Detection and Modulation of Interleukin-1 Receptor Antagonist Messenger Ribonucleic Acid and Immunoreactivity in Graves' Orbital Fibroblasts

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Purpose. To analyze the expression and regulation of intracellular and soluble interleukin-1 (IL-1) receptor antagonist (IL-1RA) in orbital fibroblasts and to determine whether a dysbalance between IL-1 receptor agonist and antagonist may promote IL-1-mediated proinflammatory actions in Graves' ophthalmopathy (GO).

Methods. Early passages of cultured orbital fibroblasts (OFs) derived from four patients with active GO and six control subjects were examined for their baseline expression of the two variants of IL-1RA, intracellular IL-1RA (icIL-IRA), and soluble IL-1RA (sIL-IRA). In addition, modulation of icIL-1RA and sIL-1RA was studied after exposure of OF to a broad range of cytokines as well as nonspecific stimulating agents such as lipopolysaccharide and phorbol-12-myristate 13-acetate. The IL-1RA gene and protein variants were analysed by reverse transcriptase–polymerase chain reaction, immunocytochemical staining, immunoblotting, and enzyme-linked immunosorbent assay.

Results. At baseline, both GO- and control OF showed low levels of constitutive icIL-1RA ribonucleic acid and absence of sIL-1RA ribonucleic acid expression. Exposure to various cytokines stimulated icIL-1RA and sIL-1RA gene expression in both groups, but generally to markedly lower levels in GO–OF compared to that of control OF (P < 0.01). Analysis of IL-1RA protein expression showed low levels of constitutive IL-1RA immunoreactivity (22 kDa) in cell lysates and absence of sIL-1RA immunoreactivity in culture supernatants derived from both GO–OF and control OF. Interleukin-1-alpha was capable of inducing expression of two variants (25 and 26 kDa) of IL-1RA immunoreactivity in supernatants, derived from control OF, and to a lesser degree, GO–OF (P < 0.01). Quantitative analysis showed markedly lower abundance of IL-1RA immunoreactivity in cell lysates and supernatants derived from GO–OF monolayers compared to that detected in control OF (P < 0.001).

Conclusions. Our results demonstrate differences in regulation of icIL-1RA and sIL-1RA and markedly lower levels of expression in cultured GO–OF compared to normal OF. Failure to generate, upon cytokine stimulation, sufficient quantities of icIL-1RA and sIL-1RA to balance agonist stimulation of the IL-1 receptor may facilitate IL-1-dependent proinflammatory and fibrogenic actions within the orbital tissue in GO. Invest Ophthalmol Vis Sci. 1997;38:1018–1028.
cent detection, within the affected connective tissue adjacent to T-cell aggregates, in OF monolayers and in conditioned media, of immunoreactivity for various cytokines including tumor necrosis factor-alpha (TNFa), interferon-gamma (IFNy), and interleukin-1-alpha (IL-1a). These studies have suggested that IL-1a, TNFa, and IFNy are released from infiltrating inflammatory cells and that IL-1a also is produced by residential connective tissue cells in an autocrine and paracrine manner.

Interleukin-1 is a potent pleiotropic cytokine with numerous immunologic and proinflammatory properties, including hematopoietic, endocrine, vascular, renal, and catabolic effects. Interleukin-1 acts on a variety of cells to upregulate and downregulate receptors and to induce or suppress gene expression. In addition, several immunoregulatory functions of IL-1 have been described, including its participation in T- and B-cell activation and its capacity, in macrophages–monocytes, to induce its own synthesis and release as well as that of various other cytokines. Interleukin-1 receptor antagonist (IL-IRA), a naturally occurring inhibitor of IL-1-mediated activities with sequence homology to IL-1a and IL-1b, recently has been described as a new member of the IL-1 family. Interleukin-1 receptor antagonist recognizes the two IL-1 cell surface receptors, acts as a competitive inhibitor of IL-1a and IL-1b, and blocks IL-1-mediated cellular activities without inducing detectable cellular responses. Two structural variants of the IL-1RA gene, a soluble (sIL-1RA) and an intracellular (iIL-1RA) form, have been shown and most likely originate from alternative splicing of an upstream exon into an internal acceptor site within the exon defining the leader sequence. Recently, iIL-1RA type II, a novel structural variant of iIL-1RA that contains a new exon of 63 base pairs, has been reported. These structural IL-1RA variants are regulated and produced differentially in various cell types.

