Chemical Characterization of High Buoyant Density 
Proteoglycan Accumulated in the Affected Skin 
of Pretibial Myxedema of Graves’ Disease

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Abstract

From three patients with pretibial myxedema (PTM) of Graves’ disease, a 
portion of the skin involved was biopsied, analyzed for proteoglycans and the 
results were compared with those obtained with euthyroid and hyperthyroid 
subjects without PTM. The tissue specimen was extracted with 4 M guanidine 
HCl and subjected to subsequent CsCl density gradient centrifugation. Glyco-
saminoglycan and protein were recovered in the heaviest density fraction in 
the three specimens obtained from patients with PTM and not from subjects 
without PTM. From the analysis by Sepharose CL-6B column, glycosamino-
glycan was present as a form of proteoglycan because alkaline borohydride 
treatment released single chain glycosaminoglycan with a molecular weight of 
77,000 or 66,000. The digestion with chondroitin ABC lyase revealed that the 
majority of proteoglycan in the skin tissue was chondroitin sulfate or der-
matan sulfate, and heparan sulfate comprised the minor component (14–34%).

The rate of proteoglycan biosynthesis was examined by 35S incorporation 
into glycosaminoglycan’s by cultured fibroblasts from PTM and normal skin. 
Incorporation of 35S into both proteoglycan and single chain glycosaminoglycan 
was observed in the fibroblasts of PTM patients as well as of those of subjects 
without PTM, although the rate of synthesis was more pronounced in the for-
ter. The rate of synthesis was influenced neither by normal serum or serum 
from a pretibial myxedema patient. Since proteoglycan accumulation was 
detected only in the affected skin of PTM patients, the impairment of local 
degradation and the proteoglycan clearance mechanism may also be involved.

Circumscribed pretibial myxedema is a 

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rare cutaneous complication of Graves’ disease characterized by an infiltration of glycosaminoglycan (= acid mucopolysaccha-
ride) to the cutaneous tissue. The presence of glycosaminoglycan, especially hyaluronic acid and chondroitin sulfate, was demonstr-
ated by means of a histochemical technique employing the changes in staining properties after digestion with chondroitin 
ABC lyase and hyaluronidase, respectively
(Lever et al., 1983). The electron microscopy of the affected skin also demonstrated the fibrils and knobs characteristic of the presence of proteoglycans (Kobayashi et al., 1976). Although there have been attempts at chemical extraction and partial characterization, the method was classical, and not suitable for the quantitative analysis of glycosaminoglycan (Cheung et al., 1978, Jolliffe et al., 1979). In those reports, the accumulation of hyaluronic acid was rather firmly established but the accumulation of sulfated high buoyant density proteoglycans was not clear. In the present study, we described the accumulation of high buoyant density proteoglycans in the affected skin of 3 different cases of circumscribed pretibial myxedema of Graves’ disease.

Materials and Methods

Diagnosis of Graves’ disease was made based on clinical grounds; serum levels of total T₄, T₃, TBG, free T₄ and free T₃ indices, and ¹²³I-24h thyroid uptake test. A diagnosis of circumscribed pretibial myxedema (PTM) was also based on clinical grounds and histological findings. Biopsy of the affected skin was performed on patients who agreed with the written form of consent.

Approximately a 0.5g specimen of biopsied skin was weighed and minced with scissors to fine pieces. The minced tissue was extracted with three times the volume of buffer comprising 5.3 M guanidine-HCl, 0.13 M 6-aminohexanoic acid, 67 mM benzamidine-HCl, 33 mM EDTA, pH 5.8 for 48 h at 4°C with continuous stirring. After the extraction, tissue fragments were removed by passing through a glass fiber filter and the filtrate was fractionated by CsCl density-gradient centrifugation. Dissociative CsCl density-gradients in 4 M guanidine HCl (initial density 1.46 g/ml) were performed in a Hitachi 65P RPS-50 rotor by centrifugation at 37,000 rpm at 10°C for 48 h as described by Hascall and his associates for cartilage proteoglycans (Hascall et al., 1974a, Hascall et al., 1974b). Four equal fractions designated as D₁ through D₄ from bottom to top were prepared using a Beckman tube slicer as described by Heinegard (Heinegard, 1972). The bottom one forth fraction designated as D₁ was dialyzed against 0.5 M sodium acetate buffer, pH 7.0 for 48 h and then against distilled water for another 48 h at 4°C. The dialyze was lyophilized and kept refrigerated until further analysis.

