Significance of serum antibodies reactive with flavoprotein subunit of succinate dehydrogenase in thyroid associated orbitopathy

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Abstract

**Aims**—Thyroid associated orbitopathy (TAO) is an autoimmune disorder of extraocular muscles and orbital connective tissue. Identification of the principal target antigens would help the understanding of the pathogenesis of the disease and possibly lead to the development of specific therapies in the future. The purpose of this study was to measure serum antibodies against the flavoprotein subunit of succinate dehydrogenase in patients with TAO and correlate their presence with factors of TAO.

**Methods**—Sera of patients with active TAO of 6 months’ duration or less were tested for antibodies against the flavoprotein subunit of succinate dehydrogenase. Clinical data were obtained by retrospective review of patients’ charts. Enzyme linked immunosorbent assay was used to test sera for serum antibodies against purified succinate dehydrogenase.

**Results**—38 patients with TAO and 32 healthy age and sex matched controls were included in the study. Anti-flavoprotein antibodies were detected in 24 out of 38 patients with TAO (63.16%) and in five out of 32 healthy controls (15.63%) (p<0.01). Neither age, sex, duration of thyroid disease, thyroid status, treatment of thyroid disease, smoking history, duration of orbitopathy, activity of orbitopathy, nor the presence of lid retraction were significantly associated with the presence of serum antibody test (p>0.05). However, the total number of rectus muscles affected in both eyes of the patients was significantly correlated with the finding of a positive antibody test (p<0.05).

**Conclusions**—Serum antibodies reactive with the flavoprotein subunit of succinate dehydrogenase are associated with extraocular muscle involvement in active TAO of recent onset.

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Thyroid associated orbitopathy (TAO) is the most common cause of unilateral or bilateral exophthalmos and orbital inflammation in adults. It has an unpredictable behaviour which may lead to blindness as a result of optic nerve compression or, rarely, corneal involvement. In addition to its disfiguring and disabling effects, the disease also can have negative influences on the patient’s quality of life.

TAO and thyroid disease are thought to be closely associated but separate disorders. The course of orbitopathy seems to be independent of the type of treatment for the associated systemic thyroid dysfunction. However, some studies propose that hypothyroidism and TSH elevation after antithyroid drug therapy or radioactive iodine may lead to the development or worsening of the eye disease.

Environmental factors are also important in TAO. Smoking seems to play a role in the pathogenesis of the eye disease either by affecting the immune system or being goitrogenic. The risk of developing orbitopathy increases in smokers. In addition, smoking reduces oxygen concentration and causes fibroblast stimulation in the eye muscles.

TAO has inflammatory and post inflammatory stages. The active stage usually lasts from 6 months to 3 years. The disease may present in different forms and treatment should be individualised. Observation, corticosteroids, intravenous immunoglobulins, cyclosporine A, plasmapheresis, and orbital radiation therapy are currently used in the treatment of this disorder. However, currently available forms of therapy for TAO are not specific and not directed at the precise immunological cause. Detection of the principal target antigens would help in our understanding of the pathogenesis of the disease. This may lead to the development of specific therapies to prevent or halt progression of TAO.

The eye muscles may be the main target tissue in TAO. Lymphocytic infiltration (mainly by T cells), glycosaminoglycan accumulation, oedema, and fibrosis of eye muscles occur during the course of the disease. Although the identity of the antigens recognised by the activated T cells is not certain, there is good evidence for serum antibodies against eye muscle membrane antigens of 63–67 kDa molecular weight (MW) in TAO. Among these antigens, the 64 kDa protein is shown to be most closely associated with TAO and is the best candidate target antigen. In recent studies, the 64 kDa protein was demonstrated to have a correct molecular mass of 67 kDa and to be the flavoprotein subunit of mitochondrial succinate dehydrogenase.

The purpose of this study was to test for serum antibodies against the flavoprotein subunit of the mitochondrial enzyme succinate dehydrogenase in patients with TAO and correlate their presence with variables of TAO. For this purpose, enzyme linked
immunosorbent assay (ELISA) was used to test sera of patients with active orbitopathy of 6 months or less and healthy subjects.

