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T Helper Type 1 and Type 2 Cytokines Exert Divergent Influence on the Induction of Prostaglandin E_2 and Hyaluronan Synthesis by Interleukin-1β in Orbital Fibroblasts: Implications for the Pathogenesis of Thyroid-Associated Ophthalmopathy

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Thyroid-associated ophthalmopathy (TAO) is an autoimmune component of Graves' disease characterized by intense inflammation in the setting of volume expansion. At the heart of orbital susceptibility to Graves' disease appears to be the peculiar phenotype of orbital fibroblasts that, when activated by IL-1β and other proinflammatory cytokines, produce excess prostaglandin E_2 (PGE_2) and hyaluronan. T helper type 1 (Th1) cytokines predominate early in TAO, whereas Th2 cytokines are more abundant later. It is currently unknown whether this transition might promote changes in tissue reactivity associated with disease progression. We report here that interferon-γ and IL-4, representative of these respective classes of cytokines, attenuate IL-1β-provoked PGE_2 production. This down-regulation is mediated by blocking the induction of prostaglandin endoperoxide H synthase-2 (PGHS-2), the inflammatory cyclooxygenase. The mechanism involves blockade by IL-4 and interferon-γ of the IL-1β-dependent activation of PGHS-2 gene promoter activity. In addition, interferon γ inhibits IL-1β-provoked PGHS-2 mRNA stability. The actions of interferon-γ and IL-4 are mediated through the Janus kinase 2/signal transducer and activator of transcription signaling pathway and could be abolished by treating with AG490, a specific inhibitor of Janus kinase 2. In contrast, the up-regulation of hyaluronan synthesis by IL-1β is enhanced by either IL-4 or interferon-γ. The latter two cytokines enhance the induction by IL-1β of hyaluronan synthase-2 expression. These unexpected findings indicate that the Th1→Th2 cytokine transition exerts equivalent influence on PGE_2 and hyaluronan production as TAO progresses from early to late stage. (*Endocrinology* 147: 13–19, 2006)

THYROID-ASSOCIATED ophthalmopathy (TAO) is a process where connective/adipose tissue of the orbit becomes inflamed and expands, a result in large part of the disordered accumulation of the nonsulfated glycosaminoglycan, hyaluronan (1). It is currently thought that lymphocytes and mast cells are trafficked to the involved tissues and that cytokines generated by these recruited cells participate in driving tissue reactivity and remodeling (2–5). Appreciable morbidity occurs in patients with severe TAO because the intense inflammation subsides, giving way to fibrosis (1). End-stage fibrosis limits extraocular muscle motility. Orbital fibroblasts are particularly susceptible to many proinflammatory cytokine actions. Exaggerated responses to these mediators are likely to emerge as the basis for the anatomically restricted manifestations of Graves' disease (6). Therefore, understanding the profile of recruited cells, the cytokines they generate, and the nature of their interaction with fibroblasts remains critical to understanding the molecular events underlying disease pathogenesis.

A number of studies have examined the cellular infiltration in TAO. T helper type 1 (Th1) and Th2 T cells, B lymphocytes, and mast cells accumulate in connective/adipose tissue and extraocular muscles (2–5). Graves' disease is associated with elevations in cytokine levels in serum (7, 8). A wide range of Th1 and Th2 cytokines has been detected in affected orbital tissues (9, 10). Recently, Aniszewski *et al.* (11) found that disease duration represented an important determinant of the T cell profile residing in the orbit. From that report, the Th1 helper cell subset predominates early, whereas Th2 cells are more abundant later in the disease. These findings have potential importance in defining the immunological processes associated with TAO and its natural course. Unfortunately, the biological consequences of this transition have not been investigated previously.

Prostaglandin E_2 (PGE_2) is an important determinant of immune responses. This prostanoid biases development of Th0 cells toward Th2 at the expense of Th1 (12, 13). Moreover, it has important impact on B and mast cell behavior (14, 15). Orbital fibroblasts produce extraordinary levels of PGE_2 when treated with proinflammatory cytokines such as IL-1β, leukoregulin, and CD154 (16–18). These effects on PGE_2 synthesis are mediated through the induction of prostaglandin endoperoxide H synthase-2 (PGHS-2), the inflammatory cy-

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Abbreviations: DRB, 5,6-Dichlorobenezimidazol; FBS, fetal bovine serum; HAS, hyaluronan synthase; Jak2, Janus kinase 2; PGE_2, prostaglandin E_2; PGHS-2, prostaglandin endoperoxide H synthase-2; TAO, thyroid-associated ophthalmopathy; Th1, T helper type 1.

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clooxygenase. The magnitude of PGHS-2 induction is substantially greater in orbital fibroblasts than that found in extraorbital cells. The molecular basis for the exaggerated expression of PGHS-2 in orbital fibroblasts concerns a defective induction of IL-1 receptor antagonist in these cells (19).

