ORIGINAL ARTICLES—Laboratory science

T cells and fibroblasts in affected extraocular muscles in early and late thyroid associated ophthalmopathy

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Abstract

***Aim*—To determine whether there are differences in the lymphocytic cell infiltrate present in affected extraocular muscles (EOM) during early and late stages of thyroid associated ophthalmopathy (TAO).**

***Methods*—17 biopsies of affected EOMs were collected from two groups of TAO patients (n=14): the first of five patients with early, active TAO, and the second of nine patients with late, inactive TAO. The control group was of EOM biopsies taken from 14 non-TAO patients undergoing squint surgery. Immunohistochemical analysis was undertaken using the relevant monoclonal antibodies and an avidin-biotin system and the three groups compared.**

***Results*—Both CD4+ and CD8+ T cells were found in the cellular infiltrate in early, active TAO specimens which were much less evident either in late, inactive stage disease or in control tissue. There was also a significant increase in both CD45RO+ and CD45RB+ cells and macrophages in early TAO compared with the others. Increased expression of HLA-DR antigen by interstitial cells including fibroblasts was detected in both early and late disease but the EOM fibres remained morphologically intact and did not express MHC class II antigens at any time.**

***Conclusion*—These results demonstrate that T cells are only significantly present in early disease but increased HLA-DR antigen expression on fibroblasts is observed at all stages. This suggests that T cells are much more involved in the early than the later stages of the disease process and that early activation of fibroblasts occurs. Early intervention with immunosuppressive therapy to downregulate cytokine production by T cells may significantly influence the sequelae caused by EOM fibrosis.**

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Thyroid associated ophthalmopathy (TAO) is a potentially sight threatening condition and can have a major effect on the extraocular muscle (EOM) function.[1] A strong association between TAO and autoimmune thyroid disease suggests that these two conditions share a common autoimmune pathogenesis. TAO is associated with Graves' disease in about 90% of patients, the remaining 10% of TAO patients having Hashimoto's thyroiditis or a subclinical form of thyroid autoimmunity, in roughly equal numbers.[2–6] However, there is considerable discussion as to whether there are shared antigens in the thyroid and orbit.[7]

EOMs are the major site of the disease process in TAO, although orbital fat/connective tissue is also often involved. Common to both sites is the orbital fibroblast, which is thought to be a key target and effector cell in the pathogenesis of TAO.[8 9] Limited histopathological studies of involved EOM in late disease or when patient has been on immunosuppressive drugs, have reported patchy lymphocytic infiltration of muscle with deposition of glycosaminoglycans (GAGs),[10–15] particularly hyaluronan[16] by activated fibroblasts in the interstitial spaces. This causes oedema leading to enlargement of the extraocular muscles with expansion of the orbital contents, resulting in proptosis, exposure keratitis, and limited ocular movements causing diplopia.[1] Fibrosis of the EOMs is a late sequela with this causing further restriction of the ocular movements and surgical intervention is often required to relieve the ensuing diplopia.

Much is known about the immune mechanisms in autoimmune thyroiditis[16] as thyroid glands are often partially excised in the treatment of thyrotoxicosis. T cells are thought to have a pivotal role in the pathogenesis of this disease. However, EOM surgery is not indicated until the late (fibrotic) phase of the disease for correction of the muscle imbalance once stable. EOM samples studied previously have come from patients either with longstanding disease, or from those who had been on long term immunosuppressive therapy.[13 15] In the present study, we have analysed 17 EOM biopsies from 14 patients with either early active or late inactive TAO using a panel of monoclonal antibodies to identify the subtypes of infiltrating cells present and the expression of HLA-DR on the EOM muscle fibres and interstitial cells. The aim was to see if T cells are involved in the pathogenesis of early compared with late disease and to determine

Table 1 Early, active TAO patient characteristics

Patient No	Age/sex	Duration of TAO (months)*	Class at time of biopsy†	Treatment
1	65/M	5	4 (4b)	None
2	57/M	5	4 (4b)	None
3	61/F	1	4 (4b,2b)	Steroids (<1 month before biopsy)
4	53/F	6	4 (4b,2b)	Steroids, azathioprine (<1 month before biopsy)
5	42/M	3	4 (4b)	None

*Duration of TAO expressed as the time elapsed since diagnosis.
†Class according to modified NO SPECS classification.[23]

