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Igs from Patients with Graves' Disease Induce the Expression of T Cell Chemoattractants in Their Fibroblasts[1]

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Thyroid-associated ophthalmopathy and dermopathy are connective tissue manifestations of Graves' disease (GD). Tissue remodeling is a prominent feature of both and is apparently driven by recruited T cells. In this study, we report that IgG isolated from patients with GD (GD-IgG) up-regulates T lymphocyte chemoattractant activity in GD-derived fibroblasts from orbit, thyroid, and several regions of skin. This chemoattractant activity, absent in fibroblasts from donors without known thyroid disease, is partially susceptible to neutralization by anti-IL-16 and anti-RANTES Abs. IL-16 is a $CD4^+$-specific chemoattractant and RANTES is a C-C-type chemokine. IL-16 and RANTES protein levels, as determined by specific ELISAs, are substantially increased by GD-IgG in GD fibroblasts. Addition of the macrolide, rapamycin, to fibroblast culture medium blocked the up-regulation by GD-IgG of IL-16, implicating the FRAP/mTOR/$p70^{s6k}$ pathway in the induction of IL-16 expression. These findings suggest a specific mechanism for activation of fibroblasts in GD resulting in the recruitment of T cells. They may provide insight into a missing link between the glandular and extrathyroidal manifestations of GD. ***The Journal of Immunology*****, 2002, 168: 942–950.**

Thyroid-associated ophthalmopathy (TAO)[3] and dermopathy are extrathyroidal manifestations of Graves' disease (GD), where connective tissue of the orbit and shin are remodeled extensively (1). The features common to both lesions are the disordered accumulation of hyaluronan, a nonsulfated glycosaminoglycan, and infiltration of bone-marrow-derived cells (2, 3). The profile of cells infiltrating the orbit includes $CD4^+$ and $CD8^+$ lymphocytes and mast cells (2, 4–6). Although the proximate factors that drive tissue remodeling in these sites remain unidentified, it is currently believed that cytokines produced by inflammatory cells play important roles in fibroblast activation. To date, several cytokines, including IL-1β, IL-4, leukoregulin, and CD154 have been shown to robustly activate the expression in cultured orbital fibroblasts of genes potentially relevant to GD (7–13).

IL-16 is a $CD4^+$ cell-specific cytokine detected in asthma (14), rheumatoid arthritis (RA) (15), Crohn's disease (16), and systemic lupus erythematosus (17). It is synthesized as a precursor molecule of 69 kDa (18, 19). The pro-IL-16 molecule undergoes cleavage by the cysteine protease, caspase-3, and the resulting 17- to 20-kDa polypeptides are released from the cell (19). Activity is conferred by the multimerization of four identical subunits to a 56-kDa molecule (20). IL-16 is a specific $CD4^+$ ligand that activates T lymphocytes, monocytes, and eosinophils displaying CD4 (21–24). $CD4^+$ and $CD8^+$ lymphocytes (25, 26), airway epithelium (27), B lymphocytes (28), and mast cells (29) can express IL-16. Recently, IL-16 expression was demonstrated in cytokine-activated fibroblasts (30). Fibroblasts express high levels of constitutive IL-16 mRNA but pro-IL-16 and mature IL-16 protein cannot be detected in unstimulated cultures (30). Another chemoattractant released by cytokine-activated fibroblasts is RANTES, a C-C-type chemokine (30–32). Induction of RANTES occurs at the pretranslational level and is mediated through substantial increases in steady-state mRNA levels (30). RANTES has been implicated in the lymphocytic infiltration associated with GD (33). This chemokine is more promiscuous than IL-16 in that it utilizes at least four GTP-protein-coupled receptors, including CCR5, and activates basophils, eosinophils, monocytes, as well as resting and activated naive and memory T lymphocytes (31, 32). In T cells, RANTES engagement of CCR5 activates Janus kinase kinases and p38 mitogen-activated protein kinase and multiple downstream signaling pathways (34). Thus, a diverse array of immunocompetent cells can be activated by these two chemoattractants that are synthesized and exported from cytokine-treated fibroblasts. These cells are now viewed as sentinels, capable of coordinating a complex interplay between immunocompetent cells and specialized tissues (35).

An important question concerns the mechanism through which immunocompetent cells are trafficked to affected tissues in GD. The identity of an anatomic site-restricted autoantigen, expressed only in thyroid, orbit, and pretibial skin, has been sought. A self-Ag expressed only in the anatomic regions manifesting the disease could account for the peculiar distribution of GD. The central autoantigen involved in the thyroidal component of GD has been established to be the thyroid-stimulating hormone receptor (TSHR) (36). This receptor has received considerable attention as a potential autoantigen relevant to TAO, and has been detected widely in cells of the fibroblast lineage (37, 38) and in several adipose tissue depots (39, 40). Recently, animal models of GD have been described, involving mouse immunization with the

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[3] Abbreviations used in this paper: TAO, thyroid-associated ophthalmopathy; GD, Graves' disease; RA, rheumatoid arthritis; TSH, thyroid-stimulating hormone; IGF-1, insulin-like growth factor 1.

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TSHR, its cDNA, or by passively transferring TSHR-primed T cells (41–43). These animals variably exhibit hyperthyroidism and thyroid histopathology consistent with the disease. Some of these reports contain histological evidence for infiltrative tissue changes and Th2 cytokine profiles in the orbits and edema of the extraocular muscles in animals manifesting TSHR-induced thyroiditis (42, 43). A potentially important relationship between circulating Igs directed against TSHR and the extrathyroidal manifestations of GD is implied by the presence of anti-TSHR Abs in patients with TAO (44). Moreover, isolated reports have appeared suggesting direct effects of GD IgGs on human fibroblasts (45). Nevertheless, there currently exists little evidence that directly links the TSHR with the pathogenesis of human TAO or dermopathy.

We report here for the first time that IgG from patients with GD (GD-IgG) activates the expression and release of T cell chemoattractant activity from their fibroblasts. This lymphocyte activity can be attributed in large part to IL-16 and RANTES. GD-IgG rapidly activates $p70^{s6k}$ in these fibroblasts. Rapamycin, a macrolide that inhibits FRAP/mTOR, attenuates the IL-16 synthesis provoked by GD-IgG. These observations suggest a novel mechanism for trafficking of T cells to connective tissue in GD.