A prominent role for IL-1 in the evolution of GO is suggested by its capacity to stimulate cell proliferation, glycosaminoglycan synthesis, and prostaglandin production and to induce various adhesion molecules, immunomodulatory proteins, and metalloproteinases in OF. In view of the predominance of IL-1-dependent proinflammatory actions in GO, we hypothesized that an imbalance between IL-1 receptor agonist and antagonist may promote IL-1-receptor-mediated proinflammatory and fibrogenic actions in GO. Therefore, we examined the expression of iIL-1RA and sIL-1RA in cultured OF derived from patients with severe, active GO and control subjects. In addition, we determined whether certain cytokines and proinflammatory agents, expressed by the retroorbital microenvironment during the active stages of GO, are capable of modulating the expression of IL-1RA gene and protein variants in OF. We report marked differences in the expression and regulation of the IL-1RA gene and its protein products, comparing OF derived from patients with severe GO and healthy subjects, respectively.

METHODS

Reagents

Human recombinant IL-1a, human recombinant TNFa, human recombinant IFNy, human recombinant interleukin-4 (IL-4), human recombinant interleukin-6 (IL-6), and human recombinant granulocyte macrophage–colony-stimulating factor (GM-CSF) were obtained from Boehringer Mannheim Biochemicals (Mannheim, Germany). Human recombinant interleukin-8 (IL-8) was purchased from Amer sham Life Science (Braunschweig, Germany), human recombinant interleukin-10 (IL-10) from PromoGen Corporation (Madison, WI), and human recombinant interleukin-12 (IL-12) from R&D Systems (Minneapolis, MN). Human recombinant transforming growth factor-beta (TGF/3) was obtained from Gibco Life Technologies (Eggenstein, Germany). Lipopolysaccharide (LPS), phorbol-12-myristate 13-acetate (PMA), and alkaline phosphatase-conjugated rabbit antigen immunoglobulin were purchased from Sigma (Deisenhofen, Germany). Antihuman IL-1RA antibody and recombinant IL-1RA were obtained from R&D Systems. Antibodies against desmin, vimentin, myosin, and 5-prolylhydroxylase were obtained from Dakopatts (Santa Barbara, CA) and an antibody against fibroblast antigen from Dianova (Hamburg, Germany). The reverse transcription kit was obtained from Gibco BRL (Gaithersburg, MD), and Thermus aquaticus deoxyribonucleic acid (DNA) polymerase (Taq DNA polymerase) was from Perkin Elmer Cetus (Vaterstetten, Germany). The 3'-End Labeling Kit and DIG Luminescent Detection Kit were purchased from Boehringer Mannheim Biochemicals.

Cell Culture

Orbital connective and extraocular muscle tissue was obtained from four euthyroid patients during transantral orbital decompression for severe active GO. These patients previously had received glucocorticosteroids (not within 3 months before surgery) but did not respond or had experienced intolerable side effects. Normal orbital connective and extraocular muscle tissue, taken from intraorbital sites comparable to those obtained from patients with GO, was derived from six subjects undergoing orbital surgery for refractory glaucoma (n = 2) and strabismus surgery (n = 4). Orbital fibroblasts were isolated and propagated as described previously and cultured in medium 199 containing 10% fetal calf serum, penicillin (100 U/
various cytokines and immunomodulatory molecules. Cells were subjected to reverse transcription using an oligo-(dt)-primer. Aliquots of the resulting complementary DNA (cDNA) were amplified using icIL-IRA-specific primer (5'CGGGCTGCAGTCACAGAA-13') and sIL-IRA-specific primer (5'TGTTGTGACGCCTTCTGAGGGTCC3') and an antisense primer (5'TGGAGACGGTTGACGACCAACC-3'), respectively.29 The IL-1RA variant-specific primers were designed to span at least 1 intron and to generate products of 750 base pairs (bp) (icIL-IRA) and 700 bp (sIL-IRA), respectively.29 Amplifications were performed with 5 μl of each cDNA template, 5 μl 10× polymerase chain reaction (PCR) buffer (20 mM Tris-HCl pH 8.0; 100 mM potassium chloride; 0.1 mM ethylenediaminetetraacetic acid; 1 mM dithiothreitol; 50% glycerol; 0.5% Tween 20; 0.5% Nonidet P40), 1 μl 10 mM dNTP-mix, 1 μl of each oligonucleotide primer (20 μM), and 2 U of Taq DNA polymerase in the final volume of 50 μl. Amplifications were conducted on an automated thermocycler (Thermodux, Wertheim, Germany) with 5 minutes of denaturation at 94°C followed by 35 cycles (1 minute, 94°C; 2 minutes, 65°C; 3 minutes, 72°C) and a final 10 minutes' extension at 72°C. To control for the integrity of cDNA templates and to rule out DNA contamination of samples, all templates were amplified with primers spanning an intron of the β-actin gene (5'TGACGGGTT-CACCCACACGTGCCCCTGCTA5') and 5'CTAGAAG-GCATTTGCCGAGCACAGTAGAGGG3'). The expected β-actin product generated by these primers from a cDNA template is 661 bp in length. Reaction conditions were as above. The amplified PCR products were resolved by electrophoresis on 1% agarose gels with 10 ng/ml ethidium bromide, visualized under ultraviolet light, and compared with a 125-bp molecular size control ladder (Gibco BRL).

