Activation of Human Orbital Fibroblasts through CD40 Engagement Results in a Dramatic Induction of Hyaluronan Synthesis and Prostaglandin Endoperoxide H Synthase-2 Expression

INSIGHTS INTO POTENTIAL PATHOGENIC MECHANISMS OF THYROID-ASSOCIATED OPHTHALMOPATHY*

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Fibroblasts derived from the connective tissue investing the orbit possess a distinctive phenotype in vitro. Orbital fibroblasts are heterogeneous with regard to the surface display of Thy-1 (1), exhibit characteristic profiles of receptor (2), ganglioside (3, 4), and plasminogen activator inhibitor type-1 expression (5–7), and respond distinctively to cytokines and hormones (8–12) when compared with other types of human fibroblasts. They have attracted substantial attention recently because of their putative role in the pathogenesis of thyroid-associated ophthalmopathy (TAO)1 (13). A small fraction of individuals with Graves’ disease develop TAO, which involves remodeling of the orbital tissue, including the perimysial connective tissue. Molecular events underlying the relatedness of the orbital tissue activation with the thyroid glandular disease process have yet to be identified. The cardinal features of orbital tissue remodeling include a disordered accumulation of hyaluronan, a non-sulfated glycosaminoglycan, and intense inflammation. Histopathological examination reveals an infiltration of lymphocytes and mast cells (14). Lymphocyte populations infiltrating the orbit have been characterized partially, and both CD4+ and CD8+ cells are present (15, 16). The mechanism whereby bone marrow-derived cells are recruited to the orbit in TAO is currently not understood. Moreover, the proximate conduit(s) for molecular cross-talk between resident cells of the orbital tissues and recruited immunocompetent cells has yet to be identified. We hypothesize that bone marrow-derived cells drive the activation of orbital fibroblasts through several cell-signaling pathways. A variety of inflammatory mediators can up-regulate orbital fibroblast target genes and alter the biosynthetic activities of these cells (2, 5, 6, 10–12).

A recently recognized pathway through which fibroblasts can be activated is the CD40/CD40 ligand bridge (17). CD40 is a member of the TNF-α receptor superfamily that was initially identified on B lymphocytes (18). It functions as a crucial conduit for the activation of lymphocytes when ligated with gp39,

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1 The abbreviations used are: TAO, thyroid-associated ophthalmopathy; CHAPS, 3-[3-cholamidopropyl]dimethylammonion)-1-propanesulfonic acid; dexamethasone, 1,4-pregnadien-9-fluoro-16α-methyl-11β,17α,21-triol-3,20-dione; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL, interleukin; MAPK, mitogen-activated protein kinase; PBS, phosphate-buffered saline; PGE2, prostaglandin E2; PGHS, prostaglandin endoperoxide H synthase; rα, receptor antagonist; TNF-α, tumor necrosis factor-α; ELISA, enzyme-linked immunosorbert assay; Ab, antibody; PGE, polyacryl-amide gel electrophoresis; kb, kilobase pairs; HAS, hyaluronan synthases.
also known as CD40 ligand or CD154. CD40 ligand is a member of the TNF-α family and is expressed on T lymphocytes and mast cells (19, 20). Fibroblasts from a variety of anatomic regions express CD40 (17), and other cell types also have been shown to display this glycoprotein, including epithelial (21) and endothelial cells (22) and keratinocytes (23). We recently showed that orbital fibroblasts, derived either from normal tissue or from that affected with TAO, display surface CD40 that can be up-regulated with interferon-γ (24). Moreover, the CD40 expression on orbital fibroblasts is functional. When engaged with CD40 ligand, there is a nuclear translocation of nuclear factor-κB and the activation of IL-6 and IL-8 expression (24). Of particular interest is our finding that thyroid fibroblasts also express functional CD40 (4).

Cyclooxygenases also known as prostaglandin endoperoxide-H synthases (PGHS) are enzymes that catalyze the conversion of arachidonate to prostaglandins and other prostanoids (25). They are bifunctional, heavily glycosylated proteins possessing heme prosthetic groups. Two isoforms of PGHS have been identified recently and their cDNAs cloned (26–29). PGHS-1 is a constitutively expressed enzyme, located on human chromosome 9, that is thought to catalyze the production of prostaglandins involved in normal physiological homeostasis. In contrast, PGHS-2 localizes to chromosome 1, is expressed ordinarily at extremely low levels but is inducible by serum, mitogens, and cytokines. PGHS-2 is an early immediate response gene and represents the inflammatory cyclooxygenase; its activities generate prostanoids believed to be involved in the inflammatory response. The up-regulation of PGHS-2 expression by cytokines is rapid and transient and can involve both increased gene transcriptional activity and an enhancement of mature mRNA stability (30, 31). The profound attenuation of prostaglandin production observed following glucocorticoid stimulation of mature mRNA stability (30, 31). This induction, mediated through the blockade of PGHS-2 expression (28, 32). We have found that orbital fibroblasts are particularly susceptible to the up-regulation of PGHS-2 expression by certain pro-inflammatory cytokines such as leukoregulin, a 50-kDa product of activated T lymphocytes and IL-1α (31). This induction, mediated through both transcriptional and post-transcriptional mechanisms, results in dramatic increases in the production of PGE2. Moreover, the cytokine-dependent PGE2 synthesis can be inhibited with PGHS-2-selective inhibitors such as SC 58125 (31). We recently reported that PGE2 production in lung fibroblasts is enhanced through the ligation of CD40, an effect mediated through the induction of PGHS-2 (33). This action of CD40 ligand on PGHS-2 expression has not been characterized until now. Moreover, whether orbital fibroblasts are similarly activated through CD40 has not been examined, but this could be of critical importance to the pathogenesis of orbital inflammatory states such as TAO.