Materials and Methods

Materials

Human recombinant IL-1β was purchased from BioSource International (Camarillo, CA). Eagle's medium, antibiotics, and FBS were supplied by Life Technologies (Bethesda, MD). An affinity-purified polyclonal rabbit anti-rIL-16 Ab was prepared from rIL-16-immunized rabbit sera as described previously (46). An ELISA for RANTES was purchased from BioSource International and neutralizing anti-RANTES Abs were purchased from R&D Systems (Minneapolis, MN). Rapamycin was obtained from Calbiochem (La Jolla, CA). Human recombinant thyroid-stimulating hormone (TSH) and dexamethasone were purchased from Sigma-Aldrich (St. Louis, MO). A radioreceptor assay kit for determining TSHR Abs was purchased from Brahms (Hennigsdorf, Germany).

Cell culture, serum collection, and IgG preparation

Human fibroblasts were obtained from individuals with GD or from donors without known thyroid disease. Orbital fibroblast strains were initiated from surgical waste. Dermal fibroblasts were derived from punch biopsies of normal-appearing skin or were purchased from American Type Culture Collection (Manassas, VA). All patients were thought to be euthyroid at the time of tissue donation. One patient with GD was, in retrospect, found to be severely hypothyroid at the time of surgery. Sera were collected from patients with GD, either without or with clinically apparent TAO and from individuals without thyroid disease (controls). These included adult men and women. The diagnosis of GD was made on clinical grounds, including suppressed TSH, elevated serum T_4 levels, the presence of anti-thyroid Abs, goiter, and typical symptoms and signs of thyrotoxicosis. Most of the individuals were euthyroid at the time of blood drawing while few were hyperthyroid. IgG was prepared by the method previously described using protein A (47). These activities have been approved by the Institutional Review Board of Harbor-University of California, Los Angeles Medical Center. Some of the culture strains were kindly provided by Dr. R. Bahn (Mayo Clinic, Rochester, MN). Fibroblast monolayers were covered with Eagle's medium supplemented with 10% FBS, antibiotics, and glutamine as described previously (48). Cultures were incubated in a 37°C, humidified, 5% CO_2 atmosphere and were serially passaged with gentle treatment with trypsin/EDTA. Fibroblast strains were used between the 3rd and 12th passage. They have been shown to not express factor VIII or smooth muscle-specific actin (49). There were no differences noted in the morphology of cultures from normal controls and patients with GD.

Chemotaxis assay

Fibroblasts were plated in 24-well plates and were allowed to proliferate to confluence. After rinsing the monolayers with PBS, cultures were shifted to medium containing 1% FBS overnight before addition of nothing (control), IL-1β (10 ng/ml), human serum (final concentration 1%), or protein A-purified human IgG to the medium. Cultures were incubated for the times indicated in the text and figure legends. At the end of these incubations, culture medium was collected quantitatively and stored at −80°C until assayed.

Chemotaxis was assessed in a modified Boyden chemotaxis chamber utilizing human NWNA-T lymphocytes as the cellular targets, as described previously (30). In brief, 50 μl of a cell suspension (10^7 cells/ml) was placed in the upper compartments of 48-well microchemotaxis chambers separated from 32-μl samples by 8-μm micropore nitrocellulose filters (NeuroProbe, Cabin John, MD). These were then incubated at 37°C in a 5% CO_2 environment for 3 h. Filters were fixed, stained with hematoxylin, dehydrated and mounted on glass slides, and viewed under light microscopy. Lymphocyte migration was quantified by counting the total number of cells migrating beyond a certain depth. This depth was set routinely to identify a baseline migration under control conditions of 10–15 cells/high-power field. Five high-power fields were counted in duplicate for each sample and the means ± SD were calculated and expressed as percentage values of baseline cell migration in control buffer alone (100%). For each set of experimental conditions, at least three separate experiments were performed. The differences between experimental and control conditions were analyzed with the Student *t* test using the absolute values obtained for lymphocyte migration, and statistical significance was accepted at the 5% level of confidence. To assess the chemoattractant activity attributable to IL-16, neutralizing experiments were conducted by incubating culture supernatants for 15 min with affinity-purified anti-IL-16 mAb (clone 14.1, 10 μg/ml, which neutralizes the chemoattractant activity of 50 ng/ml rIL-16). To determine the RANTES-dependent fraction, anti-RANTES mAb (5 μg/ml, possessing an ND_{50} of 200 ng/ml for recombinant RANTES) was added to the migration assay.

Analysis of IL-16, RANTES, and anti-TSHR Ab levels

Quantitation of IL-16 protein released from the fibroblast monolayers was accomplished by subjecting aliquots of conditioned medium to a specific ELISA, performed as described previously (30). Samples from each culture were assayed in duplicate. rIL-16 and aliquots of conditioned medium were diluted in PBS to the desired concentrations. Samples of culture medium (100 μl) were incubated in a 96-well microtiter plate (Nunc, Naperville, IL) at 37°C for 1 h. Subsequent maneuvers were performed at room temperature. With regard to RANTES, levels were determined using a commercially available ELISA and following the supplier's instructions. Anti-TSHR Ab levels were determined with a TRAK human radioreceptor assay kit purchased from Brahms following the instructions of the supplier.

Immunoprecipitation of ^{35}S-labeled IL-16

Newly synthesized IL-16 protein was quantified by incubating cultures in methionine-free medium for 18 h followed by pulse labeling with [^{35}S]methionine (500 μCi/ml) for 6 h. Medium samples were collected and subjected to immunoprecipitation with anti-IL-16 (clone 14.1, 5 μg/ml) conjugated to protein A beads. Samples were incubated for 1 h at room temperature and then the beads were centrifuged, washed, and counted.