Cytokine-activated orbital fibroblasts also overproduce hyaluronan (20). This glycosaminoglycan is synthesized at the plasma membrane as a result of the activities of three enzymes, termed hyaluronan synthase 1 (HAS1), HAS2, and HAS3 (21). These enzyme isoforms are expressed differentially in various tissues and appear to play distinct biological roles. Hyaluronan represents much of the metachromatic material accumulating in TAO (22). Its rheological properties, including an enormous molecular volume when hydrated, lead to connective tissue expansion in TAO (1). The magnitude of PGHS-2 induction and cytokine-dependent PGE_2 synthesis as well as the exaggerated production of hyaluronan by orbital fibroblasts are now believed to play important roles in TAO. Moreover, the anatomic restricted pattern of PGHS-2 and HAS expression may represent the basis for localized inflammatory manifestations of Graves' disease.

In this paper, we report that both IL-4 and interferon-γ can block the induction by IL-1β of PGE_2 production. This effect involves Janus kinase 2 (Jak2) signaling and is mediated through attenuation of PGHS-2 gene promoter activation and stabilization by IL-1β of PGHS-2 mRNA stability. In contrast, both Th1 and Th2 cytokines enhance IL-1β-dependent hyaluronan synthesis through their synergistic action on HAS2 expression. These unexpected results suggest that both IL-4 and interferon-γ exert divergent influence on the actions of IL-1β in the orbit. They suggest further that cytokines from both helper subsets can play important roles in modulating pro-inflammatory signals in TAO.

Materials and Methods

Dexamethasone, arachidonate, 5,6-dichlorobenezimidazol (DRB), and cycloheximide were from Sigma Chemical Co. (St. Louis, MO). IL-1β, IL-4, and interferon-γ were purchased from BioSource (Camarillo, CA). The full-length human PGHS-2 cDNA was a gift from Dr. Kerry O'Banion (University of Rochester, Rochester, NY). A monoclonal antibody directed against human PGHS-2 was purchased from Cayman (Ann Arbor, MI). AG490 was purchased from EMD Bioscience (San Diego, CA). The plasmid designated −1800pGL2 containing a 1.8-kb fragment of the human PGHS-2 promoter was generously supplied by Dr. Steven M. Prescott (University of Utah, Salt Lake City, UT).

Cell culture

Orbital fibroblast cultures were initiated from tissue explants obtained as surgical waste during orbital decompression surgery for severe TAO or from normal-appearing orbital tissues in patients undergoing surgery for noninflammatory conditions. The Harbor-UCLA Medical Center Institutional Review Board has approved these activities. Tissue fragments were generated by mechanical disruption of explants, and fibroblasts were then allowed to outgrow and adhere to plastic culture plates. They were covered with Eagle's medium to which 10% fetal bovine serum (FBS), glutamine (435 μg/ml), and penicillin/streptomycin were added as described previously (23). Medium was changed every 3–4 d, and monolayers were maintained in a 5% CO_2, humidified incubator at 37 C. Strains were used between the second and 12th passage from culture initiation. All experimental manipulations were conducted after a state of confluence had been reached. We have already established the purity of these cultures and found them essentially free from contamination with endothelial and smooth muscle cells (23, 24).

RNA isolation and Northern hybridization

Fibroblasts were cultivated in 100-mm-diameter plates until confluent and then treated with test agents specified in the figure legends. Cellular RNA was extracted from rinsed monolayers by the method of Chomczyski and Sacchi (25), with an RNA isolating system purchased from Biotecx (Houston, TX). The nucleic acid was subjected to electrophoresis through denaturing 1% agarose, formaldehyde gels. Purity of the RNA was established by determining the 260–280 spectroscopic ratios. In addition, electrophoresed samples were often stained with ethidium bromide and inspected under UV light. RNA was transferred to Zeta-Probe membrane (Bio-Rad, Hercules, CA), and immobilized samples were hybridized with [^{32}P]dCTP-labeled PGHS-2 cDNA probes generated by the random-primer method. Hybridization was conducted in ExpressHYB solution from BD Biosciences-Clontech (Mountain View, CA) for 1 h at 68 C. Membranes were washed under high-stringency conditions, and then the RNA/DNA hybrids were visualized by autoradiography on X-omat film (Kodak) after exposure at −80 C with intensifier screens. Bands resulting from radioactive hybrids were scanned by densitometry. Membranes were then stripped according to the instructions of the manufacturer and rehybridized with a glyceraldehyde-3-phosphate dehydrogenase cDNA probe, and band densities were more normalized to this signal. For PGHS-2 mRNA stability studies, cultures were treated with IL-1β for 4 h as pretreatment. Cells were then washed and all plates incubated with medium containing DRB (20 μg/ml), an inhibitor of gene transcription, without or with IL-1β (10 ng/ml) for the intervals indicated in the figure. Abundance of PGHS-2 mRNA was quantified by Northern blot hybridization and subjected to densitometry.

