Evidence for thyrotropin receptor immunoreactivity in pretibial connective tissue from patients with thyroid-associated dermopathy

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Abstract
Pretibial myxedema (PTM), mainly characterized by the accumulation of glycosaminoglycans in the dermis and subcutaneous tissue, is an extrathyroidal manifestation of autoimmune Graves’ disease (GD), almost always associated with Graves’ ophthalmopathy (GO). The thyrotropin receptor (TSH-R) has been proposed as the common target antigen in GD, GO and PTM, with evidence for receptor transcripts and/or protein in these locations. The aim of this study has been to investigate whether receptor protein is present in the pretibial tissues. Skin biopsies were obtained from two patients with PTM and two normal subjects without thyroid disease. A portion of each sample was fixed to produce semi-thin sections for Toluidine Blue or Periodic Acid Schiff (PAS) staining. The remainder was snap frozen to generate cryostat sections for immunohistochemical analysis using three monoclonal antibodies against TSH-R. In the skin from the two patients suffering from PTM, the dermis was infiltrated by inflammatory cells (lymphocytes, B cells, macrophages, mast cells) and adipocytes. The collagen fibers were dissociated by edema and by the accumulation of a PAS-positive material. Immunodetection of TSH-R produced positive staining on cells localized in the dermis, beneath the epidermis or close to the hypodermis. These cells were elongated and resembled fibroblasts. No immunoreactivity was observed in the dermis from control patients without thyroid disease. In conclusion, we have evidence for TSH-R immunoreactivity in the pretibium of patients with GD, GO and PTM. Further studies are needed to unambiguously identify the positive cells and determine whether the reactivity is due to the receptor itself or to a cross-reacting protein.

European Journal of Endocrinology 146 35–38

Introduction
Pretibial myxedema (PTM) is an uncommon manifestation of autoimmune thyroid disease almost always associated with Graves’ ophthalmopathy (GO) and found in an estimated 10–12% of these patients (1). Most affected patients have severe ophthalmopathy and high serum titers of thyrotropin receptor (TSH-R) antibodies. Both diseases are characterized histologically by the presence of large amounts of glycosaminoglycans (GAG), which in PTM are diffusely dispersed in the reticular part of the dermis (2). Although the pathogenesis is still controversial, the process is likely to be driven by T cells that access and infiltrate the orbital and pretibial spaces and release various cytokines capable of stimulating cell proliferation, GAG synthesis and expression of immunomodulatory molecules in microvascular endothelial cells, dendritic cells and fibroblasts. In support of this concept, orbital and pretibial fibroblasts have recently been shown to act as targets of the autoimmune process in GO and PTM (3). These histo- and immunopathological similarities might indicate the presence of a common autoantigen in thyroid, orbital and dermal tissues. Although this autoantigen has not been defined, a prime candidate is the TSH-R, an hypothesis that is supported by the demonstration of various TSH-R transcripts in cultured orbital and peripheral skin fibroblasts from patients with GO and PTM (4–10). The presence of TSH-R immunoreactivity has been described in the oculomotor muscles and adipose tissue from patients with GO (11–13) and in the dermis of one patient with PTM (14). In the present study, we show that TSH-R immunoreactivity is also present in the pretibial area in two patients with PTM, but absent in control patients without thyroid disease.