Lyophilized specimens were reconstituted with the appropriate buffers described below and subjected to either enzymatic treatments or alkaline borohydride treatment. Papain digestion was carried out at 65°C for 4 h in 0.1 M sodium acetate, pH 7.0 containing 5 mM sodium EDTA and 5 mM cysteine hydrochloride. Digestion with chondroitin ABC lyase (0.25 unit/ml) was performed in a buffer consisting of 0.1 M Tris and 0.1 M sodium acetate, pH 7.3, incubating for 3 h at 37°C. Treatment with alkaline borohydride was performed in 0.05 M NaOH at 45°C for 24 h with 1 M sodium borohydride. Excess borohydride was destroyed by neutralization of the solution with glacial acetic acid. Nitrous acid treatment was carried out at room temperature with 0.24 M NaNO₂ in 0.18 M acetic acid for 80 min as described by Lindahl (Lindahl et al., 1973). The reaction was stopped by the addition of the same volume of 2 M ammonium sulfamate. Enzyme digests, alkaline borohydride treated or nitrous acid treated samples were chromatographed immediately or after storage at −20°C. The specimens were applied to an analytical Sepharose CL-6B or 2B column (100 × 0.65 cm) and eluted with 0.5 M sodium acetate, pH 7.0. Protein was measured by the method of Lowry (Lowry et al., 1951) with bovine serum albumin (BSA) as a standard. The hexuronic acid concentration was determined by the carbazole reaction as described by Dische (Dische, 1947). The molecular size of glycosaminoglycan was estimated from a comparison of the peak elution position with that reported for glycosaminoglycan of known molecular size (Wasteson, 1971).

Biopsy specimens obtained from involved pretibial skin or adjacent uninvolved skin of PTM patient, or punch biopsy specimens (4 mm) obtained from the pretibial skin of hyperthyroid patients or apparently normal subjects without cutaneous involvement, were washed with Dulbecco’s phosphate buffered saline containing 100 U/ml of penicillin and 100 µg/ml of streptomycin (1% P/S), minced with scalpels, explanted, and grown in 9 cm Falcon culture dishes. Dulbecco’s minimal essential medium was supplemented with
10% fetei calf serum (FCS) and maintained at pH 7.2 in a 5% CO₂ — 95% air environment. When the primary cell cultures reached confluence, they were trypsinized and subcultured. When the biosynthesis of glycosaminoglycan was studied, the cells were subdivided into two sets. Each set was incubated in Ham's F12 medium containing the amount of antibiotics described and 10% FCS. The final concentration of sulfate was approximately 100 μM. On the day of the experiment, medium was enriched with [35S] sulfate concentration and further incubated. After 72 h of incubation, the medium was separated from the cell layer and an appropriate amount of guanidine HCl buffer was added to the separated incubation medium to make a final concentration of 4 M. The cell layers were treated with trypsin and the number of detached cells was counted with a Colter Counter.

When the effect of normal or PTM serum on [35S] sulfate incorporation into proteoglycan was studied, the medium was replaced with that containing the stated amount of either normal or PTM serum. The long-acting thyroid stimulator (LATS) was measured by McKenzie bioassay with slight modification (Shishiba et al., 1982). Cells were cultured with medium containing test serum for 6 days. During the last 3 days, the medium was enriched with [35S] sulfate at a concentration of 10 μCi/ml. At the end of the incubation, the medium was harvested and guanidine HCl buffer was added as described. The 4 M guanidine HCl extract was processed in a similar manner as described for chemical analysis of proteoglycans.

Results

Table 1 summarizes the results of high buoyant density proteoglycan extraction by CsCl gradient centrifugation on excised pretibial skin specimen obtained from a patient with non-thyroid disease, a hyperthyroid patient with erythematous cutaneous involvement different from PTM and three hyperthyroid patients with pretibial myxedema. Neither hexuronic acid nor protein was detected in the extracts from non-thyroidal disease or from a hyperthyroid patient without PTM. The histopathological findings of the latter were not compatible with circumscribed pretibial myxedema but apparently normal. On the other hand, there was a definite increase in hexuronic acid content and protein in the specimens obtained from three hyperthyroid patients with typical PTM. The D1 fraction of the patient #3187071 (A) contained hexuronic acid 890 μg/g and protein 143 μg/g of tissue wet weight. Glycosaminoglycan comprised about 91% of the weight of the D1 fraction of this patient, assuming that the average weight of hexuronic acid was 35% of that of glycosaminoglycan. The D1 fraction of the other two patients, #2340950 (B) and #3790576 (C), showed a similar increase in glycosaminoglycan content, namely, hexuronic acid 394 and 92 μg/g, and protein 86 and 14 μg/g, respectively.