RT-PCR analysis of HAS mRNA levels

Confluent fibroblast cultures were shifted to serum-free DMEM for 24 h and treated with IL-1β without or with either interferon-γ or IL-4 for 16 h. RNA was extracted and equal amounts were digested with RNase-free DNase 1 and reverse transcribed with oligo-dT (Invitrogen, Carlsbad, CA) as the primer using an Omniscript RT kit (QIAGEN, Chatsworth, CA). PCR was performed using *Taq* PCR Master Mix kit (QIAGEN) and using 100 ng RT product as the template. PCR for HAS1, HAS2, and HAS3 was performed using the following conditions: 94 C for 1 min, 55 C for 1min, and 72 C for 1 min for 35 cycles. Primers for HAS1 were 5′-TGTGTATCCTGCATCAGGGGT-3′ (forward) and 5′-CTGGAGGTGTACTTGGTAGCATAACC-3′ (reverse); for HAS2, 5′-GTGTTATACATGTCGAGTTTACTTCC-3′ (forward) and 5′-GTCATATTG TTGTCCCTTCTTCCGC-3′ (reverse); and for HAS3, 5′-GGTACCATCAGAAGTTCCTAGGCAGC-3′ (forward) and 5′-GAGGAGAATGTTCCAGATGCG-3′ (reverse). HAS1, HAS2, and HAS3 products were normalized using β-actin primers 5′-CCAAGGCCAACCGCGAGAAGATGAC-3′ (forward) and 5′-AGGGTACATGGTGGTGCCGCCAGAC-3′ (reverse) under the following conditions: 94 C for 1 min., 60 C for 1 min, and 72 C for 1 min for 35 cycles.

Western blot analysis of fibroblast proteins

Cellular proteins were solubilized in ice-cold harvest buffer containing 0.5% Nonidet P-40, 50 mM Tris HCl (pH 8.0), and 10 μM phenylmethylsulfonyl fluoride from fibroblast monolayers after the treatments indicated in the figure legends. Lysates were taken up in Laemmli buffer and subjected to SDS-PAGE. Separated proteins were transferred to Immobilon membrane (Millipore, Bedford, MA). Primary monoclonal antibodies were incubated with the membranes overnight at 4 C. After washes, membranes were reincubated with secondary peroxidase-labeled antibodies. The ECL (Amersham Biosciences, Piscataway, NJ) chemiluminescence detection system was used to generate signals.

PGE_2 assay

Fibroblasts were grown to confluence in 24-well plastic cluster plates covered with medium containing 10% FBS. Monolayers were shifted to serum-free medium for the final 24 h of incubation. IL-1β and the other

test compounds were added at the times and concentrations indicated in the figure legends. Medium was removed from the cultures, and the monolayers were covered with PBS in the presence of the respective agents for the final 30 min of treatment. PBS was collected quantitatively, clarified by centrifugation, and subjected to PGE_2 RIA using a commercial kit (Amersham).

Transient transfection of orbital fibroblasts with plasmids containing a PGHS-2 promoter fragment

For studies involving the transient transfection of fibroblasts, cultures were allowed to proliferate to 80–90% confluence in medium containing 10% FBS. With regard to the human PGHS-2 promoter, a plasmid designated −1800pGL2 containing −1840 to +123 and thus located 5 bp upstream from the ATG was used. Promoter constructs were transiently transfected into fibroblasts using the LipofectAMINE PLUS system (Invitrogen), and 0.75 μg pGL2 promoter DNA and 0.1 μg pRL-TK vector DNA (Promega, Madison, WI), serving as a transfection efficiency control, were mixed with PLUS reagent for 15 min before being combined with LipofectAMINE for another 15 min. The DNA-lipid mixture was added to culture medium of 80% confluent cells for 3 h at 37 C. DMEM containing 10% FBS replaced the transfection mixture overnight. Transfected cultures were then serum starved, and some received either IL-1β (10 ng/ml) for 2 h or nothing (control) as indicated in the figure legends. Cellular material was harvested in buffer provided by the manufacturer (Promega) and stored at −80 C until assayed. Luciferase activity was monitored with the dual-luciferase reporter assay system (Promega) in an FB12 tube luminometer (Zylux, Oak Ridge, TN). Values were normalized to internal controls, and each experiment was performed at least three times.

Hyaluronan ELISA

Confluent cultures were treated with nothing or one of the test cytokines indicated for 48 h. The media were removed and assayed for hyaluronan content using a specific hyaluronan ELISA, according to the manufacturer's instructions (26). Cell layers were subjected to enzymatic digestion and cell counts with a hemocytometer.

Statistics

Data were analyzed by Student's *t* test.

