compositions may be increased by

stimulating natural production or suppressing normal degradation.

A decrease in differentiation of fibrocytes from monocytes may alleviate symptoms of numerous fibrosing diseases or other disorders caused by fibrosis.

The present invention also includes assays to detect the ability of a sample to modulate fibrocyte differentiation from monocytes. In one embodiment, normal monocytes are supplied with the sample. The sample may include normal SAP. It may also include SAP or a biological fluid from a patient such as a patient with a fibrosing disease, or it may include a potential drug. In another embodiment, the sample includes normal SAP while the monocytes are derived from a patient and may be abnormal. In either type of assay, the effects on monocyte differentiation into fibrocytes may be compared with a normal control to detect any increases or decreases in monocyte differentiation as compared to normal.

## BRIEF DESCRIPTION OF THE DRAWINGS

The following figures form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description presented herein.

FIGURE 1 illustrates the effects of serum and plasma on the rapid differentiation of fibroblast-like cells.

In FIGURE 1A peripheral blood mononuclear cells (PBMC) at 2.5 x $10^5$ per 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.

In FIGURE 1B PBMC at 2.5 x $10^5$ per ml were cultured in serum-free medium for 6 days in dilutions of human plasma. Cells were then air-dried, fixed, stained, and fibrocytes were enumerated by morphology. Results are expressed as mean ± SD of the number of fibrocytes per 2.5 x $10^5$ PBMCs (n=5 experiments). Stars indicate statistically significant differences from 0 plasma.

FIGURE 2 illustrates the 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. Monoclonal antibodies used are as indicated, and identified by biotin-conjugated goat anti-mouse Ig followed by ExtrAvidin peroxidase. Cells were counterstained with Mayer's haematoxylin to identify nuclei. Positive staining was identified by brown staining, nuclei are counterstained blue. An insert for CD83 was used to indicate positive staining on a dendritic cell.

FIGURE 3 illustrates the characterization of the molecule in present in plasma that inhibits fibrocyte differentiation. Citrated plasma was treated with BaCl2 and the precipitated material was collected by centrifugation and dialyzed against 10mM sodium phosphate containing 10mM EDTA and protease inhibitors. This material was then fractionated by heparin and ion exchange chromatography.

In FIGURE 3A fractions were analyzed by PAGE on a 4-20% reducing gel and stained with coomassie blue. M

indicates molecular weight markers. Lane 1 contained plasma, lane 2 contained BaCl2 supernatant, lane 3 contained wash 1, lane 4 contained wash 2, lane 5 contained BaCl2 precipitate, lane 6 contained BaCl2 precipitate, lane 7 contained heparin flow through, lane 8 contained the heparin fraction, lane 9 contained High Q flow through, lane contained the 10 High Q fraction, lane 11 contained the gel purified fraction. Lanes 1-5 diluted 1:500 in sodium phosphate buffer, lanes 6-11 undiluted.

Active fractions eluted off the High Q ion exchange column and gel slices were analyzed by 4-20% PAGE on a native gel in FIGURE 3B and a reducing gel in FIGURE 3C. NM indicates native gel markers, RM indicates reduced gel markers. In 3C lanes 1-3 are control gel samples, lane 4 contained active fraction. In FIGURE 3D fractions were assessed by western blotting, using a rabbit anti-SAP antibody. Lanes 1-11 correspond to those in FIGURE 3A.

FIGURE 4 shows the inhibition of fibrocyte formation by SAP, but not CRP or other plasma proteins. PBMC at $2.5 \times 10^5$ per ml were cultured in serum-free medium for 6 days in the presence of commercially available purified SAP (filled square), CRP (open square), Protein S (open diamond)or C4b (open circle) and then examined for the appearance of fibroblast-like cells. Cells were then air-dried, fixed, stained and fibrocytes enumerated by morphology. Results are mean ± SD of fibrocytes per $2.5 \times 10^5$ PBMC (n=3 separate experiments).

FIGURE 5 shows the effect of depletion of SAP from plasma in a fibrocyte differentiation assay.

FIGURE 5A shows the effect on fibrocyte differentiation of depleting SAP from plasma with BioGel

agarose beads. Number of fibrocytes found in an assay supplied with either plasma (open square) or BioGel depleted plasma (filled square) at a variety of dilutions is shown. Stars indicate statistically significant differences between the two curves.

FIGURE 5B shows the number of fibrocytes in formed in assay performed with no plasma or equal dilutions of plasma, BioGel SAP depleted plasma, or anti-SAP antibody depleted plasma. Stars indicate statistically significant differences.

FIGURE 6 shows the effects of various cytokines on monocyte differentiation into fibrocytes. FIGURE 6A shows the effects of a variety of cytokines. FIGURE 6B shows the effects of IL-12 in greater detail.

FIGURE 7 shows the effects of extracellular matrix proteins on fibrocyte formation. Extracellular matrix proteins were bound to 96 well tissue culture plates for 18 hours at 4°C in 50 mM carbonate buffer pH 9.5. ProNectin-F and ProNectin-L were diluted in PBS. Plates were washed in PBS, and incubated for 60 minutes at 37°C in PBS containing 2% bovine serum albumin, to prevent non-specific binding. Plates were washed with PBS and then tissue culture medium. Peripheral blood mononuclear cells (PBMC) were then added and cultured for 4 days. Results for a variety of extracellular matrix proteins are shown in FIGURE 7A. The effects of Laminin-1, Laminin-10/11 and ProNectin-L are shown in greater detail in FIGURE 7B.

FIGURE 8 shows the effects of monomeric IgG on the ability of SAP to bind to monocytes and inhibit their differentiation. PBMC were cultured in serum-free medium in the presence of a range of concentrations of monomeric

IgG for 60 minutes. SAP, at the concentrations indicated, was then added and the cells were cultured for 4 days.

FIGURE 9 shows the effects of ligation and cross-linking of Fc receptors on monocyte to fibrocyte differentiation. Soluble immune complexes (ovalbumin-antibody), particulate immune complexes, including opsonised sheep red blood cells (SRBC) and heat-aggregated IgG were used. In FIGURE 9A PBMC cultured for 4 days with ovalbumin or anti-ovalbumin mAb alone, or ovalbumin:anti-ovalbumin immune complexes. FIGURE 9B shows the effects of SRBC alone and SRBC opsonised with rabbit anti-SRBC at 20:1 and 40:1 SRBC:monocyte ratios. Finally, FIGURE 9C shows the effects on PBMC of heat-aggregated IgG and heat-aggregated $F(ab)_2$. Stars in 9A and 9B indicate statistically significant differences.

FIGURE 10 shows the effects of anti-FcγR antibodies on monocyte differentiation. Stars indicate a statistically significant difference from control.

## DETAILED DESCRIPTION

### *Monocyte Differentiation Suppression*

The regulation of events leading to fibrosis involves the proliferation and differentiation fibrocytes. Fibrocytes are a distinct population of fibroblast-like cells derived from peripheral blood monocytes that normally enter sites of tissue injury to promote angiogenesis and wound healing. Culturing CD14+ peripheral blood monocytes in the absence of serum or plasma leads to the rapid differentiation of fibrocytes. This process occurs within 72 hours and is suppressed by

the presence of serum or plasma. The factor in serum that suppresses the rapid appearance of fibrocytes is serum amyloid P (SAP). Further, a cohort of patients with the fibrosing disease scleroderma have sera with a poor àbility to suppress fibrocyte differentiation and exhibit a correspondingly low level of SAP. These results suggest that low levels of SAP in the circulation or the peripheral tissues lead to or play a part in pathological processes such as fibrosis. Monocyte differentiation assays have also revealed that IL-12, Laminin-1 and conjugated IgG molecules also suppress differentiation of monocytes into fibrocytes.

Compositions containing one or more of the fibrocyte formation suppressors may be used to suppress fibrosis in inappropriate locations and in fibrosing disorders and chronic inflammatory conditions, *inter alia*. Compositions may be applied locally or systemically. In specific embodiments, compositions containing SAP may be operable to raise SAP concentration in target locations to approximately at least 0.5 μg/ml. In humans, $I^{125}$ radiolabelled SAP has been previously administered to study patients with amyloidosis. In the treatments, approximately 600 μg of SAP was administered to an adult human. Accordingly, administration of approximately 600 μg of SAP systemically to an adult human is safe. Higher dosages may also be safe under appropriate conditions.

SAP supplied in certain compositions of the present invention may include the entire SAP protein or a portion thereof, preferably the portion functional in suppression fibrocyte formation. In an exemplary embodiment, the functional portion of SAP is selected from the region that does not share sequence homology with CRP, which has

no effect on fibrocyte formation. For instance amino acids 65-89 (KERVGEYSLYIGRHKVTSKVIEKFP-SEQ.ID.NO.1) of SAP are not homologous to CRP. Amino acids 170-181 (ILSAYQGTPLPA- SEQ.ID.NO.2) and 192-205 (IRGYVIIKPLV-SEQ.ID.NO.3) are also not homologous. Additionally a number of single amino acid differences between the two proteins are known and may result in functional differences.

Compositions containing IL-12 may be operable to raise the IL-12 concentration in target locations to approximately 0.1 to 10 ng/ml. Compositions containing Laminin-1 may be operable to raise the laminin-1 concentration in target locations to approximately 1 to 10 μg/ml. Compositions containing aggregated IgG may be operable to raise aggregate IgG concentrations in target locations to approximately 100 μg/ml. The compositions may also be supplied in combinations or with cofactors. Compositions may be administered in an amount sufficient to restore normal levels, if the composition is normally present in the target location, or they may be administered in an amount to raise levels above normal levels in the target location.

The above compositions may be supplied to a target location from an exogenous source, or they may be made *in vivo* by cells in the target location or cells in the same organism as the target location. These compositions may be isolated from donated human tissues, including biological fluids. They may be also be made as a recombinant protein in bacteria, tissue culture cells, or any other type of cells or tissues known to the art, or in whole animals. They may also be made synthetically or by any other methodology known to the art. If these

compositions are made *in vivo*, they may be the expression product of a transgene or they may result from enhancement of production in an existing *in vivo* source. Levels of these compositions, if they are normally present in a target location, may also be raised by reducing their normal rates of degradation. Additionally, it may be possible to increase the fibrocyte differentiation suppression ability of these compositions, for instance by supplying cofactors.

Compositions of the present invention may be in any physiologically appropriate formulation. They may be administered to an organism topically, by injection, by inhalation, or by any other effective means.

*Disease targets*

The same compositions and methodologies described above to suppress monocyte differentiation into fibrocytes may also be used to treat or prevent fibrosis resulting from conditions including but not limited to: scleroderma, keloid scarring, rheumatoid arthritis, lupus, nephrogenic fibrosing dermopathy, fibrotic lesions such as those formed after *Schistosoma japonicum* infection, autoimmune diseases, pathogenic fibrosis, Lyme disease, stromal remodeling in pancreatitis and stromal fibrosis, asthma, idiopathic pulmonary fibrosis, chronic obstructive pulmonary disease, and pulmonary fibrosis. In some such fibrosing diseases fibrocytes may not represent an end-stage of fibrosis. For example, in asthma, fibrocytes further differentiate into myofibroblasts, which persist in thickened airway walls.

The invention also includes a method of inhibiting fibrocyte formation or treating or preventing a fibrosing

disease or asthma by activating any component of the Fc signaling pathway in monocytes normally activated by SAP. This pathway is described in detail in Daeron, Marc, "Fc Receptor Biology", Annu. Rev. Immunology 15:203-34 (1997). In an exemplary embodiment a portion of the pathway that is not shared with other signaling cascades or only a limited number of non-critical signaling cascades is selected for activation to minimize side-effects.

*Monocyte Differentiation Assays*

Another aspect of the invention relates to assays to detect the ability of a sample to modulate fibrocyte differentiation from monocytes. In serum-free medium, normal monocytes form fibrocytes in two to three days. Normal serum, blood or other biological fluids suppress the formation of fibrocytes from normal monocytes over a specific dilution range. Thus the assay may be used to test whether a sample can modulate differentiation of monocytes into fibrocytes in serum-free medium. It may also be used to determine whether sample monocytes differentiate normally into fibrocytes in serum-free medium and if they respond normally to serum, SAP or other factors affecting this differentiation.

In a specific embodiment, the assay may be used to determine whether a patient's biological fluid has a decreased or increased ability to suppress monocyte differentiation into fibrocytes. If suppression by SAP is to be tested, any biological fluid in which SAP is normally or transiently present may be used in the present invention, including whole blood, serum, plasma, synovial fluid, cerebral spinal fluid and bronchial

fluid. A decreased ability to suppress monocyte differentiation may be indicative of a fibrosing disease or the propensity to develop such a disease. Although in many patients a decreased ability of a biological fluid to suppress fibrocyte formation may be due to high levels of SAP, this is not necessarily the case. SAP may be present at normal levels, but exhibit increased suppressive activity due to defects in the SAP itself or the absence or presence of a cofactor or other molecule. Methods of determining the more precise nature of the suppression problem, such as use of ELISAs, electrophoresis, and fractionation will be apparent to one skilled in the art.

The methodology described above may also be used to determine whether certain potential drugs that affect fibrocyte differentiation may or may not be appropriate for a patient.

In another specific embodiment, the assay may be used to determine if a patient's monocytes are able to differentiate into fibrocytes in serum-free medium and if they respond normally to a biological fluid, SAP or another composition. More particularly, if a patient with a fibrosing disease appears to have normal levels of SAP, it may be advisable to obtain a sample of the patient's monocytes to determine if they are able to readily differentiate into fibrocytes even in the presence of serum or SAP.

Finally, in another specific example, the assay may be used to test the effects of a drug or other composition on monocyte differentiation into fibrocytes. The assay may be used in this manner to identify potential drugs designed to modulate fibrocyte formation,

or it may be used to screen for any potential adverse effects of drugs intended for other uses.

The following examples are included to demonstrate specific embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples that follow represent techniques discovered by the inventors to function well in the practice of the invention. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments that are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

## EXAMPLES

### *EXAMPLE 1: INHIBITION OF FIBROCYTE FORMATION*

While examining the possible role of cell density in the survival of peripheral blood T cells, it was observed that in serum-free medium peripheral blood mononuclear cells (PBMC) gave rise to a population of fibroblast-like cells. These cells were adherent and had a spindle-shaped morphology (Figure 1A). Approximately 0.5-1 % of PBMC differentiated into fibroblast-like cells in serum-free medium, and this occurred in tissue culture treated plasticware and borosilicate and standard glass slides.