Fig. 1 illustrates the results of Sepharose CL-6B chromatography analysis of the D1 fraction obtained from the three hyperthyroid patients with typical PTM. When the intact D1 fraction was analyzed, all the

<table>
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<tr>
<th>Materials</th>
<th>D1 (mg/g)</th>
<th>hexuronic acid (μg/g)</th>
<th>protein (μg/g)</th>
<th>glycosaminoglycan (% of D1 weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal subject</td>
<td>&lt; 0.1</td>
<td>&lt; 5</td>
<td>&lt; 14</td>
<td>—</td>
</tr>
<tr>
<td>PTM #3187071 (A)</td>
<td>2.8</td>
<td>890</td>
<td>143</td>
<td>91%</td>
</tr>
<tr>
<td>PTM #2340950 (B)</td>
<td>1.1</td>
<td>394</td>
<td>86</td>
<td>102%</td>
</tr>
<tr>
<td>PTM #3790576 (C)</td>
<td>1.1</td>
<td>92</td>
<td>&lt; 14</td>
<td>24%</td>
</tr>
<tr>
<td>Hyperthyroidism without PTM</td>
<td>&lt; 0.1</td>
<td>&lt; 5</td>
<td>&lt; 14</td>
<td>—</td>
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* g wet weight tissue
Fig. 1. Analysis of proteoglycan and glycosaminoglycan in D1 fraction by Sepharose CL-6B column. Hexuronic acid was measured by the absorption at OD 530 after carbazole reaction.
hexuronic acid positive materials were eluted at void volume (Fig. 1, top panels of A, B and C). When the D1 fraction was treated with alkaline borohydride and analyzed by means of a Sepharose CL-6B column, the peak at void volume disappeared and a new peak appeared at $K_a=0.27$ in case (A) and 0.30 in case (B) (Fig. 1, middle panels of A and B), suggesting that the glycosaminoglycan was covalently linked with protein because alkaline borohydride treatment was known to release glycosaminoglycan from proteoglycan by an elimination reaction. From the results of chromatography of chondroitin sulfate of known molecular weight (Wasteson, 1971), the molecular size of the single chain species of glycosaminoglycan was estimated to be approximately 77,000 for case (A) and 66,000 for case (B). The alkaline borohydride treatment was not performed on the specimen obtained from case (C), because of the paucity of the materials. When the D1 fraction was digested with chondroitin ABC lyase, the peaks representing a high molecular form of hexuronic acid positive materials disappeared and a big peak appeared at $V_t$ (Fig. 1, bottom panels A, B and C) suggesting that the glycosaminoglycan was mainly composed of chondroitin sulfate and/or dermatan sulfate (CS/DS). The remaining broad peak between $K_d=0.6$ and 0.9 may be heparan

![Graph showing analysis of 35S labeled macromolecules in D1 fraction by Sepharose CL-6B column.](image-url)
sulfate (HS) proteoglycans comprising 34% in case (A) and 17% in case (B) of the total proteoglycans. In case (C), no peak representing HS proteoglycan was observed. These results indicated that CS/DS proteoglycans were accumulated in all the three and HS proteoglycans in two out of three biopsied specimens obtained from the involved skin of PTM, but not in those without PTM.

Fig. 2 shows the analysis of $^{35}$S-labeled proteoglycans synthesized by fibroblasts from PTM and normal skin in cell cultures. The radioactive materials recovered in D1 fraction were analyzed by Sepharose CL-6B chromatography. When the untreated D1 fraction was applied to the column, the $^{35}$S-radioactivity was eluted in two peaks, one at void volume and the other at $K_d=0.65$ (case A with PTM) or 0.57 (case B with PTM and normal) (Fig. 2, top panels of A, B and C). When treated with alkaline borohydride and analyzed, radioactivity at void volume disappeared and that at 0.65 (case A) or 0.57 (case B and normal) was increased (Fig. 2, middle panels of A, B and normal) suggesting that the peak at void volume represents proteoglycan and that at $K_d=0.65$ (case A) or 0.57 (case B and normal) represents the single glycosaminoglycan chain. The molecular size of single chain glycosaminoglycan was approximately 17,000 for case (A), while that for case (B) and normal subjects was approximately 23,000. When the D1 fraction was digested with chondroitin ABC lyase, approximately 71% of the radioactivity was degraded in case (A), indicating the amount of CS/DS. The rest remaining 29% which resisted the enzyme (Fig. 2, bottom panel of A) probably represents HS. In case (B) and normal, the peaks of $^{35}$S at $K_d=0.57$ disappeared by the enzyme treatment (Fig. 3, bottom panel of B and normal), indicating that $^{35}$S was incorporated into glycosaminoglycan mainly composed of CS/DS proteoglycans. Approximately 8% of the radioactivity remained at the fraction between $K_d=0.6$ and 0.9 in case (B), representing HS proteoglycans. These results indicated that