Results

IL-1β-dependent PGE_2 synthesis and PGHS-2 expression in orbital fibroblasts is attenuated by IL-4 and interferon-γ

Orbital fibroblasts incubated in medium without additives such as cytokines synthesize low levels of PGE_2 (Fig. 1). When treated with proinflammatory cytokines such as IL-1β (10 ng/ml), they produce extremely high levels of this prostanoid (control, undetectable; IL-1β, 640 ± 8 pg/ml) (Fig. 1). These levels are considerably greater than those found in identically treated nonorbital fibroblasts (18). To determine whether Th1 and/or Th2 cytokines might influence the up-regulation of PGE_2 by IL-1β, either interferon-γ (100 U/ml) or IL-4 (10 ng/ml) was added to the medium of fibroblast cultures alone or in combination. The figure demonstrates that both of these cytokines increased PGE_2 production modestly when added alone, compared with the effect of IL-1β. However, both could substantially attenuate the induction by IL-1β (Fig. 1). The levels were reduced to those found in cultures receiving only IL-4 or interferon-γ [IL-1β + IL-4, 96 ± 27 pg/ml ($P < 0.01$ *vs.* IL-1β); IL-1β + interferon-γ, 198.8 ± 12 pg/ml ($P < 0.01$ *vs.* IL-1β)]. Thus, both Th1 and Th2 cytokines exert a substantial blockade of IL-1β-dependent PGE_2 production.

Because the vast majority of cytokine-provoked PGE_2 is generated as a consequence of PGHS-2 activity, the expression of that enzyme was assessed, and as expected, IL-1β markedly induced its levels (Fig. 2). Treatment with either interferon-γ (100 U/ml) or IL-4 (10 ng/ml) could substantially block this enzyme induction (Fig. 2A). This is entirely consistent with the ability of SC58125, a PGHS-2-selective cyclooxygenase inhibitor, to block the increase in PGE_2 production elicited by IL-1β (data not shown). Neither interferon-γ nor IL-4 induced PGHS-2 protein. We then tested the effects of these cytokines on the expression of PGHS-2 in several orbital fibroblast strains, each from a patient with TAO (Fig. 2B). As the data from Western analysis found in that figure demonstrate, the modulation by IL-4 and interferon-γ is demonstrated in all four strains tested. Substantial variability is found in the magnitude of response from one strain to another, consistent with our findings concerning the induction of PGHS-2 by IL-1β (18).

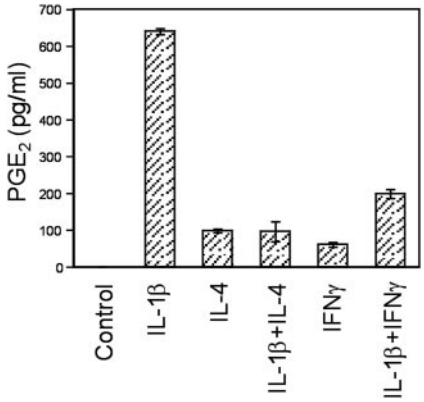

FIG. 1. IL-1β induces PGE_2 synthesis in orbital fibroblasts, whereas IL-4 and interferon-γ (IFNγ) markedly attenuate this up-regulation. Confluent orbital fibroblast cultures, in this case from a patient with severe TAO, were treated with IL-1β (10 ng/ml), IL-4 (10 ng/ml), or interferon-γ (100 U/ml) alone or in the combinations indicated for 15.5 h. Monolayers were washed and covered with PBS with the respective test agents for the final 30 min of incubation. Aliquots of PBS were then subjected to a specific PGE_2 enzyme immunoassay (Amersham) according to the instructions provided by the manufacturer. Results from triplicate wells were used, and the data are expressed as the mean ± SD from a representative experiment of two performed.

Interferon-γ and IL-4 apparently exert their blockade of IL-1β-dependent PGHS-2 expression at the pretranslational level. As the Northern blot in Fig. 3A indicates, the two cytokines attenuate the up-regulation of steady-state PGHS-2 mRNA provoked by IL-1β. The next studies were directed at determining the molecular mechanisms through which Th1 and Th2 cytokines might lower IL-1β-dependent PGHS-2 gene expression. In a series of gene promoter assays, cells transfected with an 1800-bp fragment of the PGHS-2 promoter fused to a luciferase reporter were treated with IL-1β alone or in combination with interferon-γ or IL-4 for 2 h. As the data in Fig. 3B demonstrate, both Th1 and Th2 cytokines could completely block the activation of PGHS-2 promoter activity.

