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Role of the Eye Muscles in Thyroid Eye Disease: Identification of the Principal Autoantigens

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ABSTRACT

Thyroid-associated ophthalmopathy (TAO) is a progressive orbital disorder associated with Graves' hyperthyroidism and, less often, Hashimoto's thyroiditis in which autoantibodies react with orbital antigens and lead to exophthalmos and eye muscle inflammation. Eye muscle (EM) membrane proteins initially reported as 55 and 64 kd are the best markers of ophthalmopathy. The "64-kd protein" is now shown to be the flavoprotein subunit of mitochondrial succinate dehydrogenase and to have a correct molecular weight of 67 kd. We have cloned a fragment of a novel eye muscle protein, which we call G2s, and sequenced 1.4 kb of the full length cDNA. G2s does not share any significant homologies with other reported proteins. The 5.9 kb G2s mRNA, that corresponds to a protein of approximately 220 kd, is expressed in EM, other skeletal muscle and thyroid, but not in other tissues tested. We have also cloned and sequenced a 63-kd eye muscle protein identified as the calcium binding protein calsequestrin. Antibodies against calsequestrin were found in 40% of patients with active ophthalmopathy, but in 0% of normal subjects. Finally, we have sequenced a 19 amino acid fragment of a 55-kd porcine eye muscle membrane protein that exactly matched porcine and human sarcalumenin, a 160-kd glycoprotein localized in the lumen of the longitudinal sarcoplasmic reticulum of the skeletal muscle fiber where it binds calcium. A 53-kd glycoprotein fragment of the molecule corresponds to the 55-kd protein. In a preliminary study, serum antibodies against purified sarcalumenin were detected in 40% of patients with active TAO of less than 1 year duration, but in no controls tested. We porpose that the primary autoantigen in TAO is G2s, which would also explain the association of ophthalmopathy with thyroid autoimmunity, and that antibodies against the intracellular proteins flavoprotein, calsequestrin, and sarcalumenin are secondary markers of an immune-mediated reaction in eye muscle in patients with thyroid autoimmunity.

INTRODUCTION

THYROID-ASSOCIATED OPHTHALMOPATHY (TAO) is an autoimmune disorder of the eye muscle and the surrounding orbital connective tissue and fat (1–3), although the identity and nature of the principal target antigens and the mechanism for the close link of the ophthalmopathy to thyroid disease remain unclear and controversial. The limited access to orbital tissues from patients with early eye disease, and lack of an animal model, have slowed progress in gaining an understanding of the basic immune abnormalities in ophthalmopathy. Despite this there is considerable evidence for eye muscle (EM) inflammation in TAO and serum autoantibodies reactive with one or more eye muscle antigens are detected in many patients with TAO (4–7). This review focuses on the nature of the principal EM antigens and the significance of the corresponding serum autoantibodies and concludes with a hypothesis about this complex orbital and eyelid disorder.

Clinical findings

Many of the main eye signs and symptoms, in particular blurred vision on binocular gaze, diplopia and a pulling sensation on upward gaze, result from involvement of the EM (1). These findings correlate with enlargement of the

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EM on orbital imaging in the patients with TAO or Graves' hyperthyroidism (8,9). Since the "eye muscle" comprises about 30% by volume of orbital connective tissue this swelling could result from inflammation of the EM, the orbital connective tissue, or both. In our opinion there is strong evidence for an immunologically-mediated reaction directed against the EM fiber, although orbital connective tissue inflammation, which we believe to be secondary, also contributes to both the swelling within the orbit and the clinical features.

Histology

A mixed mononuclear cell infiltration, an important marker of the autoimmune reaction, is found within the EM bundles and surrounding the individual fibers. The infiltrating mononuclear cells comprise T lymphocytes and a few B cells, antibody secreting plasma cells, macrophages and, occasionally, mast cells. The T lymphocytes are mainly activated/memory cells belonging to both $CD4^+$ and $CD8^+$ subsets (10). There is also enlargement of the fibroblasts, which display secretory hyperactivity, edema, and glycosaminoglycans accumulation and, in the later stages, increased collagen production and fibrosis (11,12).

EM damage in TAO

Because of the poor availability of surgical and biopsy material it is difficult to obtain evidence for EM inflammation and damage in the early stages of TAO. Indeed, since the first abnormality in patients with Graves' hyperthyroidism without clinically evident eye disease may be EM enlargement on orbital imaging, it may be impossible to confirm this in humans. From electron microscopic study of orbital tissue from patients with severe, but fairly recent onset of ophthalmopathy requiring orbital decompression, we observed features suggestive of early damage to the EM fibers, in the absence of orbital connective tissue inflammation or collagen overproduction (13). In some cases the mitochondria were larger than normal with clear, ballooned spaces between their cristae. In other cases, the intercristal spaces were widened. We also observed an increased number of subsarcolemmal inclusions in all muscle specimens from patients with TAO (13).

