Assessment of disease activity in Graves' ophthalmopathy
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Urinary glycosaminoglycans do not correlate with disease activity in Graves' ophthalmopathy

M.N. Gerding, J.W.C. van der Meer, M. Broenink, O. Bakker, M.F. Prummel, W.M. Wiersinga
Abstract

Context The accumulation of hydrophilic glycosaminoglycans (GAG’s) and its fractions chondroitin sulphate A (CA), dermatan sulphate (DS), and hyaluronic acid (HA)) in retrobulbar tissue lead to edema, and subsequently to the clinical features of Graves’ ophthalmopathy (GO). Edema is present in active eye disease. GAG production might thus reflect activity of the eye disease. The validity of a disease activity parameter is defined as the ability to predict a response to immunosuppression.

Objective To evaluate the predictive value of urinary GAG levels in 65 moderate severe GO patients for the outcome of treatment with retrobulbar irradiation.

Methods Urinary GAG excretion was measured both by a conventional metachromatic assay and by a highly sensitive HPLC method.

Results Thirty-four patients (53 %) responded to retrobulbar irradiation, and 31 did not respond (47%). The correlation coefficient between the urinary GAGs measured by both assays was $r = 0.44$ (p $< 0.0001$). When comparing responders and nonresponders to radiotherapy, we found no significant difference in the baseline levels of urinary GAG excretion, nor in the baseline fractions measured by HPLC. Urinary GAG levels did not correlate with the duration of GO ($r = 0.11$, p $= 0.37$), nor with the clinical activity score ($r = -0.01$, p $= 0.91$).

Conclusions Urinary GAG levels do not differ in active and inactive GO patients, and therefore measuring urinary GAGs alone is not useful in the assessment of disease activity in Graves’ ophthalmopathy.

Introduction

In Graves’ ophthalmopathy (GO) immunocompetent cells invade the retrobulbar tissues, producing cytokines which stimulate glycosaminoglycan (GAG) production by the orbital fibroblasts.1 The hydrophilic GAGs attract water causing edema. Edema is one of the characteristics of active eye disease and GAG production might thus be a parameter of disease activity. Indeed, Kahaly et al.
report higher urinary GAG levels in patients with GO compared to controls or to patients with Graves’ hyperthyroidism without eye disease.\textsuperscript{2} The data allowed discrimination between active and inactive eye disease (activity assessed by a single examination) with a positive predictive value of 68\%, and a negative predictive value of 96\%. However, other investigators could not confirm these findings and found similar urinary GAG excretion in ophthalmopathy patients and controls.\textsuperscript{3} In more recent studies, Kahaly’s group applied an HPLC method to determine GAG levels. Using this technique they again found higher levels of GAG excretion in active GO patients compared to inactive GO patients.\textsuperscript{4,5}

To determine whether GAG levels indeed correlate with disease activity, it is of great importance how disease activity is defined. The gold standard for the determination of the activity of the eye disease would be histological examination of retrobulbar tissues. Biopsies of retrobulbar tissues, however, are seldom feasible in clinical practice. Therefore we have to accept a surrogate criterion to be met by an activity parameter: its value in predicting the outcome of immunosuppressive treatment.\textsuperscript{6} A study using this criterion has not been performed previously. We therefore analyzed urinary GAG levels in 65 GO patients before and after treatment with retrobulbar irradiation and compared the values in those who responded to this therapy with those who did not. Urinary GAG excretion was measured in two ways. Urinary GAGs were determined by a conventional metachromatic assay, and by an HPLC method performed in the laboratory of Prof. Dr. G. Kahaly, Mainz, Germany.

**Patients and methods**

**Patients**

We studied 65 patients with moderately severe untreated Graves’ ophthalmopathy (defined as proptosis $\geq 25$ mm and/or evident restriction of eye muscle motility), who had been euthyroid for at least two months. Treatment consisted of retrobulbar external irradiation, given in 10 divided fractions of 2 Gy daily over a
Urinary glycosaminoglycans do not correlate with disease activity in GO

2 week period. Therapeutic outcome was assessed 26 weeks after irradiation, and was defined according to minor and major criteria, as described in chapter one. GAG levels were related to the outcome of radiotherapy, and also to the clinical activity score (CAS) and the duration of the eye disease (calculated from the onset of signs and symptoms as perceived by the patients). The CAS is based on classical signs of inflammation (pain, redness, swelling and loss of function, i.e. increase of proptosis, worsening of eye muscle motility or decrease of visual acuity); each item is scored as present (1) or absent (0), with a maximum score of 10 points.