Materials and methods

Of more than 1000 serum samples stored in the thyroid research laboratory, serum of all patients with active TAO of 6 months or less was studied. A retrospective review of the charts of these patients was performed to obtain clinical data. Patients with incomplete clinical information were excluded. None of the patients was on corticosteroid treatment at the time of blood collection. Our control group included hospital or laboratory staff who had no personal or family history of thyroid orbitopathy or other autoimmune disease. Informed consent was obtained from all patients and healthy subjects.

Ophthalmic examination included measurement of visual acuity, colour vision, pupillary function, evaluation of the eyelids, extraocular muscle movements, and performance of biomicroscopic and fundus examinations. Intraocular pressure was measured in primary gaze and upgaze using applanation tonometry. Goldmann peripapillary Hertel exophthalmometry was used to measure the degree of proptosis. Blood samples were collected from all patients and healthy subjects.

ENZYME LINKED IMMUNOSORBENT ASSAY

Serum autoantibodies against the flavoprotein subunit of mitochondrial succinate dehydrogenase were measured by ELISA. In preliminary assays, optimal concentrations of serum and antigen were determined by "checker board" analysis. As a result, a succinate dehydrogenase concentration of 0.125 mg/ml and a serum dilution of 1:50 were used in all subsequent studies.

Succinate dehydrogenase, diluted in coating buffer (0.5 M NaCO/NaHCO, pH 9.4) was added to 96 well plates (Immulon IV, Dynatec Laboratories) and incubated for 2 hours at 37°C or overnight at 4°C. Plates were washed three times in 1.0% bovine serum albumin fraction V and 0.06% Tween-20 in phosphate buffered saline (PBS). The unoccupied binding sites on the plates were blocked with 1.0% bovine serum albumin (BSA) in PBS for 2 hours at room temperature (RT). Diluted sera were added to the plates and incubated overnight at 4°C. Control wells contained coating buffer and BSA instead of antigen, and PBS instead of serum. Plates were washed before the addition of a second antibody, alkaline phosphatase conjugated goat anti-human IgG (1:2000), which was applied for 1 hour at RT. After the plates were washed again, p-nitrophenyl phosphate (p-NPP) substrate in diethylenamine buffer, pH 9.8, was added and the plates were incubated for 30 minutes at RT. Assays were read in a semi-automatic ELISA reader and the results expressed as optimal density (OD) at 410 nm. A positive test was defined as an OD greater than the upper limit of normal, which was determined as mean +2 standard deviations (SD) for a panel of eight normal subjects. The same positive and negative controls were included in each assay to serve as internal controls. All assays were performed in duplicate. A single batch of antigen was used for all studies. The method for purification of beef succinate dehydrogenase has been described in detail previously.

Results

The duration of the thyroid disease ranged from 1 to 144 months (mean 28 months). Eleven patients were euthyroid, 21 patients were hyperthyroid, and two patients were hypothyroid at the time of serum collection. At the time of serum collection 18 patients had been treated with radioactive iodine, four with propylthiouracil, and one patient had been treated with tapazole. Five patients received no treatment. The duration of orbitopathy ranged from 1 to 6 months (mean 3.8 months); 26 patients had lid retraction (68.4%) and 20 patients (52.63%) had significant eye muscle involvement.

Overall, anti-flavoprotein antibodies were detected in 24 of the 38 patients (63.16%) and in five of the 32 healthy controls (15.63%) (p<0.01, χ² test). Patients with TAO were further analysed according to the presence or absence of eye muscle dysfunction. Antibodies against the flavoprotein subunit of succinate dehydrogenase were detected in 15 (62.5%) patients with extraocular muscle restriction compared with nine (37.5%) of those without (p>0.05).

