Stimulation of Glycosaminoglycan Production in Cultured Human Retroocular Fibroblasts

J. M. Korducki, S. J. Loftus, and R. S. Bahn

Histologic examination of the retroocular connective tissues in Graves' ophthalmopathy (GO) reveals lymphocytic infiltration and an accumulation of glycosaminoglycans (GAG), hydrophilic macromolecules produced locally by fibroblasts. We studied the in vitro effect on fibroblast GAG production of several cytokines and growth factors likely to be secreted by these activated lymphocytes or macrophages. Cultures were established from retroocular connective tissue, extraocular muscle perimysium, and pretibial skin obtained from patients undergoing orbital decompression or eye muscle surgery for severe GO and from normal individuals. Confluent cultures were treated with one of the compounds and labeled with [3H]glucosamine or [35S]sulfate for quantitation of [3H]GAG or [35S]GAG accumulation. Of the various compounds examined, only interleukin-1 (IL-1) and transforming growth factor (TGF)-β significantly stimulated [3H]GAG accumulation in a dose- and time-dependent fashion. There was no difference in sensitivity to the GAG-stimulating effect of IL-1 or TGF-β between fibroblasts from the four anatomical sites studied or between normal and GO patient fibroblasts. In conclusion, both IL-1 and TGF-β are potent stimulators of [3H]GAG accumulation by retroocular connective tissue and perimysial fibroblasts, as well as by fibroblasts from the dermal sites studied. Stimulation of GAG production by these cytokines, released from lymphocytes or macrophages infiltrating the retroocular space, may play a role in the accumulation of GAG in the retroocular and perimysial connective tissues in GO.

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From a purely mechanical standpoint, most of the signs and symptoms of Graves' ophthalmopathy (GO) can be understood. The orbit is a pear-shaped bony socket that contains the globe of the eye, the supporting muscles, nerves, vessels, orbital fat, and the lacrimal gland. Because these tissues are contained within a confined space, any significant increase in their volume may result in anterior globe displacement (proptosis) and impairment of extraocular muscle function and may impede venous drainage from the orbit, resulting in periorbital, eyelid, and conjunctival edema. Although the extraocular muscle bodies are grossly enlarged in GO, the muscle cells themselves are morphologically and functionally normal. In contrast, the connective tissue surrounding and investing the muscle cells, as well as the remainder of the connective tissue within the orbit, is edematous and contains increased quantities of glycosaminoglycans (GAG), hydrophilic macromolecules produced by connective tissue fibroblasts, and mast cells. The clinical features of GO can be explained by an increase in the volume of the retroocular tissues secondary to GAG accumulation and edema within the perimysial and retroocular connective tissues. Pretibial dermopathy, a skin condition of the lower extremities associated with GO, also is characterized by local accumulation of GAG.

The purpose of our study was to investigate factors that might stimulate GAG production by retroocular connective tissue and extraocular perimysial and pretibial skin fibroblasts in Graves' disease. We demonstrated previously that interferon-gamma stimulates [3H]GAG production in retroocular connective tissue fibroblasts and not in those derived from pretibial or abdominal skin. Furthermore, it has been shown that retroocular fibroblasts are particularly susceptible to the inhibitory effects of triiodothyronine and dexamethasone in terms of [3H]GAG production. In the present study, we examined cytokines or growth factors likely to be released by the activated lymphocytes and macrophages that are known to infiltrate the orbit in GO. We studied interleukin-1 (IL-1), interleukin-2 (IL-2), interleukin-6 (IL-6), transforming growth factor (TGF)-β, tumor necrosis factor (TNF) alpha, epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), and platelet-derived growth factor (PDGF). In addition, we wanted to determine whether thyroid stimulating hormone (TSH),...
a polypeptide known to stimulate production of thyroid hormone by the thyroid gland, might stimulate production of [3H] GAG by fibroblasts from patients with GO.

Materials and Methods

Materials

[3H]Glucosamine (specific activity 10–30 Ci/mmol) and [35S]sulfuric acid (SA 1050–1600 Ci/mmol) were purchased from New England Nuclear (Boston, MA). MCDB 104 (defined medium) was obtained from Hazelton Biologies, Inc. (St. Lenexa, KS). Recombinant human IL-1, IL-2, IL-6, EGF, and bFGF were purchased from Boehringer Mannheim (Indianapolis, IN). TGF-β was purchased from Calbiochem (San Diego, CA), and Collaborative Research (Bedford, MA). PDGF was obtained from Collaborative Research. Highly purified bovine TSH (30 U/mg) was obtained from Dr. J. G. Pierce, formerly of UCLA. All reagents used were of the highest purity commercially available.

