Synovial Fibroblasts from Patients with Rheumatoid Arthritis, Like Fibroblasts from Graves’ Disease, Express High Levels of IL-16 When Treated with Igs against Insulin-Like Growth Factor-1 Receptor

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We have reported recently that IgG from patients with Graves’ disease (GD) can induce the expression of the CD4-specific T lymphocyte chemoattractant, IL-16, and RANTES, a C-C chemokine, in their fibroblasts. This induction is mediated through the insulin-like growth factor-1 receptor (IGF-1R) pathway. We now report that Abs from individuals with active rheumatoid arthritis (RA-IgG) stimulate in their synovial fibroblasts the expression of these same cytokines. IgG from individuals without known autoimmune disease fails to elicit this chemoattractant production. Furthermore, RA-IgG fails to induce IL-16 or RANTES expression in synovial fibroblasts from donors with osteoarthritis. RA-IgG-provoked IL-16 and RANTES production also appears to involve the IGF-1R because receptor-blocking Abs prevent the response. RA fibroblasts transfected with a dominant-negative mutant IGF-1R fail to respond to RA-IgG. IGF-1 and the IGF-1R-specific analog Des(1–3) also induce cytokine production in RA fibroblasts. RA-IgG-provoked IL-16 expression is inhibited by rapamycin, a specific macrolide inhibitor of the Akt/FRAP/mammalian target of rapamycin/p70S6K pathway, and by dexamethasone. GD-IgG can also induce IL-16 in RA fibroblasts, and RA-IgG shows similar activity in GD fibroblasts. Thus, IgGs from patients with RA, like those associated with GD, activate IGF-1R, and in so doing provoke T cell chemoattraction expression in fibroblasts, suggesting a potential common pathway in the two diseases. Immune-competent cell trafficking to synovial tissue is integral to the pathogenesis of RA. Recognition of this novel RA-IgG/fibroblast interaction and its functional consequences may help identify therapeutic targets. The Journal of Immunology, 2004, 173: 3564–3569.

Fibroblasts play a central role in the pathogenesis of autoimmune disorders affecting connective tissue. They both produce and respond to numerous cytokines and lipid mediators involved in inflammation. Fibroblasts synthesize extracellular matrix molecules and express degrading enzymes that are critical to tissue remodeling (1, 2). They can be activated directly by immunocompetent cells through several pathways including the CD40/CD154 bridge (3, 4). With regard to rheumatoid arthritis (RA), the invasion of cartilage and bone by the pannus formed from synovial fibroblasts leads to joint destruction (5).

Infiltrating T cells and monocytes trafficked to inflamed joints are characteristic of RA, and drive disease pathogenesis. Chemokines and other chemoattractants appear to direct this process (6). Among these molecules, IL-16 and RANTES have been demonstrated in the synovial fluid of patients with active RA (7–10). IL-16, a 17-kDa chemoattractant, binds to the D4 region of CD4 on the lymphocyte surface and induces migration to sites of inflammation (11). A principal site of IL-16 production in the joint is the CD68+ fibroblast-like cell of the synovial lining (7). When activated by IL-1β, cultured synovial fibroblasts express high levels of IL-16 (8). In synovial fluid, IL-16 concentrations correlate positively with chomotactic activity. The highest levels have been observed in patients with early RA (7). IL-16 displays both inhibitory and stimulating effects on T cell activation, leading to controversy regarding its role in disease pathogenesis (9, 12). RANTES is a C-C chemokine targeting monocytes, CD4+/CD45RO+ memory T cells, eosinophils, basophils, and mast cells (13, 14). Increased concentrations of RANTES have been found also in synovial fluid and tissue from patients with RA and osteoarthritis (OA) (10). In addition, RANTES expression can be induced in cultured synovial fibroblasts by IL-1β and TNF-α (8, 10, 15).