Another aspect of cell biology evolving rapidly and of proximate relevance to TAO relates to the regulation, synthesis, and action of hyaluronan. This non-sulfated glycosaminoglycan possesses rheologic properties similar to those of the other abundant complex carbohydrates but lacks a core protein. The extraordinary hydrophilic nature exhibited by hyaluronan suggests that its accumulation in the orbit underlies the anterior displacement of the eye in TAO. Hyaluronan accumulates in the orbital fat pad and infiltrates the perimysial connective tissue of the extraocular muscles (13). Cellular receptors for hyaluronan have been identified and include CD44 and RHAM (34, 35). The recent discovery of three distinct mammalian hyaluronan synthases (HAS), each encoded by a separate gene, has allowed rapid advancement of insight into the mechanisms involved in hyaluronan synthesis (36–42). We have reported previously that hyaluronan synthesis in orbital fibroblasts is particularly vulnerable to up-regulation by cytokines such as IL-1β, interferon-γ, and leukoregulin (43, 44). In contrast, the accumulation of abundant sulfated glycosaminoglycans is not influenced by these cytokines. Thus the up-regulation of hyaluronan biosynthetic activity in orbital fibroblasts may be an important target for the molecular signals derived from activated immunocompetent cells recruited to the orbit in TAO.

In this paper, we report studies examining the consequences of CD40 engagement by CD40 ligand in human orbital fibroblasts. When incubated with CD40 ligand, these cells exhibit substantial increases in hyaluronan and PGE2 synthesis. The effects on prostanoid production, but not those involving hyaluronan, are mediated through an enhanced expression of PGHS-2 and are partially dependent upon an intermediate up-regulation of IL-1α synthesis. Thus we have identified a previously unrecognized mechanism for the activation of orbital fibroblasts through the CD40/CD40 ligand bridge that can explain the participation of these fibroblasts in situ in the tissue remodeling observed in TAO. This fibroblast-signaling pathway may represent a highly efficient and specific means by which the immune system can activate connective tissue cells in the setting of an inflammatory response. We hypothesize that the disruption of the CD40/CD40 ligand bridge, either directly or through the attenuation of the IL-1 autocrine loop, may represent an important therapeutic target relevant to TAO and other lymphocyte-driven forms of inflammation involving connective tissue.

**Experimental Procedures**

**Materials**—Human recombinant CD40 ligand in insect membranes was prepared as described previously (45) and kindly provided by Dr. Marilyn Kerhey (Boehringer Ingelheim, Ridgefield, CT). SC 58125, a PGHS-2-selective cyclooxygenase inhibitor (46), was a generous gift of Searle, and IL-1ra was provided by Angen (Boulder, CO). Dexamethasone (1,4-pregnadien-9-fluoro-16a-methyl-11β,17α, 21-triol-3,20-dione) was from Sigma. Recombinant IL-1β and human interferon γ were from Biosource (Camarillo, CA) and Genzyme (Cambridge, MA), respectively. [3H]Glucosamine hydrochloride (specific activity 1369 GBq/mmol) was supplied by NEN Life Science Products. cDNAs encoding human PGHS-1 and PGHS-2 were kindly provided by Dr. D. A. Young (University of Rochester). HAS1 cDNA was a gift from Dr. M. J. Briskin (LeukoSite, Cambridge, MA); HAS2 cDNA was kindly provided by Dr. Y. Yamaguchi (Burnham Institute, La Jolla, CA); and HAS3 cDNA was from Drs. A. Spicer and J. McDonald (Mayo, Scottsdale, AZ). Anti-CD40 monoclonal antibodies were purchased from R & D Systems (Minneapolis, MN). PGF2α, radioimmunoassay was from Amersham Pharmacia Biotech; PGE2 ELISA was from Amgen (Boulder, CO). Anti-IL-1α neutralizing and anti-IL-1β,17α,21-triol-3,20-dione was provided by Amgen (Boulder, CO). Recombinant human recombinant CD40 ligand in insect membranes was supplied by NEN Life Science Products. Anti-IL-1ra was kindly provided by Dr. Y. Yamaguchi (Burnham Institute, La Jolla, CA). Recombinant human recombinant CD40 ligand in insect membranes was supplied by NEN Life Science Products. Anti-IL-1ra was kindly provided by Dr. Y. Yamaguchi (Burnham Institute, La Jolla, CA).

**Cells**—Orbital fibroblast cultures were initiated from tissue explants obtained during decompression surgery for severe TAO or from normal orbital tissue derived from surgical waste during procedures to correct non-orbital disease. These tissues have been approved by the Institutional Review Board of the Albany Medical College. Tissue specimens were disrupted mechanically and covered with Eagle’s medium to which fetal bovine serum (FBS, Life Technologies, Inc) was added. Medium also contained glutamine (435 µg/ml) and penicillin/streptomycin. The disrupted explants were allowed to attach to the bottom of the culture plates as described previously. Cultures were maintained in a 5% CO2-enriched air atmosphere at 37 °C. Unattached cells were removed by washing with PBS every 3–4 days. When fibroblasts were outgrown, the explants were removed, the fibroblast monolayer disrupted with trypsin/EDTA, and the cells re-plated. Culture strains were utilized between the 2nd and 12th passage and had reached a state of confluence when all experimental manipulations were undertaken. We have determined that these cells fail to express factor VIII or smooth muscle-specific actin (1),...
and thus the cultures are not contaminated with endothelial or smooth muscle cells.

