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An ultrastructural and systemic analysis of glycosaminoglycans in thyroid-associated ophthalmopathy

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Abstract

Purpose **To determine the ultrastructural localisation of glycosaminoglycans (GAGs) in the extraocular muscles (EOMs) of patients with thyroid-associated ophthalmopathy (TAO) and to see whether the quantity and type of GAGs present in blood and urine are markers of the disease.**

Methods **Biopsies of affected EOMs were taken and studied by transmission electron microscopy (TEM). These were either fixed conventionally for TEM, or in 0.5% tannic acid and others for immunogold staining. Serum hyaluronan (HA) was measured using a radioimmunoassay in patients with TAO as well as control subjects, and urinary GAG levels assessed by photometric quantitation of hexuronic acid after reaction with carbazole. The excretion pattern of the urinary GAGs was determined by discontinuous electrophoresis.**

Results **TEM showed that there is a marked expansion of the endomysial space in TAO EOM biopsies as compared with non-TAO strabismus specimens. This is caused by an increased number of collagen fibres, interspersed with a granular amorphous material surrounding striated collagen fibres shown to be hyaluronan by immunogold staining. In contrast, serum hyaluronan concentrations were similar in TAO and control patients, although there was a statistically significant difference in the urinary GAG excretion between the two groups of patients examined. By discontinuous electrophoresis, chondroitin sulphate and heparan sulphate were present in both patients and controls.**

Conclusion **GAGs and in particularly HA are present at the EOM level in patients with recently inactive TAO. However, serum levels of HA and urinary GAGs are not sensitive indicators for their presence within the EOMs.**

Key words Autoimmunity, Glycosaminoglycans (GAGs), Hyaluronan (HA), Transmission electron microscopy (TEM), Immunogold, Thyroid-associated ophthalmopathy (TAO)

Thyroid-associated ophthalmopathy (TAO) is a disfiguring and potentially sight-threatening manifestation of autoimmune thyroid disease in which glycosaminoglycans (GAGs) have been proposed to play a major role. Current therapy of TAO is limited to addressing the complications of the disease, which include chemosis and periorbital oedema, exposure keratitis, impairment of eye movements owing to extraocular muscle (EOM) inflammation, and optic nerve compression with possible loss of vision.[1] No effective means of prevention is known. Since no animal model exists for TAO, studies on the pathogenesis of the disease have to date relied on peripheral blood and urine samples with limited availability of affected EOM and retro-ocular fat/connective tissue specimens.

The strong association between TAO and autoimmune thyroid disease (ATD) suggests that these two conditions share a common autoimmune pathogenesis.[2–6] However, the exact relationship between Graves' disease and TAO is unclear, with the course of ophthalmopathy being independent from that of the thyroid disease and being largely unaffected by its treatment.[7] Massively enlarged EOMs, the most striking gross anatomical feature of TAO, are the major site of the autoimmune inflammatory process, although orbital fat/connective tissue is also involved. The few histopathological studies of involved EOMs that have been performed to date, reported patchy lymphocytic infiltration of muscle[8–10] with deposition of GAGs[8,9,11–14] by activated fibroblasts in the interstitial spaces.

Connective tissue GAGs are synthesised by the fibroblasts, and classified as chondroitins, keratans and dermatans, which are sulphated, and hyaluronan (or hyaluronic acid, HA), which is not.[15] A similar group, the heparans, are particularly associated with cell and basement membranes. All GAGs, except keratan sulphate, contain hexuronic acid as part of their molecule in a repeat disaccharide sequence with a hexosamine. In keratan sulphate the hexuronic acid is replaced by galactose. Owing to their polyanionic charge, GAGs osmotically attract

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and bind large amounts of water, playing an important role in maintaining the tissue shape as well as in the formation of oedema during inflammation. Most of the signs and symptoms of TAO are largely explicable by the enlargement of the EOMs and the retro-ocular fat/connective tissue within the confined cavity of the orbit. GAGs are likely to play a major role in this process by increasing the volume of the affected tissues.

