In vitro synthesis of glycosaminoglycans in endocrine ophthalmopathy

G Kahaly, C Stover, J Beyer and E Otto

III Department of Medicine and Endocrinology, Johannes Gutenberg University Hospital, Mainz, Germany

The effects of humoral and cell-mediated immunity on the glycosaminoglycan synthesis of retrobulbar fibroblasts was evaluated in patients with endocrine ophthalmopathy. After incubation with IgG and sera, secreted glycosaminoglycans, radiolabeled with D-6-3H-glucosamine and 35Sulfate, were precipitated with cetylpyridinium chloride and ethanol. Hyaluronic acid synthesis of human retrobulbar fibroblasts after incubation with sera and IgG and after co-culture with lymphocytes was assessed by means of a radiometric test. Patients’ IgG, compared to controls’, accounted for a higher secretory stimulation of porcine retrobulbar fibroblasts (as measured by cetylpyridinium chloride precipitation) after 24 and 48 h. Contrasting with 24 h incubation time, glycosaminoglycan values after 48 h were increased two to threefold. Patients’ and controls’ sera caused earlier and stronger, yet indistinguishable glycosaminoglycan production. Non-sulfated hyaluronic acid was the preponderant glycosaminoglycan secreted into the media by retrobulbar fibroblasts. As assessed with the radiometric test, incubation with patients’ and controls’ sera and IgG did not reveal a significant difference in stimulating the hyaluronic synthesis of patients’ and controls’ retrobulbar fibroblasts. When measuring the hyaluronic acid synthesis of controls’ and patients’ retrobulbar fibroblasts after co-cultivation of lymphocytes, however, patients’ lymphocytes had a marked ability to increase the hyaluronic acid concentration compared to controls’ lymphocytes. The hyaluronic acid concentration after incubation of a patient’s retrobulbar fibroblasts with autologous lymphocytes was markedly elevated than the intrinsic hyaluronic acid production of retrobulbar fibroblasts. In conclusion, though a significant in vitro influence of patients’ IgG and sera on the glycosaminoglycan release of both porcine and human (patients’ as well as controls’) retrobulbar fibroblasts could not be observed in this study, the indications of a marked stimulatory influence of lymphocytes on the hyaluronic acid secretion of retrobulbar fibroblasts demand further investigation.

George Kahaly, III. Department of Medicine and Endocrinology, University Hospital, Langenbeckstrasse 1, 6500 Mainz, Germany

The substantial increase of retroorbital volume which is characteristic of endocrine ophthalmopathy (EO) brings about infiltrative as well as non-infiltrative affections of the eyelids, exophthalmos, paresis of the eye muscles, and, as the disease progresses, compression of the optic nerve (1, 2). The course of the disease is influenced by thyroid status as well as by therapy regimens (3-5). Histological analysis of retrobulbar tissue in EO reveals a lymphocytic infiltration of the interstitium of muscle and of the lacrimal gland (6, 7) which includes plasma cells, macrophages and, to a lesser degree, mast cells (8). Lymphocytes in normal tissue, by contrast, were found to be quiescent due to the lack of interleukin-2 receptor and HLA class II expression (9). The activation of retrobulbar fibroblasts in EO orbits which express the HLA class II determinant (10) and the P1 blood group antigen (11) is mirrored in a stronger secretion of collagen and glycosaminoglycans (GAG), which are, with the exception of keratan sulfate, polysaccharides consisting of linear chains of repetitive disaccharide units comprising acetylated or sulfated hexosamines and hexuronic acids. The striking prevalence of hyaluronic acid and dermatan sulfate among the analysed GAG in normal retrobulbar tissue (12) has yet to be confirmed in affected EO tissue. The edematous swelling of extraocular muscles which themselves remain histologically intact (13) affects mainly the inferior and medial rectus muscles (8) and antedates, as the disease progresses, fibrosis, which is part of restrictive myopathy. Both can adequately be quantified by MR imaging (14). Based on the fact that GAG synthesis of retrobulbar fibroblasts evidently plays a crucial role in the clinical manifestation of EO yet has not sufficiently been under investigation, the aim of this study was to assess the influence of patients’ and controls’ sera, antibodies (IgG), and lymphocytes on the secretion of fibroblastic GAG as a parameter for cell activity.