Western blot analysis of phosphorylated $p70^{s6k}$

$p70^{S6k}$ activation was assessed by subjecting cellular protein from IgG-activated fibroblasts to immunoblot analysis. Fibroblasts were allowed to proliferate to confluence in 60-mm plates. Following incubations with the test compounds indicated, monolayers were solubilized in a buffer containing 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 100 mM NaCl, 0.5% deoxycholate, 1% Triton X-100, 10% glycerol, 0.1% SDS, 2 mM Na_3VO_4, 20 mM NaP_2O_4, 1 mM NaF, 1 mM microcystin, 10 μg/ml aprotinin, and 100 mM PMSF. Lysate samples normalized to their respective protein content were boiled in Laemmli buffer and subjected to SDS-PAGE as described previously (7). The separated proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). These were then incubated with primary phospho-specific anti-$p70^{s6k}$ Thr^{389} (Cell Signaling Technology, Beverly, MA). Other aliquots of the sample were electrophoresed and blotted against a pan $p70^{s6k}$ Ab (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-actin Ab (Sigma-Aldrich). Following extensive washes at room temperature, the membranes were incubated with secondary, peroxidase-labeled Abs for 1 h. Following washes, the ECL (Amersham, Arlington Heights, IL) detection system was used to generate the relevant signals. The bands were analyzed densitometrically with a scanner.

Results

Serum from patients with GD elicits the release of lymphocyte chemoattraction activity from their fibroblasts; this activity can be attributed to an IgG

Human fibroblasts maintained under basal culture conditions release very low levels of chemoattractant activity when the conditioned medium in which they are incubated is subjected to cell migration assays using NWNA-T lymphocytes as the targets. This was true for fibroblasts derived from patients with GD and from normal donors. When unfractionated serum from a patient with GD is added to the culture medium (final concentration, 1%) covering fibroblast monolayers, in this case from orbit, subcutaneous connective tissue, and thyroid derived from a single donor, T lymphocyte chemoattractant activity is dramatically increased (Fig. 1, *upper panel*). A substantial fraction of the up-regulated chemotaxis could be blocked by anti-IL-16 mAb (5 μg/ml) added to the migration assay. When fibroblast medium from GD serum-treated fibroblasts was subjected to a specific IL-16 ELISA, levels of the chemoattractant were found to be greatly elevated (Fig. 1, *lower panel*). IL-16 was undetectable in control cultures.

IL-16-inducing activity was completely adsorbed from GD sera when they were subjected to protein A column chromatography (Fig. 2). The effluent failed to influence T cell migration. Fig. 2 also demonstrates the absence of a GD-IgG effect in fibroblasts from a donor without known thyroid disease. Moreover, IgG from a control donor failed to provoke T cell migration activity in GD or normal fibroblasts. IgG preparations from 26 different patients with GD (final concentration, 100 ng/ml) were tested in a single GD-derived fibroblast strain for their ability to induce IL-16-dependent T cell migration and IL-16 protein. Twelve of the IgG samples were derived from individuals with GD but without obvious TAO. Twenty-five of these IgG preparations elicited an up-regulation of IL-16-dependent T cell migration. Moreover, these same GD-IgG preparations up-regulated IL-16 synthesis and release into the culture medium. A high degree of correlation was found between the up-regulation of IL-16-dependent T cell migration and IL-16 protein concentrations as determined by ELISA ($r = 0.914$, $p = 0.001$; Fig. 3, *left panel*). In contrast, sera and IgG from 12 of 13 individuals without known thyroid disease failed to increase the production of lymphocyte chemoattraction activity in fibroblasts or detectable IL-16 protein (data not shown). In addition, IgG preparations from two individuals with active RA and two patients with systemic lupus erythematosus failed to induce IL-16 in fibroblasts from patients with GD. Thus, there exists some degree of specificity with regard to the type of autoimmune disease affecting the donor of IgG.

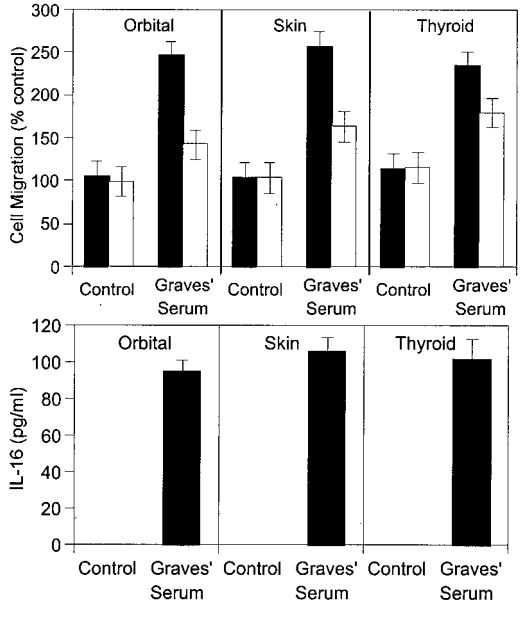

FIGURE 1. Treatment of GD fibroblasts with GD serum results in expression of T cell chemotaxis activity attributable to IL-16. *Upper panel,* T cell migration is enhanced in medium from orbital, skin, and thyroid fibroblasts when cultures were treated with nothing or 1% GD serum for 24 h. Medium was subjected to a lymphocyte chemotaxis assay without (■) or with anti-IL-16-neutralizing Abs (5 μg/ml, □). Migration of >135% was significant at 5% confidence limit. Data are expressed as the means ± SD of three independent determinations. *Lower panel,* IL-16 production is up-regulated following 24-h treatment of these fibroblasts with 1% GD serum. Limit of detection was 20 pg/ml.

GD-IgG might be ligating the TSHR and, through that potential interaction, initiating the events culminating in the induction of IL-16. We have recently reported that this receptor is expressed on fibroblasts from several anatomic regions and is competent to signal through $p70^{s6k}$ (38). We therefore incubated GD fibroblasts with high concentrations of recombinant TSH (up to 10 mU/ml) and have determined that the hormone fails to induce T cell chemoattractant activity or detectable IL-16 protein synthesis by GD fibroblasts (data not shown). When GD-IgG provoked T cell migration activity (IL-16 dependent plus IL-16 independent) from Ig-treated fibroblasts was plotted against the respective concentrations of anti-TSHR Abs (TRAb) from each serum sample, there existed no correlation ($r = 0.065$, $p = 0.62$; Fig. 3, *right panel*). These findings suggest strongly that the T cell chemotaxis and IL-16 inductions provoked by GD-IgG are not related to the anti-TSHR activity present in GD sera and that the TSHR is not mediating the up-regulation by GD-IgG of IL-16.