After secretion in the tissues, GAGs escape from the intercellular matrix into the circulation, are metabolised by the liver and finally excreted via the kidneys. The level and composition of GAGs present in serum and urine may therefore provide easily accessible indicators of the presence or activity of TAO. Kahaly *et al.*[16] have demonstrated a possible increase in urine and plasma[17] GAG levels in early TAO, but another study[18] of serum hyaluronan levels has shown no detectable change in the patients studied. To provide further information on the presence and localisation of GAGs in TAO we have taken biopsies of affected EOMs from TAO as well as non-TAO strabismus control patients and examined them by means of transmission electron microscopy (TEM) and immunogold staining using an anti-hyaluronan antibody. Serum hyaluronan (or hyaluronic acid, or HA) was measured in TAO and control patients by a radioimmunoassay. Urinary GAG levels were assessed by photometric quantitation of hexuronic acids and the excretion pattern of the urinary GAGs of the same samples was determined by means of discontinuous electrophoresis with and without previous digestion with chondroitinase ABC.

Methods

Ultrastructural immunohistochemistry

Extraocular muscle biopsies

Thirty-one biopsies of human EOM were collected from 18 TAO patients (6 males, 12 females; age 31–68 years), with their informed consent and ethics committee approval, during the course of corrective strabismus surgery where tight extraocular muscles were restricting normal ocular movements. Also 10 EOM biopsies were taken from 8 non-TAO strabismus control subjects (3 males, 5 females; age 12–55 years). TAO patients had had clinically inactive disease for at least 6 months before the biopsies were collected and had not received any immunosuppressive therapy during this period. All biopsies were obtained from the belly of the muscle (10–12 mm from the insertion) having dissected off the muscle sheath and without prior application of any diathermy.

Transmission electron microscopy

For morphological investigation specimens were fixed either in a mixture of 2.5% glutaraldehyde and 0.5% tannic acid in 0.1 M sodium cacodylate, pH 7.0 or in 3% glutaraldehyde and 1% paraformaldehyde in 0.1 M sodium cacodylate adjusted to pH 7.4 with 0.1 M HCl. Samples were osmicated for 2 h at 4 °C in 1% aqueous osmium tetroxide, dehydrated through ascending alcohols (50–100%) and propylene oxide, and embedded in Araldite resin cured at 60 °C. Semithin (1 μm) sections were cut and stained with 1% alcoholic toluidine blue for examination by light microscopy and ultrathin (70 nm) sections of selected areas contrasted by sequential staining with 1% (w/v) uranyl acetate in solution of 50% ethanol/water and Reynold's lead citrate and examined in a JEOL 1010 TEM at 80 kV by two independent researchers.

Tissue fixation for immunogold staining

Specimens for immunolabelling were fixed in 2% (w/v) paraformaldehyde and 0.05% (v/v) glutaraldehyde in 0.1 M sodium phosphate buffer for 1 h,[19] and then embedded in Lowicryl HM20 by the progressive lowering of temperature (PLT) method using a LEICA AFS unit (Leica, Milton Keynes, UK). On two occasions specimens were quick frozen in a liquid nitrogen slush (−212 °C), freeze substituted with 10 changes of dried methanol at −80 °C, and infiltrated with Lowicryl HM20 at 50 °C again using the AFS unit. Lowicryl HM20 was polymerised by UV irradiation at −50 °C within the AFS unit. Ultrathin sections were collected on formvar/carbon-coated grids for on-grid labelling.

Immunogold staining

The buffer used throughout the procedure was 50 mM Tris-HCl (pH 7.4) with 0.5 M NaCl. Ultrathin Lowicryl sections mounted on formvar/carbon-coated nickel grids were blocked by overnight incubation at 4 °C with 1% bovine serum albumin (BSA) in 50 mM Tris-HCl buffer (pH 7.4). This was followed by a 4 h incubation with the anti-hyaluronic acid antibody (raised in mice against human trophoblast membrane; Serotec, Oxford, UK) at a dilution of 1:10 at room temperature. After six 4 min changes in buffer to remove unbound primary antibody, the grids were preincubated with 1% BSA (5 min) and then incubated, for a further 1 h, with a 1:50 dilution of a secondary goat anti-mouse 10 nm gold conjugate (Auroprobe, EM GAM IgM G10, Amersham, Bucks, UK). Unbound gold conjugate was removed by three 4 min changes in Tris-HCl buffer plus 0.2% BSA, followed by three changes in Tris-HCl buffer, and washed in ultrapure distilled water. Finally, sections were stained sequentially with saturated uranyl acetate and Reynold's lead citrate before being examined in a JEOL 1010 transmission electron microscope at 80 kV by two independent researchers.