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, PBMC were cultured at $5 \times 10^5$ cells per ml in serum-free medium containing increasing concentrations of human plasma for 6 days. When plasma was present at concentrations between 10% and 0.5%, the fibroblast-like cells did not differentiate

(Figure 1B). However, at or below 0.1% serum, fibroblast-like cells rapidly developed. The activity in the serum that inhibited fibrocyte formation was retained by a 30kDa cutoff spin-filter (data not shown). If serum was heated to 56oC for 30 minutes, the efficacy was reduced 10 fold, and heating to 95oC abolished the inhibitory activity (data not shown).

These data suggest that that the inhibitory factor is a protein. As the inhibitory factor was present in human serum, it indicated that the activity was unlikely to be involved with the coagulation system. The inhibitory factor also appeared to be an evolutionary conserved protein as bovine, equine, caprine, and rat sera were also able to inhibit the appearance of these fibroblast-like cells (data not shown).

*EXAMPLE 2: CHARACTERIZATION OF FIBROBLAST-LIKE CELLS*

The differentiation of these fibroblast-like cells from peripheral blood suggested that they might be peripheral blood fibrocytes. Fibrocytes are a population derived from peripheral blood monocytes that differentiate *in vitro* and *in vivo* into fibroblast-like cells. They rapidly enter wound sites and are capable of presenting antigen to T cells. Their phenotype is composed of both hematopoietic markers such as CD45 and MHC class II and stromal markers, such as collagen I and fibronectin. However in order to identify these cells, PBMC were generally cultured for 1-2 weeks in medium containing serum.

To characterize whether the cells observed in the system were fibrocytes, PBMC were depleted of T cells with anti-CD3, B cells with anti-CD19, monocytes with

anti-CD14 or all antigen presenting cells 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 deplete fibroblast-like cells from PBMC when cultured in serum-free cultures (data not shown). Depletion of antigen presenting cells with anti-HLA class II or monocytes with anti-CD14 antibody did prevent the appearance of fibroblast-like cells, indicating that the fibroblast-like cells are derived from monocytes and not a dendritic cell population.

To further characterize the fibroblast-like cells, PBMC were cultured in serum-free medium for 5 days on glass slides. Cells were then air-dried, fixed in acetone and labeled with a variety of antibodies (Table 1 and Figure 2). Fibrocytes express CD11a, CD11b, CD45, CD80, CD86, MHC class II, collagen I, fibronectin, the chemokine receptors CCR3, CCR5, CCR7, CXCR4 and α-smooth muscle actin. In the above culture conditions, the fibroblast-like cells in the present experiment also expressed all these markers. Fibrocytes are negative for CD1a, CD3, CD19, CD38 and vWF, as were the fibroblast-like cells in the present experiment. Based on these data it appears that the fibroblast-like cells observed in the experiments were fibrocytes. Further experiments were conducted to extend this phenotype. In the above conditions, the fibrocytes expressed several β1 integrins including α1 (CD49a), α2 (CD49b), α5 (CD49e), β1 (CD29) and β3 (CD61) along with high levels of β2 (CD18), but were negative for α3, α4, α6 α4β7, αE and CLA (Figure 2 and Table 1).

Table 1: Expression of surface markers on Fibrocytes

| Marker | Alternative Name | Fibrocyte Expression |
|---|---|---|
| CD11a | LFA-1 | positive |
| CD11b | Mac-1 | positive |
| CD11c | | positive |
| CD13 | | positive |
| CD18 | β2 integrin | positive |
| CD29 | β1 integrin | positive |
| CD34 | | positive |
| CD40 | | weak positive |
| CD45 | LCA | positive |
| CD49a | α1 integrin | weak positive |
| CD49b | α2 integrin | negative |
| CD49e | α5 integrin | positive |
| CD51 | | positive |
| CD54 | ICAM-1 | positive |
| CD58 | LFA-3 | positive |
| CD61 | β3 integrin | positive |
| CD80 | B7-1 | weak positive |
| CD86 | B7-2 | positive |
| CD105 | Endoglin | positive |
| CD148 | | positive |
| MHC class II | | positive |
| CD162 | PSGL-1 | positive |
| CCR1 | | weak positive |
| CCR3 | | weak positive |
| CCR4 | | weak positive |
| CCR5 | | weak positive |
| CCR7 | | weak positive |
| CCR9 | | weak positive |
| CXCR1 | | positive |
| CXCR3 | | positive |
| CXCR4 | | weak positive |

| | | |
|---|---|---|
| Collagen I | | positive |
| Collagen III | | positive |
| Fibronectin | | positive |
| α Smooth Muscle Actin | | positive |
| Vimentin | | positive |
| CD1a | | negative |
| CD3 | | negative |
| CD10 | | negative |
| CD14 | | negative |
| CD19 | | negative |
| CD25 | | negative |
| CD27 | | negative |
| CD28 | | negative |
| CD38 | | negative |
| CD49c | α3 integrin | negative |
| CD49d | α4 integrin | negative |
| CD49f | α6 integrin | negative |
| CD69 | | negative |
| CD70 | CD27-L | negative |
| CD90 | | negative |
| CD103 | αE integrin | negative |
| CD109 | | negative |
| CD154 | CD40-L | negative |
| α4β7 | | negative |
| CLA | | negative |
| CCR2 | | negative |
| CCR6 | | negative |
| CXCR2 | | negative |
| CXCR5 | | negative |
| CXCR6 | | negative |
| Cytokeratin | | negative |
| vWF | | negative |

To obtain the data in Table 1, PBMC were cultured in the wells of 8 well glass slides at 2.5 x $10^5$ cells per ml (400 µl per well) in serum-free medium for 6 days. Cells were then air dried, fixed in acetone and stained by immunoperoxidase. Cells were scored positive or negative for the indicated antigens, compared to isotype-matched control antibodies.

*EXAMPLE 3: CHARACTERIZATION OF THE FIBROCYTE INHIBITORY FACTOR*

The initial characterization of the serum factor that prevents rapid fibrocyte differentiation indicated that the factor was a heparin-binding molecule that eluted off 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, one of these proteins was identified as C4b-binding protein (C4BP). C4b-binding protein is a 570 kDa protein, composed of seven alpha chains (70 kDa) and usually a single beta chain (40 kDa), which is involved in regulating the decay of C4b and C2a components of the complement system. C4BP also interacts with the vitamin K-dependent anticoagulant protein S. The C4BP/Protein S complex can be purified from serum or plasma using $BaCl_2$ precipitation.

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 (FIGURE 3 and Table 2). 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 off the heparin column in a peak at 200 mM NaCl (FIGURE 3 and Table 2). A slight increase in the yield suggested that this step may have removed a factor that slightly interfered with the activity of the factor.

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 indicated that the major band was a 27 kDa protein. Although the ion exchange chromatography led to a reduction in the amount of SAP recovered (FIGURE 3A, lanes 8-10 and FIGURE 3D, lane 8-10) this step did remove several contaminating proteins. After the ion exchange step the only discernable contaminant was albumin at 65 kDa (FIGURE 3A, lane 10).

The high Q fraction was concentrated and fractionated by electrophoresis on a non-denaturing polyacrylamide gel, followed by elution of the material in gel slices. A single band that migrated at approximately 140 kDa was able to inhibit differentiation (FIGURE 3B). This band had a molecular weight of 27 kDa on a reducing polyacrylamide gel, suggesting that the native conformation of the protein was a pentamer (FIGURE 3C). This band was excised from the gel, digested with trypsin and analyzed by MALDI 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 serum amyloid P.

To confirm that the active fractions contained SAP, fractions collected from column chromatography were analyzed by western blotting (FIGURE 3D). The presence of SAP at 27 kDa was detected in all fractions that inhibited fibrocyte differentiation (FIGURE 3D, lanes 6, 8, 10 and 11). A considerable amount of SAP was present in the supernatant from the BaCl2 precipitation step indicating that this procedure was inefficient, with the recovery of only approximately 10-15% of the fibrocyte inhibitory activity in the $BaCl_2$ pellet (FIGURE 3A, lane 2). In order to remove the known problem of anti-SAP antibodies binding to immunoglobulins when used with western blotting, the antibody was pre-incubated with human IgG bound to agarose. 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 in the barium precipitation, but were absent from the active fractions collected from heparin chromatography (data not shown).

Table 2: Recovery of protein and fibrocyte inhibitory activity from fractionated human plasma

| | Volume (ml) | Protein (mg/ml) | Total protein (mg) | Yield (%) |
|---|---|---|---|---|
| Plasma | 250 | 70 | 17,500 | 100 |
| $BaCl_2$ supernatant | 240 | 60 | 14,400 | 82.3 |
| $BaCl_2$ Precipitate | 31 | 1 | 31 | 0.18 |
| Heparin fraction | 4.3 | 0.25 | 1.075 | 0.006 |
| High Q fraction | 1.96 | 0.05 | 0.098 | 0.00056 |
| Gel slice | 0.075 | 0.025 | 0.0018 | 0.00001 |
| | Activity (U/ml) | Total activity (U) | Yield (%) | Specific activity (U/mg) |

| | | | | |
|---|---|---|---|---|
| Plasma | 10,000 | 2.5 x $10^6$ | 100 | 143 |
| $BaCl_2$ supernatant | 6,666 | 1.6 x $10^6$ | 64 | 111 |
| $BaCl_2$ Precipitate | 1,666 | 5.1 x $10^4$ | 2 | 1,645 |
| Heparin fraction | 500 | 2,150 | 0.086 | 2000 |
| High Q fraction | 400 | 720 | 0.029 | 7,300 |
| Gel slice | 2000 | 150 | 0.006 | 80,000 |

Plasma was fractionated by $BaCl_2$ precipitation, heparin and ion exchange chromatography. Protein concentrations were assessed by spectrophotometry at 280 nm. Inhibition of fibrocyte differentiation was assessed by morphology. 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.

SAP may also be detected by ELISA using the following methodology:

Maxisorb 96 well plates (Nalge Nunc International, Rochester, NY) were coated overnight at 4oC with monoclonal anti-SAP antibody (SAP-5, Sigma) in 50 mM sodium carbonate buffer pH 9.5. Plates were then incubated in Tris buffered saline pH 7.4 (TBS) containing 4% BSA (TBS-4% BSA) to inhibit non-specific binding. Serum and purified proteins were diluted to 1/1000 in TBS-4% BSA, to prevent SAP from aggregating and incubated for 60 minutes at 37oC. Plates were then washed in TBS containing 0.05% Tween™-20. Polyclonal rabbit anti-SAP antibody (BioGenesis) diluted 1/5000 in TBS-4% BSA was used as the detecting antibody. After washing, 100 pg/ml biotinylated goat (Fab)2 anti-rabbit (Southern Biotechnology Inc.) diluted in TBS-4% BSA was added for

60 minutes. Biotinylated antibodies were detected by ExtrAvidin peroxidase (Sigma). Undiluted peroxidase substrate 3,3,5,5-tetramethylbenzidine (TMB, Sigma) was incubated for 5 minutes at room temperature before the reaction was stopped by 1N HCl and read at 450nm (BioTek Instruments, Winooska, VT). The assay was sensitive to 200 pg/ml.

*EXAMPLE 4: SPECIFICITY OF SERUM AMYLOID P*

Serum amyloid P 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, PBMC were cultured in serum-free medium in the presence of commercially available purified SAP, CRP, C4b or Protein S. The commercially available SAP was purified using calcium-dependent affinity chromatography on unsubstituted agarose. Of the proteins tested, only SAP was able to inhibit fibrocyte differentiation, with maximal inhibitory activity at 10 µg/ml (FIGURE 4). A dilution curve indicated that the commercially available SAP has approximately $6.6 \times 10^3$ units/mg of activity (Figure 4). Serum and plasma contain between 30-50 µg/ml SAP. Fibrocytes began to appear at a plasma dilution of 0.5%, which would be approximately 0.15-0.25 µg/ml SAP, which is comparable to the threshold concentration of purified SAP. The data showing that SAP purified using two different procedures inhibits fibrocyte differentiation strongly suggests that SAP inhibits fibrocyte differentiation.

Although these data indicate that SAP is capable of inhibiting fibrocyte development and SAP purifies in a

manner that indicates that it is the active factor in plasma, it was not determined whether depletion of SAP from plasma and serum would negate the inhibition. SAP was depleted from plasma using agarose beads (BioGel A, BioRad). Plasma was diluted to 20% in 100 mM Tris pH 8, 150 mM NaCl, 5 mM $CaCl_2$ buffer and mixed with 1 ml agarose beads for 2 hours at 4oC. Beads were then removed by centrifugation and the process repeated. This depleted plasma was then assessed for its ability to inhibit fibrocyte differentiation. The control plasma diluted to 20% in 100 mM Tris pH 8, 150 mM NaCl, 5 mM $CaCl_2$ buffer had a similar dilution curve to that observed with untreated plasma. In contrast, the bead-treated plasma was less able to inhibit fibrocyte differentiation at intermediate levels of plasma. These data, along with the ability of purified SAP to inhibit fibrocyte differentiation, strongly suggest that SAP is the active factor in serum and plasma that inhibits fibrocyte differentiation. (See FIGURE 5).

Plasma was also depleted of SAP using protein G beads coated with anti-SAP antibodies. 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 antibodies ($p<0.05$) (Figure 5B). The beads coated with control antibodies 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, strongly suggest that SAP is the active factor in serum and plasma that inhibits fibrocyte differentiation.

*EXAMPLE 5: ANTIBODIES AND PROTEINS*

Purified human CRP, serum amyloid P, protein S and C4b were purchased from Calbiochem (San Diego, CA). Monoclonal antibodies to CD1a, CD3, CD11a, CD11b, CD11c, CD14, CD16, CD19, CD34, CD40, Pan CD45, CD64, CD83, CD90, HLA-DR/DP/DQ, mouse IgM, mouse IgG1 and mouse IgG2a were from BD Pharmingen (BD Biosciences, San Diego, CA). Chemokine receptor antibodies were purchased from R and D Systems (Minneapolis, MN). Rabbit anti-collagen I was from Chemicon International (Temecula, CA), monoclonal C4b-binding protein was from Green Mountain Antibodies (Burlington, VE), sheep anti human C4b-binding protein was from The Binding Site (Birmingham, UK), monoclonal anti-CRP was from Sigma (St. Louis, MO). Polyclonal rabbit anti-protein S was from Biogenesis (Poole, Dorset, UK).

