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Human orbital tissue and thyroid membranes express a 64 kDa protein which is recognized by autoantibodies in the serum of patients with thyroid-associated ophthalmopathy

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A 64 kDa protein has been identified in the membrane fraction of human eye muscle, orbital connective tissue and thyroid, by testing sera of patients with thyroid-associated ophthalmopathy in SDS-polyacrylamide gel electrophoresis and Western blotting. Antibodies to this membrane antigen seem characteristic of the early stage of ophthalmopathy. In the thyroid this newly recognized protein seems different from previously known membrane antigens. A thyroid antibody reactive with a 64 kDa membrane antigen in eye muscle could explain the very frequent association of ophthalmopathy with autoimmune thyroid disease.

Orbital antigen; Graves' disease; 64 kDa protein; Ophthalmopathy; Autoantibody

1. INTRODUCTION

Cytotoxic antibodies directed against an eye muscle cell surface antigen in antibody-dependent cell-mediated cytotoxicity (ADCC) have been identified in the serum of patients with thyroid-associated ophthalmopathy (TAO) [1]. These antibodies were demonstrated to be cross-reactive with an antigen in thyroid membranes, which was not recognized by a monoclonal antibody reactive with thyroid peroxidase (TPO) [2], now known to be the thyroid 'microsomal' antigen [3]. Here, we have investigated the nature of eye muscle (EM), orbital connective tissue (OCT) and thyroid (THY) antigens which are targets for autoantibodies in the sera of patients with TAO, using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. We were able to identify a previously unrecognized 64 kDa protein in eye muscle, orbital connective tissue and thyroid membranes against which autoantibodies, in the serum of patients with TAO but not normal subjects, react.

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2. MATERIALS AND METHODS

2.1. *Patients*

We studied 47 patients, 43 women and 4 men (19–65 years old; mean, 44 years), with thyroid-associated ophthalmopathy of whom 40 had Graves' hyperthyroidism and 7 Hashimoto's thyroiditis. The clinical severity of the eye disease was assessed using a 'clinical index' based on the classification recommended by the American Thyroid Association [4]. The disease was active in 37 patients and inactive ('burnt out') in 10. All patients had class 3 or worse disease. The eye disease was also classified according to its duration, i.e. the time, at testing, since the onset of symptoms and signs. The ophthalmopathy was assessed as (i) recent, if present for less than 12 months; (ii) 'chronic', if present for 1–3 years; or (iii) burnt-out, if present for more than 3 years, and inactive. We also studied (i) 16 patients, 9 women and 7 men (20–67 years old; mean, 35 years), with Graves' hyperthyroidism (GH); (ii) two patients, one 45-year-old women and one 36-year-old man, with Hashimoto's thyroiditis (HT), neither of whom had clinically evident eye involvement; and (iii) 12 normal subjects, 9 women and 3 men (18–67 years dd; mean, 38 years), as controls.

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2.2. *Antigen preparation*

Normal human EM and OCT were obtained from 3 subjects at autopsy less than 5 h after death. Normal human THY tissue was obtained at thyroidectomy from a patient undergoing surgery for removal of a nontoxic nodular goiter. Skeletal muscle (SKM) and skin connective tissue (SKCT) specimens were also obtained at autopsy as control antigens. Membrane fractions were prepared from homogenates by centrifugation at 800 × *g* for 15 min, to remove debris, then centrifuging the pellet at 10000 × *g* for 60 min, or at 100000 × *g* for 60 min, to obtain the cell membrane fraction and microsomes, respectively.