Two types of negative controls were incorporated into the immunogold procedure: (1) omission of the primary antibody and (2) substitution of the primary antibody with non-immune serum from the same species in which the primary was raised. Both controls were at dilutions identical to those used for the primary antibody.

Radiometric assessment of serum hyaluronan

Blood samples were obtained from 10 TAO patients (5 men, 5 women; age 33–60 years, mean 48 years), and 10 control subjects (5 men, 5 women; age 35–60 years, mean 48 years). Their clinical data are summarised in Table 1. Blood samples were collected by venepuncture into sterile, glass Vacutainer tubes with no additive and the serum separated by centrifugation (10 min, 400 *g*). Subsequently, the serum was aliquoted into 1 ml aliquots and stored at −40 °C until analysed.

The hyaluronan levels of these samples were measured radiometrically, according to the manufacturer's instructions, using the Pharmacia HA kit (Pharmacia, Uppsala, Sweden).[20] In principle, specific hyaluronan binding proteins (HABPs), isolated from bovine cartilage, which have a specific affinity for HA, are radiolabelled and used as a radiolabelled ligate. HA in the sample binds to ^{125}I-HABP in a concentration-dependent manner. Unbound ^{125}I-HABP is then absorbed by HA immobilised onto Sepharose.

Each determination was performed in duplicate for both standards and unknown serum samples. A standard of known concentration provided by the kit supplier was serially diluted for each assay. The radioactivity of the standards and samples was measured in a gamma-counter (Cobra Auto-Gamma, Model C5002, Canberra-Packard). The counts (B) for the standards and unknown serum samples were expressed as a percentage of the mean counts of the zero-standard (B_0) according to the formula: % activity bound $= 100 \times B/B_0$. Owing to the nature of the assay, the radioactivity counts are inversely correlated with the amount of HA in the sample. The HA concentration was plotted against the percentage values of the standards and a standard curve was constructed. The concentration of the unknown serum samples was assessed using the following formula: $x = 4.15 - [0.038 \times y]$, which was found by fitting a linear curve into the lin-log plot of points of the standard curve using SPSS software, where x is the log of the HA concentration of the sample and y is the percentage of the activity bound.

Urinary GAGs

Twenty-four hour urine collections were obtained from 37 different TAO patients (19 men, 18 women; age 30–85 years, mean 54 years) and 36 control subjects (17 men, 19 women; age 24–69 years, mean 47 years).

All reagents for this part of the study were purchased from Sigma, UK, and were of the highest purity available, unless otherwise stated.

Quantitative estimation of urinary GAGs by measurement of hexuronic acid content

Isolation of GAGs Initially, the undissolved constituents were centrifuged from 25 ml of the well-mixed urine (10 min; 40 *g*). One millilitre of the supernatant was used for the measurement of creatinine concentration. The hexuronic acid content of urinary GAGs was determined from another 1 ml aliquot by precipitation with cetyl pyridinium chloride (CPC) and ethanol.[21,22] The pellets were dissolved in 200 µl of distilled water prior to assay.

Hexuronic acid assay The aqueous GAGs were reacted with carbazole in the presence of borate/sulphuric acid according to the method of Bitter and Muir[23] using γ-glucuronolactone standards.

The concentration per litre was calculated from the standard curve. The value was 5 times the original urine concentration. Results were expressed in relation to the creatinine content of the urine samples, in order to take the urine concentration into account.

Identification of urinary GAG excretion pattern

Two 1 ml aliquots of the urine supernatant were precipitated in parallel, one of which was subsequently digested with chondroitinase ABC (Sigma C-2780, 100 mU/ml) as control. The enzyme digestion mixture was incubated for 1 h at 37 °C. The residual GAGs in the enzyme-treated samples and the GAGs in the undigested samples were submitted to discontinuous electrophoresis.