*EXAMPLE 6: CELL SEPARATION*

Peripheral blood mononuclear cells were isolated from buffy coats (Gulf Coast Regional Blood Center, Houston, Texas) by Ficoll-Paque (Amersham Biosciences, Piscataway, NJ, USA) centrifugation for 40 minutes at 400 x g. Depletion of specified leukocyte subsets was performed using negative selection using magnetic Dynabeads™ (Dynal Biotech Inc., Lake Success, NY) as described previously. Briefly, PBMC were incubated with primary antibodies for 30 minutes at 4oC. Cells were then washed and incubated with Dynabeads coated with goat anti-mouse IgG for 30 minutes, before removal of antibody-coated cells by magnetic selection. This process was repeated twice. The negatively selected cells were

routinely in excess of 98% pure as determined by monoclonal antibody labeling.

*EXAMPLE 7: CELL CULTURE AND FIBROCYTE DIFFERENTIATION ASSAY*

Cells were incubated in serum-free medium: RPMI (GibcoBRL Life, Invitrogen, Carlsbad, California, USA) supplemented with 10 mM HEPES (GibcoBRL/Life), 2 mM glutamine, 100U/ml penicillin and 100 µg/ml streptomycin, 0.2% bovine serum albumin (BSA, Sigma), 5 µg/ml insulin (Sigma), 5 µg/ml iron-saturated transferrin (Sigma) and 5 ng/ml sodium selenite (Sigma). Normal human serum (Sigma), normal human plasma (Gulf Coast Regional Blood Center) or fetal calf serum (Sigma), column fractions, sera and synovial fluid from patients or purified proteins were added at the stated concentrations. Patient samples were obtained from a repository available to researchers at University of Texas Medical School at Houston. This repository keeps patient information confidential, and meets all NIH guidelines.

PBMC were cultured in 24 or 96 well tissue culture plates in 2 ml or 200 µl volumes respectively (Becton Dickinson, Franklin Lakes, NJ) at 2.5 x $10^5$ cells per ml in a humidified incubator containing 5% CO2 at 37oC for the indicated times. Fibrocytes in 5 different 900 µm diameter fields of view were enumerated by morphology in viable cultures as adherent cells with an elongated spindle-shaped morphology as distinct from small lymphocytes or adherent monocytes. Alternatively cells were air dried, fixed in methanol and stained with hematoxylin and eosin (Hema 3 Stain, VWR, Houston, TX). Fibrocytes were counted using the above criterion and the

presence of an oval nucleus. Enumeration of fibrocytes was performed on cells cultured for 6 days in flat-bottomed 96 well plates, with 2.5 x 104 cells per well. 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.

*EXAMPLE 8: PURIFICATION AND CHARACTERIZATION OF SERUM AND PLASMA PROTEINS*

100 ml of frozen human serum or plasma was thawed rapidly at 37oC and 1x "Complete" protease inhibitor (Roche, Indianapolis, IN, USA), 1mM benzamidine HCl (Sigma) and 1mM Pefabloc™ (AEBSF: 4-(2-Aminoethyl)-benzenesulfonyl fluoride hydrochloride, Roche) were added. All subsequent steps were performed on ice or at 4oC. Barium citrate adsorption of plasma was performed as described previously. The precipitate was collected by centrifugation at 10,000x g for 15 minutes, 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 hours against three changes of 4 liters 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 280nm returned to baseline. Bound material was eluted with a stepped gradient of 15 mls 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) 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 gels (BMA, BioWhittaker, Rockland, ME) as described previously. 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 4oC 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% gel with 100 µM thioglycolic acid in the upper chamber (Sigma). After electrophoresis the gel was rapidly stained with Coomasie brilliant blue, destained, and the bands excised off the gel. Amino acid sequencing was performed by Dr Richard Cook, Protein Sequencing Facility, Department of Immunology, Baylor College of Medicine.

*EXAMPLE 9: WESTERN BLOTTING*

For western blotting, plasma and serum samples were diluted 1:500 in 10 mM sodium phosphate pH 7.4. Fractions from heparin and High Q columns were not diluted. Samples were mixed with Laemmeli's sample buffer containing 20 mM DTT and heated to 100oC for 5 minutes. 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 PVDF (Immobilon™ P, Millipore, Bedford, MA) membranes in Tris/glycine/SDS buffer containing 20% methanol. Filters were blocked with Tris buffered saline (TBS) pH 7.4 containing 5% BSA, 5% non-fat milk protein and 0.1% Tween 20 at 4°C for 18 hours. Primary and biotinylated secondary antibodies were diluted in TBS pH 7.4 containing 5% BSA, 5% non-fat milk protein and 0.1% Tween 20 using pre-determined optimal dilutions (data not shown) for 60 minutes. ExtrAvidin-peroxidase (Sigma) diluted in TBS pH 7.4 containing 5% BSA and 0.1% Tween 20 was used to identify biotinylated antibody and chemiluminescence (ECL, Amersham Biosciences) was used to visualize the result.

*EXAMPLE 10: 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 minutes. Endogenous peroxidase was quenched for 15 minutes with 0.03% $H_2O_2$ and then non-specific binding was blocked by incubation in 2% BSA in PBS for 60 minutes. Slides were incubated with primary antibodies in PBS containing 2% BSA for 60 minutes. Isotype-matched irrelevant antibodies

were used as controls. Slides were then washed in three changes of PBS over 15 minutes and incubated for 60 minutes with biotinylated goat anti-mouse Ig (BD Pharmingen). After washing, the biotinylated antibodies were detected by ExtrAvidin peroxidase (Sigma). Staining was developed with DAB (Diaminobenzadine, Sigma) for 3 minutes and counterstained for 30 seconds with Mayer's haemalum (Sigma).

*EXAMPLE 11: EXPRESSION OF SURFACE MAKERS ON FIBROCYTES*

PBMC were cultured in the wells of 8 well glass slides at 2.5 x $10^5$ cells per ml (400 µl per well) in serum-free medium for 6 days. Cells were then air dried, fixed in acetone and stained by immunoperoxidase. Cells were scored positive or negative for the indicated antigens, compared to isotype-matched control antibodies.

*EXAMPLE 12: RECOVERY OF PROTEIN AND FIBROCYTE INHIBITORY ACTIVITY FROM FRACTIONATED HUMAN PLASMA*

Plasma was fractionated by $BaCl_2$ precipitation, heparin and ion exchange chromatography. Protein concentrations were assessed by spectrophotometry at 280 nm. Inhibition of fibrocyte differentiation was assessed by morphology. 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.

*EXAMPLE 13: IL-12*

Experiments have shown that IL-12 is capable of promoting fibrocyte differentiation in vitro.

Specifically, peripheral blood mononuclear cells were cultured in serum-free medium in the presence of various cytokines (See FIGURE 6A). Concentrations of IL-12 above approximately 5 ng/ml inhibited the number of fibrocytes in culture. (See FIGURE 6B.) This indicates that IL-12 is capable of suppressing the differentiation of fibrocyte precursors into mature fibrocytes.

*EXAMPLE 14: LAMININ-1*

The process of crossing the endothelium and basement membrane induces activation and differentiation signals for monocytes. Therefore, experiments were performed to determine if extracellular matrix proteins had an effect on the differentiation of fibrocytes. Extracellular matrix proteins were bound to 96 well tissue culture plates for 18 hours at 4°C in 50 mM carbonate buffer pH 9.5. ProNectin-F and ProNectin-L were diluted in PBS. Plates were washed in PBS, and incubated for 60 minutes at 37°C in PBS containing 2% bovine serum albumin, to prevent non-specific binding. Plates were washed with PBS and then tissue culture medium. Peripheral blood mononuclear cells (PBMC) were then added and cultured for 4 days. Differentiation of fibrocytes was unaffected by culturing on a wide variety of ECM proteins, including collagens, fibronectin and vitronectin. However, culturing PBMC with either laminin-1 (Sigma-Aldrich, St. Louis, MO) or ProNectin-F (Sanyo Chemical Industries Inc, Kyoto, Japan) led to a significant reduction in the number of fibrocytes (See FIGURE 7A) ($p<0.0001$). ProNectin-F is a construct of silk protein and repeats of the canonical RGD adhesion sequence from fibronectin. ProNectin-L is a similar construct to ProNectin-F, with

the amino acid sequence IKVAV, from the α1 chain of laminin.

Additional experiments were performed to determine whether other laminin proteins could suppress fibrocyte differentiation. Laminin 10/11 (Chemicon, Temecula, CA) a second commercially available laminin, was not capable of inhibiting fibrocyte differentiation, compared to laminin-1. (See FIGURE 7B)

This data suggests that sequences specific to laminin-1, outside the IKVAV region, and absent from laminin-10 and -11, may be responsible for the suppressive effect on fibrocyte differentiation.

*EXAMPLE 15 – Antibody Studies*

SAP and CRP augment phagocytosis and bind to Fcγ receptors on a variety of cells. CRP binds with a high affinity to FcγRII (CD32), a lower affinity to FcγRI (CD64), but does not bind FcγRIII (CD16). SAP binds to all three classical Fcγ receptors, with a preference for FcγRI and FcγRII. Monocytes constitutively express FcγRI. Because this receptor binds monomeric IgG, it is saturated *in vivo*. In order to determine whether the presence of monomeric human IgG could prevent SAP from inhibiting fibrocyte differentiation, PBMC were cultured in serum-free medium in the presence of a range of concentrations of monomeric IgG for 60 minutes. SAP, at the concentrations indicated in FIGURE 8A, was then added and the cells were cultured for 4 days. As described in the above examples, 2.5 μg/ml SAP in the absence of IgG strongly inhibited fibrocyte differentiation. (See FIGURE 8A.) Monomeric IgG in a range from 0.1 to 1000

μg/ml, which corresponds to approximately 0.001 to 10% serum respectively, had little effect on the suppression of fibrocyte formation by SAP.

To determine whether ligation and cross-linking of Fc receptors could also influence monocyte to fibrocyte differentiation, three test samples were used; soluble immune complexes (ovalbumin-antibody), particulate immune complexes, including opsonised sheep red blood cells (SRBC) and heat-aggregated IgG. PBMC cultured for 4 days with ovalbumin or anti-ovalbumin mAb showed that the two proteins alone had a modest effect on the differentiation of monocytes compared to cultures where no reagent was added. (See FIGURE 9A.) However, the addition of ovalbumin:anti-ovalbumin immune complexes led to a significant reduction in the number of differentiated fibrocytes (See FIGURE 9A). A similar effect was observed when PBMC were cultured with opsonised SRBC. SRBC opsonised with rabbit anti-SRBC at 20:1 and 40:1 SRBC:monocyte ratios significantly suppressed fibrocyte differentiation as compared to cells cultured with SRBC alone (See FIGURE 9B). Finally, PBMC cultured with heat-aggregated IgG, but not heat-aggregated $F(ab)_2$, also showed potent inhibition of fibrocyte differentiation (See FIGURE 9C.) Together these data suggest that ligation and cross-linking of Fc receptors is suppressor of monocyte to fibrocyte differentiation.

The observation that immune complexes inhibit fibrocyte differentiation suggests that one or more FcγR influences fibrocyte differentiation. To examine the role of FcγR in fibrocyte differentiation PBMC were cultured in the presence or absence of blocking antibodies to FcγRI (CD64), FcγRII (CD32) or FcγRIII (CD16) before the addition of SAP, or as a control CRP.

When samples were pre-incubated with a blocking mAb for each of the three FcγR, SAP was later able to modestly suppress fibrocyte differentiation. However, in the absence of exogenously added SAP, the FcγRI (CD64) blocking mAb had a profound effect on fibrocyte differentiation. Incubation of PBMC with blocking mAb to FcγRI, but not FcγRII or FcγRIII, promoted fibrocyte differentiation as compared to cells cultured with isotype-matched control mAb or cells cultured with no mAb ($P < 0.01$) (See FIGURE 10). These data suggested that SAP or IgG, might have been produced by some cells in the culture system over 4 days, or that SAP or IgG was retained by cells from the blood. Western blotting failed to show the presence of SAP or IgG after cells had been cultured for 4 days *in vitro*. This suggests that the FcγRI blocking mAb has a direct effect on fibrocyte differentiation or that SAP or IgG were only present during the early time points of the cell culture.

Although only exemplary embodiments of the invention are specifically described above, it will be appreciated that modifications and variations of these examples are possible without departing from the spirit and intended scope of the invention.

SEQUENCE LISTING

<110> WILLIAM MARSH RICE UNIVERSITY

<120> METHODS AND CONDITIONS FOR SUPPRESSING FIBROCYTE DIFFERENTIATION

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<150> PCT/US2003/040957
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CLAIMS

1. Use of a Serum Amyloid P (SAP) protein in an amount sufficient to suppress fibrosis in a mammal having a fibrosing disease.

2. The use of claim 1, wherein the SAP protein forms a pentamer.

3. The use of claim 1, wherein the SAP protein is in a form for administration systemically in an amount of approximately 600 µg or more.

4. The use of claim 1, wherein the Serum Amyloid P (SAP) protein is in a form for administration in an amount at least 1.6 µg/g of mammal to the mammal for a time sufficient to suppress fibrosis at a target location in the mammal.

5. The use of claim 1, wherein the mammal has a disorder that is keloid scarring, rheumatoid arthritis, lupus, nephrogenic fibrosing dermopathy, fibrotic lesions, fibrotic lesions formed after *Schistosoma japonicum* infection, autoimmune diseases, Lyme disease, stromal remodeling in pancreatitis and stromal fibrosis, chronic obstructive pulmonary disease, pulmonary fibrosis, uterine fibroids, ovarian fibrosis, fibrocystic formations, corneal fibrosis, eye fibrosis, eye fibrosis resulting from corneal refraction surgery, fibrosis resulting from congestive heart failure, post-ischemic conditions, abdominal adhesions, wide angle glaucoma trabeculotomy, or any combinations thereof.

6. The use of claim 1, wherein the amount of SAP is sufficient to provide an average concentration of at least 0.5 µg/ml in a target location in the mammal.

7. The use of claim 1, wherein said SAP protein is conjugated to a biocompatible polymer.

8. The use of claim 7, wherein the biocompatible polymer is polyethylene glycol (PEG), a poly(amino acid), a polysaccharide; or a copolymer or combination thereof.

9. The use of claim 1, wherein a medicament selected from interleukin-12 (IL-12), Laminin-1, IgG aggregates, cross-linked IgG, and combinations thereof is also used.

10. The use of claim 1, wherein the mammal is a human.

11. The use of claim 1, wherein the SAP protein is in a form for administration topically, by injection, by inhalation, continuous release by depot or pump, or any combinations thereof.