A panel of 11 different fibroblast strains from patients with GD and 5 from individuals without known thyroid disease were challenged with GD-IgG (100 ng/ml), normal IgG (100 ng/ml), or IL-1β (10 ng/ml) for 24 h and assessed for T cell migration activity and IL-16 production (Table I). The normal and GD-IgGs used in that survey each derived from either of two individual donors. These were not pooled. GD-IgG induced IL-16-dependent cell migration in 10 of the GD fibroblast strains that included those from the orbit or various anatomic regions of skin. Included were strains from the pretibial skin as well as the abdominal wall and the neck. The latter two sites rarely manifest GD. A substantial fraction of the GD-IgG-provoked T cell migration activity in most of these strains was resistant to neutralization with anti-IL-16. We thus tested neutralizing Abs directed at other chemoattractant molecules and found that the residual activity could, in large part, be attenuated with anti-RANTES (Table I). GD-IgG up-regulated both IL-16 and RANTES proteins in eight GD-derived fibroblast strains while RANTES was undetectable in two of the GD strains exhibiting marked IL-16 inductions. GD-IgG failed to up-regulate T cell chemotaxis or to induce either IL-16 or RANTES protein in one GD strain (orbital strain 9) and in any of the five culture strains derived from donors without known thyroid disease. The control IgG failed to induce either IL-16 or RANTES expression in any of the fibroblast strains tested. In contrast, IL-1β induced T cell chemoattraction in all fibroblast strains, consistent with the previously reported findings (30).

GD-IgG induces the release of newly synthesized IL-16 and is dependent on caspase-3 activity

GD-IgG can provoke release of newly synthesized IL-16 from GD fibroblasts. [^{35}S]Methionine-labeled cultures were treated with GD-IgG for graded intervals and ^{35}S-labeled IL-16 release was

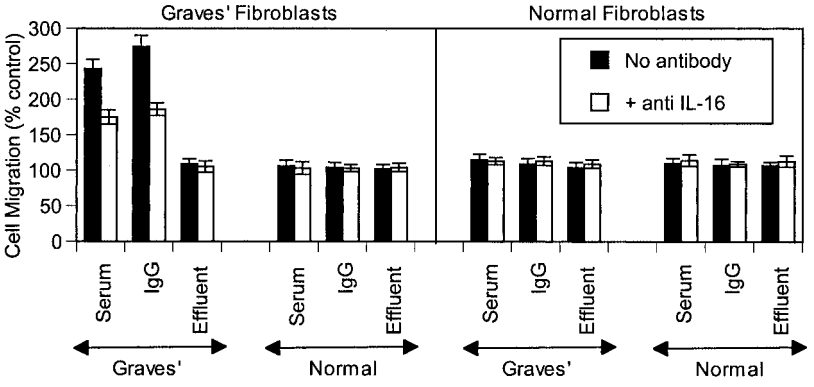

FIGURE 2. The induction of T cell chemotactic activity in fibroblasts by serum is attributable to IgG and is specific to donors with GD. Normal and GD orbital fibroblasts were treated for 24 h with serum (1%), IgG fraction (100 ng/ml), or effluent from protein A chromatography. Media were subjected to the T cell chemotaxis assay in the absence or presence of anti-IL-16-neutralizing Ab (5 μg/ml). Data are expressed as the means ± SD of three independent determinations. T cell migration >135% was significant at the 5% confidence limit.

enhanced within 12 h of IgG addition to the culture medium (Fig. 4). The maximal synthesis occurs by 30 h when it was at least 100-fold above baseline and was sustained for the duration of the study (48 h). IL-16 protein is synthesized as a pro-molecule of 69 kDa that undergoes modification to the 56-kDa active molecule which is released from the cell (18, 19). In lymphocytes, this processing involves a caspase-3 dependent cleavage (19). Moreover, in fibroblasts, the induction by IL-1β of IL-16 involves this enzyme (30). Thus, we determined whether inhibition of caspase-3 with a specific inhibitory peptide could block the GD-IgG release of IL-16 in GD fibroblasts. Addition of the peptide designated Ac-DEVD-CHO (100 μM) to fibroblast medium resulted in a dramatic decrease in the lymphocyte migration ascribable to IL-16 and to the induction by IgG of IL-16 protein as determined in the ELISA (Fig. 5). In contrast, the up-regulation of RANTES was unaffected. The caspase-1-specific peptide inhibitor, Ac-YVAD-Ald (100 μM), failed to influence either IL-16 or RANTES activity or protein release into the medium.

GD-IgG induction of IL-16 expression and release from fibroblasts involves a rapamycin-sensitive pathway

An important possibility for how GD-IgG might induce IL-16 and RANTES expression in GD fibroblasts relates to the Ig acting as a growth factor, binding an epitope on the surface of those cells, and initiating protein synthesis through the activation of one or more signaling pathways. The FRAP/mTOR pathway and the activation of $p70^{s6k}$, a serine/threonine kinase, play central roles in mediating the effects provoked by multiple factors acting at the cell surface (50). A prominent characteristic of this pathway is its susceptibility to inhibition by the macrolide rapamycin (51). We determined that this compound, at a concentration of 20 nM, can block ~50% of the chemoattractant activity elicited by GD-IgG, coinciding with an attenuation of IL-16-dependent T cell migration (Table II). The induction of IL-16 protein by GD-IgG is blocked by rapamycin, whereas that of RANTES is not. We therefore determined whether GD-IgG increased levels of activated $p70^{s6k}$. GD-IgG (100 ng/ml) elicits an increase in activated $p70^{s6k}$ as determined by Western immunoblot analysis with a primary Ab specific for $p70^{s6K}$ phosphorylated at Thr^{389} (Fig. 6). IgG from control subjects (100 ng/ml) also increased phosphorylated $p70^{s6k}$, but the levels are considerably lower than those for GD-IgG. Moreover, normal fibroblasts challenged with either GD-IgG or control IgG failed to exhibit substantial $p70^{s6K}$ activation. These findings suggest that $p70^{s6k}$ activation by IgG may alone be insufficient to up-regulate IL-16 expression. Given the ability of rapamycin to block, the activation of $p70^{s6k}$ appears to be essential for the induction by GD-IgG of IL-16 in fibroblasts.

