Immunoglobulin G of patients with circumscribed pretibial myxedema of Graves’ disease stimulates proteoglycan synthesis in human skin fibroblasts in culture

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Hyaluronic acid and proteoglycan accumulate in the affected skin of Graves’ disease patients with pretibial myxedema (PTM). We aimed to determine whether an autoantibody IgG circulating in PTM patients stimulates proteoglycan synthesis in human skin fibroblasts, resulting in PTM. IgGs were purified from 14 normal subjects, 11 Graves’ disease patients with PTM, 5 Graves’ disease patients with active ophthalmopathy and 15 Graves’ disease patients with neither PTM nor ophthalmopathy. Human skin fibroblasts were incubated with the IgGs and labeled with [35S]sulfate. The medium and cell layer were separated and the proteoglycan was extracted. The 35S radioactivity in the proteoglycan fraction was measured. Compared with normal IgGs or with those of Graves’ disease without PTM or ophthalmopathy, PTM IgGs significantly increased the incorporation of the 35S into the proteoglycan. The effect of PTM IgG was dose-dependent. As PTM IgG did not alter degradation of the 35S labeled proteoglycan, an increase in 35S incorporation reflects increased synthesis. The effect was mediated through a mechanism other than adenylate cyclase activation. The present study demonstrates the presence of an autoantibody in PTM IgG that stimulates proteoglycan production through human skin fibroblasts. This is not correlated with the thyroid stimulating antibody activity. It is suggested that the activity of this antibody leads to the development of PTM.

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Circumscribed pretibial myxedema (PTM) is a cutaneous complication occasionally associated with Graves’ disease. Histochemical examination of the biopsied specimens showed the accumulation of hyaluronic acid and proteoglycan (1, 2). In a previous paper, we examined the proteoglycans extracted from the affected skin and found that a large amount of dermatan sulfate proteoglycan had accumulated (3). It is widely accepted that fibroblasts are the source of hyaluronic acid and proteoglycan synthesis, because skin fibroblasts actively synthesize both of them in culture. It is tempting to speculate that an autoantibody IgG produced in Graves’ disease patients may stimulate skin fibroblasts to synthesize excess amounts of hyaluronic acid and proteoglycan. However, attempts to show that the sera of PTM patients stimulate proteoglycan synthesis in human skin fibroblasts in culture have yielded conflicting results (3–13). For example, Joliffe et al. (4) and Cheung et al. (5) reported that serum components of PTM patients could stimulate glycosaminoglycan synthesis in human skin fibroblasts in culture. On the other hand, Rotella and his associates reported that the IgG of PTM patients stimulated glycosaminoglycan synthesis in rat thyroid cells but not in human skin fibroblasts, although they showed that PTM IgG stimulates collagen synthesis in human skin fibroblasts in culture (6–12). A recent report by Tao et al. also showed that glycosaminoglycan synthesis in cultured human skin fibroblasts was not stimulated by the sera of PTM patients which contained autoantibodies capable of stimulating thyroid tissue (13). We wondered if there was an antibody IgG in patients with PTM capable of stimulating proteoglycan synthesis in human skin fibroblasts in culture, but that the effect was masked either by serum-derived growth factors or by limitations of the methods employed. In the present study, we examined this aspect—no serum component other than IgG was included, except for bovine serum albumin (BSA)—employing the recently developed methods for proteoglycan analysis, the quantitative features of which have been firmly established (14, 15).

Materials and methods

Diagnosis of Graves’ disease was based on clinical grounds and on laboratory findings such as serum total thyroxine (T₄), triiodo-l-thyronine (T₃) and TSH. Cir-
cumscribed PTM was diagnosed based on the clinical findings and on the histological features obtained through biopsy of the affected skin. Sera was obtained from 14 normal subjects, 11 patients with Graves’ disease associated with PTM (6F, 5M), 5 Graves’ disease patients with active ophthalmopathy but without PTM, and 15 Graves’ disease patients with neither PTM nor ophthalmopathy. Normal subjects included as controls were selected to match the PTM patients in terms of sex and age within a difference of five years. The IgGs were purified by ammonium sulfate precipitation followed by DEAE-Sephacel chromatography (16). Before the experiment, they were extensively dialyzed against the culture medium.