12. The use of claim 1, wherein the SAP protein is in a form for administration by intravenous injection.

13. The use of claim 1, wherein the mammal has scleroderma.

14. The use of claim 1, wherein the mammal has pulmonary fibrosis.

15. The use of claim 1, wherein the mammal has asthma.

16. Use of a Serum Amyloid P (SAP) protein, in an amount sufficient to suppress differentiation of

monocytes into fibrocytes in a patient having a fibrosis disease or condition.

17. The use of claim 16, wherein the SAP protein forms a pentamer.

18. The use of claim 16, wherein the SAP protein is in a form for administration systemically in an amount of approximately 600 µg or more.

19. The use of claim 16, wherein the Serum Amyloid P (SAP) protein is in a form for administration in an amount at least 1.6 µg/g of patient to the patient for a time sufficient to suppress fibrosis at a target location in the patient.

20. The use of claim 16, wherein the mammal has a disorder that is a fibrosing disease, scleroderma, pulmonary fibrosis, asthma, keloid scarring, rheumatoid arthritis, lupus, nephrogenic fibrosing dermopathy, fibrotic lesions, fibrotic lesions formed after *Schistosoma japonicum* infection, autoimmune diseases, Lyme disease, stromal remodeling in pancreatitis, stromal fibrosis, chronic obstructive pulmonary disease, uterine fibroids, ovarian fibrosis, fibrocystic formations, corneal fibrosis, eye fibrosis, eye fibrosis resulting from corneal refraction surgery, fibrosis resulting from congestive heart failure, post-ischemic conditions, abdominal adhesions, wide angle glaucoma trabeculotomy, or any combinations thereof.

21. The use of claim 16, wherein the amount is sufficient to provide an average concentration of at least 0.5 µg/ml in the target location.

22. The use of claim 1, wherein the mammal has pathogenic fibrosis.

23. The use of claim 1, wherein the mammal has post surgical scarring.

24. The use of claim 16, wherein said SAP protein is conjugated to a biocompatible polymer.

25. The use of claim 24, wherein the biocompatible polymer is polyethylene glycol (PEG), a poly(amino acid), a polysaccharide; or a copolymer or combination thereof.

26. The use of claim 16, wherein a medicament selected from interleukin-12 (IL-12), Laminin-1 ,IgG aggregates, cross-linked IgG, and combinations thereof is also used.

27. The use of claim 16, wherein the patient is a human.

28. The use of claim 16, wherein the SAP protein is in a form for administration topically, by injection, by inhalation, continuous release by depot or pump, or any combinations thereof.

29. The use of claim 16, wherein the SAP protein is in a form for intravenous injection.

30. The use of claim 16, wherein the patient has scleroderma.

31. The use of claim 16, wherein the mammal has pulmonary fibrosis.

32. The use of claim 16, wherein the mammal has asthma.

33. The use of claim 16, wherein the mammal has pathogenic fibrosis.

34. The use of claim 16, wherein the mammal has post surgical scarring.

35. Use of a Serum Amyloid P (SAP) protein in an amount sufficient to suppress fibrosis in a patient after surgery.

36. The use of claim 35, wherein the Serum Amyloid P (SAP) protein is in a form for administration in an amount at least 1.6 µg/g of patient to the patient for a time sufficient to suppress fibrosis at a target location in the patient.

37. The use of claim 35, wherein the SAP protein is in a form for injection.

38. The use of claim 35, wherein the SAP protein forms a pentamer.

39. The use of claim 35, wherein said SAP protein is conjugated to a biocompatible polymer.

40. The use of claim 39, wherein the biocompatible polymer is polyethylene glycol (PEG), a poly(amino acid), a polysaccharide; or a copolymer or combination thereof.

41. The use of claim 35, wherein a medicament selected from interleukin-12 (IL-12), Laminin-1, IgG aggregates, cross-linked IgG, and combinations thereof is also used.

42. The use of claim 35, wherein the patient is a human.

43. The use of claim 35, wherein the post-surgical fibrosis is fibrosis after wide-angle glaucoma trabeculotomy, corneal fibrosis, eye fibrosis resulting from corneal refraction surgery, fibrosis resulting from abdominal adhesion, or any combinations thereof.

44. Use of Serum Amyloid P (SAP) protein, or a portion thereof, said portion comprising one or more sequences selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:3, to suppress pulmonary fibrosis in a mammal suffering from pulmonary fibrosis, wherein the SAP or portion thereof binds to Fcγ receptors and inhibits fibrocyte differentiation from monocytes.

45. The use of claim 44, wherein prior to use the SAP or portion thereof is in a composition with a concentration in the composition of at least 0.5 µg/ml, the composition also comprising at least one excipient, diluent or carrier.

46. The use of claim 44, wherein the composition is in a form for intravenous injection.

47. The use of claim 44, wherein the mammal is a human.

48. The use of claim 44, wherein said SAP or a portion thereof is conjugated to a biocompatible polymer.

49. The use of claim 48, wherein the biocompatible polymer is polyethylene glycol (PEG), a poly(amino acid), a polysaccharide; or a copolymer or combination thereof.

50. The use of claim 44, wherein a medicament selected from the group consisting of IL-12, Laminin-1, IgG

aggregates, cross-linked IgG and combinations thereof is also used.

51. The use of claim 44, wherein approximately 1.6 µg SAP or a portion thereof per gram of bodyweight of the mammal is used.

52. The use of claim 44, wherein the SAP or portion thereof used is in an amount sufficient to approximately double a normal serum concentration of SAP or a similar portion thereof in the mammal.

53. The use of claim 44, wherein the SAP or portion thereof used is sufficient to increase a normal serum concentration of SAP or a similar portion thereof by approximately 25% in the mammal.

54. The use of claim 44, wherein the SAP protein or a portion thereof forms a pentamer.

55. The use of claim 44, wherein the SAP protein or a portion thereof in an amount of approximately 600 µg or more is used.

56. The use of claim 44, wherein the pulmonary fibrosis comprises a condition selected from the group consisting of: Adalimumab-associated pulmonary fibrosis, pulmonary interstitial fibrosis, sarcoidosis, idiopathic pulmonary fibrosis, asthma, chronic obstructive pulmonary disease, diffuse alveolar damage disease, pulmonary hypertension, neonatal bronchopulmonary dysplasia, and emphysema.

57. Use of Serum Amyloid P (SAP) protein or a portion thereof, said portion comprising one or more sequences selected from the group consisting of SEQ ID NO:1, SEQ ID

NO:2, and SEQ ID NO:3, in an amount and for a time sufficient to suppress differentiation of monocytes into fibrocytes in the kidney thereby to treat a fibrotic or fibroproliferative disorder of the kidney in a patient.

58. The use of claim 57, wherein prior to use the SAP or portion thereof is in a composition with a concentration of at least 0.5 µg/ml, the composition also comprising at least one excipient, carrier or diluent.

59. The use of claim 57, wherein the composition is in a form for intravenous injection.

60. The use of claim 57, wherein the mammal is a human.

61. The use of claim 57, wherein the SAP or portion thereof is conjugated to a biocompatible polymer.

62. The use of claim 61, wherein the biocompatible polymer is polyethylene glycol (PEG), a poly(amino acid), a polysaccharide; or a copolymer or combination thereof.

63. The use of claim 57, wherein a composition selected from IL-12, Laminin-1, IgG aggregates, cross-linked IgG and combinations thereof is also used.

64. The use of claim 57, wherein approximately 1.6 µg SAP or portion thereof per gram of mammal is used.

65. The use of claim 57, wherein the SAP or portion thereof used is in an amount sufficient to approximately double a normal serum concentration of SAP or a similar portion thereof in the mammal.

66. The use of claim 57, wherein the SAP or portion thereof used is sufficient to increase a normal serum concentration of SAP or a similar portion thereof by approximately 25% in the mammal.

67. The use of claim 57, wherein the SAP protein or portion thereof forms a pentamer.

68. The use of claim 57, wherein approximately 600 µg or more of the SAP protein or portion thereof is used.

69. The use of claim 57, wherein the fibrotic or fibroproliferative disorder of the kidney comprises a condition selected from sclerosing glomerulonephritis, diabetic nephropathy, renal tubulointerstitial fibrosis, and focal segmental glomerulosclerosis.

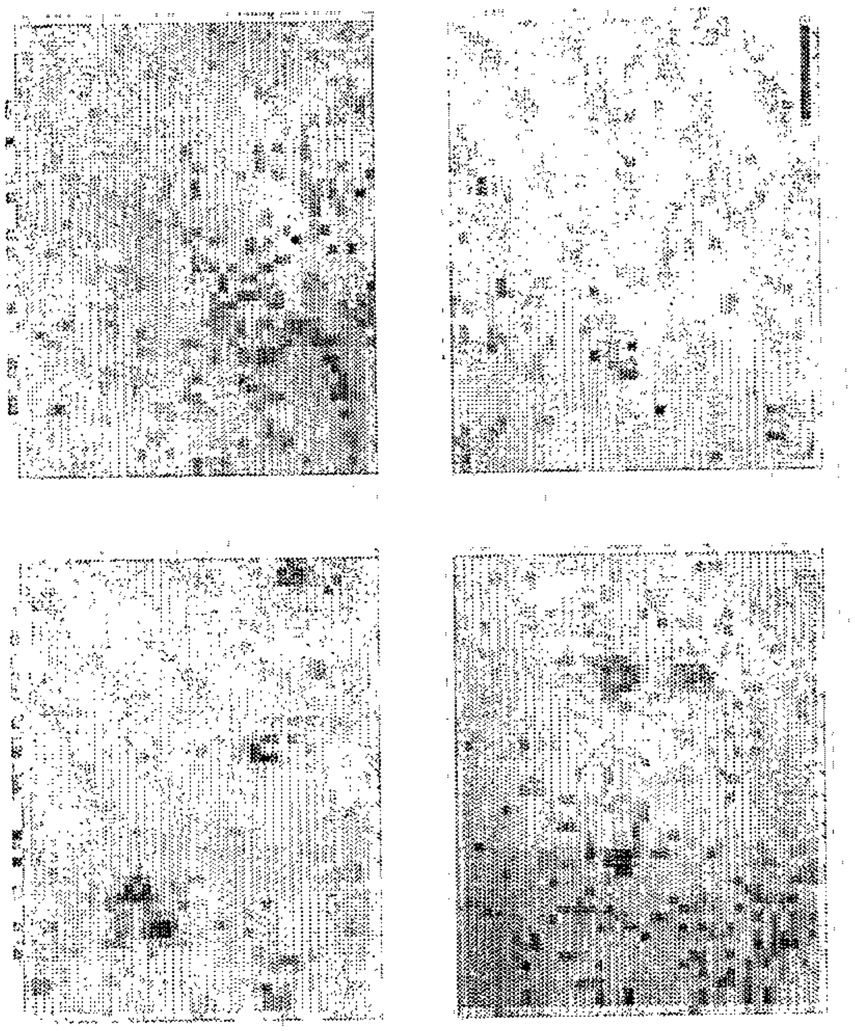



FIGURE 1A

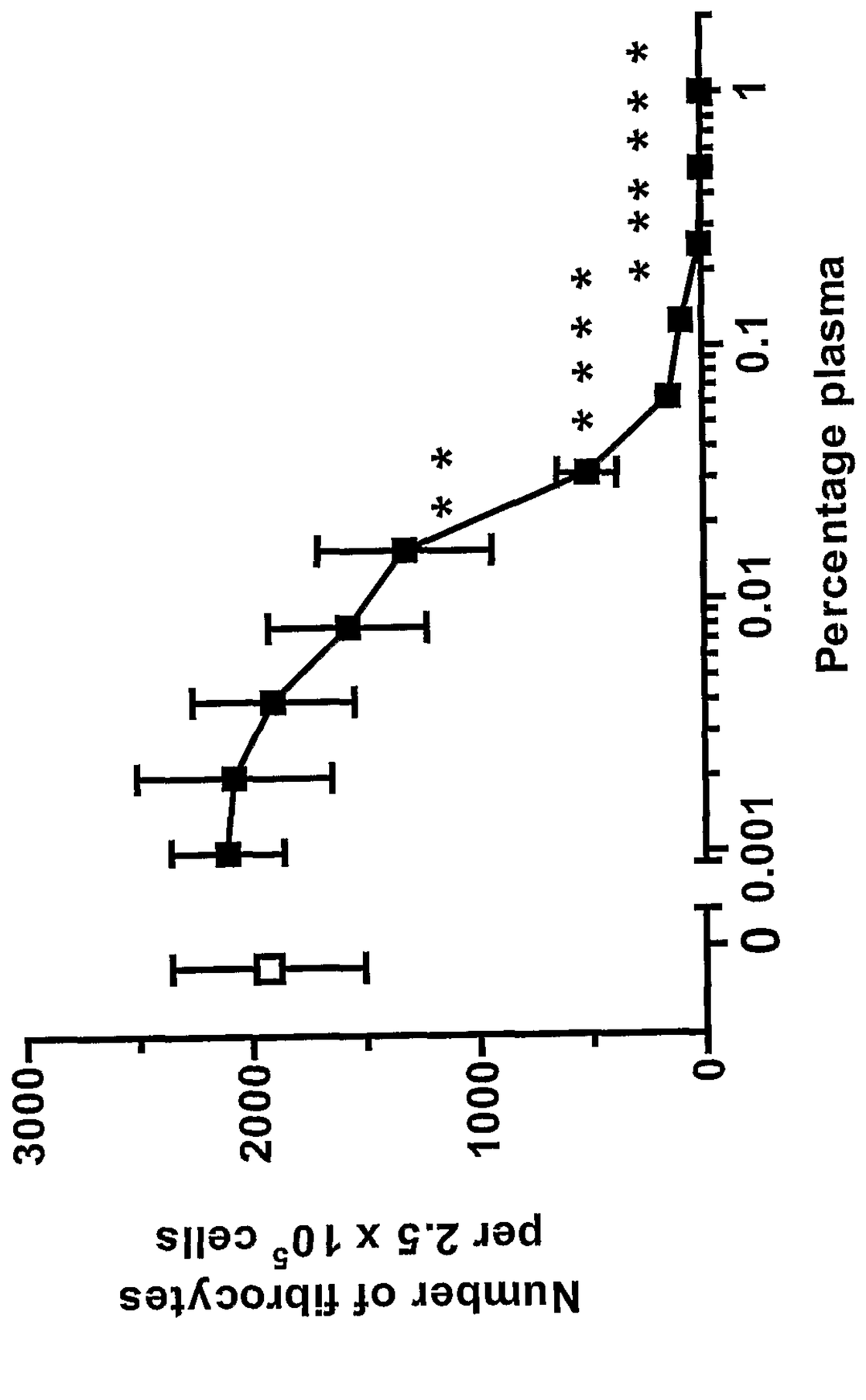

Number of fibrocytes
per 2.5 x 10$^5$ cells
3000
2000
1000
0
0
0.001
0.01
0.1
1
Percentage plasma
* * * * * * * * * * * * * * *