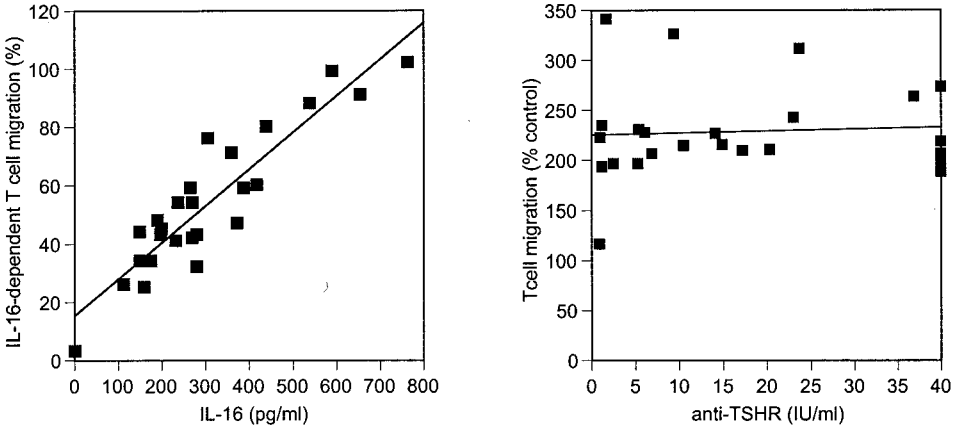

FIGURE 3. *Left panel*, Relationship between IL-16-dependent T cell chemotaxis and IL-16 protein levels provoked by the treatment of GD fibroblasts with GD-IgG (100 ng/ml) from 26 different patients. Culture wells with confluent GD fibroblast monolayers were treated for 24 h with GD-IgG, and the media were collected and subjected to the T cell chemotaxis assay or to the IL-16 ELISA. All samples were assayed in triplicate. IL-16-dependent migration represents the difference between T cell chemotaxis in the presence and absence of neutralizing Abs. Data were subjected to linear regression analysis using the equation: $f(x) = 0.1256108x + 15.37059$ ($r = 0.914$, $p = 0.001$). *Right panel*, Relationship between T cell chemotaxis activity provoked in GD fibroblasts by GD-IgG and serum levels of anti-TSHR Ab. Aliquots of sera from 26 different donors with GD were subjected to IgG preparation with protein A, were subjected to T cell migration assays (ordinate), or were subjected to a radioreceptor assay (DYNOtest-TRAK; Brahms) for TRAb, as indicated along the abscissa ($r = 0.065$, $p = 0.62$).

Table I. *Effects of IgG on IL-16 and RANTES synthesis and T lymphocyte chemotactic production in human fibroblasts*

Strain	Site	Diagnosis	Treatment	T Cell Migration[a] (% control)	+Anti-IL-16	+Anti-RANTES	IL-16[b] (pg/ml)	RANTES[c] (pg/ml)
1	Orbit	GD	Control	108 ± 9	102 ± 4	110 ± 7	ND[d]	ND
			Normal IgG	110 ± 6	104 ± 7	103 ± 6	ND	ND
			GD-IgG	179 ± 9	142 ± 8	158 ± 9	58 ± 9	83 ± 10
			IL-1β	194 ± 11	149 ± 8	167 ± 10	71 ± 9	84 ± 8
2	Orbit	GD	Control	110 ± 7	104 ± 8	107 ± 9	ND	ND
			Normal IgG	119 ± 10	109 ± 10	116 ± 11	ND	ND
			GD-IgG	181 ± 11	117 ± 12	171 ± 9	115 ± 15	ND
			IL-1β	195 ± 13	171 ± 7	170 ± 8	82 ± 9	116 ± 11
3	Abdominal skin	GD	Control	108 ± 10	101 ± 8	103 ± 6	ND	ND
			Normal IgG	110 ± 11	108 ± 7	106 ± 8	ND	ND
			GD-IgG	187 ± 10	165 ± 9	175 ± 9	90 ± 9	109 ± 12
			IL-1β	195 ± 13	171 ± 7	171 ± 8	82 ± 9	116 ± 11
4	Orbit	GD	Control	111 ± 8	105 ± 6	103 ± 4	ND	ND
			Normal IgG	109 ± 5	122 ± 10	113 ± 9	ND	ND
			GD-IgG	280 ± 12	130 ± 6	197 ± 17	213 ± 14	98 ± 9
			IL-1β	236 ± 19	175 ± 12	188 ± 9	194 ± 29	128 ± 26
5	Abdominal skin	GD	Control	93 ± 6	99 ± 7	99 ± 6	ND	ND
			Normal IgG	110 ± 9	107 ± 8	104 ± 8	ND	ND
			GD-IgG	157 ± 6	132 ± 8	142 ± 9	ND	ND
			IL-1β	144 ± 8	128 ± 8	131 ± 5	ND	ND
6	Neck skin	GD	Control	103 ± 5	100 ± 7	104 ± 6	ND	ND
			GD-IgG	195 ± 11	153 ± 12	168 ± 8	97 ± 21	198 ± 30
			IL-1β	211 ± 13	148 ± 10	178 ± 12	116 ± 27	253 ± 42
7	Pretibial skin	GD	Control	112 ± 9	109 ± 7	104 ± 8	ND	ND
			Normal IgG	108 ± 7	101 ± 6	103 ± 9	ND	ND
			GD-IgG	213 ± 11	168 ± 9	179 ± 10	168 ± 23	119 ± 17
			IL-1β	236 ± 19	175 ± 12	188 ± 9	194 ± 29	128 ± 26
8	Pretibial skin	GD	Control	109 ± 8	107 ± 8	111 ± 9	ND	ND
			Normal IgG	114 ± 9	112 ± 5	114 ± 10	ND	ND
			GD-IgG	167 ± 9	126 ± 8	150 ± 6	58 ± 24	ND
			IL-1β	204 ± 14	164 ± 10	173 ± 8	79 ± 11	ND
9	Orbit	GD	Control	109 ± 7	104 ± 8	104 ± 9	ND	ND
			Normal IgG	106 ± 8	101 ± 6	109 ± 9	ND	ND
			GD-IgG	111 ± 5	106 ± 8	104 ± 10	ND	ND
			IL-1β	243 ± 13	167 ± 11	189 ± 10	218 ± 33	78 ± 21
10	Orbit	GD	Control	105 ± 8	109 ± 10	104 ± 5	ND	ND
			Normal IgG	101 ± 9	100 ± 7	108 ± 8	ND	ND
			GD-IgG	221 ± 13	172 ± 8	189 ± 11	159 ± 28	195 ± 35
			IL-1β	248 ± 19	189 ± 14	206 ± 12	246 ± 32	214 ± 30
11	Orbit	GD	Control	106 ± 7	104 ± 8	101 ± 5	ND	ND
			Normal IgG	110 ± 10	107 ± 5	103 ± 7	ND	ND
			GD-IgG	195 ± 11	135 ± 9	159 ± 7	47 ± 7	26 ± 8
			IL-1β	210 ± 13	162 ± 11	183 ± 6	94 ± 12	69 ± 6
12	Orbit	Normal	Control	108 ± 9	109 ± 4	101 ± 7	ND	ND
			Normal IgG	104 ± 6	109 ± 5	107 ± 6	ND	ND
			GD-IgG	110 ± 9	105 ± 8	112 ± 8	ND	ND
			IL-1β	168 ± 11	143 ± 9	156 ± 9	56 ± 10	42 ± 9
13	Orbit	Normal	Control	92 ± 4	90 ± 7	94 ± 6	ND	ND
			Normal IgG	106 ± 9	95 ± 5	99 ± 8	ND	10 ± 8
			GD-IgG	92 ± 9	97 ± 6	101 ± 5	ND	ND
14	Orbit	Normal	Control	102 ± 8	104 ± 5	105 ± 7	ND	ND
			Normal IgG	108 ± 5	108 ± 4	103 ± 9	ND	ND
			GD-IgG	109 ± 8	104 ± 7	106 ± 5	ND	ND
			IL-1β	189 ± 10	154 ± 8	159 ± 9	68 ± 9	79 ± 11
15	Extremity skin	Normal	Control	103 ± 4	103 ± 8	100 ± 6	ND	ND
			Normal IgG	106 ± 9	104 ± 7	103 ± 8	ND	ND
			GD-IgG	105 ± 7	107 ± 7	101 ± 5	ND	ND
			IL-1β	197 ± 10	153 ± 9	172 ± 9	138 ± 15	173 ± 12
16	Extremity skin	Normal	Control	108 ± 8	103 ± 7	105 ± 10	ND	ND
			Normal IgG	107 ± 6	104 ± 8	103 ± 6	ND	ND
			GD-IgG	100 ± 6	106 ± 7	101 ± 5	ND	ND
			IL-1β	201 ± 7	155 ± 8	173 ± 7	98 ± 19	125 ± 9

