Poorly specific binding of thyroglobulin to orbital fibroblasts from patients with Graves’ ophthalmopathy

S. Lisi¹, R. Botta¹, P. Agretti¹, S. Sellari-Franceschini², C. Marcocci¹, A. Pinchera¹, and M. Marìnò²
¹Department of Endocrinology and ²Department of Neuroscience, University of Pisa, Pisa, Italy

ABSTRACT. It has been proposed that thyroglobulin (Tg) may be involved in the pathogenesis or the progression of Graves’ ophthalmopathy (GO). According to this hypothesis, following its release from the thyroid, Tg would reach orbital tissues, thereby eliciting an autoimmune aggression. In support of this, we recently found that intact Tg is present in orbital tissues of patients with GO, where it is complexed with glycosaminoglycans. In this study, we searched for additional Tg binding sites in orbital tissues, using primary cultures of orbital and skin fibroblasts from 7 GO patients who had undergone orbital decompression. Biotin-labeled Tg bound to both skin and orbital fibroblasts in a saturable manner, with constants of dissociation of ~75 nmol/l for skin fibroblasts and ~40 nmol/l for orbital fibroblasts. In an attempt to identify Tg binding sites, fibroblast extracts were blotted onto membranes that were incubated with biotin-labeled Tg, which bound especially to a protein migrating at ~300 kDa, present in both orbital and skin fibroblast extracts. Because no appreciable inhibition of binding of biotin-labeled Tg was produced by unlabeled Tg, we concluded that binding was poorly specific and it is unlikely to be involved in the pathogenesis of GO.

INTRODUCTION

It is generally accepted that Graves’ ophthalmopathy (GO) has an autoimmune pathogenesis, even though the autoantigen(s) involved and the mechanisms triggering the histological changes in orbital tissues are poorly known (1, 2). The majority of investigators share the view that the most reasonable candidate autoantigen is the TSH-receptor (TSH-R), because of its presence in orbital tissues and based on a recently developed murine model of GO, obtained by genetic immunization of mice with TSH-R (3, 4). In addition or as an alternative to TSH-R, it has been proposed that another thyroid autoantigen, namely thyroglobulin (Tg), may be involved in the pathogenesis or the progression of GO (5). The original hypothesis, which was formulated by Kriss in the 1970s, is that Tg would reach orbital tissues from the thyroid through a retrograde route, thereby eliciting an autoimmune aggression (5). The existence of a thyroid-orbit connection was demonstrated by Kriss himself, who showed that radiocolloid injected into the thyroid moves rapidly and retrogradly to neck lymph nodes in patients with GO (6). Following early observations suggesting the presence of Tg in orbital tissues (5, 7), more recently, we found that intact Tg is present in orbital tissues of ~70% of patients with GO (5, 8, 9).

The hypothesis that Tg is involved in the pathogenesis of GO implies the existence of specific Tg binding sites in orbital tissues, but not in other tissues. Our recent findings indicating that Tg binds to glycosaminoglycans (GAGs) that accumulate in orbital tissues of GO patients were somehow in contrast with Kriss’ hypothesis (5, 10). Thus, GAGs are expressed virtually by all tissues and their accumulation in GO is regarded as the consequence of a primary pathogenetic event, rather than being the cause of the syndrome (1). Therefore, in this study we searched for additional Tg binding sites, using primary cultures of orbital fibroblasts from GO patients, based on the knowledge that these cells are thought to be the primary autoimmune target of GO (1). Our results indicate that, although Tg binds to orbital fibroblasts, it binds also to skin fibroblasts, in both cases with poor specificity, suggesting that binding of Tg to fibroblasts is unlikely to be involved in the pathogenesis of GO.

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Key-words: Thyroglobulin, ophthalmopathy, thyroid, fibroblast, Graves’ disease.
Correspondence: M. Marìnò, MD, Dipartimento di Endocrinologia, Università di Pisa, Via Paradisa 2, 56124 Pisa, Italy. E-mail: m.marino@endoc.med.unipi.it
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MATERIALS AND METHODS

Materials

Human Tg was purified by ammonium sulphate precipitation and column size fractionation, as described previously (11). The source of human Tg was human thyroid tissue obtained at surgery from the thyroid glands of patients with Graves’ disease. Informed consent was obtained from patients. As reported previously (11), the Tg preparations were found to contain 660 and 330 kDa Tg and to be devoid of contaminating proteins or products of degradation (not shown).

BSA and p-nitrophenyl-phosphate were purchased from Sigma (St. Louis, MO, USA), alkaline phosphatase (ALP), conjugated streptavidin was from Vector (Burlingame, CA, USA) and Horseradish peroxidase conjugated-streptavidin was from Amersham (Freiburg, Germany). Tg and BSA were labelled with biotin using EZ-Link Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL), according to the manufacturer’s instructions.

Patients

Orbital tissue samples were collected from 7 GO patients who underwent orbital decompression in our Departments (Table 1). Informed consent was obtained by all patients. Before surgery, patients underwent a thyroid assessment, which included serum assays for Tg (Immulite 2000, Diagnostic Products Corporation, Euro/DPC, Gwynedd, UK) and anti-Tg autoantibodies (TgAb; Sorin Biomedica SpA, Saluggia, Italy).