Table 2 Late inactive TAO patient characteristics

Patient No	Age/sex	Duration of TAO (years)*	Treatment
6	55/M	3.8	Orbital radiotherapy (<1 month before biopsy)
7	57/M	13	Steroids (11 years before biopsy)
8	57/F	3	None
9	40/M	9	None
10	71/F	14	None
11	47/M	6.3	None
12	55/F	21	Steroids (<1 month before biopsy)
13	40/M	3.5	None
14	67/M	2.8	Orbital radiotherapy, steroids (2 years before biopsy)

*Duration of TAO expressed as the time elapsed since diagnosis.

whether fibroblasts are also activated early. Examination of the EOM fibres would also determine if they are likely to directly involved and/or damaged in this immune process.

Materials and methods

PATIENTS

EOM biopsies were obtained from two patient groups with TAO. All patients were biochemically euthyroid at the time of biopsy and defined as class 4 according to the clinical modified NO SPECS classification.[17] Informed consent and ethics committee approval was obtained for all patient groups. EOM enlargement was confirmed on computed tomography[18] or ultrasound scanning.[19] The biopsies were obtained from the belly of an affected muscle (10–12 mm from the insertion), the muscle sheath having been dissected off and without previous application of any diathermy.

Controls

Sixteen normal appearing EOM biopsies from 14 patients either undergoing routine non-thyroid related strabismus surgery (n=13) or enucleation for intraocular malignancy (n=1) provided the control specimens. EOM specimens from strabismus non-TAO appear generally morphologically normal, although functionally they behave abnormally. In two of the control patients two EOMs were biopsied. Patient ages ranged from 6 to 59 years (mean age 32.6 years).

Table 3 Mean cell/mm² counts (×400 field) of positive cells in the EOMs of early, late TAO and control specimens

Specimens	CD45RB	CD45RO	CD20	CD4	CD8	MAC	HLA-DR
Early TAO (n=5)							
Mean cell/mm² count	44.99	24.55	4.58	12.14	10.27	17.76	37.45
Range	3–130	3–72	0–18	1–43	0–37	5–39	7–80
Late TAO (n=12)							
Mean cell/mm² count	5.98	4.03	0.75	1.63	1.09	7.85	30.58
Range	1–9	0–7	0–3	0–4	0–5	1–16	10–52
Normal EOM (n=16)							
Mean cell/mm² count	2.99	1.17	0.29	0.49	0.54	4.04	19.33
Range	1–6	0–2	0–1	0–3	0–2	0–12	1–21

n = number of samples.

Early TAO

Five EOM biopsies were taken from five patients with recent onset of disease (see Table 1) who had a mean duration of symptoms of 4 months. Two patients showed evidence of active orbital inflammation despite having had treatment with steroids (no 3), steroids and azathioprine (no 4).

Late TAO

Twelve biopsies were collected from nine late, inactive TAO patients (see Table 2). Patients 7 and 12 were euthyroid from the onset of TAO and at the time of biopsy. Specimens were taken during the course of corrective strabismus surgery for tight EOMs, which were restricting normal ocular movements.

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All EOM biopsies were rapidly embedded in optimal cutting temperature (OCT) embedding medium, snap frozen in liquid nitrogen, and stored at −70°C until used. Transverse cryostat sections (5 μm) were mounted on 3-amino propyl tri-ethoxy silane (APES; Sigma, Poole) coated slides. Consecutive cryostat sections were immunostained with monoclonal antibodies to the studied inflammatory infiltrate and counterstained with haematoxylin and eosin. Primary antibody binding was visualised using an avidin-biotin system and the Vector ABC Kit (PK-4002; Vector Laboratories, Peterborough) with a few modifications.

Slides were thawed at room temperature and air dried for 30 minutes. Before immunohistochemical staining, sections were fixed in cold acetone (4°C) for 5 minutes and then washed in phosphate buffered saline (PBS; pH 7.4) for another 15 minutes. Non-specific antibody uptake was blocked by incubating the slides with normal horse serum for 20 minutes and was followed by incubation with a primary monoclonal antibody (MoAb) for 30 minutes in a humidified chamber at room temperature. After washing in PBS for 10 minutes the secondary biotinylated horse anti-mouse Ab was applied for 30 minutes. Endogenous peroxidase activity was suppressed by incubating sections in 0.3% hydrogen peroxide in 50% methanol for 20 minutes. After a further wash in PBS, sections were incubated with a preformed avidin and biotinylated horseradish peroxidase macromolecular complex for 45 minutes. The peroxidase reaction was then developed with the chromogenic substrate amino-ethyl-carbazole (AEC; Sigma). The substrate was filtered and sections developed for 2–5 minutes, washed in distilled water, and counterstained in Mayer's haematoxylin for 30 seconds. Finally, sections were washed in cold tap water for 5 minutes, mounted with aqueous based glycerol gelatine medium (Dako), and covered with a glass coverslip.

