Leukoregulin Induction of Prostaglandin-Endoperoxide H Synthase-2 in Human Orbital Fibroblasts

AN IN VITRO MODEL FOR CONNECTIVE TISSUE INFLAMMATION

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Several proinflammatory cytokines can increase prostaglandin E2 (PGE2) synthesis in a variety of cell types, constituting an important component of the inflammatory response. We demonstrate here that leukoregulin, a 50-kDa product of activated T lymphocytes, dramatically increases PGE2 synthesis in cultured human orbital fibroblasts. This up-regulation is mediated through an induction of prostaglandin-endoperoxide H synthase-2 (PGHS-2), the inflammatory cyclooxygenase. Steady-state levels of PGHS-2 mRNA are increased within 1.5 h of leukoregulin addition and are near maximal by 6 h, when they are 50-fold or higher above basal levels. The increase in PGHS-2 mRNA levels is partially blocked by cycloheximide, suggesting de novo synthesis of an intermediate protein may be required for a maximal leukoregulin response. Nuclear run-on studies indicate PGHS-2 gene transcription is up-regulated by leukoregulin 2-fold after 2 and 6 h. PGHS-2 protein, as assessed by Western blotting and two-dimensional protein gel analysis, is increased dramatically in orbital fibroblasts. This lymphokine-dependent expression of PGHS-2 is blocked by dexamethasone, and the increase in PGE2 and cAMP levels following leukoregulin treatment is also blocked by indomethacin and by SC 58125, a newly developed PGHS-2-selective cyclooxygenase inhibitor. The dramatic increase in cAMP levels causes marked alteration in orbital fibroblast morphology. PGHS-2 expression in dermal fibroblasts is also increased by leukoregulin; however, the response is considerably less robust, and these cells do not undergo a change in morphology. Both orbital and dermal fibroblasts express high levels of PGHS-1 mRNA and protein, the other abundant form of cyclooxygenase. In contrast to its effects on PGHS-2 expression, leukoregulin fails to alter PGHS-1 levels in either orbital or dermal fibroblasts, suggesting that PGHS-1 is not involved in cytokine-dependent prostanooid production in human fibroblasts.

The increased PGHS-2 expression elicited by leukoregulin in orbital fibroblasts may be a consequence of both transcriptional and post-transcriptional effects. These observations help clarify the pathogenic mechanism relevant to the intense inflammation associated with Graves’ ophthalmopathy associated with Graves’ disease (8). In that pathologic process, orbital connective tissue and extracocular muscles become hypertrophied, infiltrated with lymphocytes and other immunocompetent cells, and are often dramatically inflamed (8–11). The basis for the connective tissue and muscle enlargement is an accumulation of the nonsulfated glycosaminoglycan, hyaluronan (8). By virtue of its substantial water binding capacity, hyaluronan occupies a considerable volume and mechanically displaces orbital structures, including the eye, anteriorly causing proptosis. The presence of lymphocytes in tissues affected by Graves’ ophthalmopathy suggests a role for lymphocyte-derived cytokines in the up-regulation of hyaluronan biosynthesis in orbital tissues.

Human orbital fibroblasts represent a heterogeneous population of cells expressing phenotypes that distinguish them from skin-derived fibroblasts (1–7). These fibroblasts are believed to participate in the pathogenesis of ophthalmopathy associated with Graves’ disease (8). In that pathologic process, orbital connective tissue and extracocular muscles become hypertrophied, infiltrated with lymphocytes and other immunocompetent cells, and are often dramatically inflamed (8–11). The basis for the connective tissue and muscle enlargement is an accumulation of the nonsulfated glycosaminoglycan, hyaluronan (8). By virtue of its substantial water binding capacity, hyaluronan occupies a considerable volume and mechanically displaces orbital structures, including the eye, anteriorly causing proptosis. The presence of lymphocytes in tissues affected by Graves’ ophthalmopathy suggests a role for lymphocyte-derived cytokines in the up-regulation of hyaluronan biosynthesis in orbital tissues.

Leukoregulin is a 50-kDa cytokine product of mitogen-activated T lymphocytes that exhibits unique anti-tumor properties in a wide variety of target cells (12). Among these are rapid increases in plasma membrane permeability and antiproliferative activity restricted to neoplastically transformed cells (12, 13). Some of leukoregulin’s actions in K-562 erythroleukemia cells are mediated through the activation of protein kinase C, utilizing an apparently nontraditional signal transduction pathway (14). In normal dermal fibroblasts, leukoregulin can alter the biosynthetic repertoire of extracellular matrix components by increasing the synthesis of collagen, hyaluronan, collagenase, fibronectin, and stromelysin-1 (15–17). Some of these events are mediated at the level of gene transcription and may involve the activation of target gene AP-1 sites. Leukoregulin induces IL-8 gene expression in human dermal fibroblasts through the activation of NF-κB binding to its corresponding cis-acting element in the IL-8 promoter (18). Leukoregulin exhibits substantial activity with regard to an up-regulation of hyaluronan synthesis in orbital fibroblasts, an
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action that requires de novo protein synthesis (19). This effect is far greater in orbital than in dermal fibroblasts and is attenuated by glucocorticoids (19). In addition, leukoregulin dramatically induces expression of plasminogen activator inhibitor type-1 (PAI-1), a serine protease inhibitor, in orbital fibroblasts while down-regulating PAI-1 synthesis in fibroblasts from the abdominal wall (20). Both the increase in hyaluronan synthesis and PAI-1 expression observed in orbital fibroblasts treated with leukoregulin resemble the effects of interferon-γ (21, 22); however, the responses to leukoregulin are far greater. Thus leukoregulin appears to exhibit multiple actions in orbital fibroblasts that are quantitatively more dramatic than those observed in dermal fibroblasts. Moreover, the aggregate effects of leukoregulin in orbital cells distinguish it from several more fully characterized cytokines.

