Aggregated IgG inhibits the differentiation of human fibrocytes

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Abstract: Fibrocytes are fibroblast-like cells, which appear to participate in wound healing and are present in pathological lesions associated with asthma, pulmonary fibrosis, and scleroderma. Fibrocytes differentiate from CD14+ peripheral blood monocytes, and the presence of serum delays this process dramatically. We previously purified the factor in serum, which inhibits fibrocyte differentiation, and identified it as serum amyloid P (SAP). As SAP binds to Fc receptors for immunoglobulin G (IgG; FcγRs), FcγR activation may be an inhibitory signal for fibrocyte differentiation. FcγR are activated by aggregated IgG, and we find aggregated but not monomeric, human IgG inhibits human fibrocyte differentiation. Monoclonal antibodies that bind to FcγRI (CD64) or FcγRII (CD32) also inhibit fibrocyte differentiation. Aggregated IgG lacking Fe domains or aggregated IgA, IgE, or IgM do not inhibit fibrocyte differentiation. Incubation of monocytes with SAP or aggregated IgG inhibited fibrocyte differentiation. Using inhibitors of protein kinase enzymes, we show that Syk- and Src-related tyrosine kinases participate in the inhibition of fibrocyte differentiation. These observations suggest that fibrocyte differentiation can occur in situations where SAP and aggregated IgG levels are low, such as the resolution phase of inflammation. J. Leukoc. Biol. 79: 1242–1251; 2006.

Key Words: monocytes · inflammation · cellular differentiation · serum amyloid P · FcγR

INTRODUCTION

Tissue damage or the presence of pathogens leads to the recruitment and activation of peripheral blood monocytes [1, 2]. These cells differentiate into tissue macrophages and are a key component of the innate immune response essential for the control of many infections. After the removal of pathogenic organisms, macrophages remove apoptotic cells and promote tissue regeneration by stimulating fibroblast proliferation and extracellular matrix (ECM) production [3, 4].

The source of the fibroblasts responsible for the repair of wound lesions or the hyperplasia characteristic of chronic inflammation is still controversial. The conventional hypothesis is that local quiescent fibroblasts migrate into the affected area, produce ECM proteins, and promote wound contraction [5]. An alternative hypothesis is that circulating fibroblast-like cell precursors (fibrocyte precursors), present within the blood, migrate to sites of injury, where they differentiate into fibroblast-like cells—fibrocytes—and mediate tissue repair [6–9]. Fibrocytes express markers of hematopoietic (CD45, major histocompatibility complex II, CD34) and stromal cells (collagen I and III and fibronectin) [10, 11]. Fibrocytes, at sites of tissue injury, secrete inflammatory cytokines and ECM proteins and promote angiogenesis and wound contraction [6, 12]. Fibrocytes are also associated with the formation of fibrotic lesions after infection or inflammation and are implicated in fibrosis associated with autoimmune diseases [11, 13–17].

Fibrocyte precursors originate from ~10% of circulating CD14-positive/CD16-negative peripheral blood monocytes [6, 18, 19] (data not shown). At least two factors promote the differentiation of monocytes into fibrocytes. First, direct contact between CD14+ monocytes and T cells increases the number of fibrocytes [6]. Second, transforming growth factor-β acts as a maturation factor for fibrocytes once differentiation has occurred [6, 19].

We have found that the differentiation of fibrocytes from monocytes is inhibited by serum amyloid P (SAP), which overrules the positive effect of T cells [18]. In the absence of serum or purified SAP, monocytes differentiate into fibrocytes within 3 days. SAP, a member of the pentraxin family of proteins, which includes C-reactive protein (CRP), is produced by the liver, secreted into the blood, and circulates in the blood as stable pentamers [20–23]. SAP appears to play a role in the initiation and resolution phases of the immune response [24–26]. SAP binds to sugar residues on the surface of bacteria, leading to their opsonization and engulfment [23, 24]. 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 [25–27].