**Hyaluronan Assay—**Hyaluronan was quantitated by measuring the incorporation of [3H]glucosamine into glycosaminoglycans. Details concerning this assay have been published by us previously (8, 44, 47, 48). Briefly, sister cultures were incubated with [3H]glucosamine (1 μCi/ml) for 6 h. At the time of harvest, the cell layers were solubilized in 0.2 M NaOH, removed from the substratum with a rubber policeman, and the cells disrupted by sonication. After an aliquot was removed from the cell layer material for protein determination using bovine serum albumin as standard, the medium and cell layer material were combined and subjected to Pronase (1 mg/ml) digestion in 100 mM Tris buffer, pH 8.4, at 50 °C overnight. Samples were cooled to 4 °C, and trichloroacetic acid was added to a final concentration of 5% (w/v). After complete precipitation on ice, samples were centrifuged, and acid-soluble material was subjected to exhaustive dialysis against cold water. The retained samples were subjected to liquid scintillation counting and defined as total glycosaminoglycan.

Hyaluronan was quantitated essentially as reported previously (44, 48). Samples that had been dialyzed were lyophilized to dryness and rehydrated in 0.15 M NaCl, 0.02 M sodium acetate buffer, pH 6.0. An aliquot was subjected to digestion with Streptomyces hyaluronidase (50 μunits/ml; Calbiochem) at 37 °C for 48 h and then dialyzed against sodium acetate buffer. Samples were diluted in 10 mM Tris/HCl, pH 8.4, and layered on to 1.5 × 15 cm DEAE-Sepacel columns. These were washed with 30 ml of the same buffer, and the radiolabeled material was eluted with 50 ml of a 0–0.6 M NaCl linear gradient in Tri/HCl buffer, pH 8.4. One-ml fractions were collected and counted for radioactivity.

**PGE2 Assay—**Fibroblasts were seeded in 24-well plastic dishes and allowed to proliferate to confluence in medium containing 10% FBS. Medium was replaced with fresh medium supplemented with 1% FBS and allowed to grow for 16 h, and then cultures were treated with CD40 ligand without or with other test compounds as described in the legends to the figures. The final 30 min of the incubation was conducted by removing the medium and adding 100 μl of PBS containing the additives. At the end of the incubation period, the PBS was collected, centrifuged, and subjected to a rapid immunoenzyme assay or an ELISA for PGE2.

**Western Blot Analysis of Fibroblast PGHS Proteins—**Relative levels of PGHS proteins were determined by immunoblot analysis using monoclonal antibodies specifically directed against PGHS-1 and PGHS-2. Fibroblast cultures were allowed to proliferate to confluence in 60-mm plastic dishes covered with medium supplemented with 10% FBS. Cells were then shifted to medium with 1% FBS for 16–24 h, CD40 ligand was added without or with other test reagents as described in the legends to the figures. At the time of harvest, medium was removed, and monolayers were washed with PBS 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. Lysates were taken up in Laemmli buffer and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and the separated proteins transferred to polyvinylidene difluoride membrane (Bio-Rad). Non-specific binding sites were blocked by incubating the membranes in PBS to which 0.05% polyoxyethylene-sorbitan monolaurate (Sigma) and 10% nonfat dry milk were added at room temperature for 1 h. The primary antibodies were then added at a 1:500 dilution for 3 h at room temperature. Membranes were washed extensively and incubated with the secondary, peroxidase-labeled antibodies for 2 h.

Following three washes, the ECL (Amersham Pharmacia Biotech) chemiluminescence detection system was used to generate the signal. Resulting bands were analyzed densitometrically with a BioImage scanner (Milligen).

**Immunocytochemistry—**Orbital fibroblasts were seeded in eight-well chamber slides and cultured as indicated in the legend to the figure. Cells were fixed in 2% paraformaldehyde, blocked with 2% horse serum, and incubated overnight at 4 °C with 10 μg/ml of either anti-PGHS-1 or anti-PGHS-2 monoclonal antibodies. Sister cultures were incubated with isotype control antibodies to assess nonspecific staining. Biotinylated horse anti-mouse or anti-goat IgG (heavy + light chain, 1:200; Vector Labs, Inc., Burlingame, CA) was used as a secondary antibody followed by incubation with streptavidin-horseradish peroxidase (1:1000; Jackson ImmunoResearch Labs, Inc., West Grove, PA).

**Isolation of Fibroblast RNA and Northern Hybridizations—** Levels of the relevant transcripts were determined with standard Northern blotting techniques. Cultured fibers were alkali denatured in 1 M NaOH at 60 °C for 2 h. Total RNA was precipitated using a polyclonal anti-phosphotyrosine antibody (Transduction Labs, Lexington, KY). The RNA pellets were rinsed well with phosphate-buffered saline (PBS) that was added to the corresponding medium samples. Cell layers were solubilized in 1% SDS, 50 mM phosphate buffer, pH 8.0, at 50 °C overnight. Samples were cooled to 4 °C, and 0.2 N NaOH, removed from the substratum with a rubber policeman, and the cells disrupted by sonication. After an aliquot was removed from the cell layer material for protein determination using bovine serum albumin as standard, the medium and cell layer material were combined and subjected to Pronase (1 mg/ml) digestion in 100 mM Tris buffer, pH 8.0, at 50 °C overnight. Samples were cooled to 4 °C, and trichloroacetic acid was added to a final concentration of 5% (w/v). After complete precipitation on ice, samples were centrifuged, and acid-soluble material was subjected to exhaustive dialysis against cold water. The retained samples were subjected to liquid scintillation counting and defined as total glycosaminoglycan.