Southern hybridization was performed to verify the specificity of PCR products and to improve the sensitivity of fragment detection. Agarose gels were vacuum-blotted onto nylon membranes (Qiagen, Hilden, Germany) and the DNA immobilized by baking (80°C for 30 minutes) and ultraviolet cross-linking (1.25 J/cm²). Membranes were prehybridized in 5× SSC, 10% blocking reagent, 0.1% N-lauroylsarcosine, and 0.02% sodium dodecyl sulfate (SDS) at 65°C for 2 hours. Blots subsequently were hybridized with digoxigenin-labeled oligonucleotide probes specific for icIL-IRA or sIL-IRA, respectively. Aliquots (25 ng) of each probe were hybridized with each membrane for 16 hours at 48°C in hybridization buffer (20× SSC, 10% blocking reagent, 0.1% N-lauroylsarcosine, 0.02% SDS). Membranes were washed twice in 2× SSC, 0.1% SDS for 5 minutes at room temperature and twice in 0.1× SSC, 0.1% SDS for 15 minutes at 48°C, followed by incubation with anti-DIG-alkaline phosphatase-conjugate and luminol—purified protein derivative substrate solution. Membranes subsequently were exposed to x-ray film for 10 minutes at room temperature using an intensifying screen.

**SDS—PAGE and Immunoblotting**

Orbital fibroblast monolayers were extracted with 0.5% SDS in the presence of protease inhibitor complex containing antipain—hydrochloric acid, bestatin, chymostatin, E-64, leupeptin, pepstatin, phosphoramide, pefabloc SC, ethylenediaminetetraacetic acid, and aproitin (Boehringer Mannheim Biochemicals). Cell lysates and culture supernatants were concentrated using the Centricor-10 system (Amicon, Beverly, MA), and subjected to SDS—polyacrylamide gel electrophoresis (PAGE) under reducing conditions, as described previously.7 In brief, samples were mixed with loading buffer (Tris 0.5 M, pH 6.8, 50% glycerol, 10% SDS, 25% mercaptoethanol, bromophenol blue), heated to 95°C for 4 minutes, and loaded onto gels (4% acrylamide stacking gel and 12% acrylamide running gel) at a protein concentration of 150 μg/lane. Electrophoresis was performed with a standard buffer system at a constant current of 90 V for 2 hours. Gels were presoaked in transfer buffer (25 mM Tris, 192 mM...
glycine, 20% methanol) before electrophoretic transfer (3 hours at 90 mA) of proteins onto nitrocellulose. Nitrocellulose membranes were incubated in blocking buffer containing 1 X tris-buffered saline (TBS) (10 X TBS: 100 mM Tris pH 7.5; 1.5 M sodium chloride), 0.05% Tween 20, and 5% nonfat dry milk for 3 hours at 37°C. Goat antihuman IL-1IRA antibody was applied at a concentration of 1.5 μg/ml in 1 X TBS and 1% nonfat dry milk overnight at 4°C. After being washed, nitrocellulose membranes were incubated with alkaline phosphatase-conjugated goat immunoglobulin (1 μg/ml) for 2 hours at room temperature, washed, and the reaction products visualized using nitroblue tetrazolium and bromochloroiodindolylphosphate chromogenic substrates in developing buffer (100 mM Tris, pH 9.5, 100 mM sodium chloride, 500 mM magnesium chloride). Protein sizes were determined by comparison with a protein molecular weight marker (Amersham Life Science, Braunschweig, Germany). For quantitation of IL-1IRA reactivity, immunoblots were subjected to scanning densitometry using a dual-wave length densitometer (Shimadzu TCL Scanner; Shimadzu Scientific, Columbia, MD) connected to a data recorder (Shimadzu DR2). Intensities are expressed in arbitrary units, reflecting the relative areas under the curves of the stained bands. The assay was linear with respect to protein concentrations within the range used.