We recently observed that fibroblasts from patients with Graves’ disease (GD) are activated by IgGs obtained from these same donors (GD-IgG) to synthesize and release IL-16 and RANTES (16). This induction does not appear to involve the thyrotropin receptor, but instead is mediated through the insulin-like growth factor-1 receptor (IGF-1R) pathway (17). Moreover, GD-IgG signals through the Akt/FRAP/mammalian target of rapamycin/p70S6K pathway and is inhibited by rapamycin (16). Thus, we believe that IgGs directed against IGF-1R and found in the circulation of patients with GD may play an integral role in the trafficking of T cells to connective tissues. Activation of connective tissue in the orbit and shin is thought to underlie the extrathyroidal components of GD, namely thyroid-associated ophthalmopathy and dermopathy (2, 18).

We now report, for the first time, that synovial fibroblasts from patients with RA produce high levels of IL-16 and RANTES in...
response to RA-IgG. This induction necessarily involves signaling through the IGF-1R, and therefore resembles the observations made previously in GD fibroblasts. Our current findings could provide novel insight into the mechanism through which T cells infiltrate articular tissues in RA. Moreover, recognition of IGF-1R as a pathogenic self Ag in RA defines a potentially important therapeutic target for disease interruption.

Materials and Methods

Reagents

Human rIL-1β was purchased from BioSource International (Camarillo, CA), while rapamycin and IGF-1 were purchased from Calbiochem (San Diego, CA). IHT, a blocking Ab directed against IGF-1R, was from BD Pharmingen (San Diego, CA). IGF-1 analogues, including Des(1–3) and Leu-24, were from Gro-Pep (Adelaide, Australia). Protein A affinity-purified anti-IL-16 mAbs (clone 17.1) were used for IL-16 neutralization experiments, as described previously (19). Neutralizing RANTES Abs were supplied by R&D Systems (Minneapolis, MN).

Cell culture

Synovial fibroblasts from patients with RA and OA were generated from surgical explants and were generously provided by L. Crofford (University of Michigan, Ann Arbor, MI). These cells exhibit several phenotypic attributes similar to those from other regions of the body, including the expression of cytokine-dependent IL-16 and RANTES (8). Orbital fibroblasts from patients with GD were obtained from individuals undergoing decompressive surgery for severe thyroid-associated ophthalmopathy, as described previously (20). They have been characterized extensively (21). Briefly, these fibroblasts fail to express smooth muscle-specific actin or factor VIII. Cells were cultured in modified Eagle’s medium supplemented with 10% FBS (Invitrogen Life Technologies, Carlsbad, CA) in an atmosphere of humidified 5% CO2 at 37°C. Cells were serially subcultured with trypsin/EDTA (Sigma-Aldrich, St. Louis, MO) and were used between passages 3 and 10 from culture initiation. Identical conditions were used for the cultivation of all fibroblasts used in these studies. The phenotype of these cells remains stable over this period of culture. Serum was obtained from patients with active RA, GD, and normal volunteers. These activities have been approved by the Institutional Review Board of Harbor-University of California, Los Angeles Medical Center. Protein A fractionation of the serum isolated the IgG component using a protein A affinity column (Piper, New York, NY).

Quantification of IL-16 and RANTES-dependent T cell chemoattraction activity in conditioned medium of synovial and orbital fibroblasts

Fibroblasts were plated in 24-well plates and allowed to proliferate to confluence in medium containing 10% FBS. Monolayers were washed with PBS, shifted to fresh medium containing 1% FBS, and incubated overnight. Test compounds were added, as indicated in the figure legends, and inhibitors were added at least 1 h before addition of IgG. Cultures were incubated for 24 h, and then medium was collected, centrifuged at 13,000 × g at 4°C, and stored at −80°C until assayed. Each experiment was performed in triplicate.