EM antigens and corresponding serum autoantibodies

The main goal of our recent studies was to purify the principal antigens from porcine EM membranes and to clone them from an EM cDNA expression library using specific oligonucleotides or antibodies as probes. We cloned a novel thyroid and eye muscle protein called G2s (14) and calsequestrin, a 63-kd calcium binding muscle protein (15), and sequenced fragments of the "64-kd EM protein," which is identified as the flavoprotein (Fp) subunit of succinate dehydrogenase (16), and the "55-kd protein," identified as sarcalumenin, a 53-kd calcium binding glycoprotein localized in the sarcoplasmic reticulum of the eye muscle fiber (Gunji et al., unpublished data). We have extensively studied another 63 to 64-kd protein, called 1D, which was cloned by Dong et al. (17) from a thyroid cDNA expression library. We determined the prevalences of the corresponding serum autoantibodies in patients with TAO and thyroid autoimmunity. The main characteristics of these antigens are summarized in Table 1.

1D. A 98-amino acid fragment of 1D, called D1, was obtained by screening a thyroid cDNA library with sera from patients with Hashimoto's thyroiditis (19). Subsequently, full-length 1D was isolated and the encoded protein expressed in Chinese hamster ovary (CHO) cells. In extensive studies we showed that 1D was expressed in EM, other skeletal muscle, thyroid and testis, and in some other tissues (18,19). Serum antibodies against 1D in CHO cell membrane were detected in 52% of patients with TAO, 61% with Graves' hyperthyroidism and in 13% of normals (20). Recently, Schupert et al. (21) have demonstrated antibodies against full length 1D in 86% of patients with TAO, 10% of patients with Graves' hyperthyroidism without ophthalmopathy, and in 10% of normal subjects.

Succinate dehydrogenase. The "64-kd protein" was purified and partially sequenced and identified as the FP subunit of succinate dehydrogenase (16). Succinate dehydrogenase is a flavoenzyme consisting of a flavoprotein subunit that contains the active site and FAD cofactor of the enzyme and an iron-sulfur subunit containing three nonidentical iron-sulfur clusters, that catalyzes oxidation/reduction reactions (22,23). Its specific action is to oxidize succinate to fumarate in the mitochondrial matrix and pass the electrons directly into the ubiquinone pool of the respiratory chain. Fp has a corrected molecular weight of 67 kd as determined by immunoblotting. Serum autoantibodies reactive with purified succinate dehydrogenase were detected in 67% of patients with active TAO, 30% with more chronic, stable disease, and in 30% of patients with Graves' hyperthyroidism without ophthalmopathy, but in only 7% of normal subjects (16).

TABLE 1. CHARACTERISTICS OF THE PRINCIPAL EYE MUSCLE AUTOANTIGENS AND PREVALENCES OF CORRESPONDING SERUM AUTOANTIBODIES

		Tissue expression				*Serum antibodies*		
Antigen	*Mol wt*	*EM*	*Thyroid*	*SSM*	*Function*	*TAO*	*GH*	*Normals*
Calsequestrin	63 kd	++	−	++	Calcium binding	40%*	NT	0%
Flavoprotein	67 kd	++	?	++	Energy production	67%	30%	8%
G2s	~220 kd	++	++	+	?	66%	36%	16%
Sarcalumenin	53 kd	++	−	++	Calcium binding	40%	NT	0%
1D	63–64 kd	++	++	+	?	52%	61%	13%

EM, eye muscle; SSM, systemic skeletal muscle; TAO, thyroid-associated ophthalmopathy; GH, Graves' hyperthyroidism.
*% positive in ELISA (Fp, calsequestrin) or Western blotting (G2s, sarcalumenin, 1D).

G2s. We have cloned a fragment of a novel eye muscle protein—which we call G2s—from an eye muscle cDNA expression library, using an antibody reactive with a 55-kd protein, and sequenced 1.4 kb of the full-length cDNA. G2s does not share any significant homologies with other proteins entered into gene data banks. From Northern blotting, the parent molecule was shown to have an estimated size of 5.9 kb, which corresponds to a protein of approximately 220 kd, and to be expressed in EM, other skeletal muscle and thyroid, but not in other tissues tested. G2s appears to be a key autoantigen in TAO, for the following reasons: (1) it is expressed in both EM and thyroid, where it could be a shared target of the primary autoimmune reactions of Graves' disease (14, and Gunji et al., manuscript submitted); (2) as predicted from protein database searches, G2s should be localized in the cell membrane protein where it would be seen by cytotoxic antibodies and T cells; and (3) the corresponding serum antibodies correlate with the presence of EM disease clinically, in a preliminary study (Gunji et al. manuscript submitted). G2s and sarcalumenin, the "55-kd protein," do not share sequence homologies but are presumed to share an antibody epitope.