**Immunocytochemistry**

Orbital fibroblasts were plated directly onto multi-chamber slides and grown to near-confluence. Monolayers were incubated for 44 hours in the presence or absence of cytokines, washed with phosphate-buffered saline and fixed in 100% methanol for 10 minutes at 4°C. Air-dried slides were rehydrated in phosphate-buffered saline, and nonspecific binding was blocked with 1% bovine serum albumin. Goat antihuman IL-1IRA antibody was applied at a concentration of 1.5 μg/ml for 2 hours at room temperature. Washed cell monolayers were incubated with alkaline phosphatase-conjugated goat immunoglobulin (1 μg/ml) for 1 hour at room temperature. Slides were rinsed with TBS, incubated in developing buffer for 10 minutes, and the reaction product was visualized with nitrobluetetrazolium and bromochloroiodindolylphosphate chromogenic substrates in developing buffer. Slides were counterstained with malachite green for 5 minutes before mounting. A brown precipitate was indicative of IL-1IRA immunoreactivity. Parallel monolayers with the primary and secondary antibodies replaced, in turn, by phosphate-buffered saline and isotype matched nonimmune immunoglobulin Gs, respectively, were examined to ensure specificity and to exclude cross-reactivities between the antibodies and conjugates used.

**Human Interleukin-1 Receptor Antagonist Immunoassay**

Quantitative determination of human IL-1RA in cell culture supernatants was performed using a commercially available quantitative sandwich enzyme immunoassay (Quantikine; R&D Systems, Minneapolis, MN). This immunoassay is calibrated against a highly purified recombinant human IL-1RA and detects IL-1RA concentrations as low as 10 pg/ml. The monoclonal antihuman IL-1RA antibody used in this assay recognizes both natural and recombinant human IL-1RA, with no significant cross-reactivity or interference by a broad range of cytokines tested. Intraassay and interassay variabilities were determined for five samples of known IL-1RA concentrations, and the coefficient of variance generally was <6.9%. Data represent the means ± standard deviation of duplicate measurements derived from three separate experiments.

**Statistical Analysis**

Intraassay variabilities for measurements of IL-1RA reactivity on immunoblots were determined in untreated and IL-1-stimulated samples run in quadruplicate, and the coefficient of variance was 9.4% and 6.4%, respectively. This was determined by interassay variability, equal amounts of protein (100 μg) from the same sample were loaded onto two gels run on
FIGURE 2. Soluble interleukin-1 receptor antagonist gene expression in cultured orbital fibroblasts derived from a healthy subject (A) and from a patient with Graves' ophthalmopathy (B), as detected by polymerase chain reaction and southern hybridization. Orbital fibroblast monolayers were stimulated for 16 hours as follows: lane 1, interleukin-1-alpha (10 U/ml); lane 2, tumor necrosis factor alpha (10 ng/ml); lane 3, lipopolysaccharide (LPS; 50 ng/ml); lane 4, phorbol-12-myristate-13-acetate (PMA; 10 ng/ml); lane 5, interferon-gamma (100 U/ml); lane 6, interleukin-4 (5 U/ml); lane 7, interleukin-6 (100 U/ml); lane 8, interleukin-8 (10 ng/ml); lane 9, interleukin-10 (20 ng/ml); lane 10, transforming growth factor-beta (10 ng/ml); lane 11, granulocyte macrophage-colony-stimulating factor (50 U/ml); lane 12, LPS (50 ng/ml) + PMA (10 ng/ml); lane 13, LPS (50 ng/ml) + interleukin-10 (50 U/ml); lane 14, unstimulated (medium 199 containing 1% fetal calf serum). Experiments were repeated twice with identical results.