2.3. *SDS-PAGE and Western blotting*

The polyacrylamide gel used was 10% running gel and 5% stacking gel. Antigen preparations were applied at a concentration of 300 μg protein in 100-μl aliquots per well. In each experiment molecular mass standards (Rainbow Mix, Amersham, Arlington Heights, IL) were included. The gel was run at 7 mA overnight, then transferred onto nitrocellulose paper at 45 V for 4.5 h in transfer buffer (0.02 mol/l Trizma base, 0.2 mol/l glycine in 20% methanol-80% distilled water; pH 8.3). One strip was stained with amido black and destained with 5% methanol, 7% acetic acid in distilled water to show antigen bands. The strips were incubated in 2.5% BSA or 2% gelatin in phosphate-buffered saline (PBS), pH 7.4, at room temperature for 60 min, washed in Trizma-buffered saline (TBS: 0.2 mmol/l Tris-HCl, 0.9% NaCl; pH 7.4) for 30 min then incubated with patient or normal sera, diluted 1:50 in 0.1% BSA-PBS, for 3 h at room temperature. PBS was used instead of serum for control strips. The paper was then washed with TBS and incubated with a horseradish peroxidase-conjugated anti-human IgG (H + L), diluted 1:1500 in 0.1% BSA-PBS, for 2 h at room temperature. Strips were again washed with TBS and developed with 3,3′-diaminobenzidine tetrahydrochloride dihydrate 97% (DAB) (Aldrich, Milwaukee, WI) at 0.5 mg/ml concentration in PBS. Finally, strips were washed with distilled water for 5 min to remove excess DAB and dried on absorbing paper.

2.4. *Cell culture and antibody-dependent cell-mediated cytotoxicity*

These methods have been detailed previously [1]. Briefly, fresh normal human eye muscle, obtained at surgery from children undergoing strabismus repair, was cultured and grown as cell monolayers. A standard antibody-dependent cell-mediated cytotoxicity (ADCC) assay, incorporating ^{51}Cr-labelled eye muscle cells, unfractionated peripheral blood lymphocytes at a 25:1 effector/target cell ratio as effector cells, and heat-inactivated patient's serum at a dilution of 1:20, was used to test cytotoxicity. Results were expressed as % specific lysis, calculated from: cpm test − cpm spontaneous/cpm total − cpm spontaneous × 100 (minus natural killer cell lysis).

2.5. *Antimicrosomal and antithyroglobulin antibody assays*

Antimicrosomal and antithyroglobulin antibodies were tested for by the passive hemagglutination assay using commercial kits (Wellcome, Beckenham, England).

2.6. *Statistical analysis*

Two-variable χ^2 analysis was used to test the significance of the difference in frequencies of a 64 kDa band between patient and control groups.

3. RESULTS

SDS-PAGE and Western blotting analysis of normal and patient sera was performed using various thyroid and orbital tissue membranes. A prominent band in one or more of thyroid, eye muscle and orbital connective tissue membranes, at approx. 64 kDa (figs 1,2), was seen with the serum of 26 out of 47 patients with TAO, but with that of only three of 15 patients with Graves' hyperthyroidism without apparent eye involvement (χ^2 test, $P < 0.02$) and with none of the 12 normal subjects tested as controls (χ^2 test, $P < 0.01$) (table 1). The band was present in eye muscle membranes only, in tests with serum from 10 patients, in orbital connective tissue membranes only, with serum from one patient, and associated with a similar band in two or more of eye muscle, orbital connective tissue and thyroid in with serum from 14 patients (54%). Sera from 8 patients also recognized a 64 kDa protein in skeletal muscle and in six of them there was a reaction with a 64 kDa protein in eye muscle. In the case of two patients with TAO whose serum gave a 64 kDa band in skin connective tissue, a corresponding band was also recognized in eye muscle, thyroid and skeletal muscle in both cases. Other less prominent bands at 50,

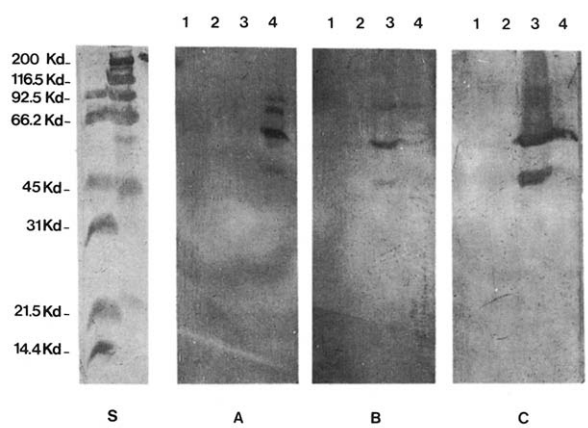

Fig.1. Reactivity with a 64 kDa protein of serum from patients with thyroid-associated ophthalmopathy, determined by SDS-PAGE and Western blotting. Lanes: 1, 100000 × *g* preparation of human thyroid membranes; 2, 10000 × *g* preparation of thyroid membranes; 3, 100000 × *g* preparation of orbital connective tissue membranes; 4, 100000 × *g* preparation of human eye muscle membranes. S, molecular mass standards. A–C show reactivity with sera from three patients with ophthalmopathy.

