

Original Research Article

Reactivity against the flavine adenosine nucleotide (FAD) cofactor of bacterial enzymes may explain positive flavoprotein antibody tests in patients without ophthalmopathy

Short title: Mitochondrial antibodies in TAO

Key words; succinate dehydrogenase, dimethylglycine dehydrogenase, sarcosine dehydrogenase, FAD, Graves' disease, thyroid-associated ophthalmopathy, Hashimoto thyroiditis, eye muscle, autoimmunity

Abstract

Background: We have re-visited a possible role of autoantibodies targeting a "64 kDa muscle membrane protein" in the pathogenesis of thyroid-associated ophthalmopathy (TAO). This antigen was identified as the flavoprotein (Fp) subunit of mitochondrial succinate dehydrogenase (SDH). We have addressed the possibility that "molecular mimicry" reactions against SDH can be explained by cross reactivity against the FAD cofactor used by this and other mitochondrial enzymes including bacterial sarcosine dehydrogenase (SarcDH).

Methods: Serum antibodies against SDH, SarcDH, dimethylglycine dehydrogenase (DMGDH) and FAD tested, in patients with TAO, Graves' Hyperthyroidism (GH) including 11 patients who developed ophthalmopathy following treatment of their hyperthyroidism, and control subjects, by enzyme-linked immunosorbent assay (ELISA) in a prospective study.

Results: Sera from 85% of patients with active TAO and 37% with eye muscle involvement contained antibodies which reacted with SDH only, compared to 37% with Hashimoto's thyroiditis, 29% with type 1 diabetes, 28% with GH and in 32% of

normal subjects (Table2). Conversely, positive responses against SDH could be explained by reaction against the FAD cofactor in over 56% of subjects without ophthalmopathy, but in only 32% of patients with active eye muscle inflammation. Anti-FAD antibodies were detected in all 11 patients (78%) in which 6 patients with GH who developed ocular myopathy after beginning anti-thyroid drug therapy and in 5 out of the 6 who developed congestive ophthalmopathy.

Discussion: Because of the wide distribution of mitochondrial enzymes in nature there are likely to be many opportunities for developing cross reactive antibodies (“molecular mimicry”) reaction in tests for anti-Fp antibodies, especially in patients with organ specific or multisystem autoimmunity.

Conclusion: Because antibodies targeting mitochondrial enzymes, Fp and FAD are unlikely to be pathogenic, as the antigens are intracellular. Future studies should focus on either the role as a marker of ophthalmopathy in patients with Graves’ hyperthyroidism.

INTRODUCTION

Although the identities of the principal target antigens and the mechanism underlying the close association of the ophthalmopathy with thyroid autoimmunity are still unclear¹⁻³, there is considerable evidence for eye muscle inflammation as serum autoantibodies against one or more eye muscle antigens are detected in the great majority of patients with thyroid-associated ophthalmopathy (TAO)⁴⁻⁶. Eye muscle membrane proteins of 63-67 kDa mol. wt. (MW) are the best markers of ophthalmopathy in patients with thyroid autoimmunity⁶⁻⁸. Of these, the flavoprotein (Fp) subunit of mitochondrial succinate dehydrogenase (SDH)⁷⁻⁹ and the skeletal muscle protein calsequestrin^{3,5,6} are most closely associated with active eye muscle inflammation. SDH comprises two Fp subunits, two iron/sulphur subunits and the covalently bound cofactor flavine adenosine dinucleotide (FAD)^{10,11}. Although serum antibodies against Fp in purified beef heart SDH are closely associated with eye muscle disease in patients with Graves' hyperthyroidism, and predict its development following treatment with anti-thyroid drugs, the antibodies are also detected in about 20% of patients with Hashimoto thyroiditis and type 1 diabetes mellitus and in 10-20% of healthy, apparently normal, subjects⁷⁻⁹. The explanation for such presumed "false positive" reactions is not known, but may reflect cross reactivity with bacterial enzymes or physiologic antibody formation secondary to muscle or liver cell damage. Indeed, Otto *et al.*¹² found antibodies against the mitochondrial matrix enzymes dimethylglycine dehydrogenase (DMGDH) and sarcosine dehydrogenase (SarcDH) in sera from patients with dilated cardiopathy and myocarditis and showed that the reactive site was the FAD cofactor¹³. We therefore tested sera from patients with thyroid