Receptors for the Fc portion of immunoglobulin G (IgG; FcγRs) are found on the surface of a variety of hematopoietic cells. There are four distinct classes of FcγR. FcγRI (CD64) is the high-affinity receptor for IgG expressed by peripheral blood monocytes and binds monomeric IgG with a high affinity [28, 29]. FcγRII (CD32) and FcγRIII (CD16) are low-affinity receptors for IgG and only bind aggregated IgG efficiently. FcγRII is expressed by peripheral blood B cells and monocytes, whereas FcγRIII is expressed by natural killer cells and a subpopulation of monocytes [30–32]. Recently, a new FcγR has been identified in mice [33]. FcγRIV is present on murine...
peripheral blood monocytes and neutrophils, macrophages, and dendritic cells and binds murine IgG2a and IgG2b antibodies efficiently. There is a putative, human FcyRIV gene, but the biological functions of the protein, such as ligand specificity and cellular expression, are as yet unknown [34].

Bacteria and proteins bound by SAP are removed by phagocytic cells, such as macrophages, as a result of the ability of these cells to upregulate (and cellular expression, are as yet unknown [34].

As SAP inhibits fibrocyte differentiation and binds to FcγR and as IgG binds to FcγR, we determined whether IgG inhibits fibrocyte differentiation [18, 35]. We find that aggregated but not monomeric IgG inhibits fibrocyte differentiation and that FcyRI and FcγRII are involved. These observations suggest that monocytes will differentiate into fibrocytes in situations where SAP and aggregated IgG levels are low.

MATERIALS AND METHODS

Antibodies, proteins, and inhibitors

Human IgA, IgG, IgM, and IgG F(ab’2)2 fragments were from Jackson Immunoresearch Laboratories (West Grove, PA). Goat F(ab’2) anti-human IgG, goat F(ab’2) anti-murine IgG, goat F(ab’2) anti-rabbit IgG, and whole mouse IgG1, whole mouse IgG2a, and mouse F(ab’2)2 IgG1 isotype control antibodies were from Southern Biotechnology Associates Inc. (Birmingham, AL). Sheep red blood cells (SRBC) and rabbit anti-SRBC were from ICN (Irvine, CA). Purified human SAP and human IgE were from Calzyme BioMedicals (San Diego, CA). F(ab’2)2 fragments of the blocking monoclonal antibodies (mAb) to FcγR (Clone 10.1, IgG1 isotype) and FcγRII (Clone 7.3, IgG1 isotype) were from Ancell (Bayport, MN) [29, 45–47]. The following primary mAb were used for immunohistochemistry: anti-CD14 [Clone M5E2, IgG2a, BD Biosciences (San Diego, CA)], anti-CD34 [Clone QBend10, IgG1, GeneTex (San Antonio, TX)], CD45 (Clone 1G10, IgG1, BD Biosciences), pan-CD45 (clone H130, IgG1, BD Biosciences), anti-prolactin 4-hyrdoside [Clone SBS, IgG1, Dako (Carpinteria, CA)], and anti-α smooth muscle actin [α-SMA; Clone 1A4, IgG2a, Sigma-Aldrich (St. Louis, MO)]. Collagen-I was detected using an affinity-purified rabbit polyclonal antibody from Rockland (Gilbertsville, PA). 4-Amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2, AG 1879), 4-amino-7-phenylpyrazolo[3,4-d]pyrimidine (PP3), and the Syk inhibitor 3-(1-methyl-1H-indol-3-yl)-methylene)-2-oxo-2,3-dihydro-1H-indole-5-sulfonamide were from Calbiochem.