Cell Culture

Cell cultures were established as described previously.10 Retrocral connective tissue was obtained in the course of orbital decompression surgery for severe Graves' ophthalmopathy. Connective tissue from the same retroocular site was obtained at necropsy or during unrelated ocular surgery in individuals without evidence of thyroid disease or orbital inflammatory conditions. Biopsies of extraocular muscles, with their investing perimysial connective tissue, were obtained in the course of eye muscle surgery in patients with GO, during strabismus surgery in patients without evidence of GO or thyroid disease, or at autopsy. Pretibial skin biopsies were obtained from patients with GO and pretibial dermopathy and from normal individuals. Abdominal skin biopsies were obtained from normal individuals and from patients with Graves' disease. Tissue explants were minced and placed directly on plastic culture dishes. Fibroblasts were allowed to proliferate in a humidified incubator at 37°C in 5% CO2 in medium 199 supplemented with Earle's salts and I-glutamine to which heat inactivated fetal bovine serum (FBS; 20% vol/vol), penicillin G (100 U/ml) and gentamycin (20 µg/ml) were added. After cells reached confluence, they were passaged and propagated in medium 199 containing 10% FBS.

Cells were incubated for 72 hr in fresh medium containing either IL-1 (1.0 U/ml) or TGF-β (22 ng/ml) prior to the addition of [3H]glucosamine (5.0 µCi/ml) or [35S]sulfuric acid (10 µCi/ml) for the final 24 hr of treatment. Dose-response studies examined the effect of IL-1 (0.25–1.0 U/ml) or TGF-β (5.0–20 ng/ml) on [3H]GAG accumulation. Time-course studies examined treatment periods between 24 and 192 hr. To examine the effect of other cytokines, fibroblast monolayers were treated for 48–96 hr with IL-2 (10 and 20 U/ml), IL-6 (100 and 200 U/ml), TNF alpha (100 U/ml), EGF (10 and 20 ng/ml) bFGF (250 ng/ml) or PDGF (2.5 and 5.0 U/ml) prior to the addition of [3H]glucosamine (5.0 µCi/ml) for the final 24 hr of incubation. For TSH experiments, confluent cultures were shifted to MCDB 104 (defined medium) to which TSH (5.0 mIU/ml) and [3H]glucosamine (5.0 µCi/ml) were added simultaneously for a labeling period of 2 hr.

All culture strains were used between the 2nd and 12th passage, with the majority of experiments performed prior to the fourth passage. We have found no morphologic, growth property, or GAG biosynthetic differences between very early (fewer than five) passage cells and cells that have undergone more than 12 passages.

[3H]GAG Quantitation

The procedure for [3H]GAG quantitation has been reported in detail.11 Culture media were quantitatively collected and monolayers were washed with phosphate buffered saline. Washed monolayers were solubilized in 0.1 N NaOH and scraped off the substratum with a rubber policeman. After the collection of an aliquot for protein determination by the method of Lowry,12 monolayers, media, and wash were combined and digested with pronase (1 mg/ml in a 100 mmol/l Tris buffer, pH 8.0) at 50°C overnight in the presence of hyaluronate and chondroitin sulfate (250 µg each), which were added as carriers. Samples were precipitated with trichloroacetic acid (5%, weight/volume, final concentration) and centrifuged at 10,000 × g. The supernatants were dialyzed extensively against water to remove unincorporated label, and an aliquot was counted by liquid scintillation spectrometry. Data are expressed as the mean ± standard error of the mean of results from triplicate or quadruplicate cultures.

Student’s t test was used to assess the level of statistical significance.