Another important component of Graves’ ophthalmopathy is a sometimes dramatic inflammatory reaction that, if not attenuated, progresses to scar formation. Insight into the molecular basis for this intense inflammation should help direct efforts in developing specific therapies. The recent identification and cloning of two distinct cyclooxygenases represent major advances in our understanding of inflammatory responses (23–34). These enzymes (prostaglandin-endoperoxide H synthase, EC 1.14.99.1, PGHS), which catalyze the rate-limiting steps in the prostaglandin, prostacyclin and thromboxane biosynthetic pathways, are encoded by two different genes and appear to be distributed differently in the cell. Prostaglandin G2 (PGG2) is generated from arachidonic acid through oxygenase activity and is subsequently converted to PGH2 by virtue of the peroxidase activity of these bifunctional enzymes (35, 36). It is currently believed that basal prostaglandin production is largely the result of PGHS-1 activity which is constitutively expressed in a large number of cell types and tissues. The transcript of the constitutive form, PGHS-1, can be processed into a full-size mRNA encoding a 68-kDa protein or can be alternatively spliced into a smaller mRNA encoding a protein lacking 37 amino acids that is presumed to be less active (25). This assumption derives from the loss in the truncated protein of a single, potential glycosylation site (25). PGHS-2 is the inducible, inflammatory cyclooxygenase, the expression of which is down-regulated by glucocorticoids and up-regulated by serum and a variety of cytokines (26–34). It is encoded by an early response gene and is ordinarily expressed at very low levels. It would appear that the cell type-dependent down-regulation by glucocorticoids of prostanoit production (37) derives from an inhibition of the expression of PGHS-2. Thus both the physiological role attributed to prostanoitds and the participation of these molecules in the inflammatory response can be understood on the basis of differential expression and activities of PGHS-1 and PGHS-2.

In this report, we present data suggesting that orbital fibroblasts are particularly susceptible to the up-regulation of PGE2 production by leukoregulin. This action of leukoregulin involves a substantial increase in steady-state PGHS-2 mRNA and protein and results in a dramatic generation of cAMP and an alteration in orbital fibroblast morphology. The alterations in cell shape induced by leukoregulin are identical to those observed previously in PGE2-treated orbital fibroblasts (38, 39). These changes can be blocked by selective inhibition of PGE2 activity and by glucocorticoids while any such effects are absent in dermal fibroblasts. Our findings define a previously unrecognized phenotypic attribute of orbital fibroblasts that may underlie this cell’s putative role in the pathogenesis of Graves’ ophthalmopathy. Moreover, the current observations suggest a potential role for leukoregulin, or a related molecule, as a trigger in initiating the inflammatory response associated with this disease process.

EXPERIMENTAL PROCEDURES

Materials—RU 38486 was generously supplied by Roussel UCLAF (Romainville, France). SC 58125 was a kind gift of Searle (Skokie, IL). Dexamethasone (1,4-pregnenadien-9-fluoro-16a-methyl-11β,17α,21-triol-3,20-dione) and cycloheximide were from Sigma and indomethacin was from Fluka. IL-1β, IL-4, and TNF-α were purchased from Biosource (Camarillo, CA), TGF-β was from Life Technologies, Inc. and recombinant human interferon-γ and TNF-β were from Boehringer Mannheim.

Leukoregulin Preparation—Leukoregulin was prepared as described previously (40) from human peripheral blood leukocytes. Briefly, normal lymphocytes were stimulated with phytohemagglutinin (Sigma) for 48 h followed by diaffiltration, anion exchange, isolectic focusing, and high performance molecular sieving liquid chromatography. The product has a relative molecular mass of 50 kDa and a pI of 5.1. The material used in these studies appeared as a single, silver-staining band on an analytical isolectric focusing gels. One unit of leukoregulin is defined as that amount causing a 50% increase in the permeability of the plasma membrane of 106 K-562 erythroblasts cells/ml after a 2-h treatment (13). The concentration range of 0.1–10 units/ml has been reported active in the wide array of effects ascribable to leukoregulin (12–20).

Cell Culture—Orbital fibroblast cultures were initiated from tissue explants obtained during decompression surgery for severe Graves’ ophthalmopathy or from normal orbital tissue obtained as waste during surgery to correct nonorbital disease. We have examined a total of five strains of orbital fibroblasts from patients with Graves’ ophthalmopathy and three strains of orbital fibroblasts from patients without the disease. Five strains of dermal fibroblasts were derived from biopsies of normal appearing skin. These activities have been approved by the Institutional Review Board of Albany Medical College. Some of the material was kindly provided by Dr. Richard Dallow (Harvard Medical School). Tissue specimens were mechanically disrupted, covered with Eagle’s medium containing 10% fetal bovine serum (FBS, Life Technologies, Inc.), glutamine (435 μg/ml), and penicillin/streptomycin and allowed to attach to the bottom of a plastic culture dish as described previously (41, 42). Cultures were maintained in a humidified incubator at 37°C in 5% CO2-enriched air. When fibroblasts were outgrown, the explant was removed, the monolayer of fibroblasts was treated with trypsin, and cells were replated. Culture strains were utilized between the 3rd and 12th passage at a state of confluence when all experimental manipulations were performed.

Phase-contrast Microscopy—Fibroblasts were inoculated on 22 × 22-mm glass coverslips at a seeding density of 104 cells/coverslip. Fibroblasts were allowed to attach for 24 h in medium supplemented with 10% FBS before any experimental manipulations. They were then shifted to fresh medium containing 1% FBS with leukoregulin (1 unit/ml), PGE2 (0.1 μM), 8-Br-cAMP (1 mM), or nothing (control) for the duration indicated. Microscopy was performed with a Nikon Labophot (Nikon Corp., Melville, NY) equipped with a 35 mm camera. Photographs were taken on Kodak Tri-Max 400 film (Eastman Kodak Co.) at a final magnification of ×100.