autoimmunity with and without ophthalmopathy, and control subjects, for antibodies against SDH, DMGDH, SarcDH and FAD.

CLINICAL SUBJECTS AND METHODS

Clinical subjects

Patients' demography and clinical details are shown in table 1. We studied;

(i) 47 patients with ophthalmopathy associated with Graves hyperthyroidism, 32 females and 15 males aged 28-77 (mean age 50 yr), none of whom were being treated with corticosteroids at the time of study, of whom 20 had active ophthalmopathy with eye muscle involvement of < 12 months duration and 27 stable disease of > 12 months duration (ii) 26 patients with Graves hyperthyroidism without ophthalmopathy or orbital ultrasound evidence for eye muscle enlargement, 22 females and 4 males aged 24-65 (mean age 43 yr), of whom 14 were studied prospectively; in these latter patient's blood was drawn initially then at approx. 3 monthly intervals for up to 24 months (iii) 30 patients with Hashimoto thyroiditis without ophthalmopathy, 25 females and 5 males aged 22 - 66 (mean age 43 yr) (iv) 14 patients with type I diabetes mellitus, 7 females and 7 males, aged 14-66 (mean age 27 yr) and (v) 37 healthy subjects, 25 females and 12 males aged 22 - 53 (mean age 39 yr) with no personal or family history of thyroid disease, ophthalmopathy or other autoimmune disease, as controls. The diagnoses of Graves' hyperthyroidism, Hashimoto thyroiditis and type 1 diabetes mellitus were made according to standard clinical criteria and confirmed by appropriate laboratory testing. Informed written consent was obtained from all patients and normal subjects studied.

Isolation of purified beef succinate dehydrogenase

Succinate dehydrogenase was solubilized by perchlorate treatment of succinate coenzyme Q oxidoreductase (Complex II of the respiratory chain), which had been isolated from beef heart mitochondria as described previously^{7,8}.

Isolation of purified DMGDH and sarcDH

SarcDH, prepared from rat liver mitochondria as described previously¹¹, was diluted in -10 mM KP04 buffer, pH 7, containing 100 mM KCl and 1mM azide. DMGDH, prepared from rat liver mitochondria as described previously^{7,8}, was diluted in 10 mM HEPES, pH 7, containing 100 mM KCl and 1 mM azide. On gels, the enzymes were shown to be > 90% pure. FAD was purchased from Sigma (St. Louis, MO).

Enzyme-linked immunosorbent assay

The method has been described in previous publications from this laboratory^{5,6}. Tests were performed in triplicate, in 96 well plates. The optimal concentration of purified SDH, DMGDH and SarcDH, and FAD, was found, in preliminary "Checker board" assays; to be 1 pg/ml for each enzyme and cofactor and optimal serum dilution was 1/50. The second antibody was an alkaline phosphatase labeled goat anti-human IgG (or IgG1, IgG2, IgG3 or IgG4 in the case of IgG subclasses) diluted 1/1500. As control, we used phosphate buffered saline (PBS) instead of antigen, serum or secondary antibody. Results were expressed as optical density (OD) at 410 nM and a positive test taken as an $OD > \text{mean} + 2SD$ for a panel of 8 normal subjects.

Statistical analysis

Prevalences of serum autoantibodies against mitochondrial enzymes and FAD were analyzed statistically using χ^2 tests with Yeats correction for small numbers. For all tests a P value of < 0.05 was considered to be significant.