Results

IL-1 was found to be a potent stimulator of fibroblast GAG production. Treatment of cultures for 48 hr with IL-1 (1.0 U/ml) stimulated [3H]GAG accumulation significantly by retroocular connective tissue, extraocular muscle perimysium, pretibial skin, and abdominal skin fibroblasts (Table 1). The stimulation reached maximal levels at 0.75 U/ml (Fig. 1). Although the degree of [3H]GAG stimulation varied...
Table 1. Effect of cytokines on \(^{3}H\)GAG accumulation

<table>
<thead>
<tr>
<th>Graves' ophthalmopathy patient fibroblast source</th>
<th>IL-1 (1.0 U/ml)</th>
<th>TGF-β (22 ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retroocular connective tissue</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No treatment</td>
<td>517 ± 42</td>
<td>411 ± 18</td>
</tr>
<tr>
<td>Treatment</td>
<td>1007 ± 138</td>
<td>709 ± 34</td>
</tr>
<tr>
<td>% Stimulation</td>
<td>+95, (P &lt; 0.01)</td>
<td>+73, (P &lt; 0.01)</td>
</tr>
<tr>
<td>Extraocular muscle perimysium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No treatment</td>
<td>502 ± 12</td>
<td>1467 ± 98</td>
</tr>
<tr>
<td>Treatment</td>
<td>1330 ± 113</td>
<td>2483 ± 71</td>
</tr>
<tr>
<td>% Stimulation</td>
<td>+165, (P &lt; 0.01)</td>
<td>+69, (P &lt; 0.01)</td>
</tr>
<tr>
<td>Pretibial skin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No treatment</td>
<td>371 ± 69</td>
<td>N.D.</td>
</tr>
<tr>
<td>Treatment</td>
<td>815 ± 210</td>
<td>N.D.</td>
</tr>
<tr>
<td>% Stimulation</td>
<td>+120, (P &lt; 0.05)</td>
<td></td>
</tr>
<tr>
<td>Abdominal skin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No treatment</td>
<td>969 ± 74</td>
<td>1194 ± 46</td>
</tr>
<tr>
<td>Treatment</td>
<td>1413 ± 197</td>
<td>1557 ± 103</td>
</tr>
<tr>
<td>% Stimulation</td>
<td>+46, (P &lt; 0.05)</td>
<td>+30, (P &lt; 0.01)</td>
</tr>
</tbody>
</table>

* Cultures were incubated for 72 hr with the factor to be studied prior to the addition of \(^{3}H\)glucosamine (5.0 \(\mu\)Ci/ml) for the final 24 hr of treatment. Data are expressed as the mean ± SEM of results obtained from three separate culture plates in the same experiment. Experiments were performed at least two times on cells from different patients with Graves' ophthalmopathy or normal individuals with similar results.

between cell strains, there were no consistent differences in the magnitude of \(^{3}H\)GAG stimulation or the sensitivity to IL-1 between cells from any of the four anatomical sites studied or between cells from normal individuals and patients with GO. Without IL-1 treatment, baseline \(^{3}H\)GAG accumulation did not differ between GO patient and normal fibroblasts. The rate of production of \(^{3}H\)GAG was maximal when cultures were incubated with IL-1 for 48–72 hr (Fig. 2).

To assess the effect of IL-1 on the accumulation of sulfated GAG, retroocular connective tissue fibroblast cultures were labeled for 24 hr with \(^{35}S\)sulfuric acid (10 \(\mu\)Ci/ml). Addition of IL-1 to the culture medium (10 U/ml) did not stimulate incorporation of the precursor into \(^{35}S\)GAG (control = 876 ± 59 cpm/\(\mu\)g protein, treatment = 904 ± 6.8 cpm/\(\mu\)g protein). In contrast, IL-1 increased the incorporation of \(^{3}H\)glucosamine into \(^{3}H\)GAG by 71% in sister cultures (control = 604 ± 107 cpm/\(\mu\)g protein, treatment = 1032 ± 93 cpm/\(\mu\)g protein). This result suggests that stimulation of chondroitin sulfate and dermatan sulfate does not account for the increase in GAG content observed with IL-1 treatment. Therefore it is likely that hyaluronate is the particular GAG stimulated by IL-1 treatment.