Cell culture and fibrocyte differentiation assay

Peripheral blood mononuclear cells (PBMC) were isolated fromuffy coats (Gulf Coast Regional Blood Center, Houston, TX) by Ficoll-Paque Plus (Amersham Biosciences, Piscataway, NJ) as described previously [38]. Cells were incubated in serum-free medium (SFM), which consists of RPMI (Invitrogen, Carlsbad, CA), supplemented with 10 mM HEPES (Invitrogen), 2 mM glu-tamine, 100 μM penicillin, 100 μg/ml streptomycin, and 1× ITS-3 [500 μg/ml bovine serum albumin (BSA), 10 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml sodium selenite, 5 μg/ml linoleic acid, and 5 μg/ml oleic acid; Sigma-Aldrich]. Normal human serum (Sigma-Aldrich) was added at the stated concentrations. PBMC were cultured in flat-bottomed, 96-well, tissue-culture plates (Type 353072, BD Biosciences Discovery Labware, Bedford, MA) in 200 μl vol at 2.5 × 10⁵ cells per ml in a humidified incubator containing 5% CO₂ at 37°C for the indicated times. Fibrocytes were identified by morphology in viable cultures as adherent cells with an elongated, spindle-shaped morphology as distinct from lymphocytes or adherent monocytes. Enumeration of fibrocytes was performed on cells cultured for 5 days. Cells were air-dried, fixed in methanol, and stained with eosin and methylene blue (Hema 3 stain, Fisher Scientific, Hampton, NH). Fibrocytes from duplicate wells were counted in five different 0.06 mm-diameter fields per well, using the above criteria of an elongated spindle shape and the presence of an oval nucleus. All cultures were counted by at least two independent observers. We observed 1.2 ± 0.6 × 10⁵ (mean±SD, n=12 healthy individuals) fibrocytes per ml peripheral blood, with a range of 3.7 × 10⁵–2.9 × 10⁶ fibrocytes per ml. These results are similar to previous data and indicate that fibrocyte precursors account for ~1% of the total PBMC [6, 17–19].

Immunohistochemistry

PBMC were cultured on eight-well glass microscope slides (Lab-Tek, Nalge Nunc International, Naperville, IL) in serum-free medium for 5 days. Cells were air-dried for at least 60 min before fixation in acetone for 15 min. Endogenous peroxidase was quenched for 15 min with 0.3% H₂O₂ in phosphate-buffered saline (PBS), and then nonspecific binding was blocked by incubation in 4% BSA in PBS for 60 min. Slides were incubated with 5 μg/ml primary antibodies in PBS containing 4% BSA for 60 min. Isotype-matched, irrelevant antibodies were used as controls. Slides were then washed in three changes of PBS over 15 min and incubated for 30 min with 2.5 μg/ml biotinylated goat F(ab’2)2 anti-mouse IgG or biotinylated goat F(ab’2)2 anti-rabbit IgG (cross-adsorbed against human Ig, Southern Biotechnology Associates Inc.). After washing, the biotinylated antibodies were detected by Extravidin peroxidase (Sigma-Aldrich). Staining was developed with diaminobenzidine (Sigma-Aldrich) for 3 min and counterstained for 10 s with Gill’s hematoxylin #3 (Sigma-Aldrich). All procedures were at room temperature.

FcγR ligation

Heat-aggregated Igs were prepared by diluting whole IgA, IgE, IgG, IgM, or IgG F(ab’2)2 fragments to 2 mg/ml in 0.9% saline and then heating to 65°C for 60 min. Large protein precipitates were then removed by centrifugation at 1000 g for 2 min. Isolation of monomeric IgG and clarification of SAP preparations were performed by ultracentrifugation at 100,000 × g for 30 min at 4°C. Monomeric IgG was cross-linked by the addition of 500 ng/ml goat F(ab’2)2 anti-human IgG for 30 min at 4°C. Opossum SRBC were prepared by incubating a 1% suspension of SRBC in RPMI 1640 with the highest concentration of nonagglutinating polyclonal rabbit anti-SRBC (generally 1/2000). SRBC were then washed three times in RPMI and added to PBMC at a range of ratios from 1:1 to 50:1, SRBC:monocyte. Monocytes were enumerated by morphology using a hemocytometer. To cross-link individual FcγR, PBMC were incubated for 30 min at 4°C with 1 μg/ml F(ab’2)2 anti-FcγRI (10.1) or F(ab’2)2 anti-FcγRII (7.3), and receptors were then cross-linked by the addition of 500 ng/ml goat F(ab’2)2 anti-mouse IgG for 30 min at 4°C. PBMC were then warmed to 37°C and cultured for 5 days. To block individual FcγR, PBMC were incubated for 30 min at 4°C with 1 μg/ml F(ab’2)2 anti-FcγRI (10.1) or F(ab’2)2 anti-FcγRII (7.3) mAb. SAP at 20 ng/ml was added to PBMC for 5 days. Incubation of Src-related tyrosine kinase (SRTK) and Syk was achieved by incubating PBMC at 4°C with 10 nM PP2, 10 nM PP3, or the Syk inhibitor for the indicated times. PBMC were then washed twice in ice-cold, serum-free medium and then cultured with anti-FcγRI or anti-FcγRII mAb, SAP, or aggregated IgG as indicated.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software (GraphPad Software, San Diego, CA). Differences between two groups were assessed by Student’s t-test. Differences among multiple groups were assessed by ANOVA using Bonferroni’s post-test. Significance was defined as P < 0.05.
RESULTS