Isolation of Fibroblast RNA and Northern Analysis—Cultured fibroblasts were cultivated in 100-mm diameter plastic culture plates to confluence, treated with the test compounds described, and total RNA extracted from rinsed monolayers by the method described by Chomczynski and Sacchi (43). For Northern blot analysis by the method of Church and Gilbert (44), RNA was electrophoresed on denaturing 1% agarose, formaldehyde gels. The integrity of the RNA was established routinely by ascertaining the 260/280 spectrophotometric ratio and by staining the electrophoresed sample with ethidium bromide and inspecting under UV light. The samples were transferred to a Zeta-probe membrane (Bio-Rad), and the immobilized RNA was allowed to hybridize with [32P]dCTP-labeled PGHS-1 and PGHS-2 probes. These were generated from 1.6- and 1.4-kb human cDNAs, respectively, that were cloned by us from WI-38 cells and were completely sequenced and shared 100% identity with previously published sequences (23, 27). Hybridization was allowed to proceed in a solution containing 5 × SSC, 50% formam-
ide, 5 × Denhardt’s solution, 50 mM phosphate buffer (pH 6.5), 1% SDS, and 0.1% sodium dodecyl sulfate. Monolayers were then washed under high stringency conditions, and then radioactive hybrids were visualized by radioautography on X-Omat film (Kodak) exposed at -70°C, and the radioactive bands were scanned with a Bioimager system (Milenage). Membranes were then stripped of radioactivity following the manufacturer’s instructions and rehybridized with probes complimentary to GAPDH for standardization.

Reverse Transcription-PCR Analysis of PGHS-1 and PGHS-2 mRNA Expression—1 µg cytoplasmic RNA obtained from control and leukoregulin-treated orbital fibroblasts was reverse transcribed using SuperScript II (Life Technologies, Inc.). The 25-µl reverse transcription reaction was diluted 1:1 with H2O, and 1 µl was amplified by PCR (45). The primers used were: PGHS-1, 5’ primer (AAA CCC TAC ACC TTC TCC T; PGHS-2, 5’ primer (GAG CAG GCA GAT GAA ATA C). Samples were amplified for 35 cycles: 94°C, 30 s; 59°C, 30 s; 72°C, 1 min.

Nuclear Run-on Reactions—Orbital fibroblasts were cultivated in 100-mm diameter plastic culture dishes to near confluence and were shifted for 40 h to medium containing 1% FBS. They were then shifted into fresh medium containing 1% FBS. The RNA was harvested and was reverse transcribed into cDNA. The cDNA was then amplified by PCR using primers corresponding to the carboxyl-terminal amino acid sequence 580-598 deduced from the human cDNA sequence and including an extra amino-terminal cysteine for protein coupling. Confuent cultured fibroblasts were shifted from growth medium containing 10% FBS to medium supplemented with 1% serum for 48 h. For the times specified in the legends to the figures, they were treated under reduced serum conditions with the test compounds. Monolayers were washed and harvested in an ice-cold buffer containing 15 mM CHAPS, 1 mM EDTA, 20 mM Tris-HCl (pH 7.5), 10 µg/ml soybean trypsin inhibitor, and 10 µM phenylmethylsulfonyl fluoride. Nuclei were lysed and extracted by incubating in Laennec buffer subjected to sonication followed by gel electrophoresis and the separated proteins transferred to polyvinylidene difluoride membrane (Bio-Rad). The primary antibodies (10 µg/ml) were incubated with the membranes for 2 h at RT, and membranes were washed extensively and reincubated with secondary, peroxidase-labeled antibodies for 2 h. Following washes, the ECL (Amersham Corp.) chemiluminescence detection system was used to generate the signal. The resulting bands were analyzed densitometrically with a BioImage scanner.

Metabolic Labeling and Two-dimensional Protein Gel Electrophoresis—Fibroblast monolayers at confluence were washed extensively with fresh RPMI medium devoid of methionine (Life Technologies, Inc.), and then fresh methionine-free RPMI 1640 medium to which [35S]methionine (DuPont NEN, specific activity, 1200 Ci/mmol) was added (100–400 µCi/ml, final concentration). Labeling was allowed to proceed for 90–180 min and was terminated by rinsing the cultures with ice-cold medium, placing the culture dishes on ice, and solubilizing the monolayers in a buffer containing 9.5 M urea, 2% (w/v) Nonidet P-40, 2% (w/v) glycerol, and 0.5% Nondet P-40 at 4°C. The suspension was vortexed for 10 s at half-maximum speed, incubated for 5 min, and homogenized with 10 strokes in an ice-cold Dounce homogenizer with a type “B” pestle, and the nuclei were pelleted at 55,000 × g. The supernatant was collected and frozen for extraction of RNA.

Nuclear pulsed-labeling experiments were analyzed by gel electrophoresis.

Nuclear Run-on Reactions—Orbital fibroblasts were cultivated in 100-mm diameter plastic culture dishes to near confluence and were shifted for 40 h to medium containing 1% FBS. They were then shifted to fresh medium containing 1% FBS without or with leukoregulin (1 unit/ml) for the times indicated. Monolayers were then scraped off the plates in ice-cold Verse (0.2 g/liter NaEDTA in phosphate-buffered saline) and pelleted at 55,000 × g.

Nuclei were then resuspended in 4 ml of a lysis buffer containing 10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 3 mM MgCl2, and 0.5% Nondet P-40 at 4°C. The suspension was vortexed for 10 s at half maximum speed, incubated for 5 min, and homogenized with 10 strokes in an ice-cold Dounce homogenizer with a type “B” pestle, and the nuclei were pelleted at 55,000 × g. The supernatant was collected and frozen for extraction of RNA. Nuclei were resuspended in 4 ml of the lysis buffer containing 10 µg/ml RNase A and incubated for 30 min at 4°C and then counted.

Approximately 107 nuclei were obtained from control and leukoregulin-treated cultures. Nuclei were pelleted and resuspended in storage buffer containing 40% glycerol (v/v), 50 mM Tris-HCl (pH 8.3), 5 mM MgCl2, 0.1 mM EDTA, and 100 units/ml RNasin (Boehringer Mannheim) and stored in liquid nitrogen until used.