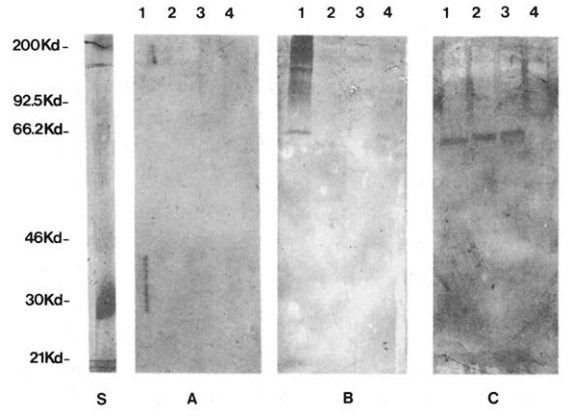

Fig.2. As in fig.1 except that (lanes) 1, eye muscle membranes; 2, orbital connective tissue membranes; 3, thyroid membranes (all 100000 × *g* preparations) and A–C show reactivity with sera from a normal subject and two other patients with thyroid-associated ophthalmopathy, respectively.

58 and 85 kDa, were also occasionally found in tests with sera from patients with autoimmune thyroid disorders with or without ophthalmopathy.

Reactivity with a 64 kDa thyroid membrane protein was demonstrated with sera from 9 patients with TAO. In all cases the band was also present in one or both orbital tissue preparations. As can be seen in table 2, in these patients the finding of a 64 kDa band was not related to the detection or titers of antimicrosomal or antithyroglobulin antibodies. A band at 105 kDa in thyroid membranes, corresponding to the microsomal (TPO) antigen, was detected in tests with serum from three patients with TAO.

Sera from 31 of the patients with TAO were also tested, in ADCC assay, for cytotoxic antibodies against eye muscle cells. Although 12 of the 19 sera giving a positive ADCC test were shown to recognize the 64 kDa protein in eye muscle mem-

Table 1

Prevalence of serum reactivity with a 64 kDa protein in human thyroid and orbital tissue membranes, as shown by SDS-PAGE and Western blotting, in patients with autoimmune thyroid disorders and ophthalmopathy

Tissue	Group			
	GO (n = 47)	GH (n = 15)	HT (n = 2)	NOR (n = 12)
EM	24(10)	3(2)	0	0
OCT	7(1)	1(0)	0	0
THY	9(0)	0	0	0
	[26]	[3] χ^2 test, GH vs GO $P < 0.02$	[0]	[0] χ^2 test, NOR vs GO $P < 0.01$

EM, eye muscle; OCT, orbital connective tissue; THY, thyroid; GO, Graves' ophthalmopathy; GH, Graves' hyperthyroidism; HT, Hashimoto's thyroiditis; NOR, normal subjects. ( ), number of patients in whom the 64 kDa band was demonstrated only in that tissue; is shown in parentheses. [ ], total number of patients in whom the 64 kDa band was demonstrated in at least one tissue

Table 2

Reactivity with a 64 kDa protein in thyroid, human eye muscle, and orbital connective tissue membranes, as shown by SDS-PAGE electrophoresis and Western blotting, and thyroid antibody titers, with serum from patients with thyroid-associated ophthalmopathy

Patient	Membrane preparation			Thyroid antibody titers[a]	
	THY	EM	OCT	AMA	ATA
CH	+[b]	+	+	1:25600	neg
SP	+	+	+	neg	neg
JT	+	+	−	1:100	neg
ML	+	+	−	1:100000	neg
LS	+	+	−	1:100	neg
GR	+	+	−	neg	neg
RV	+	+	−	1:1600	neg

[a] Antimicrosomal antibody titer; antithyroglobulin antibody titer

[b] A 64 kDa band was shown in SDS-PAGE and Western blotting with the patient's serum

Table 3

Prevalence of reactivity with a 64 kDa protein in human eye muscle membranes, as shown by SDS-PAGE and Western blotting, in relation to the duration of eye disease, with the serum of patients with thyroid-associated ophthalmopathy

Duration of ophthalmopathy	Number of patients showing reactivity with a 64 kDa protein[a]
⩽1 year (n = 37)	24 (65%)
>1 year (n = 10)	3 (30%) χ^2 test, $P < 0.05$

[a] Determined from SDS-PAGE and Western blotting

branes, 8 of the 12 ADCC negative sera also reacted with this protein, and the difference was not significant (χ^2 test, P = NS).