Chemotraction was assessed in modified Boyden chambers, as described previously (17). T cell migration was quantified by counting the total number of cells that migrated beyond a given depth, and is expressed as a percentage compared with the migration seen with buffer alone (100%). Experimental controls for migration were also performed using fibroblast conditioned medium cultured in the absence of stimuli. These are designated in the figure legends as control conditions. Preincubating the culture medium for 15 min with neutralizing Abs defined the migration attributable to either IL-16 or RANTES. Anti-IL-16 Abs (clone 17.1; 10 μg/ml) have been shown to neutralize 50 ng/ml IL-16, and anti-RANTES Abs (5 μg/ml) exhibit a neutralization dose 50 of 200 ng/ml. Levels of IL-16 and RANTES were determined by ELISA, as described (17). The lower limit of detection for the IL-16 ELISA is 5–7 pg/ml, while for RANTES is 4–10 pg/ml.

Transfection of dominant-negative (DN) mutant IGF-1R into fibroblasts

The plasmid for the IGF-1R mutant 486/STOP was a generous gift of R Baserga (Jefferson University, Philadelphia, PA). Fibroblasts were seeded in 24-well plates proliferated to ~80% confluence and then transfected with either the IGF-1R mutant or empty vector using lipofectamine PLUS (Invitrogen Life Technologies). For each well, 0.2 μg of DNA was mixed with 4 μl of PLUS reagent in 25 μl of serum-free medium and incubated at room temperature for 15 min. This mixture was combined with an equal volume of medium containing 1 μl of lipofectamine, and incubated for 15 min. The fibroblast monolayers were rinsed with PBS, and 200 μl of medium was added to each well containing transfection mix. Cells were incubated at 37°C for 3 h. Transfection medium was removed, and fresh medium containing 10% FBS was added. After 24 h, medium was exchanged for MEM containing 1% FBS, and the cells were cultured overnight before addition of RA-IgG or control IgG (final concentration 100 ng/ml).

Results

Very low levels of T cell chemoattractant activity were found in conditioned medium from untreated synovial fibroblast cultures. When fibroblasts obtained from patients with RA were treated for 16 h with RA-IgG (100 ng/ml), the cells released substantial T cell chemoattractant activity. This effect was found in all three RA synovial fibroblast strains tested to date. We previously had found that IL-1β (10 ng/ml) induces chemoattractant expression in synovial fibroblasts from patients with RA and OA (8). However, when treated with RA-IgG, only the RA fibroblasts exhibited an increase in T cell migration (Fig. 1). Medium from RA-IgG-treated OA fibroblasts failed to exhibit enhanced chemotraction (Fig. 1, lower panel). Earlier studies conducted in fibroblasts from patients with GD disclosed that the T cell chemoattractive activity induced by GD-IgG was attributable to increased levels of IL-16 and RANTES (16). Thus, the ability of neutralizing Abs directed against those two cytokines to abolish T cell migration was analyzed in conditioned medium from RA fibroblasts treated with RA-IgG. Both anti-IL-16 and anti-RANTES Abs blocked partially the chemoattractant activity exhibited in the conditioned medium (Fig. 1). Treatment of either RA or OA cultures with control IgG failed to elicit these responses (Fig. 1). Similarly, IGF-1-treated OA fibroblasts failed to exhibit either IL-16 or RANTES protein or bioactivity (data not shown). These findings indicate that RA fibroblasts exhibit an inherently different phenotype from that of OA cultures with regard to responses to RA-IgG. Moreover, they are entirely consistent with the concept that the phenotype of autoimmune fibroblasts involves distinct attributes, introduced nearly 20 years ago by Fassbender (22). A total of 10 serum samples from patients with active RA, each from a different donor, were tested, and all exhibited chemotactic activity induced in RA fibroblasts (Figs. 1 and 2). In contrast, 13 of 14 serum samples from control donors without known autoimmune disease failed to induce chemotraction in disease-derived fibroblasts (data not shown). Interestingly, sample RA-5 induced IL-16-dependent chemotraction activity (Fig. 2A), but the IL-16 levels achieved were apparently inadequate for detection with the ELISA (Fig. 2B). This may reflect the ~10-fold differences in assay sensitivity.