**Fibroblast Enzyme Assays—**Fibroblasts were incubated with isotype control antibodies to assess nonspecific staining. Biotinylated horse anti-mouse or anti-goat IgG (Vector Labs, Inc., Burlingame, CA) was used as a secondary antibody followed by incubation with streptavidin-horseradish peroxidase (1:1000; Jackson ImmunoResearch Labs, Inc., West Grove, PA).

**RESULTS**

**CD40 Engagement with CD40 Ligand on Orbital Fibroblasts**

**Results in a Substantial Up-regulation of Hyaluronan Synthesis—**Orbital fibroblasts under basal culture conditions synthesize hyaluronan, as assessed by the incorporation of [3H]glucosamine into macromolecular material. In general, the rate of incorporation in untreated orbital fibroblasts cultures is lower than that observed in orbital fibroblasts maintained in 1% serum (8, 44, 47). When the fibroblast monolayers were treated with interferon-γ for 94 h, the final 6-h period of which was incubated in the presence of [3H]glucosamine (1 μCi/ml), there was no increase in [3H]hyaluronan accumulation (Fig. 1). This treatment of orbital fibroblasts resulted in a substantial increase in the surface display of CD40 (24). Addition of recombinant CD40 li-
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**Fig. 1.** CD40 engagement in orbital fibroblasts results in a substantial up-regulation of hyaluronan accumulation. Orbital fibroblasts were allowed to proliferate to confluence, and then some cultures were pretreated with interferon-γ (500 units/ml) for 72 h. Medium was then changed in all cultures and some received CD40 ligand containing membranes (1:100) for an additional 16 h without or with interferon-γ as indicated. Cultures were then labeled with [3H]glucosamine (1 μCi/ml) for 6 h in the presence of the respective additives and were harvested as described under “Experimental Procedures.” Monolayers and media were added together and analyzed for total glucosaminoglycan accumulation. Each data point represents the mean ± S.E. n = 3–4 replicate cultures from a representative experiment.

**Fig. 2.** DEAE-Sephacel ion-exchange chromatographic analysis of the CD40 ligand-induced macromolecules incorporating [3H]glucosamine in orbital fibroblasts. The non-dialyzable macromolecular radiolabeled material was lyophilized, solubilized, and subjected to treatment with Streptomyces hyaluronidase (50 units/ml) as described under “Experimental Procedures.” Both untreated and digested materials were then subjected to ion-exchange chromatography. The material was layered on a DEAE-Sephacel column and eluted with a 0–0.6 M NaCl gradient. Squares represent undigested macromolecules, and circles refer to hyaluronidase-treated material.

HAS mRNAs may not be a prominent mechanism for the up-regulation of hyaluronan synthesis elicited by CD40 ligation in orbital fibroblasts.

Glucocorticoids can influence the synthesis of hyaluronan in human fibroblasts (8). We have reported previously that dexamethasone can block the induction of hyaluronan production by cytokines in orbital fibroblasts (44). We therefore examined whether the glucocorticoid could affect the impact of CD40 engagement on hyaluronan synthesis in these cells. In an experiment where incorporation of [3H]glucosamine into macromolecules was 94 ± 20 cpm/μg protein in control cultures, addition of CD40 ligand for 22 h increased incorporation to 381 ± 29 cpm/μg protein (p < 0.001 versus control). When dexamethasone (10 nM) was added with the CD40 ligand, incorporation was attenuated to 218 ± 48 cpm/μg protein (p < 0.02 versus CD40 ligand alone), representing a 57% decrease in the response to CD40 ligand. In contrast, addition of SC 58125 (5 μM) failed to alter the response to CD40 ligation (377 ± 27 cpm/μg protein, not significant compared with CD40 ligand alone). Thus, the effects of CD40 ligation on hyaluronan synthesis are unrelated to increases in PGE2 synthesis. This is entirely consistent with our findings concerning the up-regulation of hyaluronan synthesis by leukoregulin (44).

**CD40 Engagement in Orbital Fibroblasts Elicits a Dramatic Increase in Prostanoid Production—** Orbital fibroblasts, like most other cells in culture, fail to express high levels of PGHS-2 under un-stimulated conditions. We have reported that these fibroblasts produce lower basal levels of PGE2 than do dermal fibroblasts under identical conditions (31). When they are treated with pro-inflammatory cytokines such as leukoregulin and IL-1β, orbital fibroblasts exhibit substantial increases in PGE2 production, which are considerably greater than those observed in the dermal cultures. We therefore determined whether CD40 ligation would increase prostanoid production. As the data in Fig 4, panel A, demonstrate, there is a dramatic increase in PGE2 levels following addition of CD40 ligand to the culture medium of TAO-derived orbital fibroblasts for 16 h. PGE2 levels in control cultures were 122 ± 2.8 pg/ml (mean ± S.E.) and had increased to 1277 ± 42 pg/ml in the cultures treated with CD40 ligand. A very similar level of prostanoid was found in cultures pretreated with interferon-γ (500 units/
were then shifted to medium with 1% serum without or with interferon-γ (500 units/ml) for 72 h. A group of naïve cultures as well as interferon-γ pretreated cultures received CD40 ligand (1:100) for 8 h. At the end of the treatment period, total cellular RNA was extracted from the monolayers, and 50 µg of each sample was subjected to electrophoresis, transferred to membranes, and allowed to hybridize with cDNA probes for HAS1, HAS2, and HAS3. Membranes were stripped and rehybridized to a GAPDH probe to verify gel loading. The integrity of the HAS cDNA probes was established by demonstrating hybridization to appropriately sized transcripts in orbital fibroblasts treated with IL-1β (data not shown).