Fibroblasts obtained from the leg skin of a 14-year-old girl undergoing orthopedic surgery and employed in all of the experiments were cultured until confluence in 6-well Coster plates with DMEM medium containing 10% fetal calf serum (FCS). The concentration of FCS was decreased to 5%, 1% and 0%. BSA 1 g/l was added to the medium lacking FCS. Cells were cultured for 24 h in media at each FCS concentration. At the end of this culture, the cells were rinsed three times in the medium without FCS and the IgG to be tested was added to the culture medium at a final concentration of 2 g/l unless otherwise stated. The cells were cultured with the IgG for 72 h. During the final 24 h, 3.7 mCi/1 Bq of [35S]sulfate was added to the culture medium. After labeling the culture, the medium and cell layer were separated. Solid guanidine-HCl was added to the medium to make the final concentration 4 mol/l. Each cell layer was rinsed with the culture medium without isotopees and proteoglycans were then extracted with 4 mol/l guanidine-HCl buffer containing 50 mmol/l Na-EDTA. 50 mmol/l sodium acetate, 100 mmol/l 6-aminohexanoic acid, 5 mmol/l benzamidine-HCl and 2% Triton X-100. pH 6.0. N-acetylimaleimide dissolved in ethanol (1 mol/l) was added to the medium and cell layer extracts at a final concentration of 10 mmol/l. The extracts were eluted on Sephadex G-50 (fine) columns prepared in disposable 10 ml plastic serological pipettes (bed volume of 8 ml for a maximum of 2.0 ml sample) and equilibrated with 8 mol/l urea buffer containing 50 mmol/l sodium acetate, 50 mmol/l Na-EDTA. 0.15 mol/l NaCl and 0.5% Triton X-100. pH 6.0. The radioactive fraction excluded from the column was collected and analyzed by Q-Sepharose chromatography (2 ml bed volume, equilibrated with 8 mol/l urea buffer) as described previously (14, 15). In short, after sample application, the column was rinsed with 5 ml of 8 mol/l urea buffer and then eluted with a continuous NaCl gradient (from 0.15 to 1.5 mol/l) in the same solvent, using a total volume of 46 ml. Fractions of approximately 0.8 ml were collected at a flow rate of about 2.5 ml/h. Aliquots of each fraction were counted for radioactivity. About 80% of [35S] radioactivity was recovered in a peak eluted at NaCl concentration ranging from 0.5 to 0.8 mol/l, where standard proteoglycan was eluted.

When the effect of an IgG on cAMP generation by fibroblasts was examined, fibroblasts were cultured with the IgG for 24 h in a modified Hank’s medium, as described by Kasagi et al. (17). After the culture, the medium was separated and cAMP extracted with 6% trichloro-acetic acid (TCA). After the TCA was removed by repeated rinsing with hydrated ethyl-ether, the aqueous phase was harvested and dried. The cell layer was rinsed twice with the culture medium without IgG, and cAMP was extracted with 6% TCA as described above. After dissolving the dried residue with sodium carbonate buffer to an appropriate volume, cAMP was measured by radioimmunoassay, employing kits purchased from the Amersham Co, Tokyo. When the effect of dibutyryl cAMP (Bt2cAMP) was examined, fibroblasts were cultured with Bt2cAMP at 0, 10⁻⁴ or 10⁻³ mol/l for 24 h in the presence of [35S]sulfate and 2 g/l of normal IgG. After separation of the medium and cell layer, [35S] incorporation into the proteoglycan was measured as described.

When the effect of an IgG on FRTL cells was examined, the cells were cultured and grown as described previously (18). The cells were rinsed with medium not containing FCS and further cultured with 2 g/l of IgG solution for 72 h. [35S]sulfate was added and cultured for the last 24 h. After incubation, the proteoglycan was extracted as described.

When the effect of an IgG on the degradation of proteoglycan was examined, the cells were labeled with [35S]sulfate for 24 h (pulse label), rinsed thoroughly and then incubated in a culture medium containing the IgG for 0, 3, 8 and 24 h. Radioactivity in the medium and in the cell layer after Sephadex G-50 chromatography was counted as already described.