RESULTS

Firstly, we tested for antibodies against the mitochondrial enzymes SDH, DMGDH and SarcDH, and FAD, a cofactor used by the 3 enzymes, in serum from patients with TAO, GH without ophthalmopathy, Hashimoto thyroiditis, type I diabetes mellitus and normal subjects, in ELISA. We found a wide range of reactivity against mitochondrial enzymes and FAD in all study groups (Table 2). Antibodies reactive against all 3 enzymes and FAD were demonstrated in 10% of patients with active TAO and eye muscle disease, 22% with stable ophthalmopathy, 33% with Hashimoto's thyroiditis, 29% with type 1 diabetes, in 78% of patients with GH and in 32% of normal (Table.2) (χ^2 test, $P < 0.001$ vs. normal for SDH group and $P =$ Not Significant vs. normal for all other groups). In patients with active TAO and eye muscle involvement demonstrating positive reactivity to one or more enzymes, or FAD, reactivity was against only one enzyme (SDH, 11 patients) (Table 3) in 65% of cases ($P < 0.001$ vs. normal), compared to 50% in patients with stable TAO (SDH, 6 patients; $P =$ Not Significant), 33% in Hashimoto's thyroiditis (SDH, 6 patients), 40% in GH (SDH, two patients), 14% in patients with type 1 diabetes (SDH, one patient; $P =$ Not Significant) and in 31% of normal subjects (SDH, 4 subjects), (Table 3).

Considering reactivity against SDH, sera from 85% of patients with active TAO contained antibodies which reacted with SDH only, compared to 37% with Hashimoto

thyroiditis, 29% with type 1 diabetes, 28% with GH and 32% of normal subjects (Table2). These differences were significant, compared to normal, only for patients with active TAO (χ^2 test, $P < 0.001$) Conversely, positive responses against SDH in subjects without ophthalmopathy could be explained by reaction against the FAD cofactor (rather than Fp) in over 50% of cases, but in only 10% of patients with active ophthalmopathy. No serum contained antibodies against DMGDH only.

Next, we tested for antibodies against FAD and SarcDH in serum samples taken over a period of up to 24 months from 12 patients with newly diagnosed Graves' hyperthyroidism that developed ophthalmopathy following treatment with anti-thyroid drugs. These sera had been previously tested for anti-Fp antibodies. Six of the 12 patients developed ocular myopathy, defined as; diplopia, eye muscle dysfunction and marked eye muscle volume increase as observed by orbital ultrasound, with or without congestive changes, from 1 ½ - 6 months after beginning treatment and 6 developed severe congestive ophthalmopathy, but no eye muscle dysfunction and no or only minimal eye muscle volume increase on orbital ultrasound, after 4 1/2 - 10 months. All sera were tested in a single assay at the end of the study.

Anti-FAD antibodies were detected in all 6 patients who developed eye muscle dysfunction during treatment with anti-thyroid drugs, which was before the onset of diplopia and eye muscle dysfunction in all 6. Anti-FAD antibodies were also detected in 5 out of the 6 patients who developed connective tissue inflammation but no eye muscle disease, and predicted the ophthalmopathy in 3 of them. There was no dose correlation with anti-Fp antibodies in these patients (Fig 1A and 1B).

We also measured anti-SarcDH antibodies in some of the patients; levels were generally lower than for anti-SDH antibodies and always lower than for anti-FAD antibodies. Anti-SarcDH antibodies were detected before the onset of eye symptoms in one of the 3 patients who developed ocular myopathy, but in none of the two who developed congestive ophthalmopathy (results not shown). Mean OD values for each antibody at each time point are shown in Fig 1A and 1B.