Nuclear run on transcriptional assays were performed essentially as described elsewhere (46) with the following modifications. 5 × 106 nuclei in 125 µl of storage buffer were used in each reaction and combined with an equal volume of a solution containing 10 mM Tris-HCl (pH 8.0), 5 mM MgCl2, 0.3 mM HCl, 5 mM ATP, 5 mM CTP, 5 mM GTP, 2.5 mM [3H]UTP, and NaCl in which 10 µCi/ml [3H]UTP (3000 Ci/mmol) was included. The reaction was allowed to proceed to 30°C for 30 min, after which 3.125 µl of tritiated [3H]UTP (25 µg/ml) and 375 µl of a solution containing 100 units/ml DNase I, 10 mM Tris-HCl (pH 7.4), 0.5 mM NaCl, 50 mM MgCl2, and 2 mM CaCl2 were added and mixed well. After 5 min at 30°C, 125 µl of SDS/Tris buffer (5% SDS, 0.5 mM Tris-HCl (pH 7.4), 0.125 mM EDTA, and 8.75 µl of proteinase K 115 mg/ml) were added, and the nuclei were digested for 30–90 min at 42°C. An equal volume of RNAzol B (Cinnabiotech, Friendswood, TX) and 0.16 ml of chloroform was added for a total of 48 h of incubation. Leukoregulin and the other test compounds were added at the times indicated in the figure legends. For PGE2 measurements, medium was decanted and the monolayers covered with phosphate-buffered saline in the presence of the test compounds for the final 30 min of the incubation. PFS was collected, clarified by centrifugation, and subjected to radioimmunoassay (Amersham Corp.) to determine PGE2 release from the cell layer. For the CAMP determination, medium was removed and 1% FBS enriched medium containing the phosphodiesterase inhibitor, RO-20-1724 (0.25 mM, RBI Research Biochemicals, Natick, MA) was added for a total of 48 h. cAMP levels in the culture medium were assessed by a radioimmunoassay (Amersham Corp.).
parent after 3 h (505 ± 85 pg (mean ± S.E.) versus 29 ± 4, 18-fold increase) and was maximal after 16 h (1950 ± 329 pg, 68-fold increase). By 48 h, PGE₂ production had fallen from the maximum so that it was 11-fold (315 ± 13 pg) above baseline levels. The effects of leukoregulin were dose-dependent in the concentration range tested (0–1 unit/ml) (data not shown).

Glucocorticoids can attenuate the serum- and mitogen-dependent up-regulation of PGE₂ production in some cells (28, 33). We therefore tested the ability of dexamethasone (10 nM) to alter leukoregulin up-regulation of PGE₂ production in orbital fibroblasts. The glucocorticoid blocked the effect of leukoregulin when added to the culture medium at the same time (16 h prior to harvest) (Fig. 2). RU 38486 (100 nM), a glucocorticoid receptor antagonist, could restore substantially the leukoregulin-dependent increase in PGE₂ production (Fig. 2, bottom panel). As those data demonstrate, RU 38486 fails to influence basal PGE₂ production when present in the culture medium for 17 h. When leukoregulin and the antagonist are added together, RU 38486 partially blocks the up-regulation of PGE₂ production. These results suggest that in orbital fibroblasts, RU 38486 can act as both a glucocorticoid antagonist and as a partial agonist, consistent with its previously observed profile of activities (53, 54).

Indomethacin also inhibited the leukoregulin effect on PGE₂ synthesis, suggesting that leukoregulin was acting on prostanooid synthesis at the level of a cyclooxygenase. The newly described PGHS-2-selective inhibitor, SC 58125, was tested for its ability to inhibit leukoregulin-dependent PGE₂ production. The compound attenuated completely the cytokine’s effect on orbital fibroblast PGE₂ synthesis. In an experiment where basal PGE₂ production was 44.67 ± 4.09 pg (mean ± S.E., n = 3) and leukoregulin-treated cultures produced 586 ± 13.33 pg, cultures treated with SC 58125 alone produced 8.13 ± 0.696 pg, and in those receiving both leukoregulin and SC 58125, PGE₂ levels were 43.67 ± 1.45 pg. Thus the increase in PGE₂ production in orbital fibroblasts receiving leukoregulin appears to derive from an induction of PGHS-2. Moreover, the susceptibility of a fraction of basal PGE₂ synthesis in these fibroblasts, incubated in medium enriched with 1% FBS, may result from PGHS-2 activity contributing to nonstimulated prostanooid production. It would appear that ~93% of the PGE₂ produced in
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The increased production of PGE₂ in orbital fibroblasts after exposure to leukoregulin results in increased levels of cAMP. This increase can be blocked by inhibiting PGHS-2 activity. Orbital fibroblasts from a patient with severe Graves' ophthalmopathy were allowed to grow to confluence in 24-well culture plates in Eagle's medium with 10% FBS. They were then shifted to the same medium with 1% FBS for 48 h. Leukoregulin (1 unit/ml) was added to the culture medium for the times indicated along the abscissa (upper panel). Sixteen h before harvest, RO-20-1724 (0.25 μg/ml) was added to the medium (bottom panel). Some plates received SC 58125 (5 μg/ml) alone or in combination with leukoregulin. The medium was harvested and subjected to radioimmunoassay for cAMP. Each data point represents the mean ± S.E. of triplicate determinations in a single, representative experiment.

Leukoregulin-treated orbital fibroblasts derive from PGHS-2 activity.

Other cytokines have been shown to influence PGE₂ production in a number of cells. We therefore tested the ability of IL-1β, interferon-γ, and transforming growth factor-β to alter the synthesis of PGE₂ in orbital fibroblasts. In an experiment where leukoregulin increased PGE₂ synthesis by 30-fold, IL-1β increased production by 3.1-fold (control, 31.8 ± 4.0 pg; IL-1β, 98.4 ± 9.8 pg), and TGF-β by 2.7-fold (86.1 ± 18.9 pg), whereas interferon-γ decreased synthesis by 49% (16.3 ± 4.2 pg).

Induction of PGE₂ production by leukoregulin in orbital fibroblasts leads to a substantial increase in cAMP levels. As the data contained in Fig. 3 (top panel) suggests, this effect evolves over several hours. At 3 h cAMP levels were increased 2-fold over base line and at 6 h were 10-fold higher. They reached a maximum at 16 h when they were increased nearly 50-fold. The increase in cAMP synthesis is related to the increase in PGE₂ synthesis because SC 58125 could block completely the effects of leukoregulin on cAMP synthesis (Fig. 3, bottom panel). The relative selectivity of SC 58125 at the concentration used implies further that it is the induction of PGHS-2 specifically that drives the increase in cAMP generation.