Monomeric IgG has little effect on fibrocyte differentiation

SAP binds to cells via FcγR, with a higher affinity for FcγRI compared with FcγRII and FcγRIII [35, 36]. Monocytes constitutively express FcγRI, and as this receptor binds monomeric IgG, it is saturated in vivo [28, 31]. To determine whether the presence of monomeric human IgG could affect fibrocyte differentiation or prevent SAP from inhibiting fibrocyte differentiation, human PBMC were cultured in serum-free medium in the presence of different concentrations of monomeric, human IgG for 30 min. We cultured PBMC in a serum-free medium system to reduce any unwanted interactions between the FcγR and possible ligands present in serum, such as IgG, CRP, or SAP, which at the concentrations indicated, was then added, and the cells were cultured for 5 days. As we reported previously, 1 μg/ml SAP in the absence of IgG inhibited fibrocyte differentiation significantly (P < 0.001; Fig. 1) [18]. The addition of monomeric, human IgG in a dose range from 0.25 to 100 μg/ml, which corresponds to 0.0025–1% serum, respectively, had no significant effect on the inhibition of fibrocyte differentiation by SAP (P = 0.44). In the absence of SAP, monomeric IgG had no statistically significant effect on the differentiation of monocytes to fibrocytes (P = 0.54). To ensure that the commercial preparations of SAP were not aggregated and thus providing a multimeric rather than a pentameric FcγR-binding protein, SAP was clarified by centrifugation at 100,000 g. PBMC were then incubated in serum-free medium with clarified or the standard preparation of SAP at 1 μg/ml. There was no difference in the ability of the two preparations to inhibit fibrocyte differentiation, suggesting that the SAP was not aggregated (data not shown).

To confirm that the spindle-shaped cells we identified as fibrocytes are indeed fibrocytes, we stained the cells for known fibrocyte markers including CD14, CD34, CD43, CD45, collagen-I, and prolyl 4-hydroxylase (a key enzyme in the production of collagen), as well as α-SMA, a marker of activated fibrocytes [6, 10, 18]. Greater than 90% of the fibrocytes expressed CD34, CD43, CD45, collagen-I, and prolyl 4-hydroxylase, and approximately one-third of them expressed α-SMA (Fig. 2). A small number of the fibrocytes expressed low levels of CD14, which probably reflects their recent differentiation from CD14-positive monocytes (Fig. 2) [6, 18].

Cross-linked IgG inhibits fibrocyte differentiation

SAP is a pentameric protein with five potential FcγR-binding sites per molecule, which may cross-link FcγR effectively without additional proteins [18, 35]. To test whether IgG, when cross-linked, could also inhibit fibrocyte differentiation, we incubated PBMC for 60 min at 4°C in serum-free medium with...
increasing concentrations of monomeric, human IgG. The cells were then washed to remove unbound IgG. Goat F(ab')2 anti-human IgG was then added as the cross-linking agent, and PBMC were cultured for 5 days. In the absence of human IgG, the goat F(ab')2 had no significant effect on fibrocyte differentiation (Fig. 3A). In the absence of a cross-linking agent, monomeric IgG had no effect on fibrocyte differentiation (Figs. 1 and 3A). As the initial reagent in these experiments was monomeric, human IgG, which will only bind efficiently to FcγRI, these data suggest that cross-linking FcγRI on monocytes may be a mechanism that inhibits the differentiation of fibrocytes [28].