[a] Lymphocyte chemotaxis assay of medium from fibroblasts treated with IL-1β (10 ng/ml), GD-IgG (100 ng/ml), or IgG (100 ng/ml) from donors without known thyroid disease. Chemotactic activity is expressed as a percentage of control, the activity levels of which were found in fibroblast cultures not treated with IL-1β or IgG. IL-16- and RANTES-dependent activities are defined as the differences in chemotaxis observed in samples without and with the respective neutralizing Abs. Data are expressed as the means ± SD of three independent determinations. Migration >135% was significant at 5% confidence limit.

[b] Limits of detection, 20 pg/ml.

[c] Limits of detection, 15 pg/ml.

[d] ND, Not detected.

Glucocorticoids exert powerful modulatory actions on the expression of many proinflammatory molecules and they have an important therapeutic role in GD complicated with TAO. The impact of these steroids on the induction by GD-IgG of chemoattractant expression in fibroblasts was determined. Dexamethasone (10 nM) was added to the medium in combination with GD-IgG and

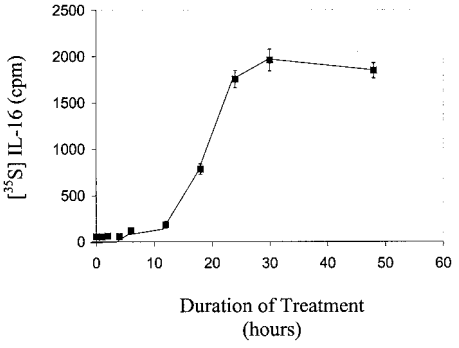

FIGURE 4. GD-IgG induces the de novo synthesis of IL-16 in GD fibroblasts. Confluent fibroblast monolayers, in this case from the skin, were treated with GD-IgG (100 ng/ml) for the durations indicated along the abscissa. They were pulse labeled with [^{35}S]methionine (500 μCi/ml) for 6 h and immunoprecipitated with anti-IL-16 (clone 14.1, 5 μg/ml) conjugated to protein A beads, washed, and counted for radioactivity. Data are expressed as the mean ± SD of three replicates.

fibroblasts were incubated for 16 h. As the data in Table II indicate, the glucocorticoid could block both IL-16 and RANTES induction by GD-IgG.

Discussion

GD is associated with trafficking of activated T lymphocytes to the thyroid gland and connective tissue where they infiltrate and, through their production of cytokines and other disease mediators, alter the behavior of resident cells (1). The presence in serum of Abs directed against TSHR appears to represent a prominent feature of GD. Although the relationship between these IgGs and thyroidal overactivity is well established, their connection with the orbital and dermal manifestations of GD is not convincing. Moreover, a correlation between Ab levels and the clinical severity/activity of the orbital disease remains controversial (44, 52–56). Implicit in the observations reported here is the identification of Abs generated in association with GD which are distinct from anti-TSHR Igs. Furthermore, these Abs have the capacity to specifically activate fibroblasts derived from patients with GD, as is indicated by the production of at least two chemoattractant cytokines. Our current findings also suggest that the IgG-fibroblast interactions that provoke IL-16 and RANTES expression require a phenotypic attribute present in fibroblasts from patients with GD but absent in cultures derived from donors without the disease. It is conceivable that these chemoattractants function to promote T lymphocyte infiltration in many tissues, including those not ordinarily manifesting the clinical disease. This finding suggests that connective tissue activation in GD may be generalized. Apparently