Methods

Patients collected 24-hour urine production on two consecutive days, just prior to the start of the irradiation, and again after 26 weeks. Total urinary volume and the total creatinine excretion was measured to assess the completeness of the urine collection. None of the urine samples had to be excluded because of incomplete collection (defined as a creatinine excretion in one 24 hr sample exceeding ≥ 150% of the creatinine excretion of the other sample). After mixing, separate urine samples of both 24 hr collections were stored at -20°C. GAGs were measured by two methods. The conventional metachromatic assay, as described by Le Phuc Thuy and Nyhan, measures GAG from the decrease in absorbance at 610 nm after adding solutions of Azure A (Sigma Chemicals Co. A2918) and Azure B (Sigma Chemicals A4043). The HPLC method quantifies GAGs and the subfractions chondroitin sulphate A (CA), dermatan sulphate (DS) and hyaluronic acid (HA), as described by Kahaly. In short, chromatography was performed at room temperature with a concave sodium phosphate gradient (100-750 mmol/l, pH 4.0; run time 40 min.) on a Bio-Sil amino disaccharide column (250x4 mm, flow rate 0.6 ml/min.). The injection volume was 10 μl for reference kit injections (pure CA, DS and HA disaccharides, Medac, Hamburg, Germany), and 40 μl for urine samples.
In both assays, the mean GAG excretion over two days was calculated from the 2x24 hour excretion using the 24 hr volume of urine voided and expressed in mg/24 hr (both assays had a coefficient of variation less than 10%). All measurements were performed without knowledge of the clinical status of the patient.

**Statistics**

The data are expressed as median (range) in case of a skewed distribution, or as mean ± SD if normally distributed. Differences between groups were analyzed by Mann-Whitney U tests, and changes after radiotherapy by the Wilcoxon’s signed rank sum test. Receiver-operating characteristic (ROC) curves were plotted and the area under the curve was calculated for the GAG levels in predicting a response to treatment.

**Results**

We included 65 patients, 20 males and 45 females; their mean (± SD) age was 53 ± 10 years. Thirty-four patients (53 %) responded to retrobulbar irradiation, and 31 (47%) did not respond (no change in 26 patients, and deterioration in 5 patients).

Urinary GAG values were lower using HPLC (median 20 mg/24 h, range 4.6-113), than in the metachromatic assay (median 30.8 mg/24 h, range 11.3-62). The correlation between both methods is given in figure 1 ($r = 0.44; p<0.0001$). When comparing responders and nonresponders, we found no significant difference in the baseline levels of urinary GAG excretion (Table 1). Also, the baseline fractions of GAGs measured by HPLC did not differ between responders and nonresponders (Table 1). The predictive values of the GAGs and their fractions (at baseline) for the outcome of irradiation were calculated by plotting receiver-operating characteristic curves. The area under the curve was maximal 0.58 (Table 2). Thus, urinary GAG levels could not predict therapeutic outcome.
When comparing the baseline and the post-treatment GAG levels in the urine, no significant changes were detected over time (Table 3). In addition, pretreatment urinary GAGs by HPLC did not correlate with the duration of GO (r=0.11, p=0.37), nor with the clinical activity score (r=-0.01, p=0.91).

Figure 1: Correlation between the pretreatment total urinary glycosaminoglycan level, measured with a conventional metachromatic assay (using Azure A and B) and by high pressure liquid chromatography (HPLC) in patients with moderately severe Graves’ ophthalmopathy. Spearman’s correlation coefficient is 0.44, p < 0.001.

Table 1. Urinary Glycosaminoglycans (GAGs) and their fractions measured by a metachromatic reaction with Azure A and B (A&B), and by HPLC in 65 patients with moderately severe Graves’ ophthalmopathy treated with retrobulbar irradiation. Data given as median and range in mg/24h.