Age, sex, duration of thyroid disease, thyroid status, treatment of thyroid disease, smoking history, duration of ophthalmopathy, activity index of ophthalmopathy, and presence of lid retraction did not correlate with the detection of anti-flavoprotein antibodies (p>0.05). However, the total number of rectus muscles affected bilaterally did significantly correlate with a positive antibody test. The mean number of rectus muscles affected in patients with positive anti-flavoprotein antibodies was 4.8 (2.8) compared with 2.5 (3.4) in patients in whom the antibodies were not detected (p <0.05) (Table 1).
Table 1  Correlations between demographic, thyroid, and orbital variables, and serum antibodies reactive with flavoprotein subunit of succinate dehydrogenase in patients with thyroid associated orbitopathy

<table>
<thead>
<tr>
<th>Variable</th>
<th>(+) anti-Fp antibodies (n=24)</th>
<th>(-) anti-Fp antibodies (n=14)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (mean (SD))</td>
<td>51.0 (14.24)</td>
<td>50.9 (8.44)</td>
<td>&gt;0.05*</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>8/16</td>
<td>2/12</td>
<td>&gt;0.05**</td>
</tr>
<tr>
<td>Current smoking (Y/N)</td>
<td>10/12</td>
<td>7/6</td>
<td>&gt;0.05**</td>
</tr>
<tr>
<td>Thyroid disease:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duration (months) (mean (SD))</td>
<td>26.8 (37.15)</td>
<td>29.0 (43.68)</td>
<td>&gt;0.05*</td>
</tr>
<tr>
<td>Status:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hyperthyroid</td>
<td>12</td>
<td>9</td>
<td>&gt;0.05*</td>
</tr>
<tr>
<td>Hypothyroid</td>
<td>9</td>
<td>2</td>
<td>&gt;0.05*</td>
</tr>
<tr>
<td>Treatment:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I131</td>
<td>11</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Propylthiouracil</td>
<td>3</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Tapezole</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>4</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Orbitopathy:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duration (months) (mean (SD))</td>
<td>3.9 (2.03)</td>
<td>3.5 (1.74)</td>
<td>&gt;0.05*</td>
</tr>
<tr>
<td>Activity (mean (SD))</td>
<td>2.8 (0.9)</td>
<td>2.5 (0.7)</td>
<td>&gt;0.05*</td>
</tr>
<tr>
<td>Lid retraction (Y/N)</td>
<td>17/7</td>
<td>9/5</td>
<td>&gt;0.05**</td>
</tr>
<tr>
<td>†EOM (mean (SD))</td>
<td>4.8 (2.8)</td>
<td>2.5 (3.4)</td>
<td>&lt;0.05*</td>
</tr>
</tbody>
</table>

*Student’s t test; **Fisher’s exact test. †EOM = number of involved extraocular muscles.

Discussion

TAO is an autoimmune disorder of the extraocular muscles and orbital connective tissue. Although the precise mechanism for development of orbitopathy in patients with Graves’ disease is unknown, cross reactivity against a thyroid and eye muscle shared antigen, such as the recently cloned membrane protein G2s, appears to be a good candidate.17

There is considerable evidence that the eye muscles are involved in the pathogenesis, since more than 90% of Graves’ patients with or without clinical signs of orbitopathy have been demonstrated to have extraocular muscle enlargement by orbital imaging techniques.14

Antibodies against eye muscle antigens in TAO were first reported by Kodama et al.18 and later by other investigators.20 21 Among these antigens, the 64 kDa protein, shown later to have a correct molecular mass of 67 kDa and to be the flavoprotein subunit of mitochondrial succinate dehydrogenase, is the best studied target antigen.14 17 Succinate dehydrogenase is a citric acid cycle enzyme and a component of the mitochondrial respiratory chain. It consists of a flavoprotein subunit that contains the active site, FAD cofactor, and an iron-sulphur subunit, containing three non-identical iron-sulphur clusters, that catalyses oxidation reactions.17 It is bound to the matrix surface of the mitochondrial inner membrane. After the flavoprotein molecule is synthesised on cytoplasmic ribosome, it is transformed into an active enzyme in the mitochondrion. Whether antibodies directed against an intracellular antigen can penetrate the cell membrane is not certain, although there is evidence that antinuclear antibodies can enter the nucleus of live cells to bind with their target antigen.22 It is also likely that the flavoprotein molecule might be presented at the cell surface before or after its conversion into an active enzyme.17 Anti-flavoprotein antibodies may cause extraocular muscle disease either by entering the cytoplasm and binding with the antigen, or by reacting with the flavoprotein molecule at the muscle cell surface. It is more likely, however, that the antibodies are secondary to extraocul-
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