Induction of IL-16 and RANTES expression in RA synovial fibroblasts by RA-IgG is mediated through the IGF-1R

The pathogenesis of RA involves dramatic tissue remodeling and fibroblast proliferation (1, 2, 5). The molecular and cellular basis for this connective tissue activation remains uncertain, but it is presumed that molecules emanating from immunocompetent cells, such as cytokines and mitogens, activate synovial fibroblasts. Thus, the potential for growth factors and their cognate cell surface receptors to participate in this disease appears great. IGF-1R is a ubiquitous protein expressed on many cell types, including human fibroblasts (23). When ligated, the tyrosine kinase receptor initiates signaling leading to multiple cell responses, including those resulting in proliferation (24). We thus determined whether IGF-1R might play a role in the RA-IgG-mediated up-regulation of IL-16
molecular disruption of its function, completely attenuates the production of IL-16 and RANTES provoked by RA-IgG.

If IGF-1R ligation by RA-IgG were central to the induction of chemoattractant expression, IGF-1 treatment should also provoke IL-16 and RANTES synthesis. Indeed, IGF-1 (10 nM) stimulates the production of both chemoattractants in synovial fibroblasts (Fig. 5), indicating that activation of the IGF-1R pathway is sufficient to induce IL-16 and RANTES. Des(1–3), an IGF-1 analog lacking the 3 terminal aa at the amino end of the polypeptide, possesses a vastly diminished affinity for the IGF-binding proteins, but retains high avidity for IGF-1R. Thus, the reagent is useful in discriminating IGF-1 actions mediated through IGF-1R from those involved with IGF-binding proteins (27). Des(1–3) (10 nM) induced IL-16 and RANTES synthesis, as that figure demonstrates. In contrast, Leu-24, an IGF-1 analog that binds and activates the IGF-1R axis, completely blocks RA-IgG-provoked T cell migration and IL-16 protein expression (Fig. 5B). Des(1–3) inhibited GD-IgG-provoked IL-16 expression in GD synovial fibroblasts (16). Thus, the ability of the compound to influence RA-IgG-induced cytokine synthesis was examined. Rapamycin (20 nM) could block RA-IgG-provoked T cell migration and IL-16 protein

**FIGURE 1.** IL-1β and RA-IgG induce T cell migration activity attributable to IL-16 and RANTES in cultured synovial fibroblasts from patients with RA, but not OA. Fibroblasts were allowed to proliferate to confluence in 24-well plates. IL-1β (10 ng/ml), RA-IgG from two different patients with active disease (100 ng/ml) or control IgG (100 ng/ml) was added to the medium overnight. Medium was collected and subjected to the T cell chemoattraction assay, as described in Materials and Methods. Medium was incubated without or with either anti-IL-16 or anti-RANTES Abs (10 and 5 µg/ml, respectively). Migration of >135% was significant at a 5% confidence limit. Data are expressed as the mean ± SD of three replicates from a representative experiment. * Statistically different migration in the presence of neutralizing Abs at the 5% confidence level.

**FIGURE 2.** RA synovial fibroblasts treated with IL-1β, IGF-1, or RA-IgG express IL-16-dependent T cell chemotraction and IL-16 protein. Cultures were treated overnight with nothing (control), IL-1β (10 ng/ml), IGF-1 (10 nM), or RA-IgG (100 ng/ml) from eight different patients with active disease. Medium was then subjected to a T cell chemoattraction assay (A) or a specific IL-16 ELISA (B). Data are expressed as the mean ± SD of three determinations. * Statistically different migration in the presence of neutralizing Abs (A) or protein production (B) at the 5% confidence level.