Discontinuous electrophoresis The presence or absence of chondroitin sulphate (CS), keratan sulphate (KS), dermatan sulphate (DS) and/or heparan sulphate (HS) in 37 TAO and 36 control subject urine samples was

Table 1. *TAO and control patients' details and serum hyaluronic acid levels*

TAO patients				Control patients			
No.	Age (years)	Sex	Hyaluronan (µg/l)	No.	Age (years)	Sex	Hyaluronan (µg/l)
1	38	M	6.39	1	39	M	10.01
2	40	M	31.81	2	45	M	60.36
3	48	M	14.46	3	49	M	20.21
4	54	M	8.15	4	54	M	48.03
5	60	M	60.97	5	59	M	19.70
6	33	F	14.42	6	35	F	6.7
7	43	F	10.80	7	40	F	61.85
8	48	F	8.22	8	47	F	14.97
9	55	F	29.86	9	53	F	25.22
10	59	F	33.18	10	60	F	20.90

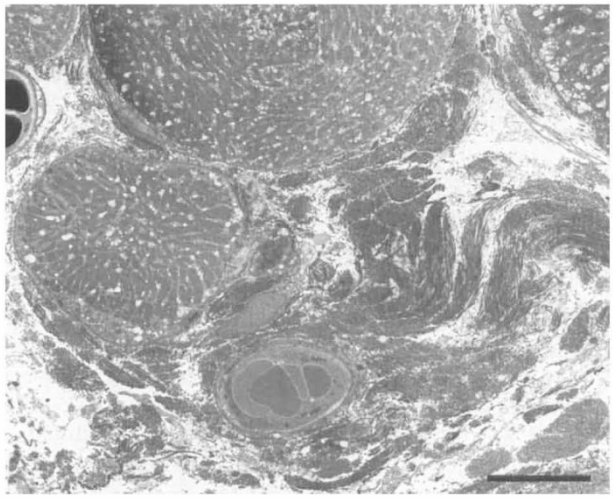

Fig. 1. *Electron micrograph of Karnovsky-fixed extraocular muscle (EOM) from a thyroid-associated ophthalmopathy (TAO) patient. Note the presence of collagen fibres within the expanded endomysial compartment of the sample without concomitant disruption of individual muscle fibres. Scale bar represents 10 μm.*

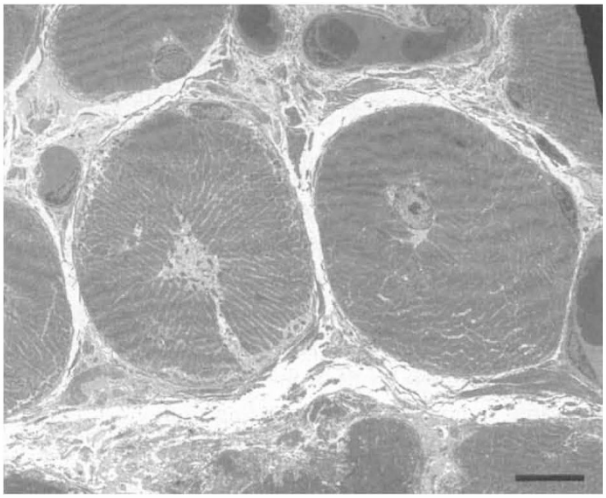

Fig. 2. *Strabismus control EOM section prepared as in Fig. 1, showing much less collagen, no enlargement of the endomysium and morphologically normal muscle fibres. Scale bar represents 10 μm.*

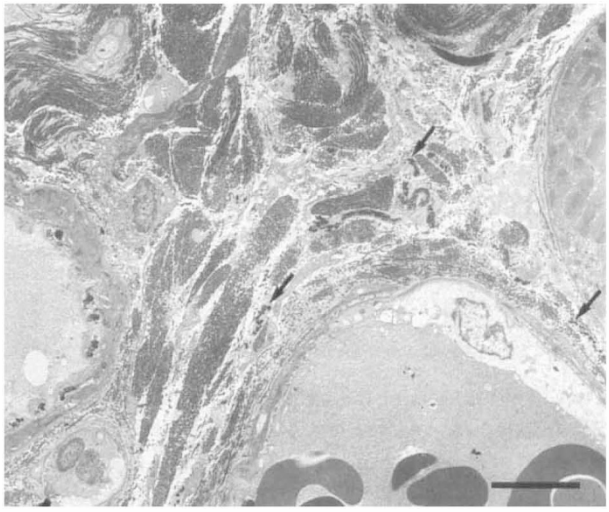

Fig. 3. *Transverse section of a tannic-acid-fixed TAO EOM biopsy showing increased endomysial collagen deposits and electron-dense glycosaminoglycan (GAG) deposits (arrows) not seen in Karnovsky-fixed samples. Scale bar represents 5 μm.*