Subjects, material and methods

Samples

Sera were obtained from 44 euthyroid patients [32 receiving thyrostatic drugs, 10 after radioactive iodine,
2 after thyroidectomy, 34F, age (range, median): 25–78 years, 49 years] suffering from clinically active, progressive EO (increase of exophthalmos, and/or of impaired eye motility, and/or of lid aperture, and/or of inflammatory parameters as well as decrease of visual acuity) and from 44 euthyroid outpatients (controls, 32F, 23–70 years, 38 years) free of any autoimmune disorder. EO was diagnosed clinically and by detection of muscle thickening in CT, sonography or by exclusion of other causes for an exophthalmos. No EO patients had undergone immunosuppressive therapy within the previous six months. IgG were purified chromatographically from sera with the help of protein A coupled sepharose (Protein A sepharose CL4B, Pharmacia). Lymphocytes were isolated from three EO patients (2F, 53–78 years, 62 years) and three controls (3F, 46–61 years, 56 years) by density gradient centrifugation (Ficoll) and immediately incubated with cultivated retrobulbar fibroblasts in 96 well plates. Retrobulbar tissue was obtained from orbital decompression with the three patients after informed consent.

**Fibroblast culture**

Porcine and human retrobulbar tissue was minced and digested mechanically and enzymatically (collagenase A. Boehringer Mannheim, 1 g/l). Filtration (50 μm filters) was followed by centrifugation of filtrate (400 × g, 10 min). Cells were incubated in medium (RPMI 1640, Boehringer Mannheim, buffered with NaHCO₃ to pH=7.4, antibiotics, 5% fetal calf serum (FCS), Gibco from tested lot) at 37°C, 5% CO₂, water saturated. Fibroblasts of a cell-line (ATCC, Maryland, CRL 7475, NBL-collection, Hs 738, eye, normal male fetus) served as control cells and were passaged with trypsin (0.1%, diluted in PBS Dulbecco) after reception before being cultivated at standard conditions.

Incorporation of ³H-glucosamine and ³⁵S into GAG

Preincubation of porcine retrobulbar fibroblasts with sulphate-free DMEM medium including antibiotics and 0.1% FCS was followed by a pulse phase with 5 ml DMEM supplemented with 250 μCi ³⁵S, 40 μCi D-6-³H-glucosamine (Amersham) and IgG (5 mg), sera (20% of the medium) respectively. After 24 and 48 h of incubation, radioactive medium was removed and a chase phase commenced. Protein content of retrobulbar fibroblast cultures was determined according to Lowry (15), while the secreted ³H/³⁵S-labeled GAG were precipitated in sterile cetylpyridinium chloride solution for 12 h at 4°C from the supernatant after the addition of 6% chondroitin sulfate (Sigma) as carrier GAG. Between intermittent centrifugation steps, the precipitate was solubilized in potassium acetate and resuspended in distilled water. The content of ³H- and ³⁵S-GAG was determined with a liquid scintillator and expressed in relation to the protein content of the cells as deintegrations per minute (dpm)/mg cell protein.

**Assessment of HA synthesis**

A radiometric test (HA-test 50, Pharmacia) was employed to measure the hyaluronic acid production of human (patients’ and controls’) retrobulbar fibroblasts (50000 retrobulbar fibroblasts/well in 24-well plates) having been incubated with IgG (1 g/l) or sera (inactivated at 56°C for 30 min, 10% of the medium) or co-cultivated with lymphocytes (5000 retrobulbar fibroblasts, 250000 lymphocytes/well in 96-well plates) for 48 h respectively. After specific detection of hyaluronic acid by a radiolabeled binding protein, any excess ¹²⁵I-binding protein is bound by sepharose-coupled hyaluronic acid. Centrifugation leads to pelleting of this complex. After decantation, radioactivity is measured in a gamma counter. The value is reciprocally proportional to the initial hyaluronic acid content of the probe (16).

**Statistical analysis**

Secretion of radiolabeled sulfated and non-sulfated GAG after incubation with patients’ and controls’ IgG and sera was analysed with the Student’s t-test for unpaired
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I 2.6
O
I
2.4
E
2.2
F-
2.0
1.8
24 h
48 h
24 h
48 h

Fig. 1. Sulfated and non-sulfated glycosaminoglycan synthesis of porcine retrobulbar fibroblasts after incubation with 22 patients' and 22 controls' (matched pairs) IgG for 24 and 48 h respectively. The values are presented as box plots indicating minimum, 25th percentile, median, 75th percentile, maximum (patients vs controls: p>0.05). □ controls; ■ patients.

observations. The influence of incubation time as well as the values measured by means of the hyaluronic acid test were evaluated by the Wilcoxon two-sample test.