FIGURE 1B

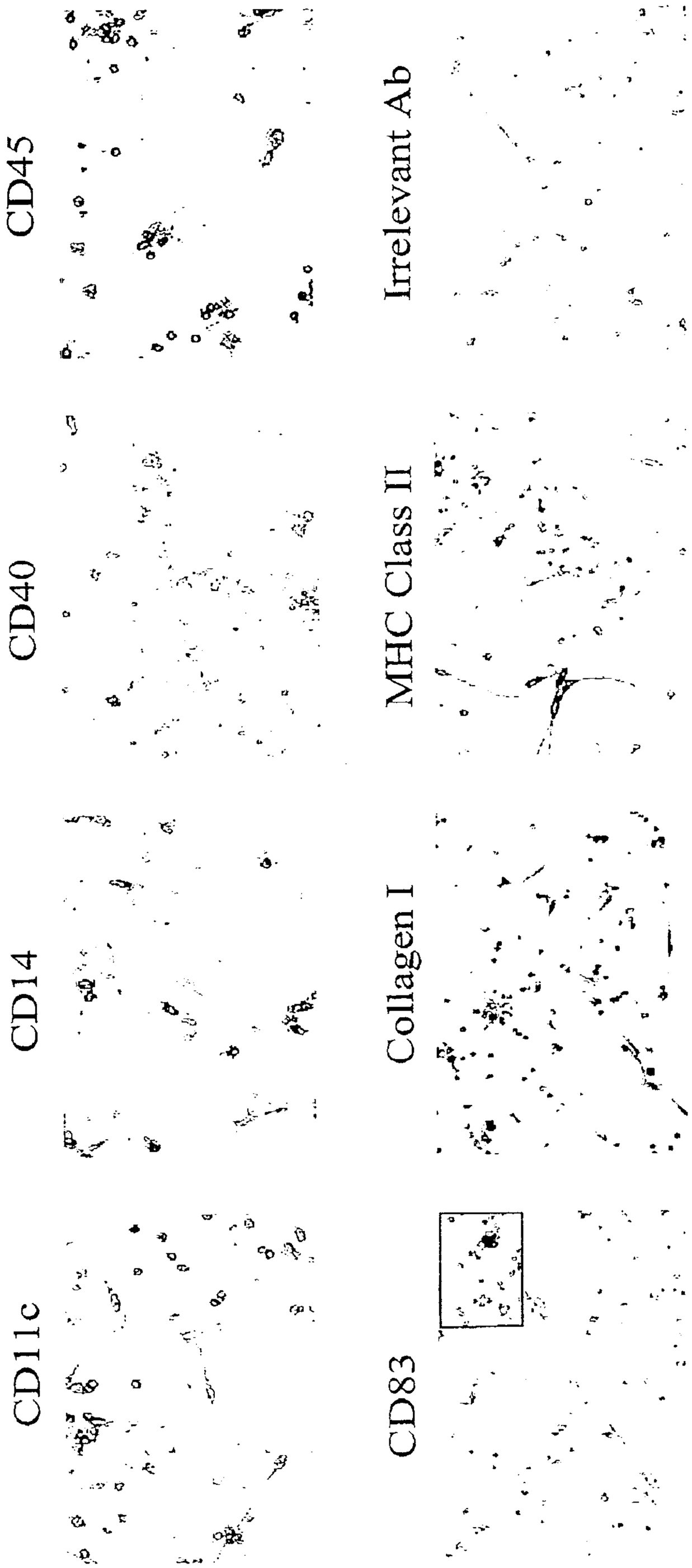

Ab
CD45
CD40
Irrelevant
MHC Class II
CD14
Collagen I
CD11c
CD83

**FIGURE 2**

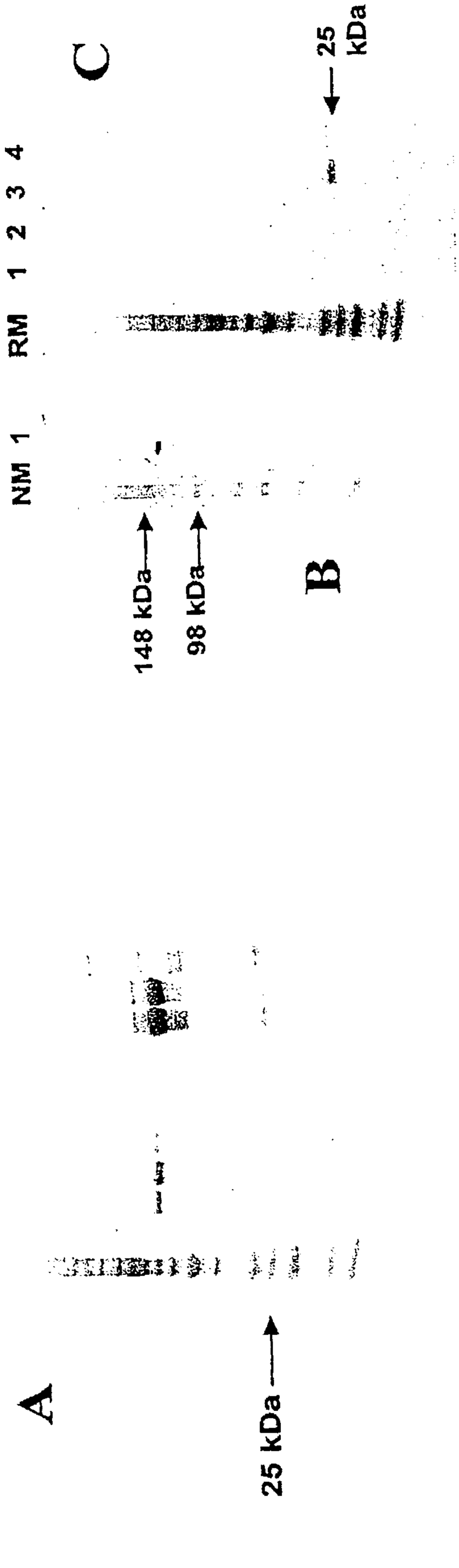

A
M 1 2 3 4
25 kDa
B
NM 1
RM 1 2 3 4
148 kDa
98 kDa
25 kDa
C
5 6 7 8 9 10 11
D
25 kDa

FIGURE 3

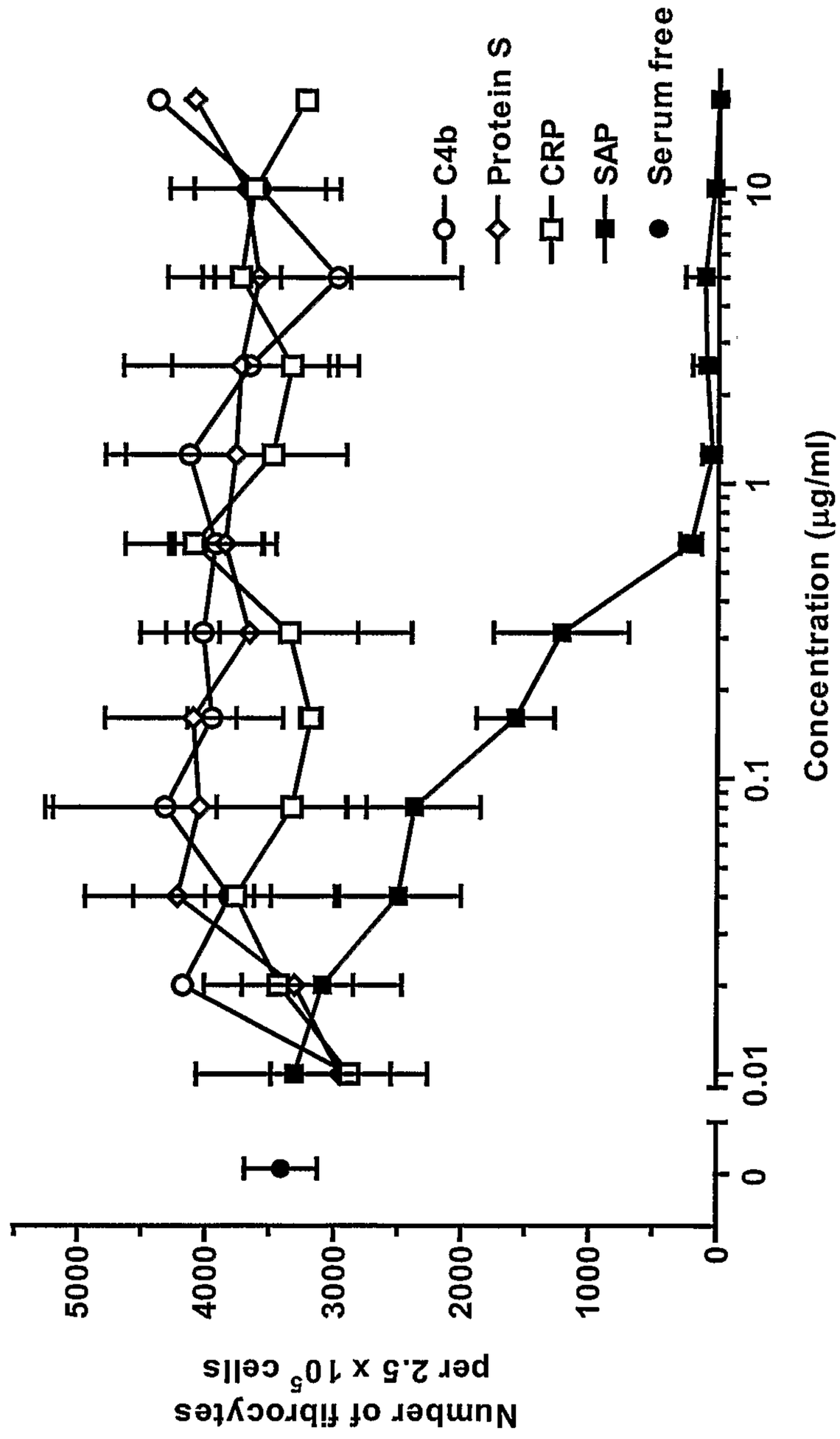

Number of fibrocytes
per 2.5 x 10$^5$ cells
5000
4000
3000
2000
1000
0
C4b
Protein S
CRP
SAP
Serum free
0
0.01
0.1
1
10
Concentration (µg/ml)

FIGURE 4

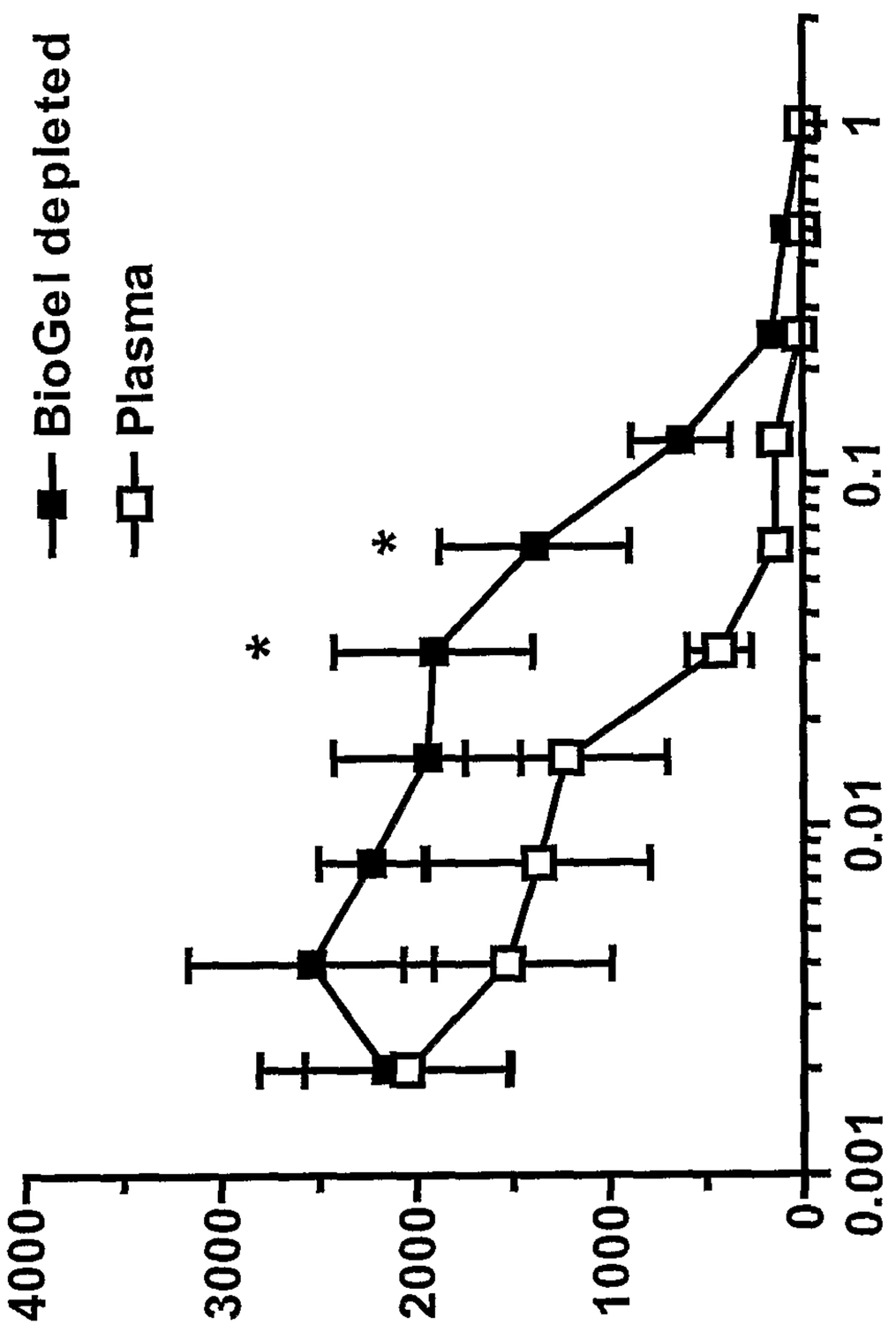

BioGel depleted
Plasma
*
*
4000
3000
2000
1000
0
0.001
0.01
0.1
1
Percentage plasma
Number of fibrocytes
per 2.5 x 10^5 cells