Finally, we measured the IgG subclasses of antibodies reactive against SDH in representative positive sera from patients with active TAO and eye muscle involvement (n = 14) and normal subjects (n=8), in ELISA using anti-IgG subclass-specific second antibodies. Whereas antibodies were mostly of the IgG3 (42%) and IgG4 (72%) subclasses in patients with TAO, they were mainly of the IgG1 (50%) and IgG2 (75%) subclasses in normal subjects. These differences were significant for IgG2 and IgG4 antibodies ($P < 0.05$, $P < 0.05$, respectively) but not for IgG1 or IgG3 antibodies.

DISCUSSION

Although many different eye muscle antigens are recognized by autoantibodies in serum from patients with TAO, the Fp subunit of mitochondrial succinate dehydrogenase the "64 kDa protein", was the first of those to be shown to be closely associated with ophthalmopathy.

The concept of molecular mimicry has been accepted as a possible mechanism of antibody generation against common antigens. To address the possibility that "molecular mimicry" reactivity in patients and control subjects with no clinical evidence for ophthalmopathy reflected targeting of the FAD cofactor which is covalently bound to SDH and other mitochondrial enzymes, we tested for serum antibodies reactive

179 against SDH, DMGDH, SarcDH and FAD in patients with TAO, and control subjects.
180 We showed that positive responses against SDH could be explained by reaction against
181 the FAD cofactor in over 50% of patients without ophthalmopathy but in only 32% of
182 patients with active ophthalmopathy and eye muscle involvement (Table 2). In patients
183 with GH who developed ocular myopathy or congestive ophthalmopathy following
184 treatment, detection of anti-FAD antibodies preceded the eye disorder in 11 out of 14
185 cases (Table 2).

186 To summarize the main results, while we found a wide range of reactivity
187 against mitochondrial enzymes and their FAD cofactor in all study groups, sera from
188 65% of patients with active TAO and eye muscle involvement contained antibodies
189 which reacted with SDH only, compared to 33% with Hashimoto thyroiditis, 14%
190 with type 1 diabetes, 40% with GH and 31% of normal subjects (Table 3). Conversely,
191 positive responses against SDH in subjects without ophthalmopathy could be
192 explained by reaction against the FAD cofactor in over 50% of cases, but in only
193 10% of patients with active ocular myopathy. In patients with GH tested serially, anti-
194 FAD antibodies were demonstrated in all 6 out of the 6 patients who developed ocular
195 myopathy, and in 5 out of the 6 who developed congestive ophthalmopathy, while low
196 levels of anti-SarcDH antibodies were detected in one out of 3 and none out of two,
197 respectively, of these patients.

198 In patients with GH who develop ophthalmopathy, the appearance of anti-FAD
199 antibodies most likely reflects release of mitochondrial enzymes and FAD from damaged
200 eye muscle cells and orbital fibroblasts, whereas anti-Fp antibodies seem to be
201 specifically associated with eye muscle necrosis.

Bacterial SarcDH and mammalian DMGDH share sequence similarities, including the covalent type of FAD attachment to a histidine residue of the enzyme^{13,14}. It is likely that many subjects are exposed to bacterial FAD and that in patients with autoimmune disorders such as type 1 diabetes and Hashimoto thyroiditis, autoantibodies are additionally produced against different epitopes on FAD and the mitochondrial enzymes following their release from damaged islet and thyroid cells, respectively, in support of the notion of different reactivity sites in the SDH protein, anti-SDH antibodies in patients with TAO were mainly of the IgG3 and IgG4 subclasses whereas, in normal subjects giving positive tests against SDH, they were mainly of the IgG1 and IgG2 subclasses. The fairly high prevalence of anti-FAD antibodies in healthy control subjects (32%), some of whom gave a history of non-specific inflammatory disorders recent musculo-skeletal trauma or past or current infections, may be explained by reactivity against FAD or enzymes released from skeletal muscle or by cross-reactivity against FAD or SarcDH in bacteria. Another likely cause of sensitization to mitochondrial enzymes, in patients with autoimmunity, is immune-mediated skeletal muscle cell necrosis. For example, we have recently shown that 5 out of 5 patients with myasthenia gravis had serum antibodies reactive against SDH¹⁵, although we did not test these sera for reactivity against FAD or other mitochondrial enzymes.