TGF-β also was a potent stimulator of \(^{3}H\)GAG accumulation by all fibroblasts studied (Table 1).
Maximal stimulation by TGF-β occurred with a concentration of 15 ng/ml (Fig. 3). The rate of production of [3H]GAG was maximal when cultures were incubated with TGF-β for 48 hr (Fig. 4). Although the degree of [3H]GAG stimulation varied between cell strains, there were no consistent differences in the magnitude of, or sensitivity to, TGF-β stimulation between fibroblasts from patients with GO and normal individuals, or between fibroblasts derived from any of the four anatomical sites studied. As was the case with IL-1, the addition of TGF-β (22 ng/ml) to the culture medium did not stimulate incorporation of [35S]sulfuric acid (10 μCi/ml) into [35S]GAG (control = 1063 ± 157 cpm/μg protein, treatment = 930 ± 33 cpm/μg protein). In contrast, TGF-β increased the incorporation of [3H]glucosamine into [3H]GAG by 34% in sister cultures (control = 1122 ± 39 cpm/μg protein, treatment = 1499 ± 37 cpm/μg protein).

Treatment with TSH (5.0 mIU/ml) did not affect [3H]GAG accumulation by fibroblasts derived from retroocular connective tissue, extraocular muscle perimysium, pretibial skin, or abdominal skin (Table 2). Stimulation did not occur after incubation of the cells with TSH (5.0 mIU/ml) and [3H]glucosamine for times of between 15 min and 5 hr. In other experiments, concentrations of TSH between 0.5–50 mIU/ml were examined and found not to stimulate [3H]GAG accumulation.

Factors having minimal or no ability to stimulate retroocular fibroblast [3H]GAG accumulation after treatment with the doses studied for incubation periods of 48–96 hr include IL-2 (10 and 20 U/ml), IL-6 (100 and 200 U/ml), TNF-alpha (100 U/ml), EGF (10 and 20 ng/ml), bFGF (250 ng/ml) and PDGF (2.5 and 5.0 U/ml). These particular doses are in the same range as doses known to be biologically active in other in vitro systems.13-19

Discussion

IL-1 is a cytokine characteristically produced by cells of the monocyte-macrophage series. However, many other cell types, including B cells, keratinocytes, brain astrocytes, and synovial fibroblasts, can produce IL-1 or molecules with IL-1 activity.20-21 IL-1 is a family of polypeptides involved in acute phase response, having an important function in the host response to infectious, inflammatory and immune challenges.22 Studies have shown that IL-1 is capable

Table 2. Effect of TSH on [3H]GAG accumulation

<table>
<thead>
<tr>
<th>Graves’ ophthalmopathy patient fibroblast source</th>
<th>Effect of TSH (5.0 mIU/ml) (cpm/μg protein)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retroocular connective tissue</td>
<td></td>
</tr>
<tr>
<td>No treatment</td>
<td>748 ± 26</td>
</tr>
<tr>
<td>Treatment</td>
<td>715 ± 32</td>
</tr>
<tr>
<td>% Stimulation</td>
<td>-4.0, NS</td>
</tr>
<tr>
<td>Extraocular muscle perimysium</td>
<td></td>
</tr>
<tr>
<td>No treatment</td>
<td>692 ± 59</td>
</tr>
<tr>
<td>Treatment</td>
<td>636 ± 55</td>
</tr>
<tr>
<td>% Stimulation</td>
<td>-8.0, NS</td>
</tr>
<tr>
<td>Pretibial skin</td>
<td></td>
</tr>
<tr>
<td>No treatment</td>
<td>1236 ± 33</td>
</tr>
<tr>
<td>Treatment</td>
<td>1245 ± 0</td>
</tr>
<tr>
<td>% Stimulation</td>
<td>+1.0, NS</td>
</tr>
<tr>
<td>Abdominal skin</td>
<td></td>
</tr>
<tr>
<td>No treatment</td>
<td>608 ± 98</td>
</tr>
<tr>
<td>Treatment</td>
<td>691 ± 55</td>
</tr>
<tr>
<td>% Stimulation</td>
<td>+14, NS</td>
</tr>
</tbody>
</table>