and RANTES expression in RA fibroblasts. Culture preincubation for 1 h with anti-IGF-1R Abs (5 µg/ml; clone 1H7) completely attenuated the induction of IL-16 and RANTES-dependent T cell migration (Fig. 3A) and protein expression (Fig. 3B) by RA-IgG. Clone 1H7 blocks IGF-1R activation by associating with IGF-1Rα and thus interfering with ligand/receptor interactions (25). In contrast, an isotype control Ab failed to influence the induction of either cytokine by RA-IgG. Another, more molecular strategy for interrupting the IGF-1R axis was then used. RA fibroblasts were transiently transfected with a DN mutant IGF-1R (486/STOP). This mutant contains a stop codon instead of the AA 486 in IGF-1R. As the data in Fig. 4 demonstrate, fibroblasts transfected with the empty vector responded to RA-IgG with an increased level of T cell chemotactic activity in the medium (Fig. 4A) and elevated IL-16 and RANTES proteins (Fig. 4B). Fibroblasts transfected with 486/STOP did not exhibit the induction of either chemoattractant following treatment with RA-IgG. Thus, interfering with the receptor, either with a blocking Ab or by the
expression. With regard to the former, migration was 105 ± 5% in controls, 197 ± 8% in RA-IgG-treated cultures, and 104 ± 5% in cultures receiving RA-IgG plus rapamycin (p < 0.01 vs RA-IgG alone). Dexamethasone had a similar effect on IL-16-dependent T cell cultures receiving RA-IgG plus rapamycin (10 nM). Medium was then subjected to either a T cell chemoattraction assay (A) or specific IL-16 and RANTES ELISA (B). Data are expressed as the mean ± SD of three determinations. *, Statistically different migration in the presence of neutralizing Abs (A) or protein production (B) at the 5% confidence level.

**Fibroblasts from patients with RA and GD respond to IgG from both diseases**

IgG from patients with GD induces IL-16 expression in fibroblast cultures from patients with that disease (16). As with RA-IgG, the actions involve the activation of IGF-1R (17). We next determined whether GD-IgG could also provoke IL-16 production in RA fibroblasts. As the data in Fig. 6 indicate, the impact on both IL-16-dependent T cell migration and IL-16 protein expression in RA and GD fibroblasts is equivalent. Moreover, GD fibroblasts also responded to both GD-IgG and RA-IgG, strongly suggesting that Igs from the two sources are equivalent with regard to their IGF-1R-activating potential.

**Discussion**

Our current findings extend observations made by us concerning GD-IgG eliciting IL-16 and RANTES expression in GD fibroblasts (16, 17). They represent the first report, to our knowledge, of RA-IgG directly activating synovial fibroblasts. Based on the findings we report in this work concerning RA and our earlier findings, it would appear that at least two human autoimmune diseases are associated with the generation of Abs directed against IGF-1R. Moreover, these Abs can activate IGF-1R and, by virtue of their enhancing the expression of IL-16 and RANTES, potentially trigger T cell migration to tissues affected by the disease. Thus, this newly recognized mechanism may explain a previously unknown bridge between B cell activity and T lymphocyte trafficking in multiple diseases. A remaining question relates to whether these findings will prove relevant to other examples of autoimmunity. Thus, additional studies will be required in experimental models of other types of human autoimmunity before the implications of these results can be fully assessed. IGF-1 exerts well-documented effects on proliferation and apoptosis of many different cell types other than those related to chemoattractant expression, should provide additional insights into the biological impact that these Abs might have on connective tissue. Although both GD-IgG and RA-IgG induce responses through interaction with the IGF-1R, this process appears to be somewhat selective as OA fibroblasts fail to respond. The mechanism for this selectivity is not fully understood, but it raises the possibility that intrinsic differences exist in the IGF-1R pathway in fibroblasts from patients with RA.