FIGURE 5A

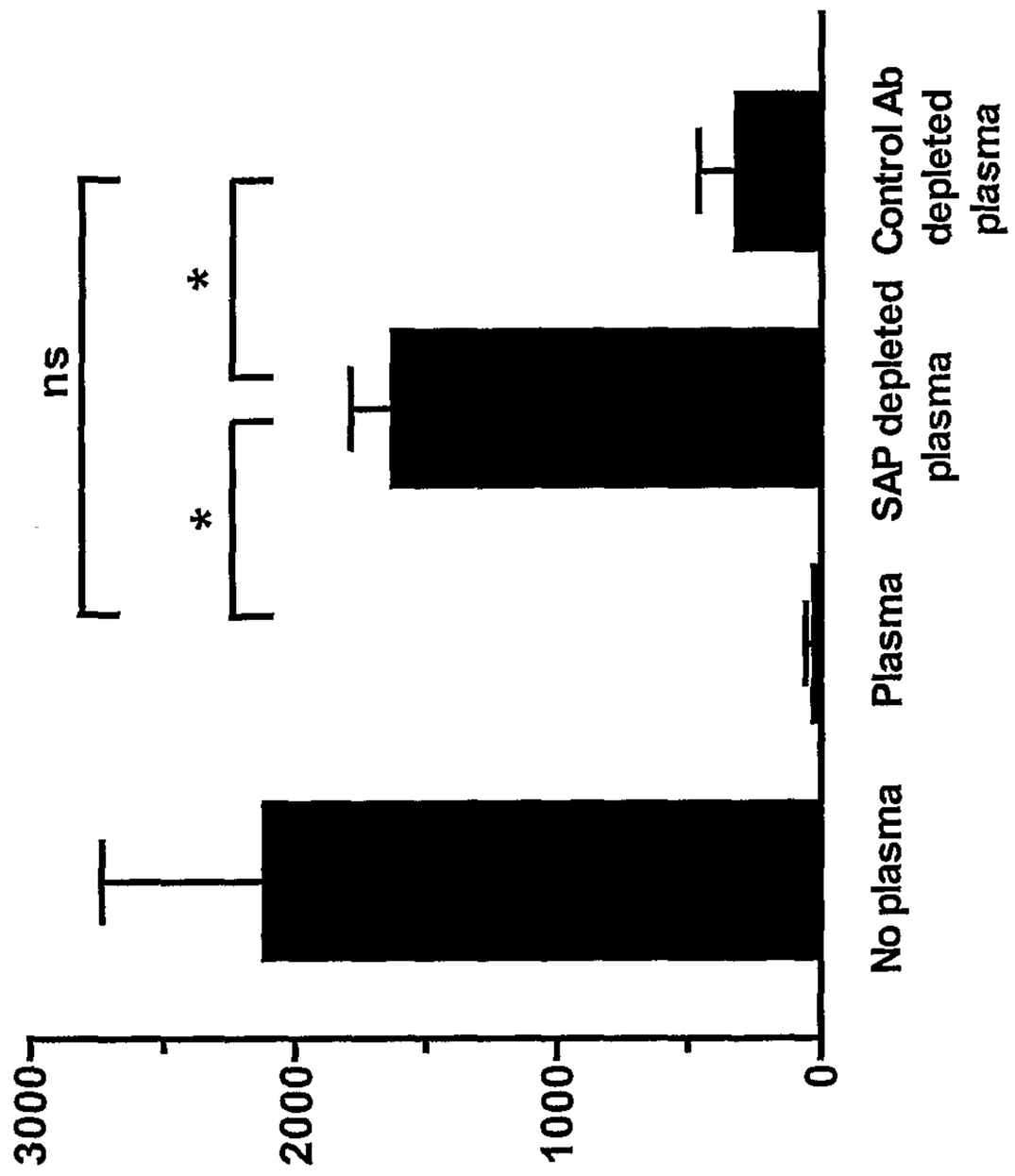

Number of fibrocytes
per 2.5 x 10^5 cells
3000
2000
1000
0
No plasma
Plasma
SAP depleted plasma
Control Ab depleted plasma
ns
*
*

FIGURE 5B

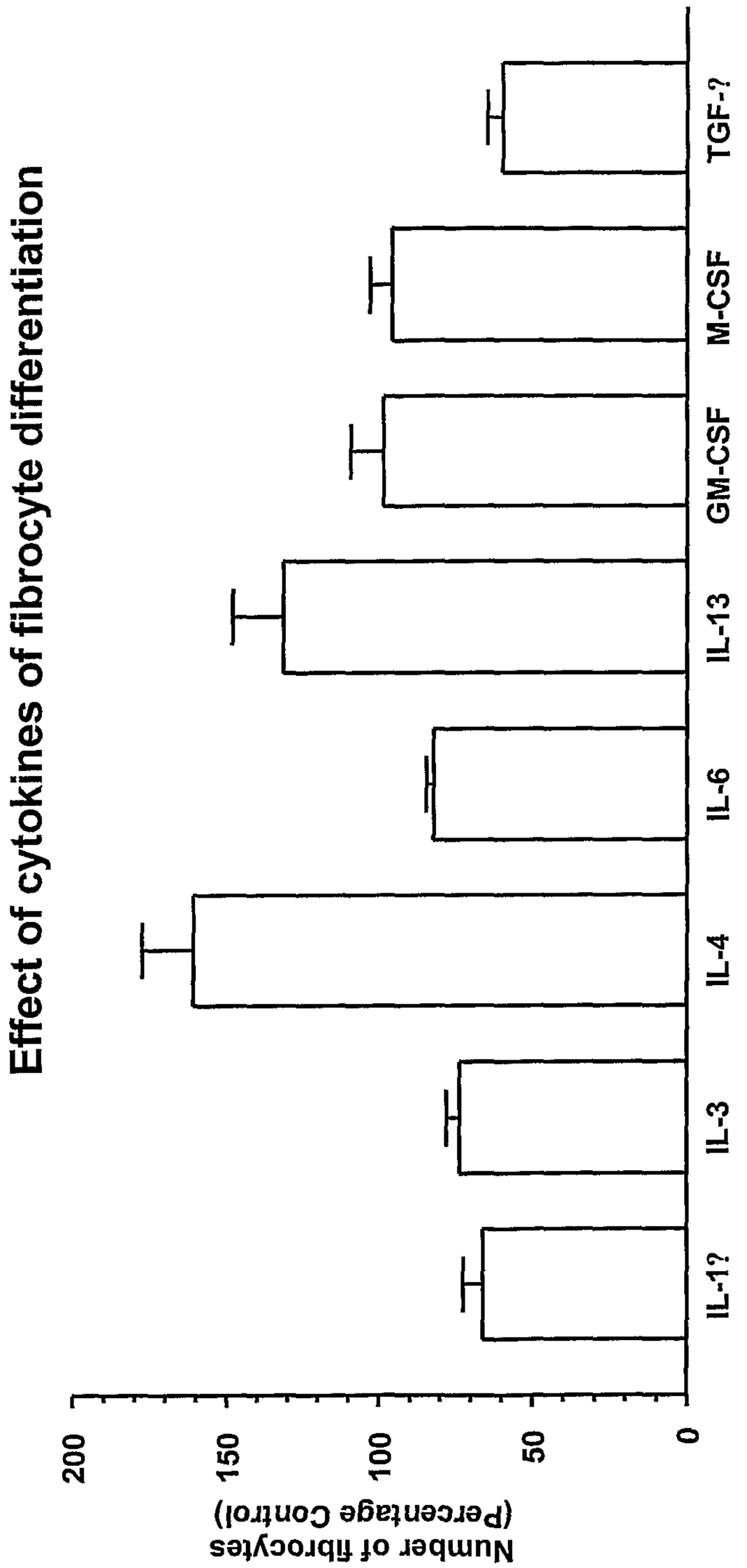

Effect of cytokines of fibrocyte differentiation
IL-6
IL-13
GM-CSF
M-CSF
TGF-?
IL-4
IL-3
IL-1?
Cytokine (1 ng/ml)
200
150
100
50
0
Number of fibrocytes
(Percentage Control)

FIGURE 6A

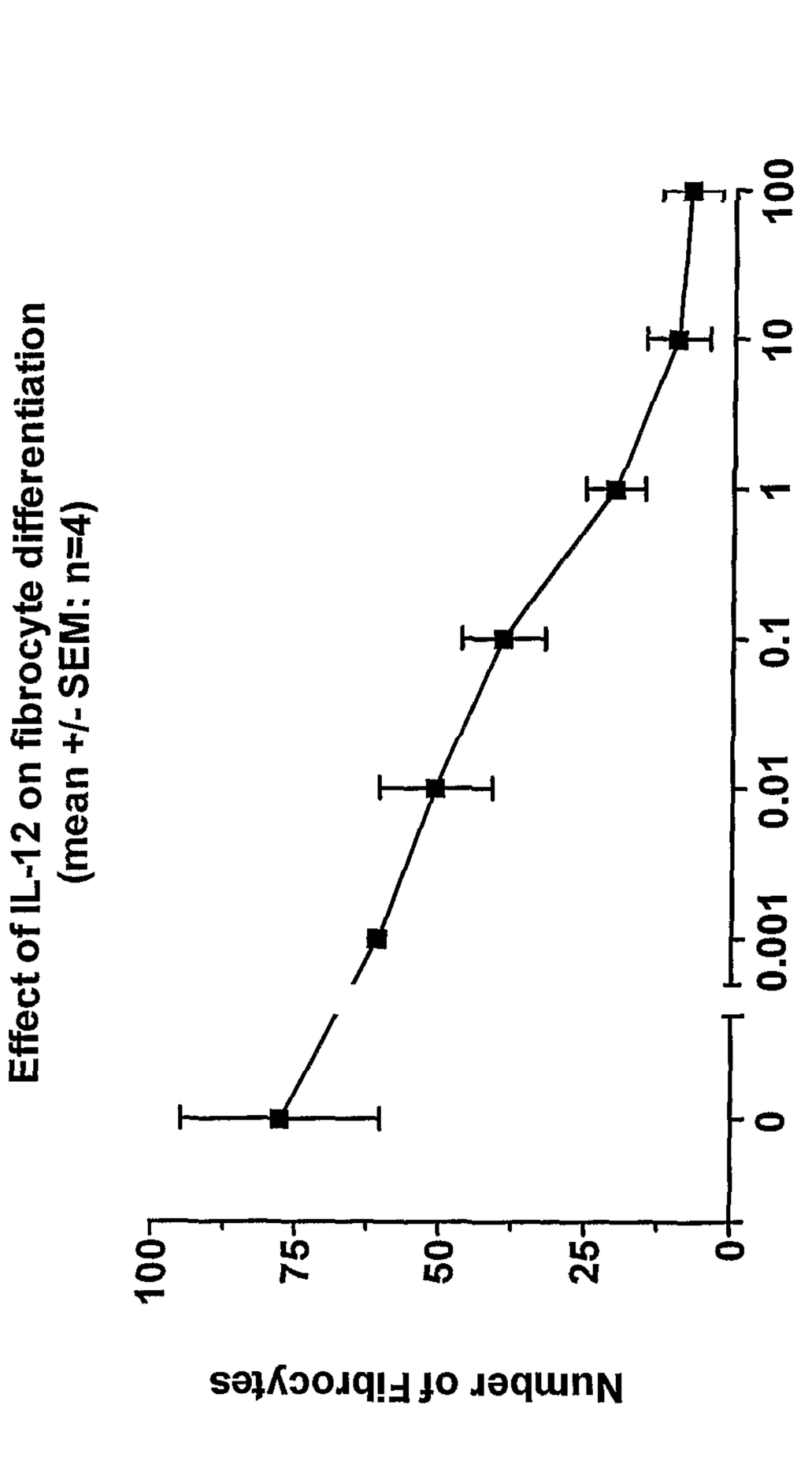
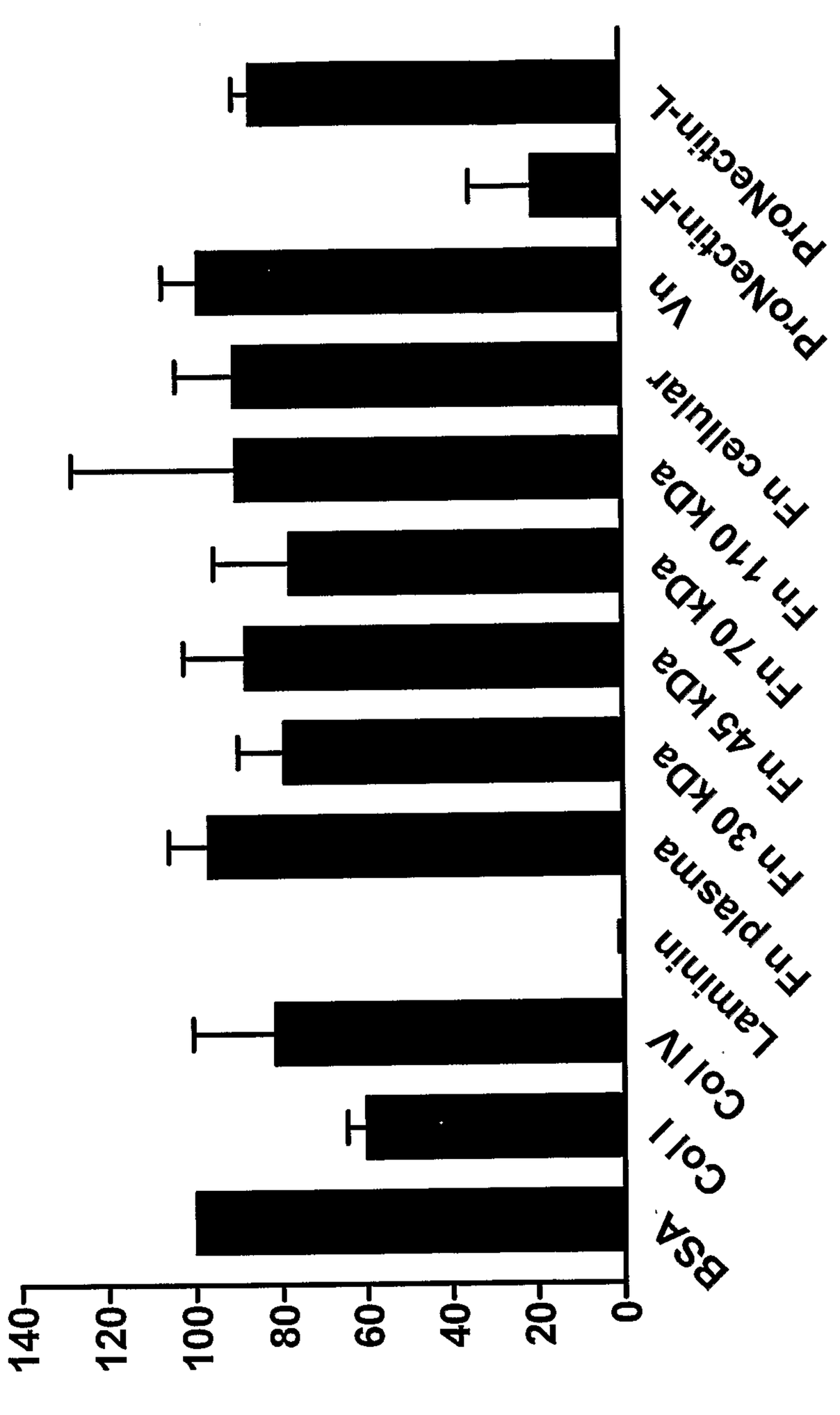