* TSH (5.0 mIU/ml) and [3H]glucosamine (5.0 μCi/ml) were added simultaneously to cultures for a labeling period of 2 hr. Data are expressed as the mean ± SEM of results obtained from three separate culture plates in the same experiment. Experiments were performed at least three times on cells from different patients with Graves’ ophthalmopathy or normal individuals with similar results.
of stimulating GAG production by human gingival, synovial, and skin fibroblasts. This stimulation is thought to act via synthesis of new hyaluronic acid synthetase. We found that IL-1 is a potent stimulator of \[^{3}H\]GAG production by retroocular connective tissue fibroblasts, as well as by fibroblasts derived from the perimysium of extraocular muscles. Similarly, IL-1 stimulates \[^{3}H\]GAG synthesis by abdominal and pretibial skin fibroblasts. We found no difference between fibroblasts from the various sites regarding the magnitude of, or sensitivity to, the stimulatory effects of IL-1. In contrast, our earlier studies showed that interferon-gamma selectively stimulates \[^{3}H\]GAG production in retroocular connective tissue fibroblasts, having no such effect in fibroblasts derived from pretibial skin. We found no differences between normal and GO patient fibroblasts with respect to baseline or stimulated \[^{3}H\]GAG production.

TGF-\(\beta\) can be produced by transformed cells as well as by normal tissues, including human T lymphocytes. Normal human fibroblasts from several sources have membrane receptors for TGF-\(\beta\). Although this factor stimulates proliferation and differentiation of some cell types, TGF-\(\beta\) demonstrates growth-inhibiting properties. In particular, TGF-\(\beta\) has been shown to inhibit TSH-stimulated iodine metabolism and cell growth in cultured porcine thyroid cells. In Graves' disease, this factor is expressed and secreted by thyroid follicular cells as well as by thyroid-infiltrating mononuclear cells. TGF-\(\beta\) has been shown to stimulate fibroblasts to produce matrix proteins, including collagen and fibronectin. We found this factor to be a potent stimulator of \[^{3}H\]GAG synthesis by cultured normal and GO patient fibroblasts derived from retroocular connective tissue and extraocular muscle perimysium, as well as from pretibial and abdominal skin.

In Graves' disease, circulating antibodies directed against the TSH receptor on thyroid follicular cells stimulate over-production of thyroid hormone. It has been suggested that these thyroid-stimulating immunoglobulins (TSI) might be responsible for stimulating the over-production of GAG, or other matrix proteins, by retroocular fibroblasts in GO. TGF-\(\beta\) has been shown to stimulate fibroblasts to produce matrix proteins, including collagen and fibronectin. We found this factor to be a potent stimulator of \[^{3}H\]GAG synthesis by cultured normal and GO patient fibroblasts derived from retroocular connective tissue and extraocular muscle perimysium, as well as from pretibial and abdominal skin.

To explain the site-selective involvement of the retroocular space and the pretibium in the extrathyroidal manifestations of Graves' disease, we sought site-specific differences between fibroblasts derived from various anatomic sites and between normal and GO patient fibroblasts. In the current study, we found no differences in response to cytokines or TSH between normal and GO patient fibroblasts or between fibroblasts from any of the four anatomic sites studied. However, in previous studies, we have shown that cultured retroocular and pretibial fibroblasts derived from patients with GO and pretibial dermopathy express heat shock protein (HSP) 72 at baseline. Abdominal fibroblasts from these same individuals, as well as fibroblasts from the same three sites derived from normal individuals, do not express HSP 72 at baseline. Treatment with IL-1 or TGF-\(\beta\) enhances HSP 70 expression in cultured normal and GO patient retroocular fibroblasts. It is unknown whether HSP 72 expression plays a primary role in the development of GO, reflects cellular stress, or is an important immunomodulatory protein in the disease.

The retroocular space in GO is selectively infiltrated with activated lymphocytes and macrophages. Our results suggest subsequent events that are likely important in the pathogenesis of the disease. Cells within this inflammatory infiltrate would secrete cytokines, including IL-1 and TGF-\(\beta\). These cytokines would stimulate the local over-production of GAG by retroocular connective tissue and extraocular perimysial fibroblasts. The accumulation of GAG in the retroocular space would lead to the clinical and histologic features of GO. The reason for the selective infiltration of activated lymphocytes into the retroocular space in Graves' disease has yet to be explained. It is possible that circulating autoreactive T cells, recognizing the TSH receptor on thyroid cells and an antigen present on retroocular fibroblasts, target fibroblasts that make up the connective tissue behind the eye and the perimysial connective tissue that invests the extraocular muscles.

Key words: Graves' ophthalmopathy, glycosaminoglycans, fibroblasts, IL-1, TGF-\(\beta\)

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References