Materials and methods
Patients
Skin biopsies from two patients with Graves’ disease (GD), GO and PTM, and from two control subjects
who underwent thyroidectomy for multinodular goiter, were obtained after the patients gave their informed consent. Case 1 is a 47-year-old man, a smoker, treated with amiodarone for 2 years. GD was diagnosed in 1994. Anti-receptor antibody titers were high (140 U/l; normal value <12). Methimazole therapy was initiated, but active ophthalmopathy appeared two months after diagnosis. Six months later, the patient developed PTM consisting of multiple nodular plaques on both lower legs with non pitting edema and irregular redness. The ophthalmopathy became very severe, with signs of optic neuropathy requiring treatment with mega doses of steroids, retrobulbar radiotherapy and orbital decompression. A pretibial skin biopsy was performed in 1997. At that time, the thyroid status was stable under methimazole, although anti-receptor antibody levels remained slightly elevated (19 U/l). Case 2 is a 71-year-old woman, a smoker. She was regularly followed for immunoglobulin A (IgA) glomerulonephritis complicated by hypertension. Her GD began in 1993, and was treated by methimazole followed by radioiodine. PTM, consisting of purple, red nodular infiltrates appeared in 1994. At that time, the anti-receptor antibodies were very high (489 U/l), their levels decreasing with time: 24 U/l (1995), 13 U/l (1996), 8 U/l (1997). In May 1996, the patient developed a painful ophthalmopathy with oculomotor disorders and diplopia, successfully treated by retrobulbar radiotherapy. The PTM remained stable and a skin biopsy was obtained in 1997.

**Morphological analysis**

Skin biopsies from the two patients with GD, GO and PTM and from the two control patients were processed either for light microscopy or for immunohistochemistry, as previously described (15). The sample designed for light microscopy was immersed for 2 h, at room temperature, in 2.5% glutaraldehyde in 0.1 mol/l cacodylate buffer, postfixed for 1 h in 1% osmium tetroxide and embedded in LX 112 resin. Sections (0.5 μm-thick) were stained with Toluidine Blue or Periodic Acid Schiff (PAS).

The sample designed for immunohistochemistry was inserted into a liver fragment, embedded in Tissue-Tek and rapidly frozen in isopentane cooled in liquid nitrogen to generate 5 μm-thick cryostat sections. The frozen sections were subjected to indirect immunoperoxidase staining (15), using three different mouse monoclonal antibodies BA8, 3G4 and NCL-TSH-R2. BA8 and 3G4 have been generated by us, by genetic immunization (16). Both antibodies recognize the native human TSH-R expressed at the surface of CHO cells, in fluorescence-activated cell sorter (FACS) analysis. BA8 is specific for conformational epitopes present in the human receptor. 3G4 recognizes a linear epitope present in human, rat, mouse and dog receptors. Both antibodies, used at a dilution of 1:250, give a positive staining on the basolateral poles of human thyroid follicles (16).

NCL-TSH-R2 from Novocastra Laboratories (Newcastle-upon-Tyne, UK) is a mouse monoclonal antibody specific for human TSH-R, within the region of amino acid residues 211 to 414. It has been used at a dilution of 1:100. For the detection of fibroblasts, we have used a mouse anti-human monoclonal antibody at a dilution of 1:100 (clone 5B5, Dako, Copenhagen, Denmark). Different immunohistochemical controls were performed: by omission of the first monoclonal antibody, by omission of the first and second antibodies, or by incubation as first antibody with IgG2a of identical isotype as BA8 and NCL-TSH-R2.

Another immunodetection method used was the En Vision technique. The secondary antibody is a goat anti-mouse immunoglobulin conjugated to peroxidase labeled-polymer (Dako En Vision+). The chromogen used for revelation was AEC (3-amino-9 ethylcarbazole) giving a red staining.

**Results**

As compared with healthy patients (Fig. 1A), the skin from the two PTM patients showed dermal modifications. The upper part of the dermis comprised more cells whereas, in the lower part, collagen bundles were dense and dissociated by edema and a material probably made of glycosaminoglycans (Fig. 1B). In the two patients, the subepithelial dermis was infiltrated by T cells and among fibroblasts, some look like young cells with more cytoplasm and a lighter nucleus (Fig. 1C). In the deeper dermis with the dissociated collagen bundles, B cells, mast cells (Fig. 1D) and adipocytes (Fig. 1E) were frequently observed.

Immunohistochemistry with the 3 monoclonal antibodies against TSH-R did not give any labeling in the skin of the two control subjects (Fig. 1F and H). On the other hand, the 3 monoclonal antibodies gave the same positive labeling in patients 1 and 2 with PTM. Positive cells were mainly located beneath the epidermis (Fig. 1G). Most were elongated (Fig. 1I) and resembled typical fibroblasts, like those detected with a specific marker (Fig. 1J). Other cells expressing TSH-R were rounded and could correspond to the ‘young’ fibroblasts observed in Fig. 1C.