Results

Radiolabeled GAG

Incubation of porcine retrobulbar fibroblasts with patients' and controls' sera (matched pairs) caused an increase of non-sulfated GAG secretion after 24 h which was augmented slightly after 48 h. Measurement of 35S-GAG synthesis after 24 h incubation time with patients' and controls' sera, however, revealed lower levels which hardly increased after incubation for 48 h. By contrast, incubation with these very patients' and controls' IgG (matched pairs) for 48 h led to higher 3H-GAG content in

Table 2. Hyaluronic acid synthesis of control’s and patient’s retrobulbar fibroblasts after incubation with 22 controls’ and 20 patients’ IgG and sera. The values are presented as medians and ranges (patients vs controls: p>0.05).

<table>
<thead>
<tr>
<th>Incubation</th>
<th>Hyaluronic acid (mg/l)</th>
</tr>
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<tbody>
<tr>
<td>Control’s RF</td>
<td></td>
</tr>
<tr>
<td>+ patients’ sera</td>
<td>2.42 (1.43–3.5)</td>
</tr>
<tr>
<td>+ patients’ IgG</td>
<td>0.71 (0.29–1.2)</td>
</tr>
<tr>
<td>Control’s RF</td>
<td></td>
</tr>
<tr>
<td>+ controls’ sera</td>
<td>2.53 (1.59–4.65)</td>
</tr>
<tr>
<td>+ controls’ IgG</td>
<td>0.97 (0.02–1.32)</td>
</tr>
<tr>
<td>Patient’s RF</td>
<td></td>
</tr>
<tr>
<td>+ patients’ sera</td>
<td>2.68 (1.24–3.92)</td>
</tr>
<tr>
<td>+ patients’ IgG</td>
<td>0.61 (0.41–1.14)</td>
</tr>
<tr>
<td>Patient’s RF</td>
<td></td>
</tr>
<tr>
<td>+ controls’ sera</td>
<td>2.82 (1.57–4.63)</td>
</tr>
<tr>
<td>+ controls’ IgG</td>
<td>0.76 (0.11–1.12)</td>
</tr>
</tbody>
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Fig. 2. Hyaluronic acid synthesis of control’s and patient’s retrobulbar fibroblasts after incubation with 22 controls’ and 20 patients’ IgG. The dotted line denotes the intrinsic hyaluronic acid production of retrobulbar fibroblasts in medium only (patients vs controls: p>0.05). □ controls; ■ patients.
the media compared to 24 h incubation time. Patients’ IgG also stimulated 15S-GAG synthesis more than controls’ IgG after 48 h, thus overcoming the lack of difference between patients’ and controls’ after incubation with IgG for 24 h (Table 1, Fig. 1, patients vs controls: p > 0.05).

**HA synthesis and sera/IgG**

When assessing the effect of sera on the synthesis of hyaluronic acid by means of the hyaluronic acid test, neither incubation of controls’ retrobulbar fibroblasts with patients’ and controls’ sera nor incubation of patient’s retrobulbar fibroblasts with the same patient’s and control’s sera made a significant difference detectable. As the intrinsic hyaluronic acid production of retrobulbar fibroblasts (in medium only) amounted to 0.55 mg/l, the incubation of retrobulbar fibroblasts (control’s and patient’s) with sera produced a 2.5 to 9-fold increase in hyaluronic acid production. Incubation of a patient’s retrobulbar fibroblasts with autologous serum was responsible for an induction of hyaluronic acid synthesis (2.56 mg/l) which was indistinguishable from that caused by heterologous inactivated sera. Incubation of 20 patients’ IgG with control’s retrobulbar fibroblasts engendered a production of hyaluronic acid which did not significantly differ from that measurable after incubation with 22 controls’ IgG. Comparable results were obtained when incubating a patient’s retrobulbar fibroblasts with both patients’ and controls’ IgG (Table 2, Fig. 2, patients vs controls: p > 0.05).