Leukoregulin Increases Steady-state Levels of PGHS-2 mRNA in Orbital Fibroblasts in Part Through Induction of PGHS-2 Gene Transcription—Two distinct cyclooxygenases have recently been cloned, each encoded by a separate gene (24, 26). To begin to identify the cyclooxygenase pathways relevant to the putative role human orbital fibroblasts might play in inflammation, total cellular RNA derived from treated and untreated orbital and dermal fibroblast cultures was subjected to Northern blot analysis with cDNA probes for PGHS-1 and PGHS-2 (Figs. 4 and 5). The PGHS-2 probe hybridized predominantly to a 4.8-kb transcript expressed in leukoregulin-treated orbital fibroblasts with at least two minor bands also visible. Virtually no PGHS-2 signal was present in control cultures. In contrast, the PGHS-1 probe recognized a ~5-kb transcript in both treated and control fibroblasts (Fig. 4, panel A). The expression of PGHS-2 mRNA in orbital fibroblasts exposed to leukoregulin is up-regulated time dependently (Fig. 4, panel B). Leukoregulin induced PGHS-2 mRNA synthesis within 1.5 h of its addition to the culture medium. At its maximum, which occurred 6 h after leukoregulin addition, the induction represented at least a 50-fold increase in steady-state PGHS-2 mRNA levels above controls. The bar graphs in the figure represent densitometric data normalized to the GAPDH signal which was invariant with respect to any experimental manipulation. The induction was transient in that, by 16 h, levels had fallen. Dexamethasone (10 μM) blocked the induction of PGHS-2 mRNA when added at the same time as leukoregulin (data not shown). PGHS-2 mRNA was also inducible in dermal fibroblasts (Fig. 5), although the magnitude of the leukoregulin-dependent increase was considerably less than that observed in orbital fibroblasts. In contrast to its influence on PGHS-2 mRNA expression, leukoregulin failed to alter steady-state levels of the PGHS-1 transcript in either orbital or dermal fibroblasts (Figs. 4 and 5). Both cell types expressed PGHS-1 mRNA at high levels under basal culture conditions. It would appear that human fibroblasts, like human endothelial cells (55) and monocytes (56), express predominately a 5-kb PGHS-1 mRNA rather than the 2.8-kb species found to be expressed in some human and animal cell types (24).

The relatively long latency period between addition of leukoregulin and the maximal up-regulation of PGHS-2 mRNA (6 h) suggested to us that intermediate de novo protein synthesis might be required in the induction. PGHS-2 is an early response gene, the expression of which is influenced by cytokines and serum within 2–3 h (28, 33). Moreover, PGHS-2 mRNA expression is superinducible by cycloheximide, a characteristic of several early response genes. As the Northern blot shown in Fig. 6 suggests, cycloheximide (10 μg/ml, a concentration that blocks 90% of protein synthesis in human fibroblasts) (57), when present in the culture medium for 8 h, appeared to up-regulate PGHS-2 expression slightly. When leukoregulin was added together with the inhibitor, the cytokine’s effects were partially blocked (~30%). Thus some requirement of ongoing protein synthesis for maximal induction of steady-state PGHS-2 mRNA by leukoregulin cannot be excluded.

Nuclear run-on studies demonstrated that the leukoregulin-dependent increase of PGHS-2 steady-state mRNA levels was partially a consequence of enhanced PGHS-2 gene transcription. An assay conducted after 30 min of leukoregulin treatment failed to demonstrate an induction of PGHS-2 transcription. As demonstrated in Fig. 7 (upper panel), the PGHS-2 signal in the untreated controls was very faint; however, after 2 h of exposure to leukoregulin, there is a 2-fold increase in PGHS-2 gene transcription. In contrast, de novo PGHS-1 mRNA synthesis was discernible in the untreated controls and was unaffected by treatment with the cytokine, as assessed by densitometry. After 6 h of leukoregulin treatment, the magnitude of the leukoregulin-dependent increase in PGHS-2 transcription remained at 1.8-fold above base line (Fig. 7, bottom panel). A total of six nuclear run-on assays examining the
effects of leukoregulin on PGHS-1 and PGHS-2 gene transcription were performed. Thus, it would appear that these modest increases in PGHS-2 gene transcription cannot fully account

for the massive up-regulation of steady-state PGHS-2 mRNA levels observed in leukoregulin-treated orbital fibroblasts.

Postnuclear RNA samples from the same nuclear run-on studies were subjected to RT-PCR. While these measurements are not quantitative, they do demonstrate the expected induction of steady-state PGHS-2 mRNA by leukoregulin at both 2 and 6 h, as the right-sided panels of Fig. 7 indicate.

Increases in Orbital Fibroblast PGHS-2 Gene Transcription and Steady-state mRNA Levels by Leukoregulin Result in Elevated Levels of Cyclooxygenase Protein—Induction of steady-state levels of PGHS-2 mRNA levels in orbital fibroblasts by leukoregulin results in a similar increase in the abundance of PGHS-2 protein recognized by monoclonal antibodies. As the Western blot analysis in Fig. 8 clearly demonstrates, treatment with leukoregulin (1 unit/ml) for 16 h resulted in a substantial PGHS-2 protein induction that was at least 40-fold above baseline. The induction was completely blocked by dexamethasone (10 nM). Moreover, the up-regulation was dose-dependent (Fig. 9, top panel) with PGHS-2 protein detectable at a leukoregulin concentration of 0.25 unit/ml and a marked induction at 1–3 units/ml. This correlates well with PGE\textsubscript{2} production (data not shown). While PGHS-2 expression was highly inducible in orbital fibroblasts, levels of PGHS-1 were invariant with respect
to either leukoregulin or dexamethasone (Fig. 8). The time course of effect of leukoregulin on PGHS-2 protein (Fig. 9, bottom panel) appeared to lag behind that on PGHS-2 mRNA (Fig. 4, panel B), as expected. A small effect was present by 3 h which gradually increased until a maximal induction occurred at 24 h. By 48 h, levels of PGHS-2 protein had returned to undetectable levels. It is of interest that the maximum increase in PGE\(_2\) levels occurred after 16 h of leukoregulin exposure (Fig. 1, bottom panel). This suggests the possibility that arachidonic acid availability may preclude further increases in production of the prostanoid despite higher levels of PGHS-2 protein at 24 h. Alternatively, the entire pool of PGHS-2 molecules synthesized following cytokine treatment may not be active. In contrast to the dramatic effects of leukoregulin in orbital fibroblasts, PGHS-2 protein expression in dermal fibroblasts was increased only slightly under identical experimental conditions (Fig. 8).