In conclusion, because of the wide distribution of mitochondrial enzymes in nature there are likely to be many opportunities for developing cross reactive antibodies which could give a "molecular mimicry" reaction in tests for anti-Fp antibodies, especially in patients with organ specific or multisystem autoimmunity.

On the other hand, in patients with GH studied prospectively, anti-Fp antibodies appear to be specific markers for eye muscle fiber necrosis. Although the significance of antibodies targeting mitochondrial enzymes and FAD, and indeed those antibodies reactive with the eye muscle protein calsequestrin are unclear they are unlikely to be the cause of tissue damage in TAO. Future studies should focus on the cellular reactions against Fp and calsequestrin which are expected to be the primary event in the development of the eye muscle component of ophthalmopathy.

Disclosure: The authors declare that they have no conflicting interests.

Table 1. Demographics and available clinical details of patients with thyroid autoimmunity,
control patients with type 1 diabetes and normal subjects

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	Total	Female	Male	Age(year)	Mean age (year)	Treatment ¹
TAO ²	47	32	15	28-77	50	None
GH ³	26	22	4	24-65	43	None
HT ⁴	30	25	5	22-66	43	None
T1D ⁵	14	7	5	14-66	27	None
Normal	37	25	12	22-53	39	None

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252 ¹With steroids or immunosuppressive drugs

253 ²TAO = Thyroid-associated ophthalmopathy

254 ³ GH = Graves hyperthyroidism

255 ⁴ HT = Hashimoto thyroiditis

256 ⁵ T1D = Type 1 diabetes

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Table 2. Serum antibodies against mitochondrial enzymes and FAD in patients with TAO, thyroid autoimmunity without ophthalmopathy and type I diabetes, and healthy subjects.

GROUP	SDH ¹	SarcDH ²	DMGDH ³	FAD ⁴
TAO ⁵ ; active + EM ⁷ disease (n = 20)	17 (85%) ⁶ P < 0.0001 ⁸	6 (30%)	4 (20%)	2 (10%)
TAO; stable, chronic (n= 27)	10(37%)	4 (15%)	6 (22%)	6 (22%)
GH ⁹ (n=14)	4 (28%)	2 (14%)	4(28%)	11 (78%)
HT ¹⁰ (n=30)	11(37%)	12 (40%)'	10 (33%)	10 (33%)
Type 1 diabetes (n=14)	4(29%)	2 (14%)	4 (29%)	4(29%)
Normal (n=37)	12 (32%)	9(24%)	6 (16%)	6 (32%)

1. SDH = Succinate dehydrogenase

2. SarcDH = Sarcosine dehydrogenase

3. DMGDH =Dimethylglycine dehydrogenase

4. FAD = Flavine adenosine dehydrogenase

5. TAO = Thyroid-associated ophthalmopathy

6. No. (%) reactive with that enzyme/FAD in ELISA

7. EM = eye muscle

8. Statistical analyses refer to differences between patients groups and normal assessed using χ^2 tests with Yeats correction for small numbers.

9. GH = Graves hyperthyroidism

10. HT = Hashimoto thyroiditis

Table 3. Spectrum of antibody reactivity against mitochondrial enzymes and FAD in patients with TAO, thyroid autoimmunity without ophthalmopathy and type I diabetes, and healthy subjects, giving positive reactivity against one or more mitochondrial enzymes or FAD.