The monoclonal antibodies used in this study were as follows: CD45RB (leucocyte common antigen, clone PD 7/26, dilution 1:50); CD45RO (pan-T cell, clone UCHL1, dilution 1:50); CD20 (B cell, clone L26, dilution 1:50); CD4 (CD4 T cells, clone MT310, dilution 1:10); CD8 (CD8 T cells,

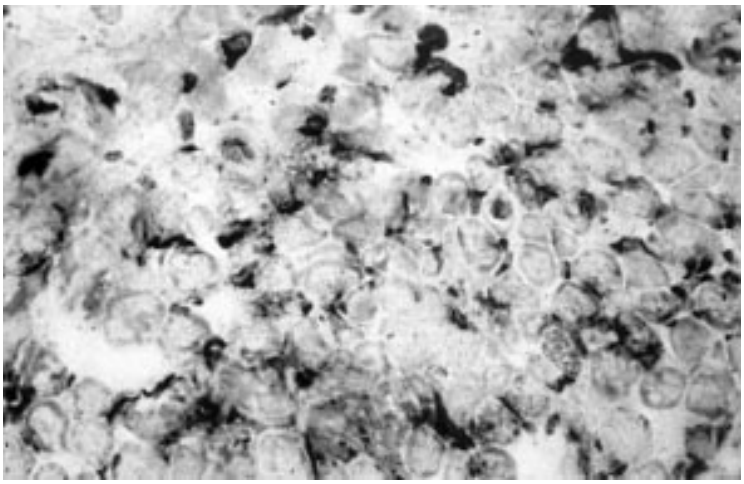

Figure 1 Light micrograph of a transversely cut extraocular muscle tissue from a patient with early TAO. Immunoperoxidase staining for CD45R0 on a cryostat section. Positive staining is seen as a black precipitate (×476).

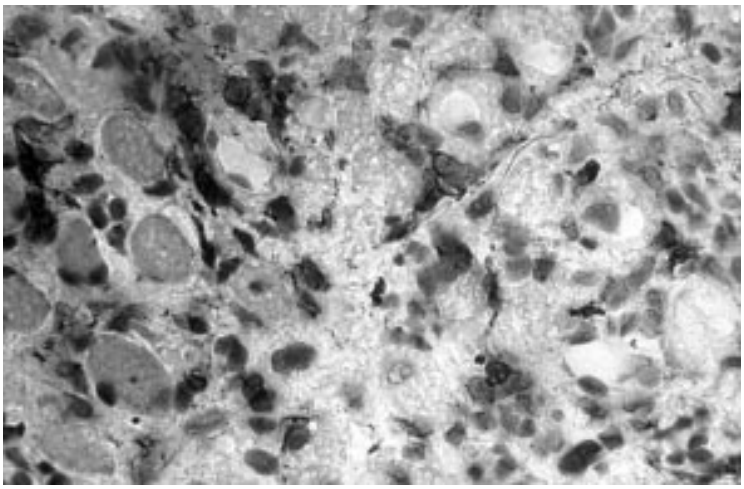

Figure 2 Light micrograph of a transversely cut extraocular muscle tissue from a patient with late, inactive TAO. Immunoperoxidase staining for CD45R0 on a cryostat section. Positive staining is seen as a black precipitate (×476).