To determine whether other IgG immune complexes could influence monocyte-to-fibrocyte differentiation, we examined the effect of particulate, opsonized SRBC complexes. PBMC were cultured for 5 days in serum-free medium with rabbit IgG bound (opsonized) to SRBC at a ratio of 20:1, SRBC:monocytes. Results are expressed as the mean ± SEM of the number of fibrocytes per 2.5 × 10⁶ cells (n=3 separate donors). Compared with SRBC (E only), SRBC opsonized with rabbit anti-SRBC (E-IgG) inhibited fibrocyte differentiation significantly (P=0.018), as determined by a Student’s t-test. (D) PBMC were cultured as above in the presence of the indicated concentrations of heat-aggregated, human IgG or heat-aggregated, human F(ab')2. Results are expressed as the mean ± SEM of the number of fibrocytes per 2.5 × 10⁶ cells (n=3 separate donors). Compared with heat-aggregated F(ab')2, heat-aggregated, whole IgG inhibited fibrocyte differentiation significantly at concentrations of 25 µg/ml and higher, as determined by a Student’s t-test. (D) PBMC were cultured as above in the presence of 20 µg/ml native or heat-aggregated, human IgA, IgE, IgG, or IgM. Only PBMC cultured in heat-aggregated IgG inhibited fibrocyte differentiation significantly (P<0.01), as determined by ANOVA (n=3 separate donors). *, P < 0.05; **, P < 0.01.
ligation of FcγRI and FcγRII is an inhibitory signal for fibrocyte differentiation [28, 49]. Together, these data suggest that cross-linked IgG inhibits fibrocyte differentiation.

Cross-linked IgG requires its Fc region to inhibit fibrocyte differentiation

IgG binds to FcγR via the Fc portion of IgG. To test the hypothesis that cross-linked IgG inhibits fibrocyte differentiation by ligating FcγR, we determined whether cross-linked F(ab’)2 IgG, which has no Fc region, could inhibit fibrocyte differentiation. We found that heat-aggregated, whole, human IgG but not heat-aggregated F(ab’)2 was a potent inhibitor of fibrocyte differentiation (Fig. 3C). These data indicate that heat-aggregated IgG also inhibits fibrocyte differentiation and that the Fc region of IgG is necessary for this effect.

Only IgG immune complexes inhibit fibrocyte differentiation

Monocytes express IgA receptors, low numbers of IgE receptors, and the recently characterized IgM receptor [49–51]. To determine if other Igs inhibit fibrocyte differentiation, we added native or heat-aggregated IgA, IgE, IgG, or IgM to PBMC. Only heat-aggregated IgG but not monomeric IgG or monomeric or heat-aggregated IgA, IgE, or IgM could inhibit fibrocyte differentiation (Fig. 3D). These data together suggest that ligation and cross-linking of FcγRs are inhibitory signals for monocyte-to-fibrocyte differentiation but that ligation of the other Ig receptors has no effect of fibrocyte differentiation.