A number of other cytokines were tested for their effects on PGHS-2 protein expression in orbital fibroblasts. While leukoregulin increased PGHS-2 levels, IL-4, interferon-\(\gamma\), tumor necrosis factor-\(\alpha\), tumor necrosis factor-\(\beta\), and transforming growth factor-\(\beta\) failed to influence the protein levels substantially as the Western analysis demonstrates (Fig. 10). IL-1\(\beta\) could induce PGHS-2 protein, but generally not as dramatically as leukoregulin (data not shown). Thus, among the cytokines tested, leukoregulin exerts the greatest up-regulation of PGHS-2 expression in orbital fibroblasts.

The glucocorticoid blockade of the up-regulation by leukoregulin of PGHS-2 was susceptible to the action of RU 38486. As Fig. 11 indicates, addition of RU 38486 (100 nM) alone to the culture medium of orbital fibroblasts failed to affect PGHS-2 expression in the absence of leukoregulin. The antagonist blunted somewhat the induction of PGHS-2 protein by leukoregulin, suggesting that it is also a partial glucocorticoid agonist in these cells. When RU 38486 was added to cultures receiving both leukoregulin and dexamethasone, the antagonist attenuated substantially the glucocorticoid effect of dexamethasone, restoring an induction of PGHS-2. Thus the actions of RU 38486 on PGHS-2 expression are consistent with its effects on PGE\(_2\) production in orbital fibroblasts (Fig. 2).
Giant two-dimensional protein gel electrophoresis allows resolution of newly synthesized PGHS-2 and therefore direct assessment of its relative abundance (30). In orbital fibroblasts, leukoregulin caused a rapid up-regulation in the accumulation of newly synthesized, radiolabeled PGHS-2 with a molecular mass coordinate of 72/74 kDa and a pI of 7.5 (Fig. 12). In contrast, a discernable protein spot corresponding to PGHS-2 was absent in control cultures. The magnitude of the induction in the leukoregulin-treated cultures was at least 40-fold above baseline. Dermal fibroblasts, obtained from the abdominal wall of the same patient with Graves’ disease who donated the orbital fibroblasts, also exhibited an induction of the PGHS-2 protein (Fig. 12); however, the magnitude of the response was considerably smaller than that seen in orbital fibroblasts.

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**Induction of PGHS-2 in Orbital Fibroblasts by Leukoregulin**

**Fig. 9.** Dose and time dependence of the induction of PGHS-2 protein by leukoregulin in orbital fibroblasts. Confluent cultures were treated with leukoregulin at the concentrations indicated along the abscissa for 16 h (top panel) or with 1 unit/ml for the times indicated (bottom panel), and then the monolayers were processed for Western blot analysis (10 μg of protein per sample) as described under “Experimental Procedures.” The films were scanned with a densitometer. IOD, integrated optical density.

**Fig. 10.** Western analysis of PGHS-2 expression in orbital fibroblasts treated with leukoregulin, IL-4, interferon-γ, TNF-α, TNF-β, and TGF-β. Confluent cultures of orbital fibroblasts were shifted to medium containing 1% FBS for 48 h, the last 16 h to which the cytokine indicated was added. Cell layers were processed and subjected to Western analysis (10 μg of protein per sample) as indicated under “Experimental Procedures.”

**Fig. 11.** The effects of dexamethasone and RU 38486 on the induction of PGHS-2 protein by leukoregulin in orbital fibroblasts. Confluent cultures were treated with the test compounds (dexamethasone, 10 nM; RU 38486, 100 nM) for 16 h, and then monolayers were subjected to Western blot analysis of 10 μg of protein per sample as described under “Experimental Procedures.”

**Fig. 12.** Identification of leukoregulin-induced protein spots corresponding to PGHS-2 in orbital and dermal fibroblasts on two-dimensional gels. Orbital and abdominal wall dermal fibroblasts obtained from a single patient with severe Graves’ ophthalmopathy were grown to confluence and quiescence in 35-mm culture dishes as described under “Experimental Procedures,” and some were treated with leukoregulin (1 unit/ml) for 16 h. Cells were metabolically labeled with [35S]methionine, and total cellular proteins were separated on giant two-dimensional gels. Separated radioactive proteins were visualized by autoradiography. The encircled areas denote PGHS-2 protein inductions and have a molecular mass coordinate of 72/74 kDa and a pI of 7.5.

**Results in a Dramatic Change in Cellular Morphology—Exogenous PGE₂ alters the cellular morphology of orbital fibroblasts (38, 39). Fibroblasts treated with the prostanoid become more stellate and develop increased numbers of cytoplasmic processes. The effects are highly stereoselective in that a number of related molecules fail to elicit similar changes. Moreover, it would appear that either the human EP₂ receptor recently identified and cloned (59) or the EP₃ receptor (60) mediates the effect of PGE₂ on orbital fibroblast morphology. We tested the ability of leukoregulin to alter cell shape and as the phase contrast micrographs contained in Fig. 13 suggest, the cytokine, when present for 6 h at a concentration of 1 unit/ml, produced changes that were identical to those seen with exogenous PGE₂ (0.1 μM) and 8-Br-cAMP (1 mM). In contrast, none
of these compounds altered dermal fibroblast shape under the same experimental conditions. Both SC 58125 and dexamethasone could block the effects of leukoregulin on cell morphology (data not shown). Orbital fibroblasts with shape alterations in response to leukoregulin reverted completely to normal by 48 h, at which time both PGHS-2 (Fig. 9, bottom panel) and PGE$_2$ (Fig. 1, bottom panel) levels had returned to base line.