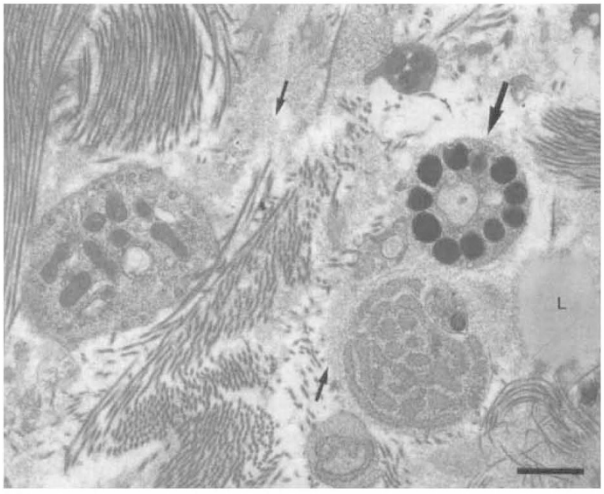

Fig. 4. *Selective enlargement of the abnormally expanded, collagen-filled endomysial space from an EOM TAO biopsy showing a lipid droplet (L), a mast cell (large arrow) and diffuse amorphous material (small arrows). Karnovsky fixation. Scale bar represents 1 μm.*

studied. The constituent GAGs were identified using discontinuous electrophoresis as described by Hopwood and Harrison.[24]

A standard mixture of GAGs containing CS, KS, DS and HS was electrophoresed in a single lane and the identity of the GAGs in the unknown samples determined by comparison with the mobilities of the standards. The quantity of each GAG band was graded as (+++), (++), (+), (±) and (−) relative to the intensity of the staining of relevant standards, graded as (+++).

Statistical analysis

Student's *t*-test was carried out on the serum hyaluronan and urinary hexuronic acid data with SPSS software, and a p value <0.05 was considered significant in the comparisons between control and disease samples.

Results

Ultrastructural immunohistochemistry

Extraocular muscle biopsies: transmission electron microscopy

Transmission electron microscopy failed to reveal any significant differences between the ultrastructural organisation of transversely sectioned extraocular muscle fibres themselves in the TAO biopsies (Fig. 1). However, in all TAO specimens there was a marked expansion of the endomysial space in comparison with the non-TAO biopsies (Fig. 2). This space was filled by an increased amount of collagen fibres interspersed with an amorphous material of low electron density. Sections from biopsies fixed in tannic acid also revealed in the same region an additional highly electron-dense material, most probably GAGs (Fig. 3). In addition, isolated myofibroblasts were seen in elevated numbers in