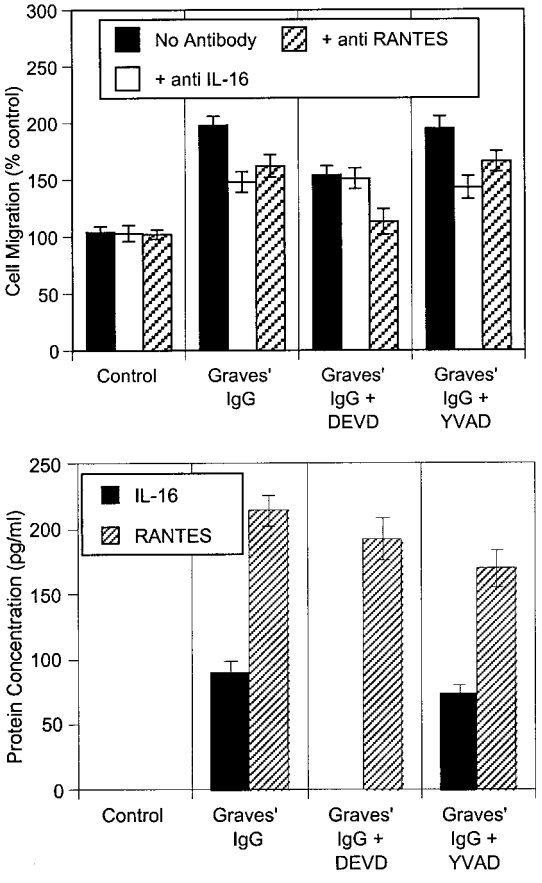

FIGURE 5. Induction of IL-16-dependent T cell migration and IL-16 protein by GD-IgG in GD fibroblasts is dependent upon caspase-3 activity. Inhibition of caspase-3 failed to influence RANTES expression. Confluent fibroblast monolayers were treated with GD-IgG (100 ng/ml) alone or in combination with the casapase-3 inhibitory peptide (Ac-DEVD-CHO, 100 μM) or a caspase-1 inhibitory peptide (Ac-YVAD-Ald, 100 μM). Media were collected and subjected to either the T cell migration assay (*upper panel*) or IL-16 or RANTES ELISAs (*lower panel*). Migration was determined in the absence or presence of anti-IL-16 (5 μg/ml) or anti-RANTES (5 μg/ml) Abs. Data are expressed as the means ± SD of triplicate determinations.

Table II. *Effects of rapamycin and dexamethasone on IL-16 and RANTES synthesis and T lymphocyte migration activity provoked by IgG in fibroblasts from patients with GD*[a]

	Lymphocyte Migration[b] (% control)	+Anti-IL-16	+Anti-RANTES	+Both Abs	IL-16[c] (pg/ml)	RANTES[d] (pg/ml)
Expt. 1						
GD-IgG	274 ± 16	186 ± 9	217 ± 9	162 ± 8	538 ± 51	462 ± 68
GD-IgG + rapamycin	190 ± 10	184 ± 12	157 ± 9	155 ± 11	ND	449 ± 73
Expt. 2						
GD-IgG	195 ± 11	135 ± 9	159 ± 7		47 ± 7	26 ± 8
GD-IgG + dexamethasone	121 ± 8	117 ± 9	118 ± 8		ND	ND

[a] Confluent monolayers of fibroblasts were treated with nothing, GD-IgG (100 ng/ml) without or with rapamycin (20 nM), or dexamethasone (10 nM) for 24 h. The conditioned medium was then subjected to the T cell migration assay, as described in *Materials and Methods* without or with anti-IL-16 (10 μg/ml) and/or anti-RANTES-(5 μg/ml) neutralizing Abs. Another aliquot of medium was subjected to the cytokine-specific ELISAs described. Data are expressed as the means ± SD of three independent determinations.

[b] Migration is expressed as a percentage of control, the activity levels of which were found in untreated fibroblast cultures. Migration of >135% was significant at 5% confidence limit.

[c] Limits of detection, 20 pg/ml.

[d] Limits of detection, 15 pg/ml.

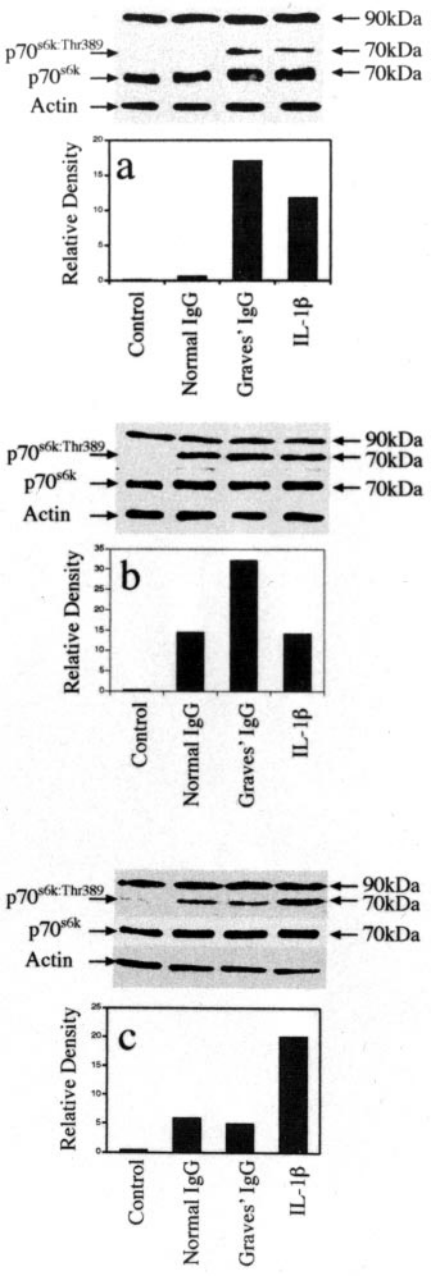

FIGURE 6. GD-IgG rapidly activates p70^{s6k} Thr^{389} phosphorylation in GD fibroblasts. Confluent monolayers of orbital fibroblasts from two patients with GD (*a* and *b*) or a single donor with normal orbital tissue (*c*) were incubated with nothing (Control), normal IgG (100 ng/ml), GD-IgG (100 ng/ml), or IL-1β (10 ng/ml) for 15 min, and cells were rinsed and harvested in lysis buffer. Equivalent amounts of protein were subjected to Western blot analysis with pan anti-p70^{s6k}, phospho-specific anti-p70^{s6k} Thr^{389}, or anti-actin Abs. Signals were generated with the ECL method. Column heights represent p70^{s6k} Thr^{389} signal densities corrected for their respective actin levels.

the GD-IgG responsible for provoking chemoattractant expression in fibroblasts is present in sera from patients with GD, regardless of whether they manifest clinically obvious TAO. The particularly robust responses observed in fibroblasts from the orbit to T cell-derived cytokines such as leukoregulin, IL-1, and CD154 and their expression and surface display of functional CD40 may underlie the susceptibility of orbital connective tissue to the anatomic site-selective manifestations associated with GD (7–12). Supporting this concept is the distinct profile of proteins expressed by cytokine-activated orbital and pretibial fibroblasts (57). On the other hand, dermopathy can occur, on occasion, in areas of the skin distant from the shin, and these cases have often been associated with antecedent trauma (1).