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Target antigen	Acute TAO+ EM disease	Chronic TAO	GH	HT	Type diabetes ¹	Normal
one enzyme	11 (65%) ¹ p < 0.001 ²	6 (50%)	2 (40%)	6 (33%)	1 (14%)	4 (31%)
one enzyme + FAD	0 (0%)	1 (8%)	0 (0%)	1 (5%)	2 (29%)	1 (8%)
2 enzymes	2 (13%)	0 (0%)	2 (40%)	2 (11%)	0 (0%)	2 (15%)
2 enzymes + FAD	0 (0%)	2 (16%)	0 (0%)	0 (0%)	1 (14%)	0 (0%)
3 enzymes	2 (13%)	1 (8%)	1 (12%)	0 (0%)	0 (0%)	1 (8%)
3 enzymes + FAD	2 (13%) p < 0.05	3 (25%)	0 (0%)	7 (39%)	1 (14%)	5 (38%)
FAD only	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Number	17	12	5	18	7	13

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332 1. No. (%) reactive in ELISA

333 2. Statistical analyses refer to differences between patients groups and normal

334 assessed using χ^2 tests with Yeats correction for small numbers.

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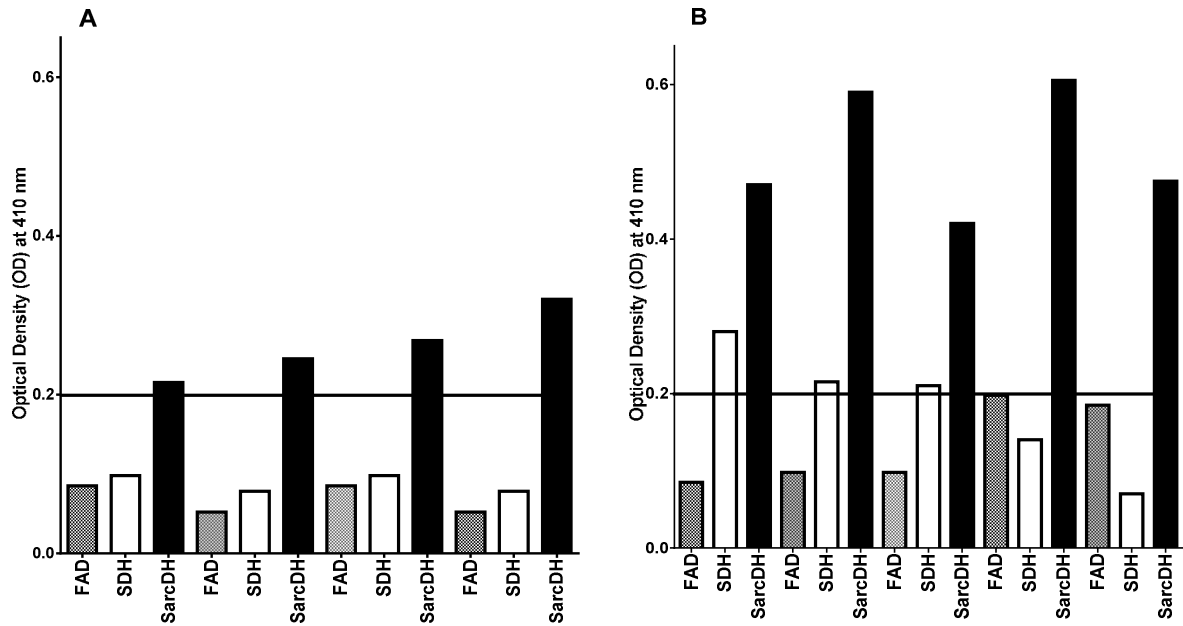


Fig. 1 Serum Levels of antibodies against FAD (shaded column), SDH (open column) and SarcDH (black column) in patients with Graves' hyperthyroidism studied prospectively following treatment, measured in enzyme linked immunosorbent assay. The results are expressed as mean OD at 410 nM. In (A) are levels in 6 patients who developed eye muscle disease. In (B) are levels in 5 patients who developed congestive ophthalmopathy but no evident eye muscle disease. The hatched horizontal line, at 0.200 OD, is the upper limit of normal, for all 3 antigens, calculated as mean + 2SD for a panel of 8 age and sex matched normal subjects tested concurrently.

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