Our finding that IL-16 and RANTES are expressed abundantly by RA synovial fibroblasts activated by RA-IgG has substantial
is very likely that an important interplay exists between the two receptors. Because RANTES uses the former, among other receptors, it modulates receptor desensitization and cross-desensitization of CCR5 and CXCR4 (33, 34). IL-16 causes cross-desensitization of CCR5 and CXCR4 (33, 34), promoting, as a consequence, anergy and enhanced Fas expression (35). It renders pre-exposed T cells refractory to antigenic stimulation, an implication for the nature of the mononuclear infiltrate that might be provoked in vivo. For instance, IL-16 induces cell cycle progression from G0 to G1 and enhances the display of the IL-2R (31). The current findings raise some important questions. If both RA-IgG and GD-IgG can induce IL-16 in orbital and synovial fibroblasts, why don’t patients with either disease exhibit both arthritis and thyroid-associated ophthalmopathy? The answer is uncertain, but could well relate to the other pathogenic features driving each disease. For instance, orbital fibroblasts from patients with GD, like synovial cells from donors with RA, exhibit peculiar phenotypic characteristics (37). With regard to the former, these cells are considerably more susceptible to the actions of proinflammatory cytokines such as IL-1β, leukoregulin, and CD154 than are fibroblasts from other anatomic regions (38–40). Moreover, GD is almost invariably associated with activating Abs directed at the thyrotropin receptor as well as other Ags (41). Importantly, these and other anti-Thy1 Abs are frequently detectable in patients with RA (42). Their presence is often associated with thyroid dysfunction, which may be 3-fold more frequent than in individuals without chronic inflammatory arthritis (43). Thus, it is possible that the convergence of multiple pathogenic factors is necessary for the clinical expression of either disease. We postulate that anti-IGF-1R Abs may be necessary, but are in themselves inadequate for disease development. This hypothesis is further supported by the observation that these activating Abs are present systemically, but inflammation is limited to confined anatomic regions. It is also possible that anti-IGF-1R Abs arise as the consequence of these diseases rather than causing them. Clearly, sorting out these possibilities will require further investigation.

Surprisingly, the current findings implicate the IGF-1/IGF-1R pathway in T cell trafficking and tissue infiltration associated with RA. Limited precedent exists for disease-associated IgG directly activating nonimmune cells such as fibroblasts. For instance, IgG4 from patients with a pemphigus variant can induce production of IL-8 in keratinocytes (44). IgG from patients with GD has been shown previously to increase the expression of ICAM-1 (45) and collagen production (46) in human fibroblasts. We have also found that GD-IgG can induce hyaluronan synthesis in orbital fibroblasts from patients with the disease, but not in control fibroblasts (our unpublished observation). In patients with GD, IgGs bind and activate the thyrotropin receptor displayed on thyroid epithelial cells. This provokes the generation of cAMP, which results in the overproduction of thyroid hormones (47). A recent report demonstrated that Abs directed against the acetylcholine receptor can up-regulate expression of monocyte chemoattractant protein 1 in muscle cells (48). The presence, in patients with GD and RA, of autoantibodies recognizing and activating IGF-1R underscores the potential importance of this mitogenic receptor in the pathogenesis of multiple autoimmune processes. Thus, investigating sera from patients with type I diabetes mellitus, multiple sclerosis, and other

![Figure 5](image.png)

**FIGURE 5.** The induction of IL-16 and RANTES in RA fibroblasts by IGF-1 and RA-IgG is mediated through IGF-1R, and not by the IGF binding proteins. Cultures were treated with IL-1β (10 ng/ml), IGF-1 (10 nM), Des(1–3) (10 nM), Leu-24 (10 nM), or RA-IgG (100 ng/ml) overnight, and the medium was collected and subjected to T cell migration assay (A) or ELISA (B). Data are expressed as the mean ± SD of three determinations. *Statistically different migration in the presence of neutralizing Abs (A) or protein production (B) at the 5% confidence level.

![Figure 6](image.png)

**FIGURE 6.** IL-16-dependent chemoattraction and cytokine expression in GD orbital fibroblasts and RA synovial fibroblasts are induced by both GD-IgG and RA-IgG. Cultures were treated with nothing (control), IGF-1 (10 nM), GD-IgG (100 ng/ml), or RA-IgG (100 ng/ml) overnight, and the medium was collected and subjected to T cell migration assay (upper panel) or ELISA (lower panel). Data are expressed as the mean ± SD of three determinations. *Statistically different migration in the presence of neutralizing Abs (upper) or protein production (lower) at the 5% confidence level.
collagen vascular diseases for these IgGs might prove enlightening. Furthermore, interruption of the IGF-1/IGF-1R axis may represent an attractive therapeutic strategy for modifying the activity of these diseases.

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References