When [3H]-thymidine uptake into DNA was studied, cells were cultured with the IgG for 48 h and the medium replaced with thymidine-free Ham’s F12 medium containing 37 K Bq of [3H]-thymidine and the IgG to be tested. The cells were further incubated for 24 h. After incubation, the medium was removed and the cell layer rinsed three times in a medium without isotopes. DNA was precipitated with 6% TCA as described (19). The [3H]-radioactivity in the DNA fraction was measured with a liquid scintillation counter. The TSH-binding inhibiting immunoglobulin of serum was measured with kits purchased from the Travenol Co., Ltd., UK. The activity was expressed as TBI% (20). The thyroid-stimulating antibody activity (TSA) was measured as described by Kasagi et al. (17), employing dispersed porcine thyroid cells, and the results were expressed as µU TSH equivalent. The concentration of Plasma-Derived Growth Factor (PDGF) in a PTM-IgG sample (no. 3) was measured with the radioimmunoassay employing anti-PDGF antibody purchased from Collaborative Research, USA and 125I-PDGF from the Amersham Co. Ltd., UK. The concentration of insulin-like growth factor-1 (IGF-1) in the same IgG sample was measured after acid ethanol extraction (21) with a radioimmunoassay method des-
Results

Table 1 summarizes clinical data obtained from 11 patients with Graves' disease associated with PTM (6F, 5M). Of these PTM patients, cases 5, 6, 7, and 10 were not thyrotoxic at the time of diagnosis for PTM and blood sampling. TBII in serum was positive in 10 patients. The IgGs of 11 PTM patients were examined in four separate experiments in order to obtain an appropriate number of observations for each experiment.

Fig 1A, B and C summarizes the results of the three experiments in which 9 out of 11 PTM IgGs were examined. IgGs (2 g/l) from PTM patients significantly increased 35S incorporation into proteoglycan compared to those from normal subjects, as shown in panels A, B and C (p<0.01, p<0.001 and p<0.005, respectively) and to those from patients with Graves' disease without PTM, as shown in panels A and B (p<0.05 and p<0.02, respectively). In the experiment summarized in panel C, three IgGs from patients with Graves' disease without PTM but with active ophthalmopathy were included. The latter IgGs showed significantly increased incorporation of 35S into proteoglycan similar to PTM-IgGs.

Fig. 2 summarizes the results of the experiment in which the effects of IgGs from a larger number of PTM patients were compared with those from normal patients, from patients with Graves' disease with active ophthalmopathy and from those with neither ophthalmopathy nor PTM. As shown, 35S incorporation into proteoglycan by PTM IgGs (designated as B in Fig. 2) significantly exceeded that by normal IgGs (A, p<0.02) and that by the Graves' IgGs with neither ophthalmopathy nor PTM (D, p<0.05). IgGs from patients with active ophthalmopathy (C) increased 35S incorporation into proteoglycan, significantly exceeding that of normal (A, p<0.05). There was no statistical difference between the activity of IgGs with and without ophthalmopathy because of considerable overlap (C vs. D, p>0.05).

The dose–response relationship was examined by taking the IgG of PTM patient no. 3. The effect of the IgG was dependent on its concentration (as shown in Fig. 3). In this experiment, the concentration of PTM IgG was balanced by adding normal IgG to make a final concentration of 2 g/l. The effect of PTM IgG (no. 3) was significantly different from that of normal IgG at doses above 1.5 g/l. We examined the possibility that cAMP mediates the effect as a second messenger. Generation of cAMP in fibroblasts cultured with PTM IgG no. 3 was examined and plotted in Fig. 3. The 2 g/l dose of the PTM IgG only slightly increased the cAMP content. In support of this finding, Bt2cAMP, at both 10^-4 and 10^-3 mol/l, was ineffective in increasing 35S incorporation (Fig. 4). Thus, the ability of IgG to stimulate 35S incorporation into proteoglycan is not mediated through a cAMP-dependent mechanism.