Our current observations suggest that the GD-IgG responsible for eliciting chemoattractant expression in fibroblasts is directed at a molecule(s) other than the TSHR. Although the identity of the relevant self-Ag remains to be determined, a very limited number of candidates have thus far been suggested that could function as the receptor relevant to these Abs. Once occupied, the receptor could initiate the signaling which results in IL-16 and RANTES expression. A 23-kDa fibroblast protein is recognized by Igs from some patients with TAO (58). This Ag is expressed by fibroblasts from the orbit and skin. It appears to localize predominantly to the cytosol rather than the plasma membrane. Consequently, its relevance as a surface Ag with signaling potential may be limited. Weightman et al. (59) have demonstrated that IgG from patients with GD, without or with TAO, inhibits ^{125}I-labeled insulin-like growth factor 1 (IGF-1) binding to sites on orbital fibroblasts derived from extraocular muscles of donors without GD. Their results suggest that anti-IGF-1 receptor Abs might be present in patients with GD. This potential self-Ag is a particularly attractive candidate because a number of the molecular events mediated through the IGF-1R are susceptible to inhibition with rapamycin (60, 61) as is the induction by GD-IgG of IL-16 (Table II).

Earlier studies have demonstrated effects of Igs from patients with GD on fibroblasts. Rotella et al. (45) observed that IgGs from the majority of patients with TAO could enhance collagen production in skin (arm) fibroblasts from individuals without known thyroid disease. Fifty percent of these IgG preparations were unassociated with thyroid-stimulating Ab activity in a thyrocyte cAMP assay. Moreover, the collagen-enhancing activity was absent in the vast majority of IgGs tested that derived from patients with GD but not manifesting TAO. Heufelder and Bahn (62) have shown that GD-IgGs can up-regulate ICAM-1 in GD-derived orbital fibroblasts but not in fibroblasts from control donors. Another report contained results suggesting that IgG from patients with GD and obvious dermopathy failed to increase glycosaminoglycan accumulation in dermal fibroblasts to a greater extent than that observed with control Abs (63). The IgGs also failed to influence total protein synthesis or [^{3}H]thymidine incorporation in these fibroblasts. We have noted similar negative results in orbital fibroblasts treated with serum from patients with TAO (our unpublished observations). None of these earlier studies identifies the binding sites on fibroblasts that GD-IgGs might be associating with.

Although the mechanisms through which IL-16 and RANTES are up-regulated in fibroblasts by GD-IgG are incompletely elucidated, the current studies do begin to define the cell signaling pathway utilized. Differential susceptibility to rapamycin inhibition suggests divergence with regard to signaling pathways upstream from IL-16 and RANTES: the IL-16 induction appears to involve the FRAP/mTOR/p70^{s6k} pathway whereas that of RANTES is rapamycin resistant. Up-regulation of IL-16 synthesis requires caspase-3 activity and inhibiting this enzyme completely blocks the release of mature cytokine from the fibroblast. This finding is consistent with the necessary role for caspase-3 in the processing of mature IL-16 in lymphocytes (19) and cytokine-activated fibroblasts (30). In contrast, the induction of RANTES by GD-IgG appears to be independent of caspase-3 activity.

Our unexpected findings define a previously unrecognized interaction between fibroblasts and GD-IgG that has proximate relevance to GD. The concept of specific pathogenic Abs has been established for another autoimmune disease, RA. Matsumoto et al. (64) have reported that in a murine model of RA, pathology of the disease is driven predominantly by specific IgGs. Our data would

suggest the possibility of a similar paradigm in GD, where the presence of specific IgG correlates with disease and directly activates fibroblasts, resulting in the production of two inflammatory cytokines. Along these lines, a very recent report has appeared containing evidence that Igs from the IgG4 subclass from patients with a pemphigus variant can induce IL-8 in keratinocytes and enhance neutrophil recruitment (65). Although that report offered little insight into the mechanisms involved, the concept that disease-specific Abs might provoke the recruitment of immunocompetent cells through the enhanced expression of chemoattractants has obvious relevance to our findings. Although our studies only examined the production of T cell chemoattractant cytokines in IgG-stimulated fibroblasts, activation of $p70^{s6k}$ in these cells likely results in the production of other inflammation-related molecules.

A role for IL-16 or RANTES in the development of inflammation in GD has not as yet been established. However, IL-16 is an activator of CD4-bearing T lymphocytes. Binding of IL-16 to CD4 results in the up-regulation of the IL-2R and thereby modulates the actions of IL-2 on $CD4^+$ lymphocytes (23). In contrast, the cellular targets for RANTES include mononuclear cells, resting, and activated naive and memory $CD4^+$ lymphocytes (32). It should also be noted that RANTES has been implicated in thyroidal GD (33). Thus, in the context of the cellular microenvironment, IL-16 and RANTES are likely to exert an important bias over localized cytokine production. There exists evidence for a complex interplay between IL-16 and chemokines such as RANTES (66) that could underlie the particular profile of immunocompetent cells infiltrating the orbit and other tissues in GD, and therefore defining the cytokine environment. The demonstration that GD-IgG can induce the expression of both chemoattractants in fibroblasts suggests a possible mechanism through which a diverse array of immunocompetent cells might be directed to connective tissue. The current results raise the possibility that IL-16 in concert with RANTES, the expression of which is driven in GD by IgG, orchestrates T lymphocyte infiltration of many tissues, including the thyroid. The contributions of B cell activity and fibroblast susceptibility implicit in our findings conform well to the understanding that GD involves a complex interplay of genetic and environmental factors.

Acknowledgments

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