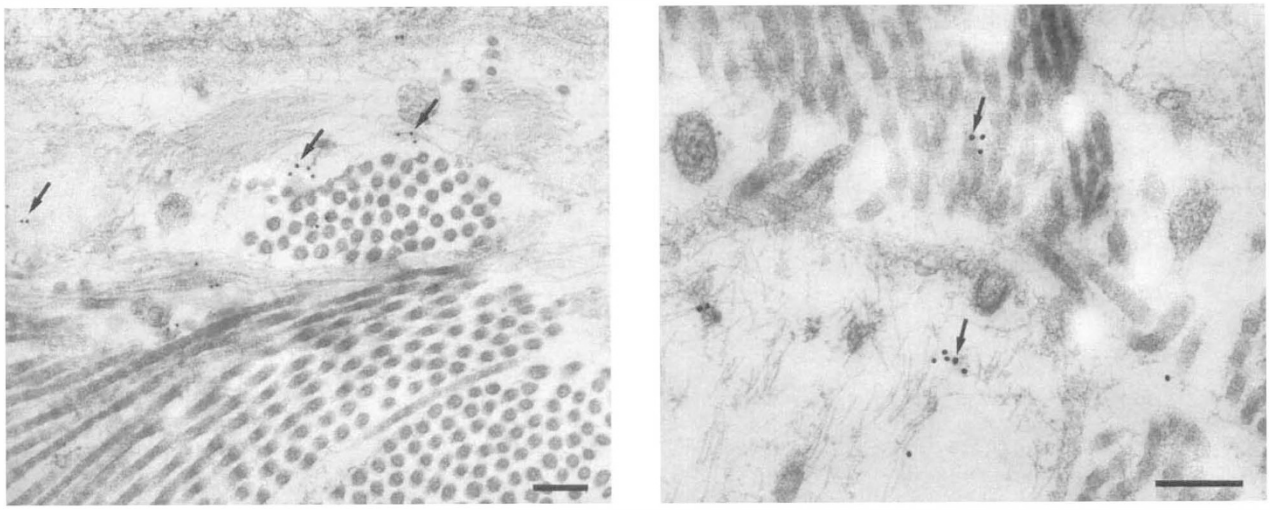

Fig. 5. *(a), (b) Ultrathin sections of paraformaldehyde-fixed, Lowicryl HM20 embedded EOM specimen from a TAO biopsy. Arrows show hyaluronic acid labelling of amorphous material. Scale bars represent 200 nm.*

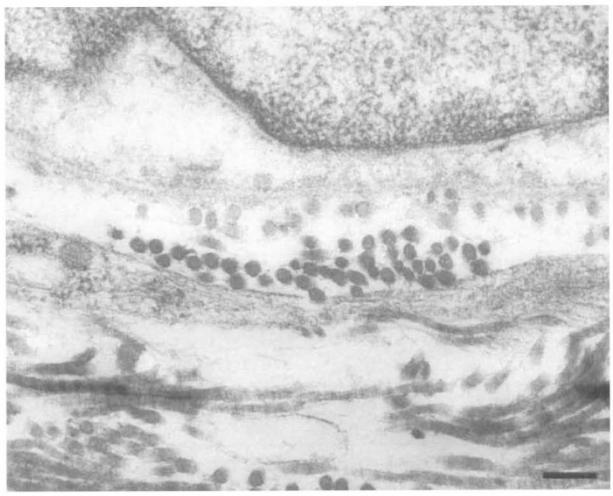

Fig. 6. *Ultrathin section of a non-TAO, strabismus EOM biopsy prepared as in Fig. 5. There is no identifiable hyaluronic acid labelling. Scale bar represents 200 nm.*

the TAO specimens, as well as mast cells and fat cells in the endomysial space (Fig. 4). Perivenular inflammation was observed in TAO specimens (as shown previously[25]). The degree of inflammation correlated generally with the amount of collagen fibres and amorphous material detected in the extracellular matrix between muscle fibres. Electron microscopy revealed evidence of both lymphocytes and monocytes within the inflammatory cuffs.

Extraocular muscle biopsies: immunogold staining

Post-embedding immunogold staining of ultrathin sections of Lowicryl-embedded EOM biopsy material from TAO patients revealed positive staining for hyaluronan associated with the amorphous material surrounding striated collagen fibres (Fig. 5). Concomitant immunogold staining of ultrathin sections previously treated with hyaluronidase was not feasible owing to the small size of EOM biopsy material. Non-TAO strabismus control sections of EOM biopsies were negative (Fig. 6).

Radiometric assessment of serum hyaluronan

The HA concentration for each patient is shown in Table 1. In the 10 TAO patients studied, the mean serum concentration of HA was 21.8±17.2 (SD) μg/l. This value is not statistically significantly different from that of the control subjects, whose mean HA concentration was 28.7±20.3 (SD) μg/l (p>0.5; Student's t-test).