RESULTS
Detection of Interleukin-1 Receptor Antagonist Ribonucleic Acid in Normal Orbital Fibroblasts

Unstimulated OF derived from normal subjects showed low levels of constitutive icIL-1RA gene expression (Fig. 1A). In contrast, sIL-IRA-specific transcripts were not detected under baseline conditions (Fig. 2A). Time courses of IL-1RA gene expression were studied using reverse transcriptase (RT)–PCR amplification. Normal and GO–OF subjects were treated with IL-1α (10 U/ml) for various periods up to 24 hours before RNA extraction. Induction of icIL-1RA RNA expression first was detected at 10 hours, and maximal abundance was observed at 16 hours of IL-1α stimulation. Expression of icIL-1RA RNA still was detectable after 24 hours; however, the abundance of expression declined between 16 and 24 hours (Fig. 3A). Equal loading of samples and comparable efficiency of amplification were confirmed by RT–PCR amplification using β-actin-specific primers on identical cDNA-templates (Fig. 3B). Similar time-dependent induction was observed for sIL-IRA gene expression. Moreover, when IL-1α was replaced by TNFa or LPS for stimulation of OF monolayers, similar results of time-dependent icIL-1RA and sIL-1RA gene expression were obtained (data not shown).

Stimulation of OF monolayers with various cytokines including IL-1α, IL-4, IL-6, IL-8, IL-10, TNFa, IFNy, TGFβ, GM-CSF, LPS, and PMA for 16 hours showed marked differences of icIL-1RA and sIL-1RA gene expression. Of all cytokines and agents tested, only IL-1α, TNFa, IFNy, TGFβ, LPS, and PMA were capable of inducing icIL-1RA gene expression in normal OF (Fig. 1A, Table 1). Further, induction of sIL-1RA-specific transcripts in normal OF was observed after stimulation of monolayers with IL-1α, TNFa, and LPS (Fig. 2A, Table 1). Combined treatment of normal different days, and the resulting immunoblots were quantitated by densitometry. Pair plotting (day 1 versus day 2) showed that all data points were within ±10% of the line of identity, and five of seven data points were within ±5% of the line of identity. Student’s test for analysis of paired and unpaired data was used to establish levels of significance (P < 0.05).

Investigations adhered to the tenets of the Declaration of Helsinki. Informed consent was obtained from the subjects after the nature and possible consequences of the study were explained to them. The research protocol was approved by the Institutional Human Experimentation Committee.
TABLE 1. Modulation of icIL-IRA and sIL-IRA Gene Expression in Orbital Fibroblasts by Cytokines and Nonspecific Stimulatory Agents

<table>
<thead>
<tr>
<th>Treatment</th>
<th>icIL-IRA</th>
<th>sIL-IRA</th>
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<tr>
<td></td>
<td>GO-OF/normal OF</td>
<td>GO-OF/normal OF</td>
</tr>
<tr>
<td>Medium alone</td>
<td>(+)/(+))</td>
<td>1/1</td>
</tr>
<tr>
<td>+IL-1α</td>
<td>+/+</td>
<td>(+)/+</td>
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<td>+TGFβ</td>
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</tr>
<tr>
<td>+GM-CSF</td>
<td>(+)/+</td>
<td>+/+</td>
</tr>
<tr>
<td>+LPS</td>
<td>+/+</td>
<td>+/+</td>
</tr>
<tr>
<td>+PMA</td>
<td>—/(-)</td>
<td>(+)/+</td>
</tr>
<tr>
<td>+LPS + IL-10</td>
<td>—/(-)</td>
<td>(+)/+</td>
</tr>
<tr>
<td>+LPS + IFNγ</td>
<td>—/(-)</td>
<td>ND</td>
</tr>
<tr>
<td>+LPS + PMA</td>
<td>—/(-)</td>
<td>(+)/+</td>
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icIL-IRA = intracellular interleukin-1 receptor antagonist; sIL-IRA = soluble interleukin-1 receptor antagonist; GO-OF = Graves’ ophthalmopathy-derived orbital fibroblasts; normal = normal orbital fibroblasts; — = undetectable; (+) = weak; + = moderate; ++ = strong; ND = not done; IL = interleukin; IFN = interferon; TNF = tumor necrosis factor; TGF = transforming growth factor; GM-CSF = granulocyte-macrophage colony-stimulating factor; LPS = lipopolysaccharide; PMA = phorbol myristate acetate.