Finally, the prevalence of detectable reactivity with a 64 kDa protein, as determined from SDS-PAGE and Western blotting, was correlated with the duration of the ophthalmopathy. The results are summarized in table 3. The band was found in a significantly higher proportion with patients' sera obtained during the first 12 months of the eye disease (24 out of 37) compared to that with sera obtained more than 1 year after the onset (3 of 10, χ^2 tests, $P < 0.05$).

4. DISCUSSION

In this study, using SDS-PAGE and Western blotting, we have confirmed the existence of autoantibodies in the serum of patients with thyroid-associated ophthalmopathy which are directed against membrane antigens in human eye muscle, orbital connective tissue and the thyroid. Among these antigens, a 64 kDa protein present in the membrane fraction of orbital tissue, and also expressed in the thyroid, appears of major relevance to the pathogenesis of TAO. Recently, Ahmann et al. [5] reported the presence of heterogeneous and non-specific antigen determinants of 50, 64 and 73 kDa in porcine eye muscle membranes as determined by reactivity, in SDS-PAGE, with antibodies in the serum of patients with Graves' disease, with or without eye disease, but also of normal controls. Bahn et al. [6] identified a 23 kDa antigen in orbital connective tissue when the serum of TAO patients was tested in SDS-PAGE incorporating orbital fibroblasts. Interestingly, autoantibodies to a 64 kDa pancreatic islet cell protein have also been reported in newly diagnosed diabetic children [7] although the authors were not able to determine whether this was an intracellular or a cell surface component. In the present study the detection of antibodies reactive with a 64 kDa membrane protein was significantly associated with the presence of ophthalmopathy, in particular when the disease was of recent onset and active.

The existence of antibodies reactive with a 64 kDa antigen in two or more of thyroid, eye muscle and orbital connective tissue membranes, in 50% of TAO sera tested, suggests that the same protein is present in all three tissues and (perhaps also in skin, other skeletal muscle and pancreas) and that the antibodies may be cross-reactive. Failure to demonstrate a band, at 64 kDa, in all three tissues with all sera reacting with at least one membrane preparation tested, may be due to the variable expression of the antigen on the different membrane preparations used. Indeed, in the present study, positive sera occasionally gave negative results when tested with membranes prepared from a different normal subject. Absorption experiments, currently in progress, are needed to confirm the presence of cross-reactive antibodies [8] and to determine the full tissue distribution of the 64 kDa protein.

We have also confirmed the existence of a previously unrecognized thyroid-directed antibody, reacting with a 64 kDa membrane antigen which seems to be different from other known thyroid membrane antigens such as TPO [3] and the TSH receptor [9]. A cytotoxic antibody, reactive with a cell surface antigen expressed on both eye muscle and thyroid cells, has been recently described by our group [2]. Furthermore Hari et al. [10] produced a cytotoxic monoclonal antibody, reactive with a 64 kDa pancreatic islet cell surface protein, from lymphocytes of non-obese diabetic mice, leading them to postulate an ADCC mechanism in the pathogenesis of type I diabetes. In our study the presence of 64 kDa protein-reactive antibodies in the serum of patients with TAO did not correlate with positive ADCC activity against eye muscle cells. In order to determine the clinical and pathogenetic significance of antibodies reactive with the 64 kDa protein we are testing, in a prospective study, ADCC and complement-mediated killing of eye muscle and thyroid cells, and performing SDS-PAGE with membrane preparations of these tissues, on the sera of newly diagnosed patients with Graves' hyperthyroidism, some of whom are expected to develop ophthalmopathy during the course of the study. The existence of a cytotoxic thyroid antibody directed against a cell wall derived 64 kDa protein which is also expressed on eye muscle cells, and perhaps orbital fibroblasts, would certainly offer a plausible explanation for the very close association of ophthalmopathy with autoimmune thyroid disorders [8].

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