Urinary GAGs

Quantitative estimation of urinary GAGs by measurement of hexuronic acid content

A statistically significant difference (p = 0.03) was found between the TAO patients (6.08 μmol/l hexuronic acid/mmol/l creatinine; SEM 0.456) and the control group

Table 2. *Urine glycosaminoglycan analysis results in control specimens*

Patient details			Urine analysis					
No.	Sex	Age (years)	Hex Ac./Cr. (μmol/mmol)	C4S	C6S	KS	DS	HS
1	F	24	3.2	+		–	–	+2b[a]
2	F	24	4.4	+		–	–	–
3	F	24	4.2	+++		–	–	++
4	F	25	3.2	++		–	–	+
5	F	25	6.1	+		–	–	–
6	F	28	3.6	+++		–	–	+
7	F	30	3	++		–	–	+
8	F	35	5.4	–		–	–	+2b[a]
9	F	36	5	+		–	–	±
10	F	40	6.8	–		–	–	+
11	F	40	4.9	–		–	–	–
12	F	44	3.8	+		–	–	–
13	F	48	6.6	++		–	–	+
14	F	49	7.2	+		+	–	±
15	F	52	5.9	+		–	–	–
16	F	53	3.8	+		–	–	+2b[a]
17	F	65	6.1	++	++	–	–	+
18	F	67	5.5	+		–	–	+
19	F	69	10	–		–	–	–
20	M	40	4	++		–	–	+
21	M	42	4.3	–		–	–	–
22	M	46	5.7	–		–	–	–
23	M	48	3.5	+		+	–	–
24	M	48	5.6	–		–	–	–
25	M	51	4.3	–		–	–	+
26	M	51	2.7	+		–	–	–
27	M	54	3.4	–		–	–	–
28	M	55	4.4	++	++	–	–	+
29	M	57	5.6	+		–	–	+
30	M	59	2.8	++		–	–	+
31	M	59	5.3	–		–	–	–
32	M	60	3.1	+		–	–	–
33	M	61	4.8	–		–	–	–
34	M	62	3.3	–		–	–	+
35	M	63	5.2	+++		–	–	+
36	M	63	9.5	–		–	–	–

Hex. Ac./Cr., hexuronic acid/creatinine; C4S chondroitin-4-sulphate; C6S, chondroitin-6-sulphate; KS, keratan sulphate; DS, dermatan sulphate; HS, heparan sulphate.
[a]2b, two bands.

(4.8 μmol/l hexuronic acid/mmol/l creatinine; SEM 0.283) in urinary GAG excretion. In the TAO patients studied the hexuronic acid/creatinine ratio ranged from 2.0 to 11.8 μmol/mmol, whereas in the control subjects studied it ranged between 2.7 and 10 μmol/mmol.

Identification of urinary GAG excretion pattern

Chondroitin sulphate was present in both TAO and control specimens although the frequency of appearance in the TAO samples as well as the intensity of the revealed bands was greater. No bands were detected in the aliquots treated with chondroitinase prior to discontinuous electrophoresis. Heparan sulphate was present in both TAO and control specimens with a pattern similar to that of chondroitin sulphate. Both control and TAO samples were mostly negative for keratan and dermatan sulphate. These results are summarised in Table 2 and 3.

Discussion

Previous electron microscopical studies of EOMs in TAO by Kroll and Kuwabara[11] have shown that the primary change in the EOMs is an interstitial inflammatory oedema and that alterations in muscle cells, when they occur, are probably a secondary phenomenon. This was confirmed a few years later by Riley.[12] Kroll and Kuwabara also noted that, in addition to inflammatory cells, fibroblasts were plentiful. Our morphological data from transmission electron microscopy confirm previous findings by demonstrating a marked expansion of the endomysial space. We have now added to and expanded this earlier work by studying a larger number of TAO EOM biopsies and also by comparing them with control non-TAO strabismus specimens. In addition, in this study we have demonstrated that the enlargement of the endomysial space was caused by the presence of an increased amount of fibrous collagen, interspersed with a granular amorphous material surrounding striated

Table 3. *Urine glycosaminoglycan analysis results in early and late TAO specimens*