Next, we assessed the influence of these cytokines on PGHS-2 mRNA stability. When untreated, the $t_{1/2}$ for the transcript was approximately 0.5 h (Fig. 3C). When IL-1β was added to the culture medium, the mRNA decayed at a sub-

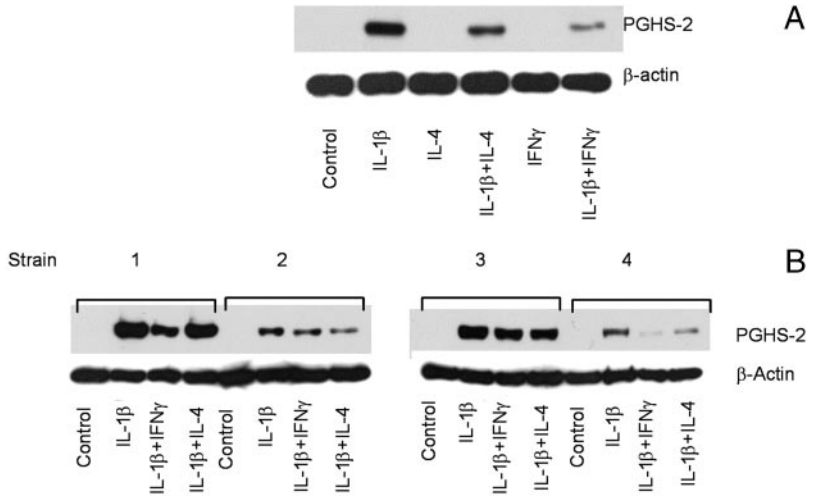

FIG. 2. Effects of IL-1β, IL-4, and interferon-γ (IFNγ) on PGHS-2 protein expression in orbital fibroblasts from a single donor with TAO (A) and a survey of four strains, each from a different patient with TAO (B). Confluent cultures were treated with IL-1β (10 ng/ml) alone or in combination with IL-4 (10 ng/ml) or interferon-γ (100 U/ml) for 16 h. Cell layers were rinsed extensively, solubilized, and subjected to Western blot analysis for PGHS-2 protein levels. Blots were then reprobed with an antibody against β-actin. Relative densities corrected for their respective β-actin signals were as follows: A, Control was 0, IL-1β was 91.2, IL-4 was 0.6, IL-1β + IL-4 was 31.3, interferon-γ was 0, and IL-1β + interferon-γ was 2.85; B, strain 1, control was 0, IL-1β was 127, IL-1β + interferon-γ was 57, and IL-1β + IL-4 was 102; B, strain 2, control was 0, IL-1β was 25.8, IL-1β + interferon-γ was 14.5, and IL-1β + IL-4 was 3.2; B, strain 3, control was 0, IL-1β was 80.3, IL-1β + interferon-γ was 55.6, and IL-1β + IL-4 was 52.3; B, strain 4, control was 0, IL-1β was 16, IL-1β + interferon-γ was 0.1, and IL-1β + IL-4 was 1.7. The results presented are representative of three experiments performed.

stantially slower rate so that the $t_{1/2}$ was increased to 1 h. Addition of interferon-γ to the IL-1β-treatment attenuated the latter cytokine's ability to promote transcript stability. Thus, the effects of the former on PGHS-2 induction are mediated through both gene transcription and mRNA stability.

The Jak/signal transducer and activator of transcription pathway is used to signal many actions attributed to both interferon-γ and IL-4 (27). We therefore determined whether inhibition of Jak2, prominent in the mediation of both cytokines' influence on gene expression, would attenuate the down-regulation of IL-1β-dependent PGHS-2 induction. As the Western blot in Fig. 4 demonstrates, addition of the specific Jak2 inhibitor, AG490 (75 μM), could completely restore the induction by IL-1β of PGHS-2.

IL-4 and interferon-γ enhance IL-1β-dependent hyaluronan synthesis

Because the signaling pathways provoking macromolecular synthesis appear to be shared in orbital fibroblasts with those involved in the up-regulation of prostanoid production, we next assessed the potential impact of IL-4 and interferon-γ on the induction by IL-1β of hyaluronan synthesis. As the data in Fig. 5 indicate, IL-1β (10 ng/ml) could increase hyaluronan accumulation by 3-fold when added alone (control, 37 ± 1.74 ng/10^5 cells, *vs.* IL-1β, 110 ± 6.4 ng/10^5 cells; $P < 0.05$). In contrast, IL-4 and interferon-γ failed to increase synthesis of the macromolecule as single agents over a 48-h treatment period. When these were added to cultures receiving IL-1β, both interferon-γ and IL-4 enhanced its impact on hyaluronan synthesis [110 ± 6.4 *vs.* 203 ± 30 ng/10^5 cells ($P < 0.05$) and 110 ± 6.4 *vs.* 146 ± 24 ng/10^5 cells ($P < 0.05$), respectively]. We have reported previously that hyaluronan degradation in human orbital fibroblasts is nil under the culture conditions we use (28). Thus, the effects of these cytokines can be attributed to their impact on glycosaminoglycan synthesis.