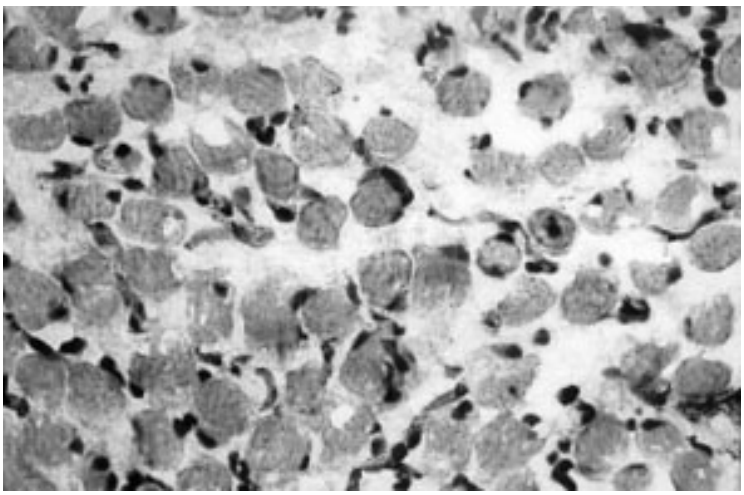

Figure 3 Light micrograph of immunoperoxidase staining for macrophages on a cryostat section of intraocular muscle tissue from a patient with early, active TAO (black precipitate) (×476).

clone DK25, dilution 1:50); MAC (macrophages and activated monocytes, clone Ber-MAC3, dilution 1:100); HLA-DR (MHC class II, clone CD3/43, dilution 1:100); von Willebrand factor (capillary endothelial cells, dilution 1:200). All monoclonal antibodies were obtained from Dako except for von Willebrand factor which was obtained from Serotec (Kidlington, Oxford).

The primary antibody was omitted to provide a negative control, and palatine tonsil tissue sections from tonsillectomy specimens used to confirm optimal staining concentration for monoclonal antibodies used. Positively stained cells were counted per ×400 microscope field, by two independent readers masked to the origin of the specimen and the monoclonal antibody. The mean result from at least three fields was calculated.

The EOM fibres and interstitial cells were examined morphologically in detail and fibroblasts identified by their fusiform appearance.

STATISTICAL ANALYSIS

The Mann Whitney U-Wilcoxon rank sum W test was carried out on the immunohistochemistry data using SPSS software, and a p value <0.05 was considered significant in the comparisons between control and disease sample. The UNISTAT Statistical Package, Version 4.54 (London) was used to carry out the assessment of kappa coefficient for the evaluation of the interobserver agreement.

Results

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The two authors who made the counts of inflammatory markers and control antibodies were in very good agreement in their results as assessed by kappa statistics ($\kappa = 0.96$).

Normal EOM

Examination of normal appearing EOM biopsies from non-TAO patients showed that these contained few CD45RO+ cells, with less than one cell counted per field (Table 3). Cells were found throughout the tissue and in a perivascular distribution. Equal numbers of CD4+ and CD8+ cells were present (mean cell/mm^2 count 1.09 compared with 1.63 mean cell/mm^2 count respectively). Very few CD20+ cells were found. Macrophages were observed in all of the control specimens, situated in the interstitial tissue and adjacent to the muscle fibres. The muscle fibres themselves did not stain with any of the monoclonal antibodies used, including that to HLA class II antigens, although interstitial cells did stain positively.

Early TAO

The EOM fibres were histologically intact. Two of the biopsies were small with few muscle fibres and infiltrating cells present (patients 2 and 5). All early disease specimens contained increased numbers of all cell types compared with specimens from patients with late TAO and from control patients. In particular, an increase in CD45RO+ (Figs 1 and 2), CD4+, CD8+, and MAC+ cells (Figs 3 and 4) were

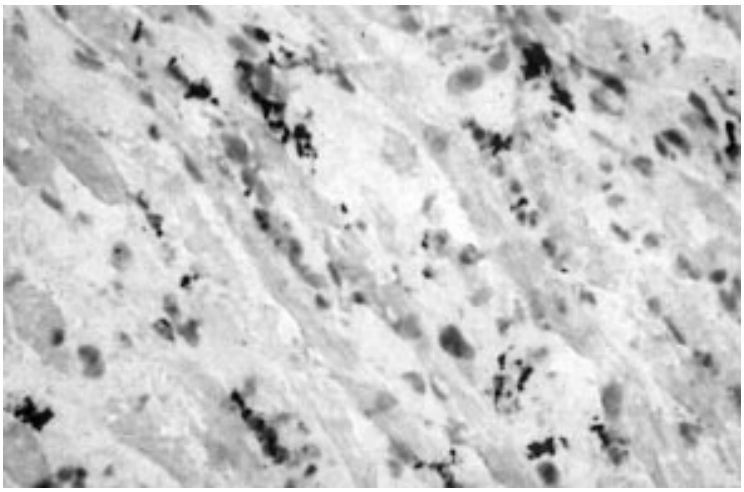

Figure 4 Light micrograph of immunoperoxidase staining for macrophages on a cryostat section of extraocular muscle tissue from a patient with late, inactive TAO (black precipitate) (×476).