At baseline, both GO and normal OF showed low levels of constitutive icIL-IRA RNA expression in the absence of IL-1RA-specific transcripts. In contrast, treatment with various cytokines and agents induced markedly lower levels of icIL-IRA RNA expression in OF derived from all patients with GO compared to those of normal subjects (P < 0.01) (Figs. 1A and 1B, Table 1). Further, compared to those levels of normal OF, where IL-1α, TNFα, and LPS stimulated sIL-1RA gene expression, comparable levels of sIL-1RA RNA expression in GO–OF were induced only by LPS, whereas markedly lower levels of sIL-1RA gene expression were induced by treatment of GO–OF with IL-1α (P < 0.001) and TNFα (P < 0.01) (Figs. 2A and 2B, Table 1). In addition, whereas IL-4, IL-6, IL-10, and PMA inhibited icIL-IRA gene expression in GO–OF, these cytokines had no inhibitory effect on icIL-IRA gene expression in normal OF monolayers (Figs. 1A and 1B, Table 1). On combined stimulation, IFNγ, IL-10, and PMA suppressed LPS-induced sIL-1RA gene expression only in GO–OF but not in normal OF (Figs. 2A and 2B, Table 1). No differences were observed between either group of OF with respect to time courses of cytokine-stimulated icIL-1RA and sIL-1RA RNA expression (data not shown). Moreover, no differences were noted between fibroblasts derived from orbital connective tissue or extraocular muscle and between the second or seventh passage of fibroblasts, respectively.

Expression and Production of Interleukin-1 Receptor Antagonist Protein by Orbital Fibroblasts

Immunocytochemical staining with a monoclonal antihuman IL-1α-antibody showed low levels of cytoplasmic IL-1α-specific immunoreactivity in untreated GO-OF compared to normal OF monolayers. Treatment of GO and normal OF monolayers with IL-1α and LPS markedly enhanced the abundance of IL-1α-specific immunoreactivity (Figs. 4A, 4B, and 4C) but generally to much lower levels in GO–OF (Fig. 4D) compared to those of normal OF. The IL-1α-specific immunoreactivity in normal and GO–OF was lost when IL-1α antibody was preabsorbed with recombinant human IL-1α (Fig. 4E) or when replaced by isotype-matched nonimmune immunoglobulin G (Fig. 4F), confirming the specificity of the immunostaining procedure. In addition, IL-1α expression in cell lysates (icIL-1α) and cell culture supernatants (sIL-1α) of OF monolayers was assayed by immunoblot analysis, using recombinant hIL-1α (17 kDa) as a positive control. Constitutive expression of icIL-1α protein was approximately 22 kDa) was detected in lysates of untreated OF derived from normal subjects (Fig. 5A). Expression of icIL-1α protein in normal OF was enhanced by treatment with IL-1α, not affected by treatment with TGFβ, TNFα, and LPS, and inhibited by treatment with IL-4 (Fig. 5A). By contrast, sIL-1α protein expression was not detected in supernatants of untreated normal OF monolayers (Fig. 5B). After exposure of OF monolayers to various cytokines for 44 hours, only IL-1α was found to strongly induce expression of sIL-1α protein in supernatants derived from normal OF monolayers (Fig. 5B). Soluble IL-1α-specific immunoreactivity in cell culture supernatants was detected as two bands of approximately 23 and 26 kDa in molecular weight, indicating cytokine-stimulated production of sIL-1α variants by normal OF.
FIGURE 4. Localization of interleukin-1 receptor antagonist (IL-1RA) immunoreactivity in untreated (A, B) or interleukin-1-alpha (IL-1α)-treated (C, D; 10 U/ml, 44 hours) orbital fibroblast monolayers derived from a normal subject (A, C) and a patient with active Graves' ophthalmopathy (B, D), using monoclonal anti-human IL-1RA antibody. Similar results were obtained in orbital fibroblast (OF) derived from two other patients with Graves' ophthalmopathy and two other healthy subjects. The IL-1RA immunoreactivity in IL-1α-stimulated normal OF monolayers was not detected when IL-1RA antibody had been preabsorbed with recombinant human IL-1RA (E), or when IL-1RA antibody was replaced by an isotype matched nonimmune immunoglobulin G (F).
IL-1 Receptor Antagonist in Graves' Ophthalmopathy