ml) for 72 h and then treated with CD40 ligand (1312 ± 49 pg/ml). Thus there is an absence of an apparent contribution from pretreatment with interferon-γ to the PGE₂ level achieved. We have found previously that in lung fibroblasts, interferon-γ pretreatment enhances PGE₂ production in response to CD40 ligand substantially (3–4-fold) (33). Fig. 4, panel A, also demonstrates a similar induction of PGE₂ synthesis by CD40 ligation in orbital fibroblasts from normal tissue.

The up-regulation of PGE₂ production by CD40 ligand is time-dependent, detectable within 4 h, and continues to increase so that at 24 h the levels are approximately 240-fold above control levels (control, 34 ± 20 pg/ml; 24 h CD40 ligand, 8190 ± 850 pg/ml, Fig. 4, panel B). The increase in prostanoid levels can be blocked by approximately 80% when dexamethasone (10 nM) is added concomitantly with CD40 ligand (Fig. 4, panel C). SC 58125 (5 µM) can also inhibit the PGE₂ synthesis elicited by CD40 ligand (Fig. 4, panel C), supporting the role of an enhanced level of PGHS-2 expression in the up-regulation of prostanoid biosynthesis. We have demonstrated previously that this concentration of inhibitor used has near-absolute specificity for PGHS-2 in orbital fibroblasts (31).

FIG. 3. Northern analysis of HAS mRNA inducibility by CD40 ligation in human orbital fibroblasts. Orbital fibroblasts from a patient with severe TAO were allowed to proliferate to confluence in 100-mm culture plates in medium supplemented with 10% FBS. They were then shifted to medium with 1% serum without or with interferon-γ (500 units/ml) for 72 h. A group of naïve cultures as well as interferon-γ pretreated cultures received CD40 ligand (1:100) for 8 h. At the end of the treatment period, total cellular RNA was extracted from the monolayers, and 50 µg of each sample was subjected to electrophoresis, transferred to membranes, and allowed to hybridize with cDNA probes for HAS1, HAS2, and HAS3. Membranes were stripped and rehybridized to a GAPDH probe to verify gel loading. The integrity of the HAS cDNA probes was established by demonstrating hybridization to appropriately sized transcripts in orbital fibroblasts treated with IL-1β (data not shown).

FIG. 4. CD40 engagement in orbital fibroblasts results in a dramatic increase in PGE₂ synthesis. Orbital fibroblasts were allowed to proliferate to confluence in 24-well plates in medium supplemented with 10% FBS. Monolayers were then shifted to 1% FBS–enriched medium. Panel A, some cultures were pretreated with interferon-γ (500 units/ml) for 72 h, and then some received CD40 ligand (1:100) without or with interferon-γ for 16 h. For the final 30 min of incubation, PBS with the respective additives was added and then collected for PGE₂ determinations as described under “Experimental Procedures.” Fibroblasts used in this study were from a patient with severe thyroid-associated ophthalmopathy (TAO) or from a donor without orbital disease (normal). Panel B, cell layers from a patient with TAO were treated as in panel A, except cultures were not pretreated with interferon-γ, and the duration of treatment with CD40 ligand was varied as indicated along the abscissa. Panel C, cultures from a patient with severe TAO received CD40 ligand alone or in combination with dexamethasone (10 nM) or SC 58125 (5 µM). Data are presented as the mean ± S.E. of triplicate wells from representative studies.

72-kDa band after 8 h of CD40 ligand treatment. The induction is transient in that the PGHS-2 signal has returned nearly to control levels by 16 h. The up-regulation of PGHS-2 by CD40 ligand could be attenuated by concomitant addition of dexamethasone (10 nM) to the culture medium. Orbital fibroblasts are heterogeneous, and thus we assessed whether the induction of PGHS-2 by CD40 ligand uniformly involved the fibroblast population. We treated cultures with CD40 ligand for 20 h, and the cells were then fixed and stained with anti-PGHS-1 and anti-PGHS-2 antibodies. As the photomicrographs in Fig. 6 demonstrate, a majority of the CD40 ligand-treated cells exhibit marked PGHS-2 staining which appears to be both perinuclear...
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CD40 Ligand Induces PGHS-2 Expression at a Pretranslational Level—Our next studies were directed at assessing whether PGHS-2 up-regulation following CD40 engagement was mediated through an increase in the steady-state levels of PGHS-2 mRNA. Cultures were pretreated with nothing or interferon-γ for 72 h to up-regulate CD40 display, and then some cultures received CD40 ligand for 8 h. As the Northern blot analysis contained in Fig. 7 demonstrates, PGHS-2 transcript is not detectable under control conditions. CD40 ligand elicits a substantial increase in PGHS-2 mRNA levels. This transcript appears as a 4.8-kb band, similar to the pattern we have found previously following treatment with pro-inflammatory cytokines (31). Moreover, the interferon-γ pretreatment fails to induce the transcript alone or to augment the effects of CD40 ligand on PGHS-2 mRNA expression. PGHS-1 mRNA, migrating as a 5.0-kb transcript, is expressed at similar levels under control, interferon-γ, and CD40 ligand treatment conditions, as expected. We have reported previously that the predominant PGHS-1 transcript expressed by human fibroblasts is 5.0 kb (31), similar to that observed in endothelial cells and monocytes (55, 56) but different from some other human and animal cells that express a 2.8-kb PGHS-1 mRNA (26).