Effect of IL-12 on fibrocyte differentiation
(mean +/- SEM; n=4)
Number of Fibrocytes
100
75
50
25
0
0
0.001
0.01
0.1
1
10
100
IL-12 (ng/ml)

FIGURE 6B




ECM Proteins (@ 10 µg/ml: Vn @ 1 µg/ml)

FIGURE 7A

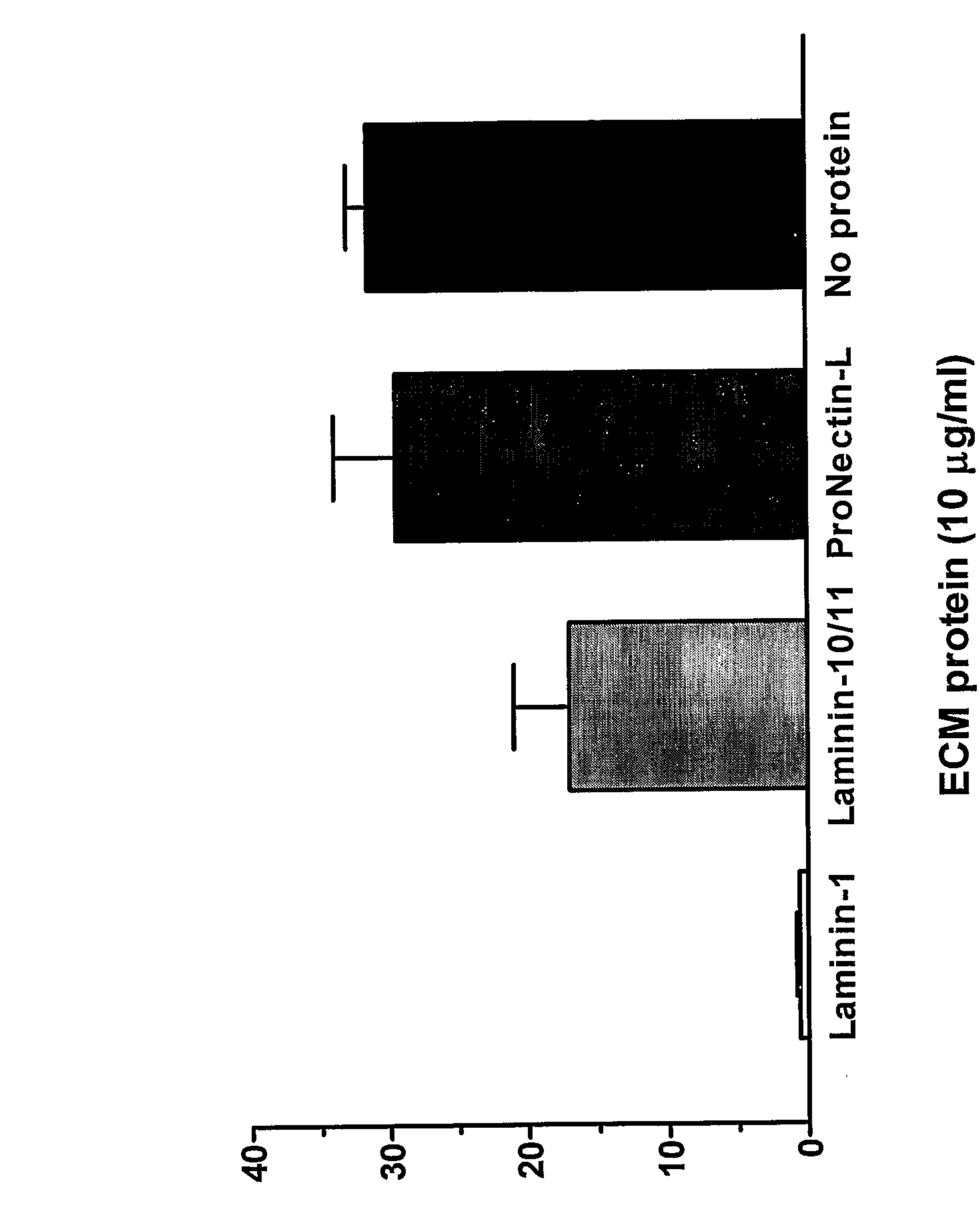

Number of fibrocytes
0
10
20
30
40
Laminin-1
Laminin-10/11
ProNectin-L
No protein
ECM protein (10 µg/ml)

FIGURE 7B

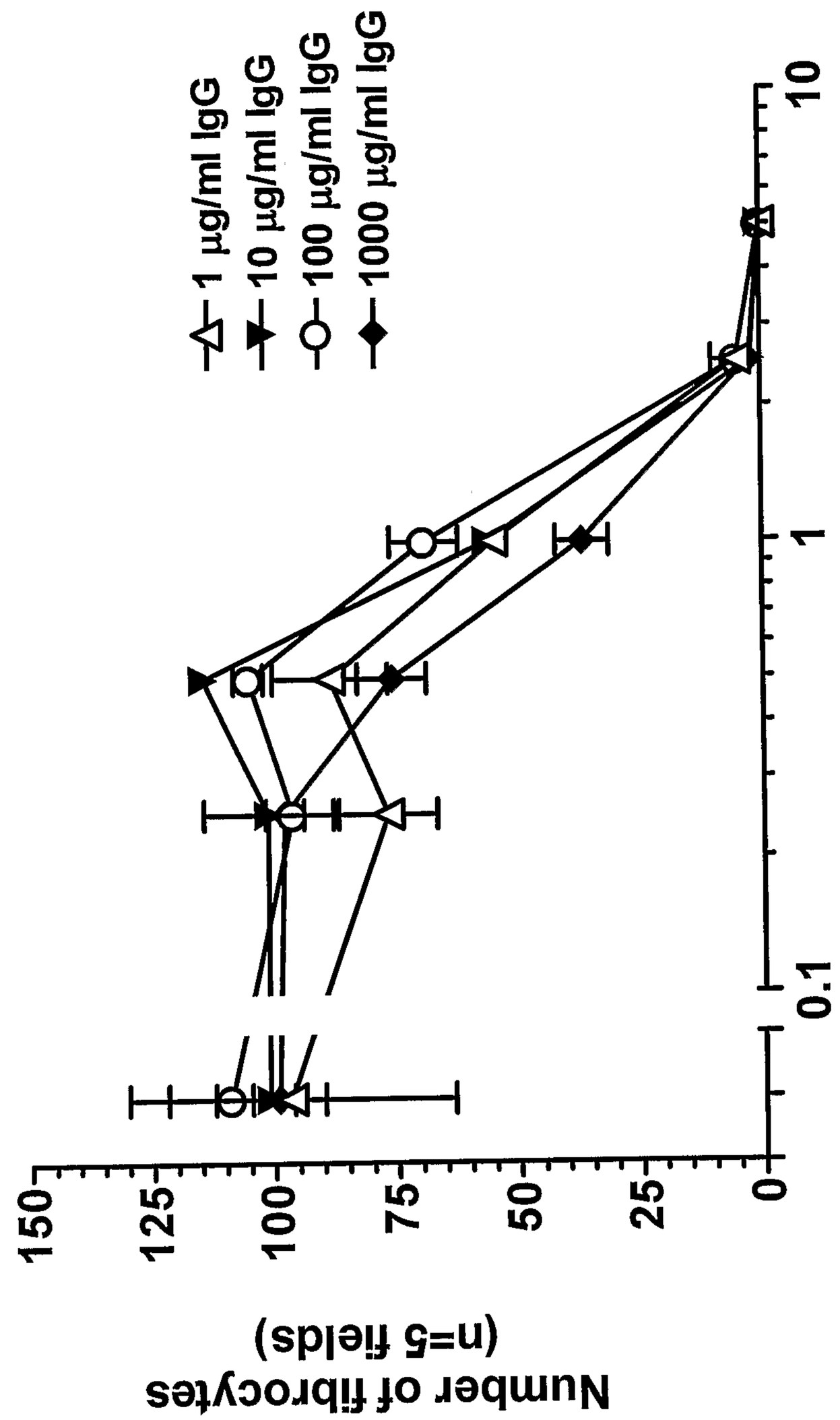

1 µg/ml IgG
10 µg/ml IgG
100 µg/ml IgG
1000 µg/ml IgG
SAP (µg/ml)
10
1
0.1
0
25
50
75
100
125
150
Number of fibrocytes
(n=5 fields)

FIGURE 8

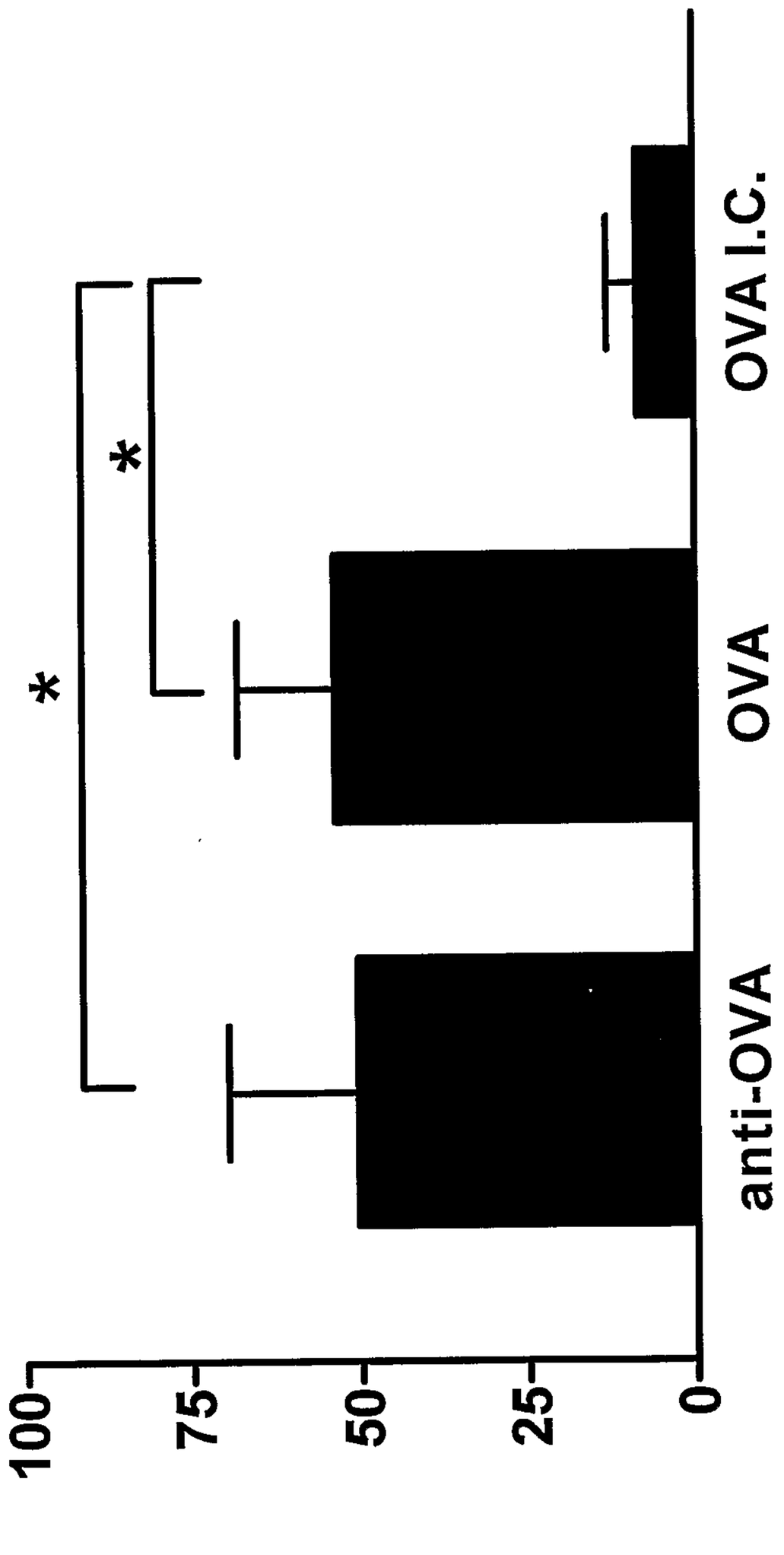

Percentage of fibrocytes
compared to control
100
75
50
25
0
anti-OVA
OVA
OVA I.C.
*
*