<table>
<thead>
<tr>
<th></th>
<th>All patients n=65</th>
<th>Responders n=34</th>
<th>Nonresponders n=31</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>total GAGs (A&amp;B)</td>
<td>30.8 (11.3-62)</td>
<td>30.5 (11-62)</td>
<td>30.8 (12-47)</td>
<td>.77</td>
</tr>
<tr>
<td>total GAGs (HPLC)</td>
<td>20.2 (4.6-113)</td>
<td>22.2 (4.9-55)</td>
<td>18.9 (4.6-113)</td>
<td>.51</td>
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<tr>
<td>Hyaluronic acid (HPLC)</td>
<td>4.0 (0.3-94)</td>
<td>4.2 (0.5-29)</td>
<td>3.5 (0.3-94)</td>
<td>.26</td>
</tr>
<tr>
<td>Chondroitine acid (HPLC)</td>
<td>8.7 (2-32)</td>
<td>8.6 (2-20)</td>
<td>8.7 (2-32)</td>
<td>.67</td>
</tr>
<tr>
<td>Dermatan sulphate (HPLC)</td>
<td>5.4 (0.2-16)</td>
<td>5.5 (0.2-16)</td>
<td>4.5 (0.3-11)</td>
<td>.26</td>
</tr>
</tbody>
</table>

*p-value using the Mann-Whitney U test.
Table 2. Area under the curve (AUC) from receiver operating characteristic (ROC) curves for total urinary glycosaminoglycans (GAGs), measured by Azure A & B (A&B), and by HPLC (GAG subfractions: hyaluronic acid (HA), chondroitinic acid (CA), dermatan sulphate (DS)).

<table>
<thead>
<tr>
<th></th>
<th>AUC ± se</th>
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<tr>
<td>GAGs (A)</td>
<td>0.52 (0.08)</td>
</tr>
<tr>
<td>GAGs (HPLC)</td>
<td>0.45 (0.07)</td>
</tr>
<tr>
<td>HA</td>
<td>0.42 (0.07)</td>
</tr>
<tr>
<td>CA</td>
<td>0.47 (0.07)</td>
</tr>
<tr>
<td>DS</td>
<td>0.58 (0.07)</td>
</tr>
</tbody>
</table>

Table 3. Urinary glycosaminoglycans (GAGs), measured by a metachromic reaction using Azure A and B (A&B), expressed in mg / 24 hr as median and range, at baseline and 26 weeks after retrobulbar irradiation, in 65 patients with moderately severe Graves' ophthalmopathy.

<table>
<thead>
<tr>
<th></th>
<th>All (n=65)</th>
<th>Responders (n=34)</th>
<th>Non Responders (n=31)</th>
</tr>
</thead>
<tbody>
<tr>
<td>t=0: total GAGs (A&amp;B)</td>
<td>30.8 (11-62)</td>
<td>30.5 (11-62)</td>
<td>30.8 (12-47)</td>
</tr>
<tr>
<td>t=26: total GAGs (A&amp;B)</td>
<td>31.3 (16-48)</td>
<td>32.7 (16-48)</td>
<td>31.3 (19-45)</td>
</tr>
<tr>
<td></td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

Discussion

In this study we examined prospectively 65 consecutive patients with moderately severe GO, who were not selected for activity of the eye disease, and found that the total amount of urinary GAGs nor the levels of its fractions were useful in predicting whether a patient would benefit from immunosuppression. We also found no significant change in any of the GAG measurements 26 weeks after retrobulbar irradiation, nor a correlation between the urinary GAGs and clinical
Urinary glycosaminoglycans do not correlate with disease activity in GO. It thus seems that urinary GAG levels do not reflect the activity of Graves' eye disease.

Our data are in disagreement with the studies reported by Kahaly et al., in which a correlation between GAG levels and the CAS was found. In our present study, we also could not confirm these promising findings when evaluating the response to radiotherapy as a "gold standard" for detecting disease activity. This was independent of the method used for determination of GAGs. Pre-analytical factors seem to be ruled out as they were exactly the same for all measurements. We have to conclude that measuring urinary GAG excretion has very little clinical significance in predicting the outcome of immunosuppressive therapy in moderately severe GO. It might still be that plasma GAG levels could have a better prognostic value. Plasma GAGs were found to be elevated in untreated, active GO patients in contrast to normal GAG levels in inactive GO patients. However, Pappa et al., could not find any correlation of the serum GAG levels with tissue GAG levels measured in orbital samples from ten patients with GO.

References