Ligation of FcγRI (CD64) or FcγRII (CD32) inhibits fibrocyte differentiation

To determine if cross-linked IgG activates FcγR to inhibit fibrocyte differentiation, PBMC were cultured for 5 days in serum-free medium in the presence or absence of free or cross-linked F(ab’)2 antibodies to FcγRI or FcγRII. Cross-linking FcγRI or FcγRII alone inhibited fibrocyte differentiation significantly (Fig. 4). However, there was no additional inhibition when both receptors were cross-linked together, suggesting that no synergistic interaction occurs. These experiments show that we could inhibit fibrocyte differentiation to ≈50% by the addition of 1 μg/ml mAb. Greater inhibition could be achieved by incubating PBMC with higher concentrations of mAb (5 and 10 μg/ml); however, these concentrations of mAb also led to significant cell death (data not shown). Although not statistically significant, as determined by ANOVA, there was also a trend that bivalent F(ab’)2 mAb, especially anti-FcγRI, were able to inhibit fibrocyte differentiation (Fig. 4). If these data are analyzed by Student’s t-test comparing PBMC cultured with F(ab’)2 FcγRI mAb with PBMC cultured with control mAb, then the data are significantly different (P=0.043). These results suggest that ligation and cross-linking of FcγRI or FcγRII can inhibit fibrocyte differentiation.

In an attempt to determine if SAP inhibits fibrocyte differentiation preferentially through FcγRI or FcγRII pathways, we preincubated PBMC with F(ab’)2-blocking mAb to FcγRI or FcγRII before the addition of an intermediate concentration of SAP (0.5 μg/ml). As expected, the addition of SAP inhibited fibrocyte differentiation significantly by ≈60% (P<0.001, comparing control to SAP-treated PBMC; data not shown). Preincubation of PBMC with blocking mAb to FcγRI, FcγRII, or FcγRI combined with FcγRII had no effect (data not shown). As discussed above, even the presence of bivalent F(ab’)2 mAb to FcγRI or FcγRII inhibits fibrocyte differentiation when compared with an IgG1 F(ab’)2 control mAb (Fig. 4). Therefore, using this technique, we were unable to determine which FcγR is ligated preferentially by SAP to inhibit fibrocyte differentiation.

To determine if SAP and aggregated IgG have an additive effect with respect to inhibiting fibrocyte differentiation, we cultured PBMC in the presence or absence of 1 μg/ml SAP and 20 μg/ml aggregated IgG. As expected, SAP and IgG inhibited fibrocyte differentiation significantly when added individually to PBMC as determined by ANOVA (P<0.001), but there was no additive or synergistic interaction when these molecules were used together (P>0.05; Fig. 5). This apparent lack of synergy may be because SAP binds aggregated IgG, and this may have partially reduced the ability of the SAP or IgG to inhibit fibrocyte differentiation [52].

Inhibition of fibrocyte differentiation is Syk- and Src kinase-dependent

FcγR activation leads to a cascade of signaling events initiated by two main kinases. The initial events following FcγR aggregation involve the phosphorylation of intracellular ITAMs present on the cytoplasmic tail of FcγRII or the FcγRII chain associated with FcγRI by SRTK [43]. In monocytes, the main Src kinases associated with FcγRI and FcγRII are hck and lyn [53–56]. The phosphorylated ITAMs then recruit cytoplasmic Src homology 2-containing kinases, especially Syk, to the ITAMs, and Syk then activates a series of downstream signaling molecules [43, 57].

To determine the roles of SRTK and Syk in the regulation of fibrocyte differentiation, we preincubated PBMC with the spe-
were then air-dried, fixed, and stained, and fibrocytes were enumerated by flow cytometry using anti-human CD45 and anti-human CD14 antibodies. Serum-free medium for 5 days in the presence or absence of 1 or 10 µg/ml heat-aggregated IgG, or SAP and heat-aggregated IgG together. Cells were then air-dried, fixed, and stained, and fibrocytes were enumerated by morphology. Results are expressed as the mean ± SEM of a number of fibrocytes per 2.5 × 10⁶ cells. Compared with PBMC, cultured in the presence of SAP or IgG individually, cells cultured in the presence of SAP and IgG had no significant difference in fibrocyte differentiation, as determined by ANOVA (n=3 separate donors).