The synthesis of hyaluronan is catalyzed by the activities of three enzymes in the HAS family (21). We have previously reported that HAS2 appears to play the major role in the synthesis of hyaluronan in orbital fibroblasts (29). We next determined whether either IL-4 or interferon-γ could alter the expression provoked by IL-1β of HAS mRNAs. As the RT-PCR results shown in Fig. 6 demonstrate, all three isoforms are induced by IL-1β. The effects of interferon-γ and IL-4 appear to be isoform specific. With regard to HAS3, neither affects IL-1β up-regulated expression. Both cytokines down-regulate IL-1β-dependent HAS1 mRNA levels while up-regulating HAS2 mRNA. Thus, the effects of Th1 and Th2 cytokines on IL-1β-dependent PGHS-2 and HAS2 expression in orbital fibroblasts appear to diverge and are therefore likely to involve distinct patterns of cell signaling.

Discussion

Our results demonstrate for the first time an interaction between both Th1 and Th2 cytokines and IL-1β-driven PGHS-2 and HAS gene expression in orbital fibroblasts. These findings have important implications with regard to the cytokine milieu existing in TAO. Specifically, IL-1, IL-4, and interferon-γ have been detected in affected orbital connective tissues (9). Moreover, the profiles of cytokines and infiltrating immunocompetent cells evolve as a function of disease stage/duration (11). Because cytokine-activated orbital fibroblasts produce extremely high levels of PGE_2 when compared with other fibroblasts, a better understanding of what impact local prostanoid generation exerts on the biology of orbital connective tissue is needed. PGE_2 exerts strong influence on immunity through its actions on T and B lymphocytes as well as mast cells. It biases the commitment of naive (Th0) T cells away from the Th1 phenotype and enhances Th2 development (12, 13). PGE_2 enhances the synthesis of IL-5 in Th2 cells while down-regulating steady-state IL-2 mRNA levels in Th1 lymphocytes. It plays a critical role in the activation of mast cells (14). Thus, in the context of the orbit, the substantial increase in PGE_2 production by resident fibroblasts would be expected to condition the immune response by influencing the profile of infiltrating immunocompetent cells and therefore the cytokines that are generated locally.

We have reported previously that IL-4 and interferon-γ can act on orbital fibroblasts. Specifically, IL-4 is generated