FIGURE 5. Immunoblot analysis of interleukin-1 receptor antagonist (IL-1RA) expression in cell lysates (A) and cell culture supernatants (B) of orbital fibroblast (OF) monolayers derived from a normal subject, using monoclonal anti-human IL-1RA antibody. Confluent OF monolayers were treated for 44 hours as follows: lanes 1 and 7, medium 199 alone; lanes 2 and 8, transforming growth factor-beta (10 ng/ml); lanes 3 and 9, IL-4 (5 U/ml); lanes 4 and 10, lipopolysaccharide (50 ng/ml); lanes 5 and 11, tumor necrosis factor alpha (10 ng/ml); lanes 6 and 12, IL-1a (10 U/ml). Recombinant human IL-1RA was loaded for comparison (far right). Cell lysates and supernatants were concentrated 10-fold and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions.

tent cells, predominantly T-lymphocytes and macrophages. Various cytokines have been detected within the orbital tissues in GO, including IL-1a, TNFalpha, and IFNgamma, and are thought to play an important role in the evolution and propagation of GO.

Although some of these cytokines derive mainly from infiltrating activated lymphocytes and macrophages, others such as IL-1 alpha also are produced locally by resident cells such as OF. The prominent role of IL-1 in the orbital immune process is highlighted by its capacity to stimulate several important functions in OF, including cell proliferation, glycosaminoglycan synthesis, and various immunomodulatory molecules expressed by OF. The IL-1-dependent biologic activities require binding to specific cell surface receptors and normally are balanced by IL-1RA, which acts competitively to inhibit binding of IL-1 to its receptors.

Recently, it was shown that exogenous addition of recombinant IL-1RA can inhibit IL-1-induced stimulation of glycosaminoglycan synthesis in cultured human OF, suggesting that certain IL-1-mediated effects in GO may be countered by IL-1RA in vitro. Our study was designed to determine whether cultured human OFs are capable of producing IL-1RA, how IL-1RA gene and protein expression is regulated, and whether there exist any differences in the expression and production of IL-1RA between OF derived from patients with active GO and healthy subjects. As shown by RT-PCR and southern hybridization, both cultured normal OF and GO-OF constitutively express low levels of icIL-1RA RNA. Exposure of cell monolayers to various cytokines induced sIL-1RA and
TABLE 2. Modulation of Soluble IL-IRA
Levels in Orbital Fibroblast Conditioned Media by Cytokines and Lipopolysaccharide

<table>
<thead>
<tr>
<th>Treatment</th>
<th>sIL-IRA (pg/ml)</th>
<th>P value</th>
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<tr>
<td>Medium alone</td>
<td>0.00</td>
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<tr>
<td>GO-OF (baseline)</td>
<td>15.67 ± 4.41</td>
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<tr>
<td>+IL-1α</td>
<td>27.77 ± 3.65</td>
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<tr>
<td>+TNFα</td>
<td>21.22 ± 6.29</td>
<td>0.28*</td>
</tr>
<tr>
<td>+TGF/β</td>
<td>25.65 ± 3.61</td>
<td>0.059*</td>
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<td>+LPS</td>
<td>22.75 ± 6.07</td>
<td>0.18*</td>
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<tr>
<td>Normal OF (baseline)</td>
<td>43.35 ± 14.70</td>
<td>0.021*</td>
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<tr>
<td>+IL-1α</td>
<td>214.00 ± 39.34</td>
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<td>94.33 ± 19.55</td>
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<td>+LPS</td>
<td>118.00 ± 33.87</td>
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Same abbreviations as in Table 1.
* Compared with baseline.
† Compared with GO-OF receiving identical treatment.

An important result of our study is the detection of marked differences in expression and modulation of IL-IRA gene and protein between cultured normal OF and GO-OF. Lower levels of both icIL-IRA and sIL-1RA gene expression were detected consistently in OF derived from all patients with GO as compared to those of normal OF. Treatment with IL-4, IL-10, and PMA inhibited expression of icIL-1RA-specific transcripts in OF but had no effect on normal OF. Further, in contrast to normal OF, where IL-1α, TNFα, and LPS were capable of inducing sIL-1RA gene expression, only PMA acted to stimulate potentially sIL-1RA in GO-OF. Similarly, abundance of constitutively produced icIL-1RA protein consistently was lower in GO-OF as compared to control OF. Moreover, after stimulation with IL-1α, TNFα, and LPS, respectively, significantly lower levels of both forms of IL-1RA protein were detected in GO-OF. Diminished production of IL-1RA protein and significantly lower IL-1RA/IL-1β ratio recently have been reported in rheumatoid arthritis synovial tissues. Diminished production of IL-1RA protein by GO-OF appears to represent a unique condition, differences of cytokine receptor density on the cell surface, and differences in postreceptor signaling may contribute to varying sensitivities of cells to respond to certain cytokines.