Employing the same IgG at a dose of 2 g/l, the effect on the degradation of the 35S labeled proteoglycan was examined. The cells were labeled with [35S]sulfate for 24 h and chased for 0, 3, 8 and 24 h in the presence of either normal IgG or the PTM IgG (no. 3). The radioactivity of the proteoglycan in the medium and in the cell layer was measured. The 35S radioactivity of the proteoglycan in the cell layer decreased with time, but increased with time in the medium (data not shown). The PTM IgG did not influence the process of degradation and secretion of proteoglycan at all, so the result is not shown here. In a situation in which degradation of proteoglycan is not influenced, an increase in 35S incorporation into proteoglycan reflects the increase in proteoglycan synthesis.

Fig. 5 summarizes the effect of IgG on 3H-thymidine incorporation into DNA in which the effects of four IgGs from PTM patients were compared with those of four normal IgGs (this experiment was carried out simultaneously with that shown in Fig. 1C). As illustrated, PTM IgGs failed to significantly increase 3H-thymidine incor-

<table>
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<th>Patient</th>
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<th>Age</th>
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<th>T3 (nmol)</th>
<th>TBII (%)</th>
<th>TSAb (μU TSH)</th>
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<td>38</td>
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Normal range: (60–140) (0.9–2.4) (<14) (<5)

* Assay was not performed.
correlation in the two, although $^{35}$S incorporation into FRTL cells is a good indicator of thyroid stimulation correlating with TBI activity, as shown in Fig. 9. The concentration of PDGF and IGF-1 in the PTM IgG sample (no. 3) was less than the detectable limits of the respective radioimmunoassay methods.

Discussion

We have demonstrated that the IgGs of Graves' disease patients with PTM stimulate human skin fibroblasts to increase proteoglycans synthesis. In the affected skin of PTM patients with Graves' disease, accumulation of proteoglycan has been demonstrated histopathologi-

Fig. 2. Effect of IgGs from 9 normal subjects (A), from 9 PTM patients (B), from 5 patients with Graves' disease with active ophthalmopathy (C) and 15 patients with Graves' disease with neither ophthalmopathy nor PTM (D). The concentration of the IgG tested was 2 g/l. Statistical difference is as follows. A vs B: $p < 0.02$, A vs C: $p < 0.05$, A vs D: not significant, B vs D: $p < 0.01$.

Fig. 3. Effect of the dose of a PTM IgG (patient no 3) on $^{35}$S incorporation into proteoglycan (indicated by solid circles) and cAMP generation (indicated by open circles). The concentration of IgG was balanced to obtain 2 g/l by adding normal IgG. Bars indicate the standard deviation obtained with three observations.
Fig. 4. Effect of Bt2cAMP on $^{35}$S incorporation into proteoglycan at the concentration of $10^{-4}$ and $10^{-1}$ mol/l. Fibroblasts were incubated with the medium containing Bt2cAMP in the presence of 2 g/l of normal IgG. Solid circles and bars indicate the mean $\pm$ SD of three observations.

Fig. 6. The relationship between TBI\(\%\) measured on 50 $\mu$l serum and the activity to FPSAb measured on the 2 g/l of IgG sample. There was no significant correlation between the two activities.

serum component other than IgG stimulates glycosaminoglycan synthesis in human fibroblasts in culture (4). Cheung and his associates also reported that the IgG of PTM patients stimulates glycosaminoglycan synthesis in human skin fibroblasts in culture (5). However, the methods employed were not necessarily adequate, because in these studies glycosaminoglycan synthesis

Fig. 7. The relationship between TSAb activity measured on 50 $\mu$l serum and expressed in terms of $\mu$U TSH equivalent and FPSAb measured on 2 g/l of IgG sample. There was no statistically significant correlation between the two activities ($p > 0.1$).

Fig. 5. Effect of PTM IgG (patient no. 3) at a concentration of 2 g/l on $^3$H-thymidine incorporation into DNA.
was traced either by $[^3]H$acetate or $[^14]C$acetate incorporation into macromolecules sensitive to pronase and then precipitated with cetylpridinium chloride. With this method, precipitation of glycosaminoglycan was largely influenced by the concentration of sodium chloride in the solution (24). Furthermore, possible changes in specific activity of labeled precursor by the alteration of endogenously generated acetate were not taken into account. In their study, separation of glycosaminoglycan into hyaluronic acid and sulfated proteoglycan was not possible. On the other hand, Rotella, Alvarez, Kohn, Toccafondi and their associates reported that glycosaminoglycan synthesis was stimulated by PTM IgGs in FRTL cells, but not in human skin fibroblasts (6–12). Similar results were reported by Tao and his associates, too (13). The glycosaminoglycan synthesis in thyroid cells including FRTL cells is a good indicator of thyroid stimulation mediated through TSH receptor, as described previously (18). As patients with PTM usually have a high titer of thyroid stimulating antibody, such as classical LATS, it is not surprising that sera or IgG of PTM patients stimulate this aspect. The issue here is whether PTM IgG stimulates glycosaminoglycan synthesis in human skin fibroblasts instead of rat thyroid cells.