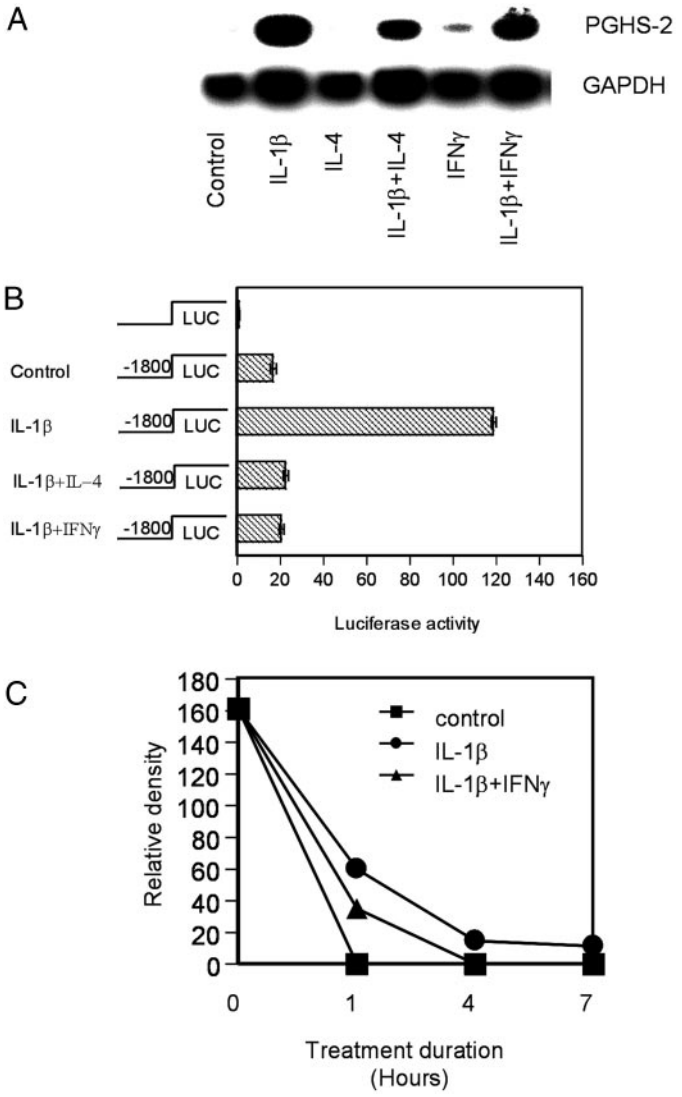

FIG. 3. Effects of IL-1β (10 ng/ml) alone or in combination with IL-4 (10 ng/ml) or interferon-γ (IFNγ) (100 U/ml) on PGHS-2 mRNA levels (A), gene promoter activity (B), and mRNA stability (C) in orbital fibroblasts. Northern blot analysis was performed on RNA extracted from confluent cultures from a patient with TAO that had been treated with the test compounds indicated for 16 h. For the promoter studies, semiconfluent cultures were transiently transfected with an 1800-bp nt PGHS-2 promoter fragment fused to a luciferase promoter gene construct. Cultures were then treated with the test compounds indicated for 2 h, and monolayers were harvested and subjected to luciferase assay. Data are expressed as the mean ± SD of triplicate independent determinations. Stability studies were performed by pretreating confluent cultures with cycloheximide (10 μg/ml) for 5 h, washing monolayers, and adding fresh medium containing the agents indicated in combination with DRB (10 μg/ml) for the time intervals shown. Cultures were harvested, and RNA was extracted and subjected to Northern blot analysis using a PGHS-2 cDNA probe. Membranes were stripped and rehybridized with a probe for glyceraldehyde-3-phosphate dehydrogenase, and the signals were normalized. Studies in A and B were performed three times, whereas the mRNA stability assay in C was conducted twice.

by mast cells and can induce low-level PGE_2 synthesis in cocultured orbital fibroblasts, and this appears to represent the molecular conduit through which the cells cross-talk (30). We have preliminary evidence that the modest effects of IL-4

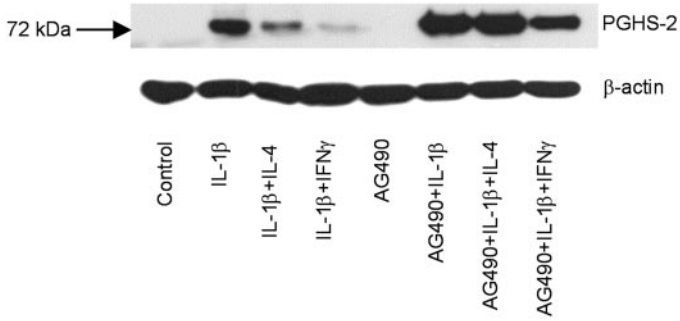

FIG. 4. The inhibition of IL-1β-dependent PGHS-2 expression by IL-4 and interferon-γ (IFNγ) is blocked by interfering with Jak2 activity in orbital fibroblasts. Confluent fibroblasts from a patient with TAO were treated with the test compounds for 16 h, and the monolayers were then washed, lysed, and subjected to Western blot analysis for PGHS-2 protein expression. Membranes were stripped and reprobed for β actin. The relative PGHS-2 densities, corrected for their respective β-actin signals, were as follows: control, 0; IL-1β, 112.3; IL-1β + IL-4, 31.5; IL-1β + interferon-γ, 3; AG490, 0.3; IL-1β + AG490, 220; IL-1β + AG490 + IL-4, 223; and IL-1β + AG490 + interferon-γ, 139. These studies were performed three times.

and interferon-γ added alone are mediated through alterations in secretory phospholipase A_2 expression rather than by increasing PGHS-2 levels (unpublished observations of the authors). Heufelder *et al.* (31) reported that interferon-γ can induce human leukocyte antigen-DR locus molecules in orbital fibroblasts and that considerably higher levels are achieved in cultures from patients with TAO. From the current studies, it would appear that IL-4 generated by infiltrating mast cells could exert an important influence on both PGE_2 production and hyaluronan synthesis in the orbit, resulting from local IL-1β expression and action.

Our studies to date with orbital fibroblasts suggest that they are particularly responsive to IL-1β with regard to PGE_2 synthesis (16–19). The findings we report now, indicating the modulation of that action by Th1 and Th2 cytokines, suggest a potentially important control mechanism for limiting pro-

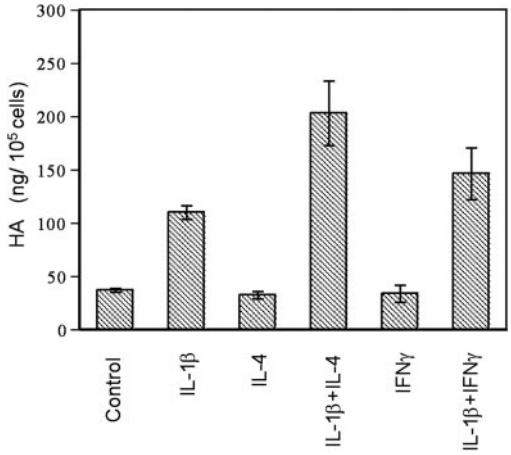

FIG. 5. IL-1β induces hyaluronan (HA) production in orbital fibroblasts, and IL-4 and interferon-γ (IFNγ) enhance this up-regulation. Confluent fibroblast cultures from a patient with TAO were treated for 48 h. with the cytokine indicated. Media samples were collected and subjected to analysis with a specific ELISA for hyaluronan. Cell layers were disrupted and subjected to cell counts. The data are presented as the mean ± SD of three independent replicates.