FIGURE 9A

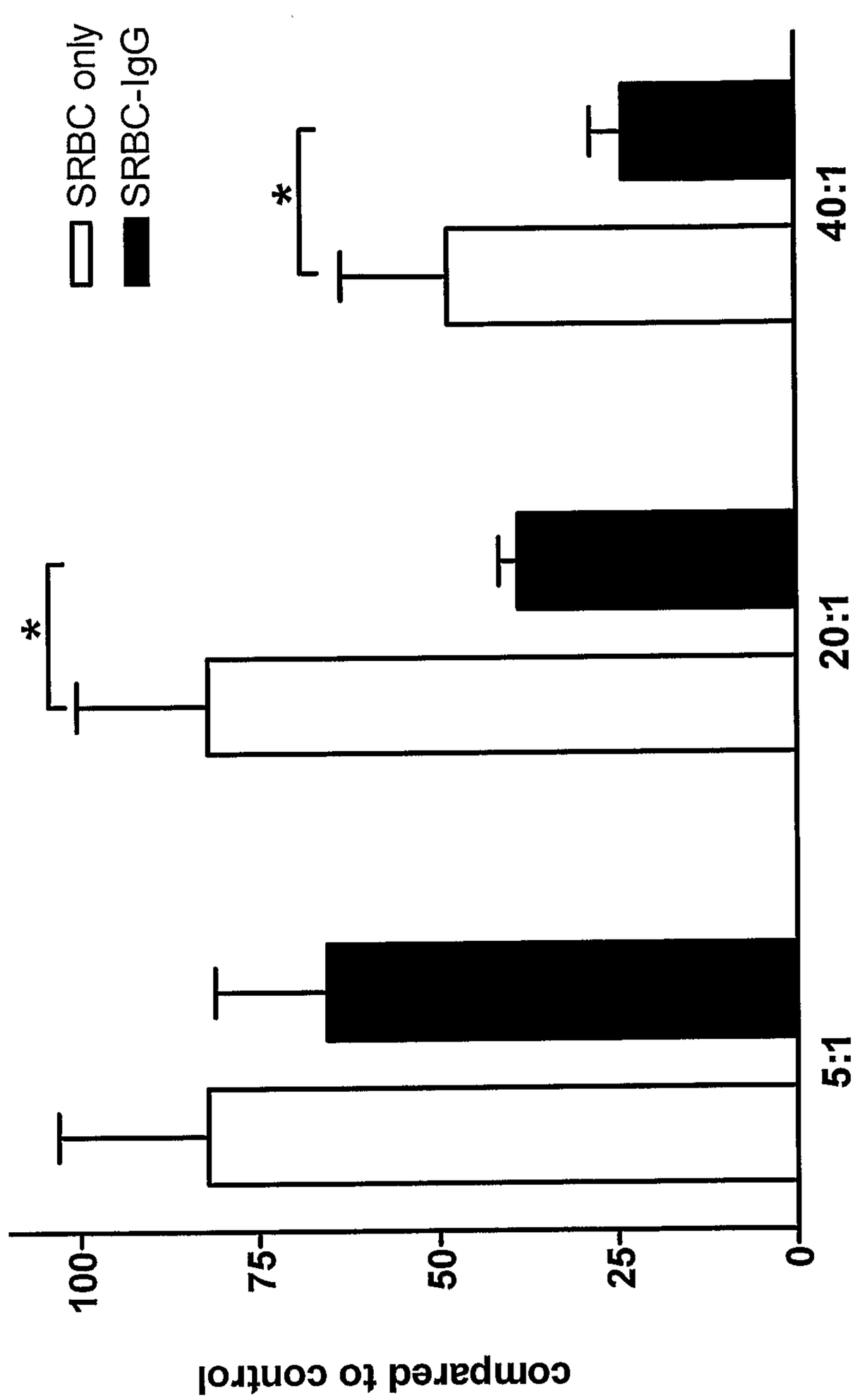

SRBC only
SRBC-IgG
*
*
20:1
40:1
Ratio SRBC:monocytes
5:1
100
75
50
25
0
Number of fibrocytes
compared to control

FIGURE 9B

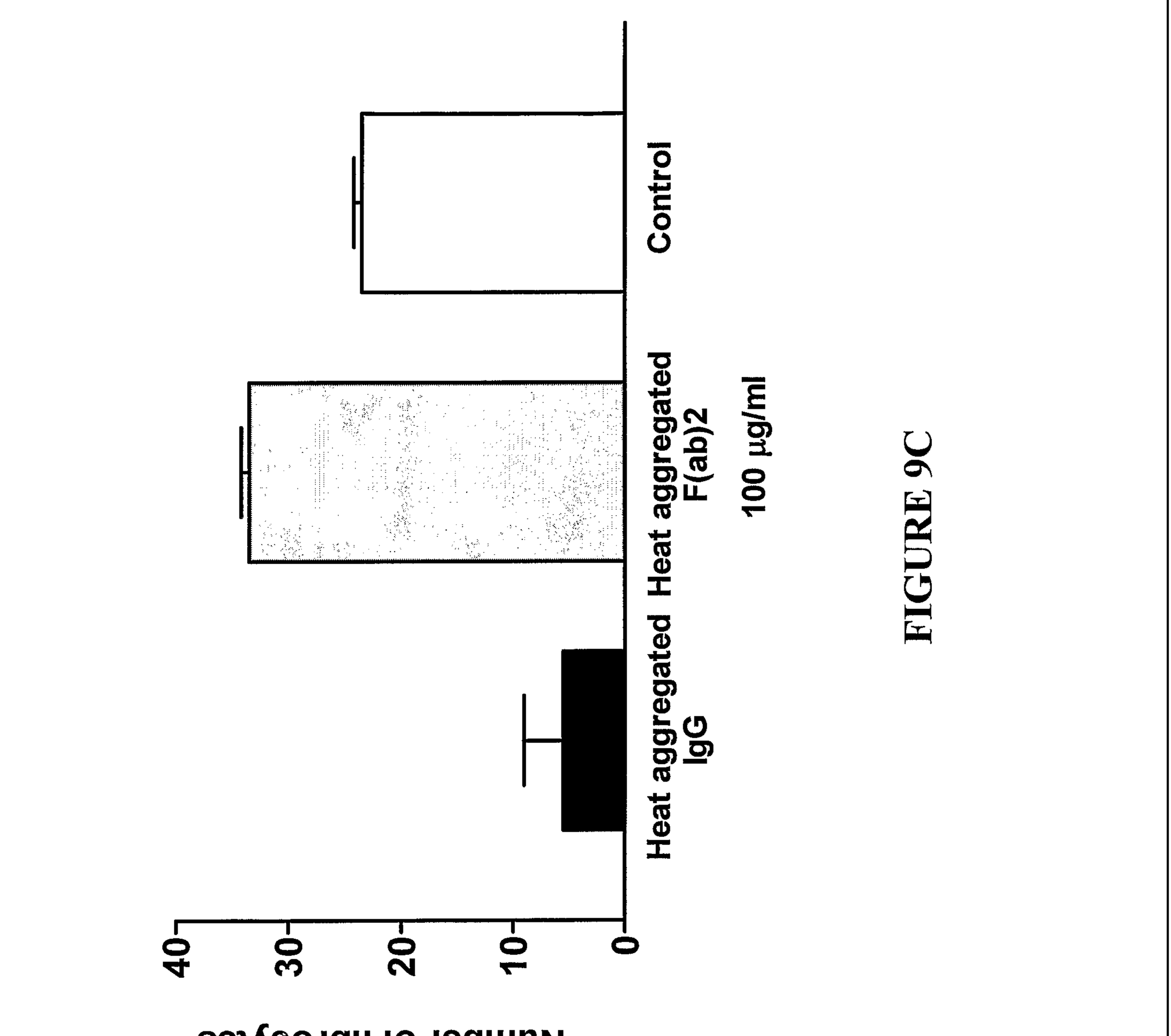

Number of fibrocytes
40
30
20
10
0
Heat aggregated IgG
Heat aggregated F(ab)2
100 µg/ml
Control

FIGURE 9C

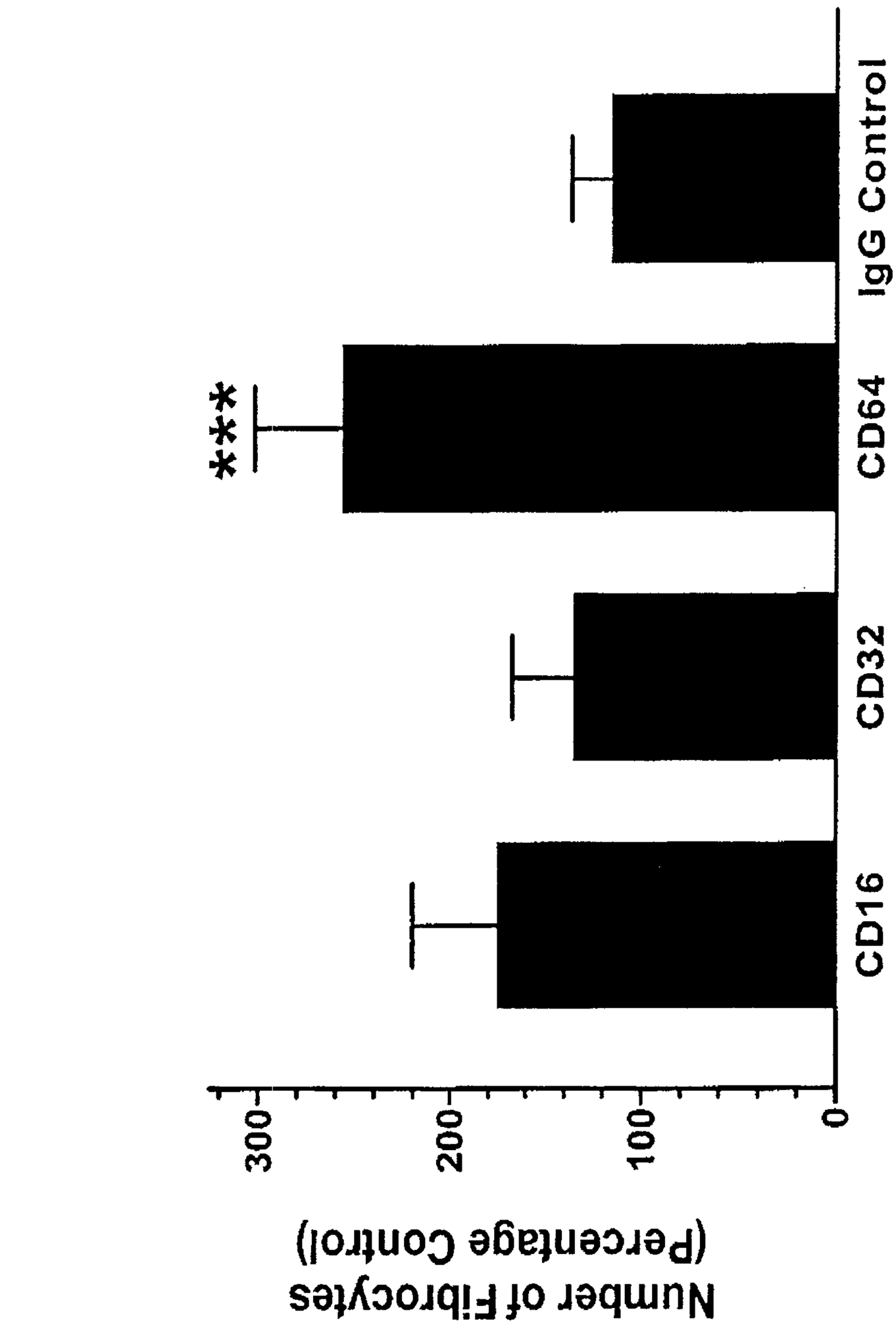
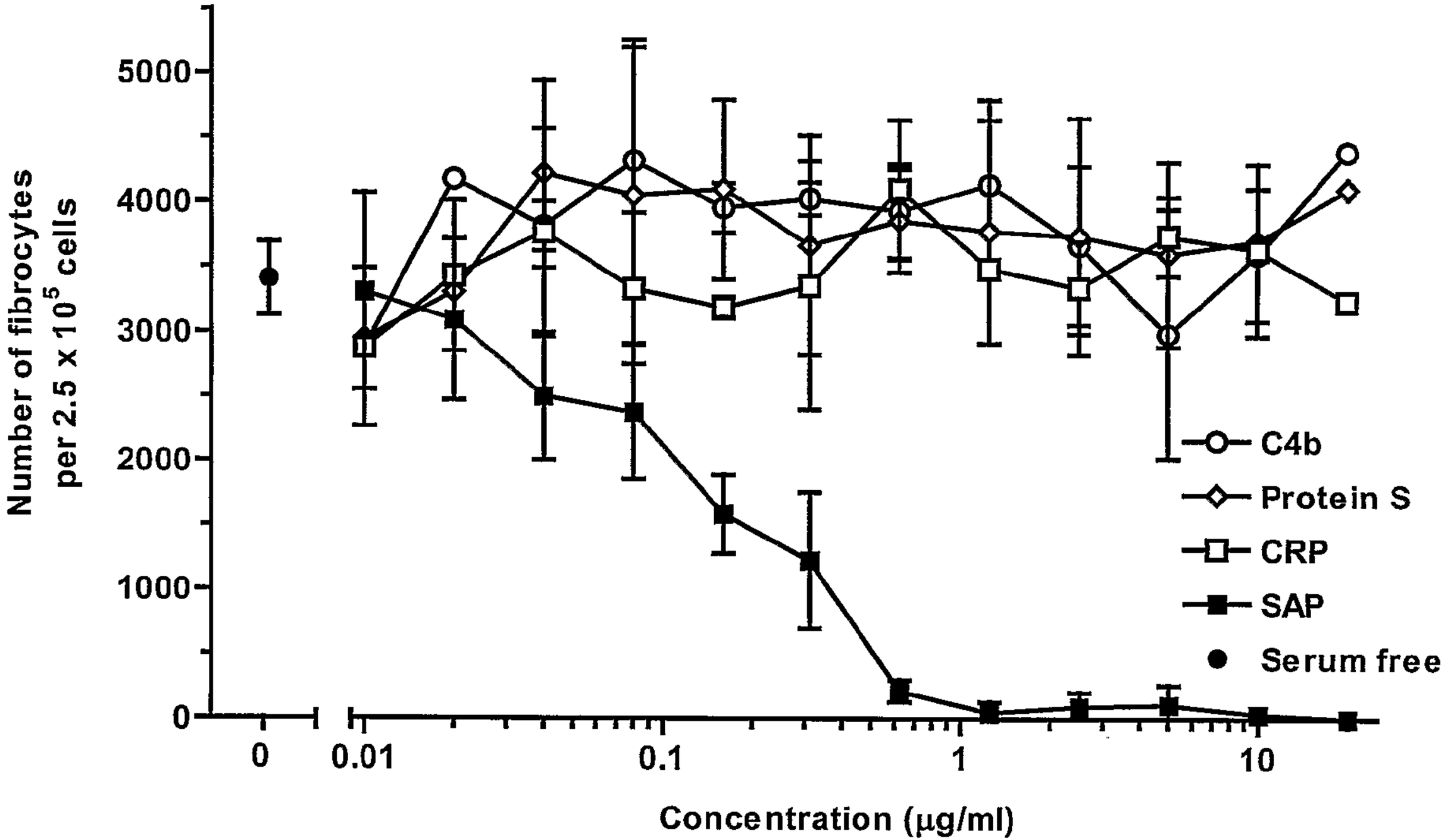

****
CD64
IgG Control
CD32
CD16
300
200
100
0
Number of Fibrocytes
(Percentage Control)

FIGURE 10


Number of fibrocytes per 2.5 x 10^5 cells
5000
4000
3000
2000
1000
0
0
0.01
0.1
1
10
Concentration (μg/ml)
C4b
Protein S
CRP
SAP
Serum free