**DISCUSSION**

The pathogenic basis for the dramatic and characteristic tissue remodeling associated with Graves' ophthalmopathy most likely resides in the interaction of fibroblasts with immunocompetent cells, including B and T lymphocytes, mast cells, and monocytes (10, 11, 61). These are trafficked to the orbit through mechanisms that remain undefined. Once in the orbit, these cells apparently initiate processes leading to fibroblast activation and the accumulation of hyaluronan and perhaps other glycosaminoglycans (8, 9). It is the profound hydropathicity of hyaluronan that results in the partial extrusion of the orbital contents and the manifestation of proptosis. We have reported previously that orbital fibroblasts are considerably more susceptible to the up-regulation of hyaluronan synthesis by leukoregulin than are dermal fibroblasts (19). Leukoregulin can increase hyaluronan synthesis by greater than 15-fold above base line in orbital fibroblasts. The magnitude of this increase in hyaluronan production is unprecedented in mammalian cell systems and implicates leukoregulin as a candidate molecular trigger in the activation of orbital fibroblasts in Graves' ophthalmopathy.

Another striking feature of the active phase of ophthalmopathy is an intense inflammatory reaction. We were prompted to assess the effects of leukoregulin on prostanoid production in orbital fibroblasts because of this cytokine's ability to enhance the distinction between orbital and dermal fibroblast biosynthetic phenotypes. The massive induction of PGHS-2 expression in orbital fibroblasts seen following leukoregulin treatment, considerably greater than that with the other cytokines tested, further implicates leukoregulin, or a molecule with similar biological actions, as an activating factor in orbital inflammation. The presence of this induction in fibroblasts from normal orbital tissue as well as that from patients with Graves' ophthalmopathy implies that the exaggerated response likely reflects an inherent property of orbital fibroblasts rather than an attribute conveyed by the disease process and retained in culture. Examination of many additional strains of fibroblasts from diseased and normal tissue will be necessary to exclude, however, some contribution of the disease to an enhanced cellular responsiveness. It would be of great importance to determine the levels of leukoregulin in tissues from individuals with normal orbits and those with Graves' ophthalmopathy.

PGHS-2 appears to represent the product of an early response gene (29). The relatively long period between addition of leukoregulin to the culture medium and maximal induction of the steady-state PGHS-2 mRNA levels suggests that this action may be mediated through intermediate step(s) requiring de novo protein synthesis. The partial attenuation by cycloheximide of the leukoregulin-dependent increase in PGHS-2 mRNA levels (Fig. 6) is consonant with the possibility that a minor component of the response may require on-going protein synthesis. However, the time-course of PGHS-2 induction is consistent with that found in cytokine-treated endothelial cells where intermediate protein synthesis was apparently not involved (62).

The mechanism for the dramatic increase by leukoregulin in orbital fibroblast steady-state PGHS-2 mRNA levels (at least 50-fold above control levels) is incompletely understood. On the basis of the run-on assays, it would appear that an up-regulation of de novo synthesized PGHS-2 transcript (2-fold) cannot fully account for this increase. Our results suggest rather that some aspect of post-transcriptional processing or transcript stabilization plays the dominant role. In fact, we have preliminary data supporting a role for leukoregulin in the stabilization of PGHS-2 mRNA in orbital fibroblasts. Thus it would appear that the up-regulation of PGHS-2 by leukoregulin in the orbital fibroblast differs somewhat from the induction of that enzyme by IL-1$\alpha$ observed in the ECV304 immortalized cell line derived from human umbilical vein endothelial cells (62). In ECV304 cells, IL-1$\alpha$ increased gene transcription up to 17-fold above control levels. In addition, IL-1$\alpha$ inhibited the decay of PGHS-2 mRNA in these cells pretreated with cycloheximide. Thus, the effects of IL-1$\alpha$ on steady-state levels of PGHS-2 mRNA derived from both appreciable up-regulation of gene transcription and mRNA stabilization. Results obtained to date suggest that PGHS-2 expression and regulation in established cell lines differ from that observed in primary cells in culture such as those used in the present study (62, 63).

Glucocorticoids can block the up-regulation by leukoregulin of hyaluronan synthesis in orbital fibroblasts (19). Moreover, they can block the cytokine's induction of PGHS-2 (Figs. 8 and 11) and the production of PGE$_2$ (Fig. 2) in orbital fibroblast

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8 H. J. Cao and T. J. Smith, unpublished observations.
cultures. These findings are entirely consistent with the clinical benefits associated with high-dose glucocorticoid therapy in Graves’ ophthalmopathy (64). The pathways through which dexamethasone attenuates these leuokregulin-mediated effects on prostanoid biosynthesis is uncertain. Our finding that PGHS-1 mRNA and protein levels are uninfluenced by cytokines and dexamethasone suggests strongly that PGHS-2 is the target for the actions of both leuokregulin and glucocorticoid. Obvious glucocorticoid response elements have not been demonstrated in the promoter region of the human PGHS-2 gene (58). Recent evidence supports a direct interaction between the glucocorticoid receptor and the nuclear transcriptional factors AP-1 and NF-κB (65, 66). These interactions, termed cross-coupling, may provide the molecular rationale for the glucocorticoid repression of certain genes encoding cytokines the promoters of which lack glucocorticoid response elements and could be relevant to the present study. Alternatively, the lack of glucocorticoid activity in a reporter system involving the murine PGHS-2 promoter suggests that the steroids could also influence post-transcriptional events (67). Such is the case with regard to the induction by glucocorticoids of fibronectin in HT-1080 fibrosarcoma cells where the transcript accumulates in the nucleus as an unspliced precursor (68). In that model, dexamethasone is presumably inducing the synthesis of a nuclear transcript stabilization factor acting on the fibronectin pre-mRNA. With regard to PGHS-2 expression in NIH 3T3 cells, dexamethasone blocks the up-regulation by serum of steady-state mRNA levels through transcript destabilization and not by altering PGHS-2 gene transcription (69). In the current studies, RU 38486 blocks substantially the dexamethasone attenuation of the induction by leuokregulin of PGHS-2 (Fig. 11). This indicates that glucocorticoid action on cyclooxygenase expression in orbital fibroblasts is mediated through the classical receptor pathway. We have detected the expression of mRNA encoding the glucocorticoid receptor in human orbital fibroblasts.3 RU 38486 can exert glucocorticoid agonist-like effects, a property that is enhanced in the setting of protein kinase A activation (53, 54). It is presumed that protein kinase A is activated in leuokregulin-treated orbital fibroblasts. Thus the presence of a partial attenuation by RU 38486 of leuokregulin-up-regulated PGHS-2 expression and PGE₂ production in orbital fibroblasts suggests that the compound is also behaving as a weak glucocorticoid agonist, consistent with these earlier reports.