Patient details			Urine analysis					
No.	Sex	Age (years)	Hex Ac./Cr. (μmol/mmol)	C4S	C6S	KS	DS	HS
1	F	30	8.6	++		–	–	–
2	F	42	4.5	++		–	–	+
3	F	42	3.4	+		–	–	–
4	F	42	3.4	+		–	–	–
5	F	43	8.6	++		–	–	±
6	F	46	4.7	++		–	–	+
7	F	51	5.3	±		–	–	+
8	F	51	4.5	++		–	–	–
9	F	52	16	+		–	–	–
10	F	55	8.1	–		–	–	–
11	F	57	4	++		–	–	–
12	F	57	5.6	++		–	–	+
13	F	59	6.8	++		+	–	–
14	F	59	4.9	+		–	–	+
15	F	64	5	+++		–	–	+
16	F	66	5	++		–	–	+
17	F	66	3.7	–		–	–	+2b[a]
18	F	85	4.3	++		–	–	+2b[a]
19	M	30	6.9	++	++	–	–	++
20	M	34	4.8	–		–	–	±
21	M	40	2	+		–	–	–
22	M	42	10.2	–		–	–	–
23	M	43	5.8	–		–	–	±
24	M	46	7.5	–		–	–	+
25	M	48	6.1	–		–	–	–
26	M	49	2.3	+		–	–	–
27	M	53	5.3	++	++	–	–	++
28	M	54	3.3	–		–	–	–
29	M	58	9.3	+		–	–	+
30	M	59	6	+		–	+	+
31	M	60	3.8	–		–	–	–
32	M	62	8.3	++		–	–	+
33	M	63	4.4	+		–	–	+
34	M	66	11.8	+		–	–	++
35	M	67	6.5	+		–	+	+
36	M	69	6.3	+		–	–	+
37	M	72	8.3	±		–	–	±

Hex. Ac./Cr., hexuronic acid/creatinine; C4S, chondroitin-4-sulphate; C6S, chondroitin-6-sulphate; KS, keratan sulphate; DS, dermatan sulphate; HS, heparan sulphate.
[a]2b, two bands.

collagen fibres. The amorphous granular material was proven by immunogold staining to have hyaluronan immunoreactivity in TAO not seen in control specimens.

Orbital fibroblasts obtained from TAO patients have been demonstrated to produce 2.9 times more HA in culture than those obtained from other non-TAO patients undergoing orbital surgery.[26] However, at the serum level, no statistically significant difference in the HA level was observed between the TAO and control subjects. The range of values obtained was similar in the two groups, in agreement with the results of Imai *et al.*[18] and the observations of Kahaly *et al.*[17] who showed that in treated clinically inactive TAO patients normal plasma GAG values were found. A possible explanation for these results is that the amount of HA overproduced locally might not be enough to influence the serum HA concentration, as has been shown in rheumatoid arthritis.[27,28] Another possibility is that there could be an impaired removal mechanism for HA into the systemic circulation in TAO, owing to reduced venous drainage in the TAO orbits, and this might also be important in the accumulation of HA rather than solely enhanced production.

A statistically significant difference in urinary GAG excretion was observed between the clinically inactive TAO and control patients studied, in agreement with previous findings for a similar group of patients.[16,29] Specimens from active untreated TAO patients were not available to us for examination during this study. In addition, analysis of both TAO and control urine specimens by the qualitative technique of discontinuous electrophoresis, showed chondroitin sulphate and heparan sulphate to be present in both groups. However, the frequency of appearance in the TAO samples as well as the intensity of the revealed bands was greater. Treatment of the same samples with chondroitinase ABC resulted in the disappearance of the bands. This disappearance of the small GAG fragments produced by the enzymatic treatment may be due to either their increased solubility in the electrophoretic buffer or their failure to stain. The enzymatic loss of the heparan

sulphate band may be owing to the observed digestion by either chondroitinase ABC or hyaluronidase to a small degree, even at room temperature, immediately after addition of the enzyme to the sample (J. Stone, personal observation). In only 3 of the male TAO patients and in none of the control specimens studied were dermatan-sulphate-positive bands observed. The significance of these bands is not known.

In conclusion, we have demonstrated by transmission electron microscopy that there is consistently marked expansion of the endomysial space in TAO EOM biopsies as compared with non-TAO strabismus specimens. This expansion is caused by an increased number of collagen fibres, interspersed with a granular amorphous material surrounding striated collagen fibres shown to be hyaluronan by immunogold staining. Serum hyaluronan concentration was similar in the TAO and control specimens studied. In contrast, a statistically significant difference in urinary GAG excretion was observed between TAO and control patients examined. However, discontinuous electrophoresis showed chondroitin sulphate and heparan sulphate to be present in both groups.

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