CD40 Engagement Results in a Substantial Up-regulation of IL-1α Expression in Orbital Fibroblasts—We have found that orbital fibroblasts can express high levels of both IL-1α and IL-1β when treated with pro-inflammatory cytokines such as leukoregulin and IL-1 itself (57). In contrast, the magnitude of IL-1α induction by exogenous IL-1 was substantially less than that found in dermal fibroblasts. Moreover, IL-1 is a potent inducer of PGHS-2 in orbital fibroblasts as well as in many other cell types. Thus, we examined the effects of CD40 engagement on IL-1 expression. As the data in Fig. 8 (panel A) indicate, IL-1α expression is increased by CD40 engagement. The increase is time-dependent (control, <15.6 pg/10 μg of protein, n = 3) and maximal at 8 h when it reaches 110 ± 12 pg/10 μg of protein. By 16 h, the levels of IL-1α have begun to decline. IL-1α expression in response to CD40 ligation was assessed in four orbital strains, three from patients with TAO and one from normal tissue. All expressed undetectable basal IL-1α levels but achieved a level of 48 ± 9 pg/10 μg (mean ± S.E., n = 3) after 8 h of treatment. In contrast, the levels of IL-1β appear uninduced or are up-regulated only slightly by CD40 ligation, suggesting a divergence in these cells away from the use of IL-1β (Fig. 8, panel B). Orbital fibroblasts are capable of expressing IL-1β when treated with other stimuli. When cultures from this same cell strain were treated with exogenous IL-1α (10 μg/ml), the level of IL-1α increased to 28 ± 1 pg/10 μg of protein from undetectable levels. This failure of CD40 ligand to elicit a substantial increase in IL-1β production was verified in four orbital fibroblast strains. The mean IL-1β level achieved after 8 h of CD40 ligand treatment was <15.6 pg/10 μg of protein compared with 106 ± 50 pg/10 μg in cultures treated with exogenous IL-1α (10 ng/ml). As the data in Fig. 8 (panel C) indicate, CD40 engagement elicits a modest increase in IL-1ra levels, which begin to increase at 16 h and have tripled by 24 h. The up-regulation of IL-1α and IL-1ra following CD40 engagement were completely blocked with dexamethasone (10 μM). In an experiment where IL-1α levels were <15.6 and 90 ± 15 pg/10 μg of protein in control and CD40-treated cultures, respectively, addition of dexamethasone resulted in an IL-1α level below the limits of detectability (<15.6 pg/10 μg of cellular protein). With regard to IL-1ra expression, the control levels were 476 ± 33 pg/10 μg of protein and achieved a level of 1292 ± 11.4 pg/10 μg (p < 0.0001 versus control) following CD40 ligation. Concomitant addition of dexamethasone with CD40 ligand resulted in IL-1α levels of 400 ± 10 pg/10 μg of protein.

The Induction of PGHS-2 Expression but Not Hyaluronan Production Elicited by CD40 Engagement Is Dependent upon Intermediate IL-1α Production—Because we have found that IL-1 induces PGHS-2 expression and hyaluronan synthesis in orbital fibroblasts, we examined the impact of neutralizing the action of the cytokine on PGE2 production and PGHS-2 expression elicited by CD40 ligand. That up-regulation of PGE2 and PGHS-2 was substantially attenuated with the addition to the culture medium of either anti-IL-1α antibodies or exogenous IL-1α (Fig. 9, panels A and B). Addition of IL-1α Abs (1 μg/ml) or IL-1α (500 ng/ml) attenuated the increase in PGE2 by 63 and 73%, respectively, and PGHS-2 expression by 78 and 79%, respectively. These findings strongly implicate the intermediate induction of IL-1α in the up-regulation by CD40 ligand of PGHS-2 expression and PGE2 synthesis. In contrast, when IL-1α was added to cultures that were then CD40 engaged and analyzed for [3H]hyaluronan synthesis, the cytokine receptor antagonist exerted no influence on the production of the macromolecule (control 94 ± 20 cpm/μg of protein, CD40 ligand; 381 ± 29 cpm/μg of protein, CD40 ligand + IL-1α; 491 ± 103 cpm/μg of protein). Thus it would appear that some but not all of the actions of CD40 ligation in orbital fibroblasts are dependent upon an intermediate induction of IL-1α.

CD40 Engagement in Orbital Fibroblasts Enhances MAPK Activation—MAPK has been implicated in the signal transduction pathway utilized by certain cytokines, such as IL-1, in their induction of PGHS-2 expression (58). We therefore determined whether the engagement of CD40 on orbital fibroblasts by CD40 ligand could influence the activation of this important kinase pathway. As the data contained in Fig. 10 (panel A) indicate, CD40 ligand up-regulates nuclear MAPK activation in a time-dependent manner in orbital fibroblasts that is substantial at 8 h, maximal at 16 h, and had decreased to near base line at 24 h. Because dexamethasone can block the induc-
tion by CD40 ligand of PGHS-2 expression, we added the glucocorticoid (10 nM) at the same time as CD40 ligand treatment was initiated and found that the increase in nuclear MAPK activation was entirely blocked after 16 h (Fig. 10, panel B). Apigenin (25 nM), a specific inhibitor of MAPK (59), could also substantially attenuate the effect of CD40 engagement on MAPK activation, as the figure attests. To determine whether these increases in MAPK activation were related to the induction of PGHS-2, we assessed whether inhibitors of two enzymes in the MAPK cascade influenced cyclooxygenase induction. Addition of geldanamycin (10 nM) or apigenin (25 nM) to the culture medium at the time of initiation of CD40 ligand treatment results in a dramatic attenuation of PGHS-2 induction (Fig. 10, panel C). Geldanamycin inhibits the action of Raf1 by causing its dissociation from Hsp90 (60). Thus, interruption of the MAPK pathway at two different levels attenuates PGHS-2 induction mediated by CD40 engagement.