**HA synthesis and lymphocytes**

Patients’ lymphocytes, after co-cultivation with control’s and patient’s retrobulbar fibroblasts, had a marked ability to increase the hyaluronic acid concentration (range: 2.42–8.09 mg/l, median: 3.39) compared to controls’ lymphocytes (2.07–2.87 mg/l, 2.46). The hyaluronic acid concentration measured after incubation of a patient’s retrobulbar fibroblasts with autologous lymphocytes (3.10 mg/l) markedly exceeded both the hyaluronic acid synthesis achieved after co-cultivation of this very patient’s lymphocytes with control’s retrobulbar fibroblasts (2.42 mg/l) as well as the intrinsic hyaluronic acid production of retrobulbar fibroblasts (in medium incl. 10% FCS. 1.95 mg/l) (Fig. 3).

**Discussion**

Though EO is considered to be of autoimmune origin, it has not yet been clarified whether dysregulation of either humoral or cellular immunity (17) is responsible for the onset of the disease, or both, which leads to a perpetuation of the retro- and peribulbar affection. In this sense, the question whether stimulation of retrobulbar fibroblasts and increased release of GAG occur as consequences of primary immune reaction or as secondary phenomena remains unanswered (18). The potential singularity of retrobulbar fibroblasts in terms of ontogenetic development, physiology and HLA class II expression having been realized (8, 12), the effects of humoral as well as cellular immunity were to be assessed on retrobulbar fibroblasts in this study. While regulatory influence of distinct lymphokines on lung fibroblast GAG production has been reported (19), using retrobulbar fibroblasts, a significant binding of EO patients’ IgG compared to controls’ was shown (20) apart from a possibly modulatory function of IGF-I in the presence of IgG (21). The relevance of a 23 kDa protein isolated from retrobulbar fibroblasts (22) and of a 72 kDa heat shock protein expressed on the surface of retrobulbar fibroblasts (23) remains to be elucidated. Though the first experiments in this study were conducted on porcine retrobulbar fibroblasts, they nevertheless reveal a certain tendency of patients’ IgG and sera to enhance radiolabeled GAG secretion compared to controls’ IgG and sera respectively. The major portion of secreted GAG
was non-sulfated hyaluronic acid. Due to the presence of growth factors, the stimulatory influence of serum on retrobulbar fibroblasts, however, is less specific than that of isolated IgG and consequently, patients’ and controls’ sera do not differ in their ability to stimulate retrobulbar fibroblasts, as shown by others, too (24). In spite of a 2.5 to 9-fold increase of intrinsic hyaluronic acid secretion of retrobulbar fibroblasts after incubation with human sera detected by means of the hyaluronic acid test, neither the values of the patients’ group as a whole nor the concentration measurable after incubation with autologous serum differed from those of the control group.

Co-cultivation of patient’s and control’s retrobulbar fibroblasts with patients’ lymphocytes, however, produced higher detectable concentrations of hyaluronic acid compared to incubation with controls’ lymphocytes, which can be interpreted as an effect resulting from the stimulation of retrobulbar fibroblasts by lymphocytes. This can only be made intelligible in the presence of traditional antigen presenting cells such as, e.g. macrophages, which are also isolated by density gradient centrifugation, or of retrobulbar fibroblasts, which, in conjunction with intrinsic ILA class II molecules, might function as antigen presenting cells themselves. Lymphokines have been shown to stimulate the GAG synthesis of human fibroblasts (19, 25). Recent data, however, indicate that lymphocytes themselves are capable of secreting chondroitin- and heparan sulphate proteoglycans with certain preferences for T- and B-cells (26). Furthermore, it may be speculated that lymphocytes, as they do possess receptors for GAG such as hyaluronic acid, chondroitin sulfate, and heparin (27), might be homed in retrobulbar tissue of EO in a dose-dependent manner. Thus, further factors might be indispensable for autologous lymphocytes to significantly increase the GAG synthesis of retrobulbar fibroblasts. It may be added that there is some implication for muscle cells being able to produce GAG as well (28).

In conclusion, the in vitro experiments conducted in this study do not favor a significant role of humoral immunity on the GAG synthesis of retrobulbar fibroblasts. This finding seems to be in accordance with previous studies in which the activity of mitochondrial enzymes in retrobulbar fibroblasts was equally influenced by patients’ and controls’ sera and IgG (29). However, the marked increase of fibroblastic hyaluronic acid synthesis engendered by patients’ lymphocytes and—from the immunological point of view more noteworthy—by autologous lymphocytes implies that further assessment of cell-mediated immunity on retrobulbar fibroblasts seems to be of not negligible importance in gaining further understanding of the immunopathological features of EO.

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