Cell cultures

To prepare primary cultures of fibroblasts, tissue samples were minced, dispersed in Medium 199 (Sigma) containing 20% fetal bovine serum (Invitrogen Corporation, Carlsbad, CA, USA), penicillin (Sigma) and gentamicin (Sigma). Cells were kept in cell culture dishes in cell incubators at 37°C for 3-4 weeks. Then, cells were expanded up to 12-15 passages and kept in Medium 199 containing 10% fetal bovine serum and antibiotics until use.

Binding assays

Fibroblasts were cultured in 96 well plates until 80-100% confluence. Cells were incubated for 4 h at 4°C with various concentrations of biotin-labeled Tg or BSA, in 100 μl of binding buffer (Medium 199 containing 0.5% BSA). Biotin-labeled Tg was applied alone or in the presence of various concentrations of unlabeled Tg. Following incubation, cells were washed with phosphate buffered saline (PBS) and incubated for 1 h at 4°C with 0.5 M NaCl in PBS, to detach bound proteins from cell membranes. The NaCl wash was then collected, and bound biotin-labeled proteins were measured by enzyme-linked immunosorbent assay (ELISA), as reported below.

ELISA

Ninety-six well microtiter plates were coated overnight at 4°C with samples to be tested for biotin-labeled proteins and then blocked for 3 h at 4°C with 1 mg/ml of BSA. Wells were then incubated for 1 h at room temperature with ALP-conjugated streptavidin (1:4000 in PBS containing 0.05% Tween-20 and 0.5% BSA), followed by p-nitrophenyl-phosphate (Sigma). Absorbance was determined at 405 nM. The amounts of biotin-labeled proteins were estimated using standard curves obtained by coating microtiter wells with biotin-labeled Tg or BSA.

Preparation of cell extracts

Cells in culture dishes were incubated for 1 h on ice with 50 mM Tris pH 6.8, containing 10% glycerol, 2.5% SDS, 10 DTT, and 10% protease inhibitor cocktail solution (Roche Diagnostics, Mannheim, Germany). Cells were then scraped and extracts were vortexed for 30 sec, briefly sonicated and finally spun for 10 min at 14,000 x g. The pellets were discarded and the supernatants were collected.

Ligand blot binding assays

Cell extracts were subjected to SDS-PAGE and blotted onto nitrocellulose membranes. Membranes were incubated with biotin-labeled Tg (100 μg/ml) overnight at 4°C, followed by horseradish peroxidase-conjugated streptavidin (1:1,000).

RESULTS

We used primary cultures of orbital and skin fibroblasts obtained at surgery from 7 patients with GO. Individual clinical and serological information is re-

Table 1 - Clinical and serological data of the Graves’ ophthalmopathy (GO) patients from whom fibroblasts were taken. The thyroid and GO treatments indicated were performed prior to orbital decompression.

<table>
<thead>
<tr>
<th>Age (yr)</th>
<th>Gender</th>
<th>Thyroid status</th>
<th>Thyroid treatment</th>
<th>GO treatment</th>
<th>Serum Tg (ng/ml)</th>
<th>Serum TgAb (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>F</td>
<td>eu</td>
<td>Tx and then L-T₄₄</td>
<td>None</td>
<td>&lt;1</td>
<td>6</td>
</tr>
<tr>
<td>28</td>
<td>M</td>
<td>eu</td>
<td>Tx and then L-T₄₄</td>
<td>GC</td>
<td>&lt;1</td>
<td>122</td>
</tr>
<tr>
<td>27</td>
<td>F</td>
<td>eu</td>
<td>¹³¹I and then L-T₄₄</td>
<td>GC and ORT</td>
<td>&lt;1</td>
<td>5</td>
</tr>
<tr>
<td>25</td>
<td>F</td>
<td>eu</td>
<td>Tx and then L-T₄₄</td>
<td>GC</td>
<td>&lt;1</td>
<td>7</td>
</tr>
<tr>
<td>29</td>
<td>F</td>
<td>eu</td>
<td>¹³¹I and then L-T₄₄</td>
<td>GC</td>
<td>&lt;1</td>
<td>1349</td>
</tr>
<tr>
<td>49</td>
<td>F</td>
<td>eu</td>
<td>¹³¹I and then L-T₄₄</td>
<td>GC and ORT</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>30</td>
<td>M</td>
<td>eu</td>
<td>¹³¹I and then L-T₄₄</td>
<td>GC</td>
<td>13</td>
<td>308</td>
</tr>
</tbody>
</table>

F: female; M: male; L-T₄₄: levothyroxine; ¹³¹I: radioactive iodine; NR: normal range; NV: normal value; eu: euthyroidism; GC: glucocorticoids; ORT: orbital radiotherapy. Tg: thyroglobulin; TgAb: anti-Tg autoantibodies; Tx: thyroidectomy.