For the proteoglycan analysis, we adopted the method of extraction and purification recently developed, the quantitative features of which have been firmly established. We examined the rate of synthesis of proteoglycan by studying $[^35]S$ incorporation from sulfate into proteoglycan. By this method, the changes in specific activity due to an endogenous supply of sulfate from amino acid sulfur were negligible, as already shown for FRTL cells (18). With this incubation condition and method of analysis we have demonstrated that the IgGs of the 11 PTM patients stimulated $[^35]S$ incorporation in proteoglycans. In a situation where degradation of the $[^35]S$ proteoglycan is not altered, enhanced incorporation of $[^35]S$ into the proteoglycan reflects either an increase in synthesis or an increase in the extent of sulfation. Although the latter aspect was not directly examined in the present study, the possibility is unlikely because in
none of the situations we examined was this the case. Changes in $^{35}$S incorporation into proteoglycan from sulfate precursor were observed in FRTL cells, in human skin fibroblasts, and in osteoblastic cells when they were stimulated by TSH, thyroid hormones and calcitriol, respectively. However, the changes were not due to alteration in the extent of sulfation but to alteration in the rate of proteoglycan synthesis (18, 25, 26 and 27). Thus, it is fairly safe to speculate that $^{35}$S incorporation into proteoglycan reflects an increase in proteoglycan synthesis.

As PTM IgGs increased proteoglycan synthesis to the level significantly exceeding that of normal IgGs and that of IgGs from Graves’ disease patients having neither PTM nor ophthalmopathy, this effect of IgG is specific for PTM and may be responsible at least in part for the accumulation of proteoglycan in PTM.

The precaution was taken to minimize the effect of serum-derived growth factors by repeated rinsing of ammonium sulfate precipitant in the buffer and subsequent chromatography. In effect, the concentration of IGF-1 and PDGF was less than the detectable limits. However, their possible contribution to the present result due to contamination was not completely ruled out although it is unlikely that IGF-1 and PDGF in the PTM IgG sample are higher than those in IgGs of control subjects and patients with Graves’ disease with neither PTM nor ophthalmopathy.

The next question to be answered is whether this activity of PTM IgG to stimulate fibroblast proteoglycan synthesis is related to thyroid stimulating activity or not. As shown in Figs. 7 and 8, we could not detect the correlation of the two activities. We also examined the correlation employing the data summarized in Fig. 1. TBII and TSAb activities in sera. The result was the same as already shown. On the other hand, there was statistically significant correlation between TBII and TSAb activity (as shown in Fig. 10) and between TBII and the activity to stimulate proteoglycan synthesis in FRTL cells (Fig. 9). These results suggest the possibility that the antibody is different from the thyroid-stimulating autoantibody, although the likelihood of developing such an antibody is higher in patients who have a thyroid-stimulating autoantibody. This notion conflicts with the reports of Tao et al. (13), who concluded that the antibody activity leading to PTM is part of the thyroid stimulating antibody. The reason for the discrepancy is not apparent at present, but is possibly due to the difference in incubation conditions and to the methods of glycosaminoglycan analysis described above.

The mechanism by which PTM IgGs stimulate proteoglycans synthesis remains to be elucidated, but our results (shown in Figs 4 and 5), indicate that it is something other than the cAMP-dependent mechanism. Whatever it is, our result, together with our previous report demonstrating that thyroid hormone excess stimulates the synthesis of proteoglycans (27), favors the view that an autoantibody capable of stimulating skin proteoglycan synthesis, together with the effect of thyroid hormone excess, triggers development of the cutaneous changes leading to PTM.

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