Specific SRTK inhibitor PP2 or the specific Syk inhibitor 3-(1-methyl-1H-indol-3-yl-methylene)-2-oxo-2,3-dihydro-1H-indole-5-sulfonamide before the addition of SAP or aggregated IgG [58, 59]. We used this Syk inhibitor instead of the standard PKC, myosin light-chain kinase, tumor necrosis factor-induced nuclear factor-κB activation, and interferon-α-mediated signaling via signal transducer and activator of transcription proteins [60–63]. Inhibition of fibrocyte differentiation by aggregated IgG was abolished by PP2 and the Syk inhibitor (Fig. 6A). Inhibition of fibrocyte differentiation by SAP was SRTK-dependent, as PP2 but not the control compound PP3 abolished the ability of SAP to inhibit fibrocyte differentiation. However, the Syk inhibitor was unable to inhibit the effect of SAP (Fig. 6B). Inhibition of fibrocyte differentiation by activating FcγRI or FcγRII alone or both receptors together was dependent on SRTK and Syk, as the inhibition was lost when PBMC were preincubated with PP2 or the Syk inhibitor (Fig. 6C). These data suggest that aggregated IgG inhibits fibrocyte differentiation through a pathway involving Syk and SRTK and that SAP appears to signal through a SRTK-dependent but Syk-independent pathway.

**Ligation of FcγR on monocytes inhibits fibrocyte differentiation**

To determine whether the inhibition of fibrocyte differentiation by FcγR ligation is specific to monocytes, we stimulated monocytes in the absence of other cell types. PBMC were allowed to adhere to the tissue-culture plates for 60 min, and the nonadherent cells were then removed. This routinely produces a population of cells, which is more than 98% monocytes [64–66]. The monocytes were then incubated on ice for 60 min in the presence or absence of the SRTK inhibitor PP2, the control compound PP3, or the Syk inhibitor. Cells were then washed twice before the addition of aggregated IgG or SAP for 60 min at 4°C. Monocytes were then washed twice, and the nonadherent lymphocytes were added back, as fibrocyte differentiation is a T cell-dependent process [6]. As observed with whole PBMC, aggregated IgG was able to inhibit fibrocyte differentiation when added to monocytes specifically (Fig. 7A). The SRTK inhibitor PP2 and the Syk inhibitor abolished the ability of aggregated IgG to inhibit fibrocyte differentiation (Fig. 7A). Monocytes incubated with SAP for 1 h also led to the inhibition of differentiation into fibrocytes (Fig. 7B). As observed with whole PBMC cultures, inhibition of fibrocyte differentiation by SAP was SRTK-dependent but Syk-independent (Figs. 6B and 7B). These data suggest that a 1-h exposure to agents, which activate FcγRI in a SRTK- and Syk-dependent manner, will inhibit monocyte-to-fibrocyte differentiation.

**DISCUSSION**

We found that cross-linked or heat-aggregated IgG inhibits the initial differentiation of fibrocytes. This inhibition is dependent on the Fc portion of IgG, as F(ab')₂ fragments of IgG could not inhibit fibrocyte differentiation. Monomeric IgG and monomeric or cross-linked IgA, IgE, or IgM were also incapable of inhibiting fibrocyte differentiation.

The mechanisms by which SAP and aggregated IgG inhibit fibrocyte differentiation appear to be distinct but related. The inhibition of fibrocyte differentiation by SAP was dependent on SRTK, as this inhibition was PP2-sensitive. By comparison, aggregated IgG inhibited fibrocyte differentiation by a SRTK- and Syk-dependent process. These data suggest that SAP and IgG activate one or more SRTK, but the subsequent events are divergent. Alternatively, the differences observed could be explained if SAP preferentially binds FcγRI, as FcγRII signaling is more resistant to Syk antagonists [55, 67, 68].

Recently, it has been shown that binding of CRP, the protein highly related to SAP, to FcγRII cannot inhibit IgG binding [41]. This suggests that pentraxins may bind to FcγRI and especially FcγRII outside the IgG-binding site. The binding of pentraxins outside the IgG-binding sites could explain our observation that monomeric IgG does not prevent SAP from inhibiting fibrocyte differentiation. Therefore, reagents that block IgG binding to FcγRI may not be useful for assessing the effects of SAP on FcγRI and may also explain some of the controversy regarding the specificity of SAP and CRP for FcγRI [39–41, 69, 70]. Manolov et al. [70] showed that although CRP does bind directly to FcγRII, the use of anti-CRP antibodies enhanced CRP binding to FcγRII and led to false positives. Therefore, antipentraxin antibodies or anti-FcγRI antibodies may not be suitable reagents for investigating the interaction of pentraxins with FcγRI.