The molecular weight of icIL-1RA protein present in cell lysates of OF (22 kDa), as detected by immunoblots, differed from the protein size of cell culture supernatant-derived sIL-1RA, where two molecular variants of IL-1RA immunoreactivity were found to migrate at 23 kDa and 26 kDa, respectively. To date, various isoforms of IL-1RA have been reported, which mainly differ in the degree of N-glycosylation of a single peptide of approximately 17 kDa predicted molecular weight. The molecular size of icIL-1RA protein detected in OF is in agreement with that reported for cell-associated IL-1RA produced by dermal fibroblasts and is slightly larger than the 18 to 20 kDa form of icIL-1RA protein expressed by cultured keratinocytes and lung epithelia. Variable protein sizes of classical icIL-1RA (type I) have been reported by several investigators, including 18 kDa, 20 kDa, and 22 kDa variants. Variabilities in migration patterns of IL-1RA variants on SDS–PAGE may be explained, at least in part, by the different molecular weights of nonglycosylated icIL-1RA and sIL-1RA, most of which appear to be glycosylated. Moreover, in addition to classical icIL-1RA (type I), a novel 25-kDa icIL-1RA (icIL-1RA type II) recently has been detected in fibroblast and keratinocyte cell lines.

An important result of our study is the detection of marked differences in expression and modulation of IL-1RA gene and protein between cultured normal OF and GO-OF. Lower levels of both icIL-1RA and sIL-1RA gene expression were detected consistently in OF derived from all patients with GO as compared to those of normal OF. Treatment with IL-4, IL-10, and PMA inhibited expression of icIL-1RA-specific transcripts in GO-OF but had no effect on normal OF. Further, in contrast to normal OF, where IL-1α, TNFα, and LPS were capable of inducing sIL-1RA gene expression, only PMA acted to stimulate potentially sIL-1RA in GO-OF. Similarly, abundance of constitutively produced icIL-1RA protein consistently was lower in GO-OF as compared to control OF. Moreover, after stimulation with IL-1α, TNFα, and LPS, respectively, significantly lower levels of both forms of IL-1RA protein were detected in GO-OF. Diminished production of IL-1RA protein and significantly lower IL-1RA/IL-1β ratio recently have been reported in rheumatoid arthritis synovial tissues. Diminished production of IL-1RA protein by GO-OF appears to represent a unique
feature rather than a generalized metabolic alteration, because GO-OF previously have been reported to express enhanced, rather than reduced, quantities of several immunomodulatory molecules such as HLA-DR, intercellular adhesion molecule-1, and 72-kDa heat shock protein.

In conclusion, we have observed markedly reduced levels as well as differences in regulation of icIL-1RA and sIL-1RA gene and protein expression in GO-OF as compared to normal OF. Attenuated production of icIL-1RA may enhance cellular responses to extracellular IL-1 by limiting competition for IL-1R internalized by endocytosis or by increasing the levels of IL-1R available on the cell surface, thus enhancing autocrine and paracrine stimulation by IL-1 in cells producing IL-1 such as OF. Although it must be remembered that our data were obtained in vitro and, therefore, should be extrapolated cautiously to the situation in vivo, impaired production and release of IL-1R by OF residing in the orbital connective tissue may contribute to the predominance of IL-1-dependent proinflammatory actions in GO. Because IL-1RA appears to be capable of inhibiting IL-1-induced glycosaminoglycan synthesis in cultured OF, strategies designed to raise IL-1RA concentrations systematically or locally within the orbital tissue may inhibit IL-1R activation, thus providing a novel approach to attenuation or prevention of IL-1-mediated proinflammatory and fibrogenic effects in GO.

Key Words
cytokines, Graves' disease, immunomodulation, interleukin-1, thyroid-associated ophthalmopathy

References
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