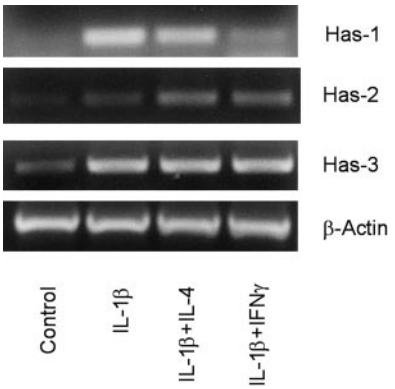

FIG. 6. RT-PCR analysis of HAS1, HAS2, and HAS3 mRNA levels in orbital fibroblasts from a patient with TAO. Cultures were treated with IL-1β (10 ng/ml) alone or in combination with IL-4 (10 ng/ml) or interferon-γ (IFNγ) (100 U/ml) for 16 h. RNA was extracted and subjected to RT-PCR using isoform-specific primers, as described in *Materials and Methods*. The relative signal intensities are as follows: HAS1, control was 0, IL-1β was 74.56, IL-1β + IL-4 was 63.65, and IL-1β + interferon-γ was 19.1; HAS2, control was 6, IL-1β was 21, IL-1β + IL-4 was 49.9, and IL-1β + interferon-γ was 42; HAS3, control was 14, IL-1β was 94.3, IL-1β + IL-4 was 95.2, and IL-β + interferon-γ was 100.4. The experiment shown is representative of three studies performed.

stanoid generation and assuring that signals provoking PGHS-2 expression are governed. The powerful down-regulatory actions of both interferon-γ and IL-4 on PGHS-2 expression and PGE_2 synthesis suggest a potential role for these and related cytokines in modulating the tissue reactivity and remodeling occurring in the orbit. Our findings are somewhat surprising in that the vast majority of actions attributed to Th1 and Th2 cytokines oppose each other (32). We have recently reported in orbital fibroblasts that both interferon-γ and IL-4 can block the induction of tissue inhibitor of metalloproteinase-1 by IL-1β (33). On the other hand, we have found that interferon-γ can exert a dramatic attenuation of IL-4-dependent 15-lipoxygenase-1 gene expression in these cells, an action that is mediated at the level of mRNA stability (Chen, B., S. Tsui, and T. J. Smith, unpublished observation). Thus, it would appear that a complex relationship exists between the actions of Th1 and Th2 cytokines in orbital fibroblasts. Moreover, these might occur *in situ*, and thus our findings could provide an important clue about orbital immunity.

The impact of PGE_2 on the clinical course of TAO remains unexplored. There have been to date no controlled prospective studies examining nonsteroidal antiinflammatory agents and their potential to modify the outcome or severity of the disease. Moreover, assessment of cyclooxygenase inhibitors as antiinflammatory therapy in Graves' disease is limited to anecdotal reports. Glucocorticoids are a powerful modulator of PGHS-2 expression and PGE_2 production (16, 17), and thus the possibility remains that at least some of the antiinflammatory benefit of steroid therapy in TAO is mediated through the down-regulation of prostanoid generation/action in the orbit. A very recent study using microarray gene analysis of orbital tissues from patients with TAO has demonstrated that PGHS-2 is overexpressed in this disease (34).

Although attenuating the induction of PGHS-2, IL-4 and interferon-γ synergistically enhance the IL-1β-dependent production of hyaluronan. The synthesis of this abundant glycosaminoglycan has been implicated in the increased orbital volume associated with TAO (22). Thus, enhanced synthesis of hyaluronan might result in greater tissue volume through expansion of water binding in the extracellular matrix. It would appear that HAS2 represents the isoform, the expression of which is increased when either Th1 or Th2 cytokine is added to IL-1β treatment. In contrast, IL-1β-dependent HAS1 expression is attenuated by IL-4 and interferon-γ. Because the biological consequences of the HAS isoforms may differ, a shift from HAS1 to HAS2 expression might result in a qualitatively different extracellular matrix being generated as a result of these modulating cytokines. A recent insight into the effects of short-chain hyaluronan on gene expression and behavior of target cells (35) makes a shift in HAS isoform expression in the orbit potentially important to the nature of tissue reactions occurring there. Clearly, a careful analysis of the hyaluronan synthesized under these various treatment conditions, including assessment of chain length, is warranted. In any event, our data strongly suggest that HAS2 activity plays an important role in orbital fibroblast hyaluronan production.

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