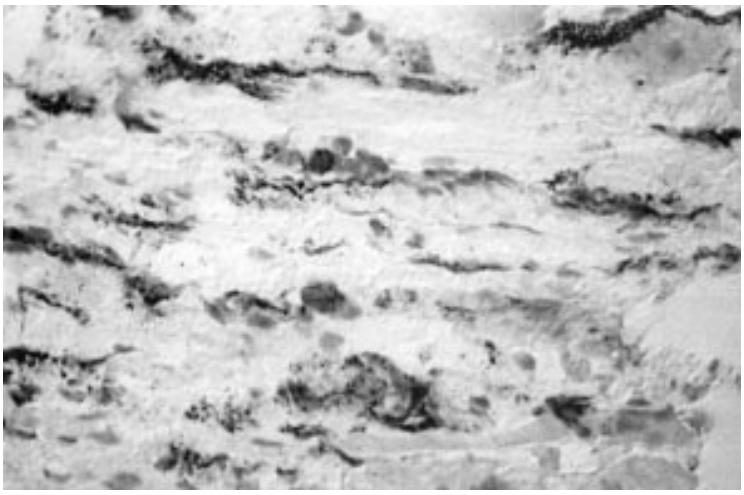

Figure 5 Light micrograph of immunoperoxidase staining for HLA class II (DR) antigen on a cryostat section of extraocular muscle tissue from a patient with early, active TAO. Positively stained cells and their processes are located in the endomysium and perimysium (black precipitate) (×476).

observed in the interstitial tissue. The EOM biopsy from patient 3, who had the shortest and most acute history, was heavily infiltrated with CD45RO+.

In general, the early and late disease EOM had similar numbers of DR+ cells (mean cell/mm^2 count 37.45 in early TAO, mean cell/mm^2 count 30.58 in late TAO) (Table 3) (Fig 5). The EOM fibres, from all patients with early TAO, were negative for HLA-DR. Capillary endothelial cells, identified by positive staining for von Willebrand factor, were DR+. The numbers of CD45RO+, CD45RB+, CD4+, and MAC+ cells in early EOM biopsies were significantly increased when compared with those in the control and late TAO specimens ($p<0.001$;Table 4).

Table 4 Statistical analysis of mean cell counts (×400 field) of positive cells in the EOMs of early, late TAO and control specimens using the Mann-Whitney U-Wilcoxon rank sum W test

Specimens	*CD45RB*	*CD45RO*	*CD20*	*CD4*	*CD8*	*MAC*	*HLA-DR*
Early TAO (n=5)							
Mean rank	18.50	19.00	10.00	16.50	13.30	18.20	13.00
Normal EOM (n=16)							
Mean rank	8.66	8.50	11.31	9.28	10.28	8.75	10.38
p Value	0.0004	0.0001	0.7190	0.0194	0.3539	0.0012	0.4451
Late TAO (n=12)							
Mean rank	19.58	19.50	15.50	17.75	14.42	18.25	17.17
Normal EOM (n=16)							
Mean rank	10.69	10.75	13.75	12.06	14.56	11.69	12.50
p Value	0.0037	0.0044	0.59	0.0735	0.9818	0.0373	0.1457

n = number of samples.
Statistical analysis refers to comparisons between samples from TAO and normal EOM using the same Ab.

Late TAO

Cells positive for CD45RB+, CD45RO+, and MAC+ were found in much smaller numbers in the EOM from patients with longstanding disease and were not significantly different from controls apart from the DR+ expression which was increased although not significantly when ranked (Table 3). CD45RO+ cells were seen scattered throughout the tissue and in a perivascular distribution. These specimens contained few CD20+ cells, with less than one cell counted per field. The EOM fibres were intact and were not DR+. In general, there was no obvious difference in the lymphocyte infiltrate in the biopsies collected from pretreated and non-pretreated TAO patients.