**DISCUSSION**

Human orbital fibroblasts can be activated through the CD40/CD40 ligand bridge to express substantially greater levels of hyaluronan and PGE2, as the current studies have demonstrated. These two features of the cellular response to CD40 engagement are consonant with the remodeling known to occur...
in orbital connective tissue in TAO. They potentially represent the molecular basis for fibroblast activation in that disease process and explain multiple aspects of the complex interplay between the immune system and connective tissue. The up-regulation of hyaluronan by CD40 ligation is apparently independent of the induction of PGHS-2 expression and PGE2 production as is evidenced by the lack of effect of SC 58125 on that cellular response. We have reported previously that CD40 engagement on orbital fibroblasts results in dramatic increases in the expression of IL-6 and IL-8 (24), and thus it would appear that the expression of a number of potentially important genes and their products is enhanced through the CD40/CD40 ligand bridge. This previously unrecognized pathway for the activation of orbital fibroblasts suggests a pathway through which CD40 ligand-displaying cells such as T lymphocytes and mast cells could directly and specifically cross-talk with orbital fibroblasts. We hypothesize that the CD40/CD40 ligand bridge is a widespread conduit utilized for the activation of connective tissue by the immune system. Both lymphocytes and mast cells have been found in the orbital connective tissue in patients with TAO (14). This has led us and others to hypothesize that bone marrow-derived cells are responsible for driving the profound tissue remodeling in this disease.

We have reported that lung (61), gingival (62), and thyroid-derived (4) fibroblasts express CD40 and that this expression can be substantially induced by interferon-γ. Moreover, PGHS-2 expression and PGE2 production are substantially up-regulated by CD40 ligation in lung fibroblasts (33). There appear to exist important differences between fibroblasts in the orbit and those from the lung with regard to CD40-mediated signaling. In lung fibroblasts, the induction of PGHS-2 by CD40 ligand in cultures not pretreated with interferon-γ is modest (33). When the cells are pretreated with the cytokine and treated with CD40 ligand, the magnitude of PGHS-2 induction in lung fibroblasts is enhanced substantially. In orbital fibroblasts, the up-regulation by CD40 ligand of PGHS-2 mRNA and PGE2 synthesis is near maximal in cultures not pretreated with interferon-γ (Figs. 4, panel A and Fig. 7). It is possible that interferon-γ is enhancing the CD40 induction of PGHS-2 in lung fibroblasts by increasing the number of CD40 ligand-binding sites on the surfaces of these cells, as we have demonstrated previously (24, 61). Alternatively, the cytokine might be enhancing the cyclooxygenase induction through an up-regulation of relevant components of intracellular signaling pathways utilized by CD40 engagement in lung fibroblasts. In any event, it would appear that orbital fibroblasts are inher-
ently more susceptible to the up-regulation by CD40 ligation of PGHS-2 expression than are lung fibroblasts. This finding further supports our view that fibroblasts represent a heterogeneous population of cells (1, 2, 5, 7, 11).

Fibroblasts from the human orbit exhibit distinctive phenotypic attributes that render the connective tissue susceptible to as yet unidentified pathogenic factors associated with TAO. We have shown previously that orbital fibroblasts are capable of an extraordinary capacity to synthesize hyaluronan when treated with a variety of pro-inflammatory cytokines, including interferon-γ, IL-1, and leukoregulin (43, 44). These cytokine-dependent increases in hyaluronan synthesis exhibited by orbital fibroblasts are considerably more robust than those observed in dermal fibroblasts under the same experimental conditions. Glucocorticoids such as dexamethasone can attenuate the up-regulation of hyaluronan synthesis elicited by cytokines (44) and can inhibit basal hyaluronan synthesis in dermal fibroblasts (8). Our finding that CD40 engagement can also enhance hyaluronan synthesis in orbital fibroblasts and that this up-regulation can be blocked partially by glucocorticoids suggests that regulation of glycosaminoglycan production in orbital fibroblasts is complex. Coupled with the finding that dexamethasone can block CD40 ligand-de-
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FIG. 11. Schematic of the proposed pathways utilized by CD40/CD40 ligand bridge in the activation of PGHS-2 expression and hyaluronan synthesis in human orbital fibroblasts. Uncertain is the target(s) for CD40 activation of the hyaluronan biosynthetic pathway.