<table>
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<tr>
<th>Source of skin fibroblast</th>
<th>Number of cells (10^6 cells per well)</th>
<th>$[^{35}S]$ sulfate incorporation per well</th>
<th>$[^{35}S]$ sulfate incorporation per 1×10^6 cells</th>
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</thead>
<tbody>
<tr>
<td>Control subjects^{(2)}</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10% normal serum</td>
<td>0.7</td>
<td>0.025</td>
<td>0.040</td>
</tr>
<tr>
<td>10% PTM serum^{(3)}</td>
<td>0.7</td>
<td>0.030</td>
<td>0.043</td>
</tr>
<tr>
<td>Affected portion of skin of case (A)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10% normal serum</td>
<td>1.0</td>
<td>0.049</td>
<td>0.049</td>
</tr>
<tr>
<td>10% PTM serum</td>
<td>1.0</td>
<td>0.038</td>
<td>0.038</td>
</tr>
<tr>
<td>Apparently normal portion of skin of case (B)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10% normal serum</td>
<td>1.2</td>
<td>0.036</td>
<td>0.031</td>
</tr>
<tr>
<td>10% PTM serum</td>
<td>1.0</td>
<td>0.030</td>
<td>0.030</td>
</tr>
<tr>
<td>Affected portion of skin of case (B)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10% normal serum</td>
<td>1.4</td>
<td>0.076</td>
<td>0.053</td>
</tr>
<tr>
<td>10% PTM serum</td>
<td>1.0</td>
<td>0.067</td>
<td>0.067</td>
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(1) Proteoglycans were isolated by CsCl density gradient, dialyzed and analyzed by Sepharose CL-6B chromatography (see text).
(2) Fibroblasts were obtained from the incised skin during orthopedic surgery on the knee.
(3) Pooled serum from a patient with hyperthyroid PTM, whose serum T₄ 14.0 μg/dl, T₃ 386 ng/dl, TBG 18.6 μg/ml and LATS was 1046% in McKenzie bioassay.
fibroblasts obtained from the skin of normal subjects and those hyperthyroid patients with PTM were equally capable of synthesizing CS/DS proteoglycans. In two hyperthyroid patients with PTM, CS/DS and HS proteoglycans were possibly synthesized.

Table 2 compares the extent of the incorporation of $^{35}$S into proteoglycans by fibroblasts obtained from two normal subjects and two different thyrotoxic patients with PTM (case A and B). The effect of PTM patient’s serum whose LATS titer in McKenzie bioassay was 1046% was also studied. As shown, fibroblasts from control subjects incorporated 0.04% of $^{35}$S per 10^6 cells. When incubated with 10% PTM serum, neither the number of the cells nor the rate of $^{35}$S incorporation was changed. Fibroblasts obtained from the affected portion of the skin of patients grew faster (1.0 x 10^6 cells vs 0.7 x 10^6 cells) and incorporated more $^{35}$S into proteoglycan (0.049%) but when expressed in terms of the number of cells, the increase in the rate of $^{35}$S incorporation was not clear.

We then studied the $^{35}$S incorporation into proteoglycans by fibroblasts obtained from an apparently normal portion of the skin adjacent to the affected portion. In the presence of 10% normal human serum, the incorporation was 0.031% per 10^6 cells. When the fibroblasts were incubated with 10% PTM serum, neither the growth rate nor the incorporation rate of $^{35}$S was affected. Fibroblasts obtained from the affected portion of the skin and incubated with 10% normal human serum grew faster and incorporated a larger amount of $^{35}$S (0.053% per 10^6 cells). Again, the effect of PTM serum on the rate of growth and $^{35}$S incorporation was not clear.

**Discussion**

Our results demonstrated that CS/DS or HS proteoglycan accumulated in the involved skin of PTM. The presence of hyaluronic acid was reported but the accumulation of sulfated proteoglycans was not clear in previous studies (Watson et al., 1947, Cheung et al., 1978 and Jolliffe et al., 1979). In the present investigation we have adopted the method by which isolation and characterization of cartilage proteoglycan was carried out (Hascall et al., 1974 and 1974b). Namely, dissociative extraction of proteoglycans with 4M guanidine buffer containing detergent and protease inhibitors was employed. And then, sulfated glycosaminoglycan and proteoglycans were isolated with CsCl density gradient centrifugation. The covalant binding of glycosaminoglycan with protein was demonstrated by the decrease in molecular size after alkaline borohydride treatment which releases single chain glycosaminoglycan by the elimination reaction. The molecular size of the glycosaminoglycan was estimated from comparison of the peak elution position with known molecular weight glycosaminoglycans (Wasteson, 1971). The constituents of glycosaminoglycan were examined in relation to its sensitivity to chondroitin ABC lyase.