Discussion

In this study EOM biopsies from patients with either recent onset or longstanding disease have been compared. Although orbital fat and levator muscle from the upper lid have been studied from patients with active disease, EOM from patients with acute disease is rarely available for analysis, limiting our understanding of the early events in this disease. Although the small size of the specimens limits what can be examined at any one time and the patchy distribution of disease makes quantitative analysis difficult, important conclusions can be drawn from these findings.

The findings in longstanding TAO are similar to those reported in previous studies, with some T cells present and an increase in class II expression.[12 13] In the specimens from patients with early TAO however, a much greater CD4+ T cell infiltrate was observed with only a few CD20+ B cells. In late TAO specimens, even fewer CD20+ B cells were identified. The muscle fibres themselves were found to be morphologically intact, in keeping with previous findings.[13 15] However, at all stages in our study, the muscle fibres in all specimens remained negative for HLA DR. This finding is in contrast with that reported by Hiromatsu *et al* [20] who reported that HLA-DR expression was detected on EOM fibres in four out of 38 patients. We did, however, see DR+ cells in the interstitial spaces where fibroblasts and macrophages are found—these may previously have been confused with EOM fibres as well as with capillaries on which adhesion molecules are also upregulated.[21] Activation of fibroblasts results in deposition of GAGs by the fibroblasts in the interstitial spaces between the muscle fibres causing enlargement of the EOMs.[15] Normal extraocular muscle has been reported to contain a few T cells, mostly of T cell suppressor type, and macrophages in the

interstitial spaces which were distributed evenly along the length of the extraocular muscle.[22] Our control specimens showed a similar picture with a small number of T cells, macrophages, but practically no B cells. Cells staining for HLA-DR were found in the interstitial tissue and resembled fibroblasts morphologically.

Of particular interest is the significant increase in macrophages in early and less so in late disease compared with control tissue. Macrophages are found in normal EOM and, as professional antigen presenting cells (APC), may play a part in presentation of local antigens to the T cells. Interestingly, in the thyroid gland, it was originally hypothesised that since the thyroid follicular cells (TFC) expressed HLA-DR they might be able to present local antigens to T cells and professional APC would be less important.[23] However, it has now been demonstrated that TFC do not express the co-stimulatory molecules B7-1 and B7-2 necessary for full antigen presentation and therefore this is likely to be the role of cells such as macrophages.[24]

The fact that the inferior and medial recti of normal individuals contained the greatest number of macrophages[22] has been put forward to explain the increased frequency of clinical involvement in these muscles. However, as the inferior rectus and inferior oblique muscles also have an extensive and well developed connective tissue system, this is an alternative explanation for the frequent clinical involvement of the inferior rectus.[25] The increased number of HLA-DR+ cells detected within diseased EOMs are likely be the result of T cell activation and local release of cytokines such as interferon-γ (IFN-γ), an important inducer of HLA class II expression. T cell lines derived from these cells in the EOM have been demonstrated to produce a variety of cytokines.[26] Interestingly, it has been recently reported that thyroid hormone itself potentiates IFN-γ induced HLA-DR expression through a cell hormone binding site causing enhanced activation of STST1α and induction of the class II trans-activator CIITA.[27] This provides further evidence for the importance of good control of thyroid function in these patients. In addition, orbital fibroblasts have been demonstrated to express CD40, a cell surface antigen which allows direct interaction with activated T cells through the ligand CD40L on the T cell surface. CD40 expression is upregulated by IFN-γ and triggering of CD40 through the T cell ligand results in fibroblastic activation—as evidenced by nuclear translocation of nuclear factor kappa B and induction of the proinflammatory and chemoattractant cytokines IL-6 and IL-8.[28]

When stimulated by T cells, fibroblasts also produce GAGs[29] which result in enlargement of the EOMs in acute TAO by osmotically attracting thereby causing protrusion of the globes and optic nerve compression in severe disease.[3] As the disease progresses, there is further proliferation of fibroblasts, which is likely to be independent of the infiltrating T cells as few are then present. The end result of the increased fibroblastic activity is the fibrosis and scarring of the EOMs which can then no longer function normally[30] and muscle imbalance results.

This study has demonstrated that there are far more T cells infiltrating affected EOM in the early stage of TAO than the later stages. With the production of cytokines, these cells are likely to be key to the disease process and downregulating their activity is likely to be helpful, but only in the early stages of the disease. More aggressive immunosuppressive therapy in the early stages may help prevent the disease from progressing to the stage where fibrosis of the EOM is inevitable.

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