The mechanisms involved in the enhancement by CD40 engagement of hyaluronan synthesis are not completely defined. Unlike the CD40 ligand actions on PGHS-2 expression, the induction of hyaluronan is independent of IL-1α, as is suggested by the inability of IL-1ra to influence the response. We have been unable to demonstrate a consistent impact of CD40 ligation on the expression of any of the three HAS mRNAs (Fig. 3) in orbital fibroblasts. In our studies to date, the predominant, inducible hyaluronan synthase isoform mRNA expressed in confluent, adult human fibroblasts is HAS2, and this transcript is up-regulated severalfold by both leukoregulin and IL-1β (55). In some fibroblast strains, we have been able to detect induction by IL-1β of HAS1 and HAS3 mRNAs as well, although the abundance of these transcripts, on the basis of Northern analysis, appears considerably lower than that of HAS2. It is possible that some other step(s) in the hyaluronan biosynthetic cascade is being induced/activated as a consequence of CD40 engagement. For instance, Spicer et al. (66) have reported very recently the cloning of the human UDP-glucose dehydrogenase and the induction of its mRNA by IL-1β in cultured orbital fibroblasts from patients with severe TAO. Thus it is possible that a step upstream from HAS, such as the UDP-glucose dehydrogenase, might act as the target for CD40-dependent up-regulation of hyaluronan biosynthesis. On the other hand, we may have as yet failed to define the proper conditions under which some HAS induction may be occurring. Our findings are consistent with those reported recently in microvascular endothelial cells where IL-1β, TNF-α, and bacterial lipopolysaccharide failed to increase steady-state HAS mRNA levels despite their up-regulation of hyaluronan in those cells (65). Clearly, additional studies are now warranted to define the molecular basis for the up-regulation of hyaluronan we report here.

Orbital fibroblasts express extraordinarily high levels of PGHS-2 expression and produce PGE₂ in response to pro-inflammatory cytokines such as leukoregulin and IL-1 (31). The elevated levels of prostanoïd result in a dramatic alteration in cellular morphology, which appears to be mediated through EP₂ receptors (67). With regard to earlier studies involving cytokines, it appeared that alterations in PGHS-2 mRNA stability account for the majority of the increases in enzyme levels as PGHS-2 gene transcriptional rates were increased only modestly (2–3-fold) (31). From the results reported here, it would appear that CD40 engagement represents another potentially important pathway through which prostanoïd production can be up-regulated in orbital fibroblasts. Steady-state PGHS-2 mRNA and protein levels are rapidly elevated, and the up-regulation of PGE₂ production can be abolished with SC 58125, a PGHS-2-selective cyclooxygenase inhibitor (46) and by dexamethasone. The mechanism involved in the PGHS-2 induction elicited by CD40 ligand has been partially identified in these studies. It would appear that the intermediate synthesis of IL-1α is important for the full impact of CD40 engagement on PGHS-2 expression because neutralizing the IL-1α, with either a specific antibody or with exogenous IL-1ra, can substantially attenuate the induction (Fig. 9). A recent study has demonstrated that CD40 engagement in vascular smooth muscle and endothelial cells results in the activation of caspase-1 and the synthesis of IL-1β (68). Thus, there is precedent for an up-regulatory role for CD40 engagement on IL-1 production in other cell types. Despite the capacity of orbital fibroblasts to express IL-1β, the CD40-dependent pathway appears to utilize only IL-1α. We have reported previously that murine lung fibroblasts express high levels of IL-1α but fail to synthesize IL-1β when activated with TNF-α (69). It may be that different fibroblast subpopulations utilize members of the IL-1 family of genes selectively, with regard to particular activational triggers. Our demonstration of an autocrine loop comprised of IL-1α mediating, at least in part, the induction of PGHS-2 in orbital fibroblasts represents a potentially powerful new insight concerning the mechanism through which CD40 ligand exerts its actions in non-lymphoid tissues. These findings suggest that disruption of IL-1 expression and/or action at the level of the fibroblast could attenuate the impact of CD40 ligation in these cells. On the other hand, the inability of IL-1ra to influence the impact of CD40 ligation on hyaluronan synthesis suggests a divergence of intermediate pathways relevant to the CD40 activation domain in fibroblasts.

The signal transduction pathways utilized by CD40 have been examined recently, and the issues appear to be complex and cell type-specific. In B lymphocytes, CD40 is coupled to both ERK and Jun kinase pathways (70–72). Nuclear MAPK activation is enhanced by CD40 engagement in orbital fibroblasts (Fig. 10A), and the interruption of the MAPK cascade can partially block PGHS-2 up-regulation (Fig. 10C). This was accomplished with geldanamycin which depletes the cell of Raf-1 and therefore acts at a level up-stream of MAPK (59) and with apigenin, a compound which inhibits MAPK directly (60). We have proposed a pathway through which the CD40/CD40 ligand bridge might activate orbital fibroblasts in a schematic (Fig. 11). Our findings are entirely consistent with a very recent report demonstrating the role of MAPK in the induction by IL-1 of PGHS-2 in fibroblasts and human umbilical vein endothelial cells (58). In contrast to the effects of CD40 engagement on PGHS-2 expression and PGHS-2-dependent PGE₂ production, PGHS-1 levels appeared to be invariant with regard to CD40 engagement.

The molecular trigger(s) that drives the hyaluronan synthesis and inflammation in TAO is uncertain but is presumed to emanate from the immunocompetent cells that are trafficked to the orbit (14). Lymphocytes and mast cells have been shown to express high levels of CD40 ligand (19, 73), and thus our finding that orbital fibroblasts display surface CD40 (24) and

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can be activated by CD40 ligand is of particular mechanistic importance. Whether the CD40 bridge constitutes a functionally important component of fibroblast signaling in situ under normal physiologic conditions or in disease states will require further studies, such as those utilizing conditional disruption of CD40 or CD40 ligand gene expression. Interruption of this intercellular signaling conduit may represent an important therapeutic target for modifying the natural course of TAO.

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