**Discussion and Conclusions**

In the present study, we demonstrate the presence of TSH-R immunoreactivity in pretibial tissue of two patients with PTM, GD and GO.

The thyrotropin receptor has been proposed as the common target auto-antigen in GD, thyroid eye disease (TED) and PTM (4). PTM, like GO, can develop following radioiodine treatment of Graves’ disease. In such cases, the levels of serum TSH-R antibodies are dramatically increased, lending further support to the idea that TSH-R is the target antigen (1). TSH-R expression is
Figure 1 (A and B) Sections of skin (0.5 μm-thick, Toluidine Blue stained) from a control subject (A, × 130) and from a patient with PTM (B, × 130). The upper part of the dermis comprises numerous cells whereas, in the lower part, collagen bundles are dense and dissociated by edema. (C–E) Sections of skin (0.5 μm-thick, Toluidine Blue stained) from patients suffering from thyroid dermopathy (× 300). The subepithelial dermis is infiltrated by T cells (*) and comprises ‘young’ fibroblasts (arrow) (C, × 400). In the deeper dermis, mast cells recognized by their dense granules (arrow), and B cells (*) (D, × 400) and adipocytes (E, × 300) are frequent. (F and G) Immunohistochemical labeling with 3G4 mAb. In the control patient, there are no positive cells (F, × 130). In the patient with PTM, positive cells are mainly located in the upper part of the dermis (G, × 130). (H–J) Immunohistochemical labeling with NCL-TSH-R2 (H,I) and with anti-fibroblasts mAb (J). In control subjects, fibroblasts do not express TSH-R (H, × 300). In patients with PTM, cells expressing TSH-R (I, × 400) are either elongated, like fibroblasts, specifically detected in (J) (× 200) or are more rounded.
increased in orbital tissue of GD patients with ophthalmopathy but not in normal orbital tissue (17). Evidence for TSH-R immunoreactivity in pretibium provides a strong argument in favor of that pathophysiological mechanism. As in GO (11), similar results are obtained with three different monoclonal antibodies. This increases the probability that the immunoreactivity is due to the receptor itself and not to a cross-reacting protein. Contrary to the observation of Rapoport et al. (14), no immunoreactivity against the TSH receptor was observed in the two control subjects. This difference may be related to the different mAbs used.

In GO, the presence of TSH-R transcripts provides a further argument for the up-regulation of expression (10). Similar information is difficult to obtain from the pretibium, as it would require fibroblast culture which in itself could modify gene expression.

Some investigators have suggested that dermopathy could be a generalized disorder accompanying GD (18). This view is supported by the fact that GAG levels are dramatically increased in the urine of patients with Graves’ disease and ophthalmopathy (19), an increase which is difficult to explain by production in the orbit and pretibium only.

In GO, the location and the morphology of TSH-R-expressing cells were considered evocative of their preadipocyte nature (11). This suggested a sequential process with proliferation of fibroblasts, GAG production, differentiation into preadipocytes and then into adipocytes. In the pretibium, the situation seems to be slightly different, as GAG production is evident but fat cells, although present, are less numerous. Possibly, the cytokines elaborated by lymphocytic infiltrate in the two locations might account for these differences.

In summary, our results provide complementary evidence that the TSH-R is the common autoantigen in GD, GO and PTM, but they provide no answers to the following questions: why are the orbital and pretibial tissues apparently the sole extrathyroidal sites involved in the pathological process? Conversely, why are GO and PTM not present in all cases of GD? Recently, Rapoport et al. (14) suggested that the autoimmune response to the TSH-R is necessary but not sufficient for the development of extrathyroidal manifestations. Graves’ dermopathy and ophthalmopathy follow the superimposition of local physical and anatomical factors (trauma, edema) on a background of a usually subclinical systemic connective tissue inflammation. This hypothesis was also supported by Davies (20).

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Received 3 April 2001
Accepted 26 September 2001