From the result described, we can conclude that PTM tissue accumulated proteoglycans with a molecular weight greater than a million (because it was excluded from Sepharose CL-6B) having a glycosaminoglycan chain size of approximately 70,000 M.W. The majority of the proteoglycan was CS/DS but a small amount of HS proteoglycans was observed as well. The accumulation of proteoglycan was demonstrated exclusively in the affected skin of PTM but not in the identical area of the hyperthyroid patient without PTM or of the patient with non-thyroid disease.

As the chondroitin sulfate proteoglycan is produced by skin fibroblasts and is mainly distributed in connective tissue, we have examined the biosynthesis of proteoglycan by cultured fibroblast employing $[^{35}S]$sulfate incorporation into proteoglycans using the
same analytical methods. The concentration of stable sulfate in the medium was kept constant at 100 μM among dishes in order to minimize the difference in specific activity. As shown in Fig. 2, fibroblasts in culture were capable of synthesizing proteoglycans and single chain glycosaminoglycans. The size of single chain glycosaminoglycan in the medium of fibroblast culture was smaller than that obtained from biopsy. This result may reflect the difference in the capability of fibroblasts to synthesize glycosaminoglycans in vivo and in vitro. As shown in Table 2, fibroblasts obtained from normal subjects or from apparently normal portion of the skin of patients with PTM were capable of synthesizing sulfated proteoglycans. In view of the fact that there was no accumulation of proteoglycans in normal or hyperthyroid skin without PTM and there was abundant accumulation in PTM skin, the mechanism of local degradation of cutaneous proteoglycans in vivo in PTM patients may be impaired. The 35S incorporation was more pronounced in fibroblasts from the affected skin of PTM patients than in those of normal subjects or the unaffected skin of PTM patients. But the difference is not so remarkable as to explain the accumulation of proteoglycans only in affected skin of PTM patients. In our hands, the incorporation of 35S into proteoglycans was not increased by the serum of PTM patients whose LATS was 1046% in McKenzie bioassay, either in fibroblasts from normal subjects or in those from the affected skin of PTM patients (Table 2).

The present result concerning the effect of PTM serum on glycosaminoglycan synthesis by fibroblasts is contradictory to the report of Cheung et al. (1978) in which they reported that constituents of PTM serum were capable of stimulating 35S incorporation into glycosaminoglycan. However, in their study, glycosaminoglycan was defined as the 35S-radioactivity precipitated with ethanol and insensitive to pronase digestion. They did not provide any information on the recovery of radioactivity in each extraction step. The effect of alkaline borohydride and that of chondroitin ABC lyase by which the nature of glycosaminoglycan was to be characterized were not studied. Furthermore, they did not provide any information on whether fibroblasts from normal tissue were capable of synthesizing sulfated glycosaminoglycan. Our result is also contradictory to the report of Jolliffe et al. (1974) in which they reported that [3H]-N-acetylhexosamine incorporation into fibroblast was enhanced by PTM serum. [3H]-N-acetylhexosamine is not easily incorporated into cell compared to [3H]hexosamine and hence is not a good labeling precursor. Furthermore, their method of glycosaminoglycan extraction was repeating freezing and thawing whose efficiency for extraction of glycosaminoglycan associated with cell membrane is unknown. Our methods have been rigorously examined by Hascall and his associates (1974 a, b) and were accepted as a quantitative method for extraction and isolation of glycosaminoglycans and proteoglycans. Further study is required to determine the reason for the discrepancy concerning the effect of serum.

Despite the use of strong denaturing conditions of 4 M guanidine HCl and protease inhibitors throughout the extraction and purification procedure, a part of the 35S was incorporated into glycosaminoglycan present as single chain species in in vitro fibroblasts culture, suggesting proteolytic degradation of proteoglycans in the in vitro condition. This may represent the difference between in vivo conditions, where there is a clearance mechanism eliminating degraded proteoglycans, and in vitro conditions, where such mechanisms do not operate. It is obvious that further study is required to elucidate the mechanism of proteoglycan accumulation in affected skin of PTM patients.
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References