The observations that bivalent F(ab')₂ mAb to FcγRI and FcγRII have some ability to inhibit fibrocyte differentiation suggest that cross-linking pairs of FcγRI is sufficient to inhibit fibrocyte differentiation. It would have been preferable to add...
cytes and thus aid tissue regeneration and wound healing [4, 44]. Our data suggest that these complexes would inhibit the differentiation of fibrocytes. However, during the resolution phase, IgG and many other serum proteins such as SAP are cleared from the site of infection by four main mechanisms: restoration of hemostasis following repair of blood vessels, drainage of the wound fluid through the lymphatic system, engulfment by phagocytic cells, and degradation by proteases [71–74]. Assuming that SAP and immune complexes are cleared from the tissue during the resolution phase, the resulting low levels of SAP and immune complexes would create an environment favorable for the differentiation of fibrocytes and thus aid tissue regeneration and wound healing [4, 75].

Immune complexes are also found in chronic inflammatory conditions. In rheumatoid arthritis, immune complexes are present mainly in the synovial fluid and on the superficial layers of the joint [76, 77]. Immune complexes are absent frequently from the interior of the tissue (pannus) invading the cartilage [78, 79]. The leading edge of the invading pannus is populated mainly by macrophages, whereas the cells in the main body are more fibroblast-like [79]. Given our observation that immune complexes inhibit fibrocyte differentiation, a possible explanation for this distribution of cells in the pannus is that the presence of immune complexes at the leading edge of the pannus would block fibrocyte differentiation. As these cells at the leading edge internalize and clear the immune complexes, the deeper layers of cells would have lower levels of these complexes. These lower levels may facilitate monocyte-to-fibrocyte differentiation. In vitro, we were unable to detect IgG or SAP after culturing PBMC for 3 days in serum-free medium, suggesting that any residual IgG or SAP present on the surface of the cells ex vivo was catabolized, removing ligands that might inhibit fibrocyte differentiation (data not shown).
Fig. 7. Direct ligation of FcγR on monocytes leads to the inhibition of fibrocyte differentiation. PBMC at 2.5 × 10^6 per ml were incubated for 60 min at 37°C, and nonadherent cells were then removed by pipetting. (A) The adherent monocytes were incubated for 60 min at 4°C in the presence or absence of 10 nM PP2, PP3, or Syk inhibitor. Monocytes were then washed twice, and the nonadherent cells were replaced to a final concentration of 2.5 × 10^5 cells (n=3 separate donors). Compared with monocytes cultured in SFM, aggregated IgG inhibited fibrocyte differentiation significantly (P<0.01), as determined by ANOVA. Compared with monocytes incubated with 10 μg/ml aggregated IgG, preincubation with PP2 (P<0.01) or the Syk inhibitor (P<0.05) significantly inhibited the ability of IgG to inhibit fibrocyte differentiation as determined by ANOVA. (B) Adherent monocytes were incubated for 60 min at 4°C in the presence or absence of 10 nM PP2, PP3, or Syk inhibitor. Monocytes were then washed and incubated for 60 min at 4°C in the presence or absence of 0.5 μg/ml SAP. Cells were then washed, the nonadherent cells were replaced, and the cells were cultured for 5 days. Compared with monocytes cultured in SFM, SAP inhibited fibrocyte differentiation significantly (P<0.05), as determined by ANOVA. Compared with monocytes cultured in 0.5 μg/ml SAP, preincubation with PP2 significantly inhibited the ability of SAP to prevent fibrocyte differentiation (P<0.05), as determined by ANOVA. Results are expressed as the mean ± SEM of the number of fibrocytes per 2.5 × 10^5 cells (n=3 separate donors). *, P < 0.05; **, P < 0.01.

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