Sarcalumenin. The "55-kd protein" was purified from porcine EM membrane and sequenced, in the same way as the 64-kd protein. This antigen is identified as sarcalumenin, a 160-kd calcium binding glycoprotein localized in the lumen of the longitudinal sarcoplasmic reticulum of the skeletal muscle fiber (Gunji et al., unpublished data). The amino acid sequence of the 160-kd protein is identical to that of a 53-kd glycoprotein except that it contains an in-frame insertion of 436 amino acids near its 3′ end, apparently resulting from alternative splicing. Both proteins are involved in calcium transport. In a preliminary study, antibodies against purified sarcalumenin were detected in sera from 4 out of 10 patients with active TAO of less than 1 year duration, but in 0 of 10 age- and sex-matched normal subjects, by immunoblotting (Gunji et al., unpublished data).

Cytotoxic antibodies against EM in TAO

Antibodies that play a role in autoimmune-mediated tissue damage are typically cytotoxic to target cells, expressing antigen at or near the cell surface, either in association with killer cells in antibody-dependent cell-mediated cytotoxicity (ADCC) or through a complement-mediated cytotoxic mechanism. We found cytotoxic antibodies against human EM cells, measured in an ADCC assay, in serum from 50% to 60% of patients with TAO and levels of cytotoxicity, expressed as % specific lysis, correlated with the clinical status, assessed as intraocular pressure and ATA class (24). The greatest prevalence of cytotoxic antibodies, 75%, was found in patients with "euthyroid ophthalmopathy," ie, ophthalmopathy associated with subclinical thyroiditis, from an iodine deficiency area (25).

Using a new nonradioactive assay for measuring cell lysis, we confirmed cytotoxicity against cultured EM cells in the majority of patients with TAO, and showed that positive reactivity against the orbital fibroblasts was a rare finding (26). It is not known whether antibodies reactive with any of the EM antigens so far identified are cytotoxic to human EM cells *in vivo*.

Autoimmunity against Muller's muscle and levator palpebrae in patients with eyelid disease

Eyelid disease, manifesting as stable lid lag and retraction, is very common in patients with ophthalmopathy and thyroid autoimmunity (1) and associated with functional and cosmetic disabilities that sometimes require surgical correction. Although most often found in patients with active TAO, lid abnormalities may be an isolated finding in patients with Graves' hyperthyroidism or Hashimoto's thyroiditis without active ophthalmopathy (27). Muller's muscle from patients with TAO and eyelid disease is sometimes infiltrated with lymphocytes and other mononuclear cells (28), although the nature and consequences of the reaction are poorly understood. We tested for serum antibodies reactive against Muller's muscle membrane antigens by immunoblotting. Antibodies targeting a 67-kd protein were detected in 30% of patients with lid lad and, overall, one or more antibodies reactive with proteins of 50 to 110 kd molecular weight were detected in 75% of patients with lid lag (29). We also tested for cytotoxic antibodies against Muller's muscle cell targets in ADCC. Tests were positive in 50% of patients with eyelid lag, with EM cells, in 44% with Muller's muscle cells and in 31% with (abdominal) skeletal muscle cells (29).

Hypothesis

Our working hypothesis for the pathogenesis of ophthalmopathy and its association with thyroid autoimmunity is that, in the context of thyroid inflammation, T lymphocytes and antibodies reactive with a thyroid and eye muscle shared antigen(s), of which the membrane protein G2s is a good candidate, cause myositis and damage to the eye muscle fiber, manifesting as increased muscle volume on orbital imaging and double vision. In the course of eye muscle inflammation and fiber necrosis, intracellular proteins including succinate dehydrogenase, sarcalumenin and calsequestrin, and possibly 1D, are released, and recognized by autoantibodies. Of these, antibodies reactive against Fp are the best markers of an immune-mediated EM fiber damage in patients with Graves' hyperthyroidism. We additionally postulate that immune attack against unknown antigens in Muller's muscle and levator palpebrae causes eyelid lag and retraction in patients with thyroid autoimmunity and ophthalmopathy and that targeting of succinate dehydrogenase is a marker of this reaction. Finally, we propose that "TAO" comprises at least two disorders namely, *ocular myopathy* in which eye muscle damage, in association with antibodies against Fp and G2s, occurs in the absence of connective tissue inflammation, and *congestive ophthalmopathy*, which reflects an autoimmune reaction in the orbital connective tissue and fat, but not eye muscle, and low prevalences of serum eye muscle antibodies. Some patients may have a mixture of these two processes.

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