The substantial induction of PGHS-2 expression in orbital fibroblasts elicited by leuokregulin results in a dramatic change in cellular morphology (Fig. 13). It appears to involve the loss of F-actin stress fibers in the affected cells, the partial relinquishing of focal contacts on the ventral surface of the fibroblasts and retraction of the cytoplasm.4 This effect of leuokregulin is mediated through the generation of PGE₂ and cAMP and appears identical to that seen after orbital fibroblasts are exposed to exogenous PGE₂ (38, 39) and 8-Br-cAMP. PGE₂ has been shown to elicit similar changes in synovial fibroblasts from patients with rheumatoid arthritis (70) and in corneal endothelial cells, the latter also being mediated through the generation of cAMP (71). On the basis of rank-order of receptor isom-specific agonists and antagonists, we surmise that PGE₂ is acting through its binding to the recently cloned EP₂ prostanoid receptor subtype (59) by virtue of the ability of Butaprost to mimic the PGE₂ effects (39). The influence of leuokregulin on fibroblast shape is not seen in dermal fibroblasts. Whether this reflects differences in expression of relevant cytokine receptors and signal transduction pathways in the two fibroblast subtypes or whether the cytoarchitecture of orbital fibroblasts is inherently more plastic remains to be determined.

The physiological or pathological implications of this susceptibility to PGE₂-induced morphology change are unknown but, given the absence of these effects in dermal fibroblasts, might represent an important and unusual phenotypic attribute of orbital fibroblasts. In A431 cells, HeLa cells, and rat-1 fibroblasts, epidermal growth factor causes cortical actin polymerization through an activation of the lipooxygenase pathways and actin stress fiber breakdown mediated through cyclooxygenases (72). These effects result in substantial changes in cell morphology. Thus, eicosanoid pathways may produce metabolites important in the regulation of cell shape. A recent report described studies in which rat intestinal epithelial cells, permanently transfected with a PGHS-2 expression vector, over-expressed PGHS-2 and demonstrated increased adhesion to extracellular matrix proteins and resistance to butyrate-induced apoptosis (73). Both of these effects could be blocked with an inhibitor of PGHS. Thus, it would be of interest to assess additional aspects of orbital fibroblast behavior with regard to PGHS-2 induction and the PGHS-2-dependent alterations in cell morphology.

Our current findings imply that fibroblasts derived from the orbit possess a PGHS-2 that is particularly susceptible to induction by leuokregulin. Moreover, the magnitude of the response to leuokregulin in orbital fibroblasts with regard to prostanoid production appears to distinguish this cytokine from others. These findings suggest that the potential to mount an inflammatory response in orbital connective tissue may differ from that in tissue from other anatomic regions of the human body. Because components of this response are probably integral to normal tissue maintenance and remodeling, such regional variations could help explain the propensity of the orbit to manifest Graves’ disease locally. The emerging and complex physiological and pathological roles played by fibroblasts include an ability to synthesize and release a wide array of small regulatory molecules such as growth factors and cytokines. Moreover, fibroblasts lay down distinctive extracellular matrix. These fibroblast products undoubtedly influence the behavior of neighboring cells and thus the finding of fibroblast diversity suggests another layer of complexity with respect to cell regulation and tissue specialization. Recent reports of mice in which PGHS-1 (74) or PGHS-2 (75) gene disruption was accomplished by homologous recombination suggest potentially powerful models for further defining the roles of these isoenzymes as determinants of fibroblast phenotype diversity. It would appear from the PGHS gene disruption studies (75, 76) that a model where PGHS-2 is solely involved in inflammation and not “housekeeping” functions may be incorrect. There is little doubt, however, that this enzyme’s activities are proximate to mature, fully expressed inflammatory reactions in most tissues.

PGE₂ has been shown recently to bias the commitment of naïve T lymphocytes (Tₜ₀) away from the Tₜ₁ phenotype and toward Tₜ₂ (77, 78) and can also influence B lymphocyte behavior (79). PGE₂ can increase IL-5 synthesis in Tₜ₂ lymphocytes and down-regulates steady-state IL-2 mRNA levels in Tₜ₁ lymphocytes (77). Moreover, the prostanoid plays an important role in the activation of mast cells (80). Thus, in the case of orbital fibroblasts, the substantial increase in PGE₂ production observed in response to leuokregulin indicates the potential for these cells to condition the immune response within the orbit by influencing the relative proportion of Tₜ₁ and Tₜ₂ lymphocytes present and thus the cytokine milieu.

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3 T. J. Smith, unpublished observation.
4 T. J. Smith, H.-S. Wang, and C. S. Izzard, manuscript in preparation.
Leukoregulin Induces PGHS-2 in Human Orbital Fibroblasts

Our findings may have substantial therapeutic implications. Graves' ophthalmopathy remains a vexing clinical problem currently treated with high dose glucocorticoid steroids and in severe cases with radiation and surgery. None of these therapeutic modalities is wholly satisfactory. If the anatomic site propensity for the induction of PGHS-2 in fibroblasts by leukoregulin or other cytokines represents a key and initiating component of the intense orbital inflammation observed in this disease process, it is possible that the highly selective cyclooxygenase inhibitors such as SC 58125 and other compounds of the disease process, it is possible that the highly selective cyclooxygenase inhibit may play important therapeutic roles. To our knowledge, large, well controlled prospective studies evaluating the efficacy of nonsteroidal anti-inflammatory drugs in Graves' ophthalmopathy have not yet been undertaken.

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