Why no simple relationship between thyroid peroxidase activity-inhibiting immunoglobulins and thyroid function in autoimmune thyroid disease?

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Abstract. We have reported that some anti-thyroid peroxidase antibodies inhibit the activity of thyroid peroxidase in vitro. These thyroid peroxidase activity-inhibiting immunoglobulins seem to inhibit thyroid function in some patients, but the relationship between thyroid peroxidase activity-inhibiting immunoglobulins and thyroid function is not simple. We designed this study to explore this lack of a simple relationship. We stained immunoglobulin G deposits by immunofluorescence staining or the peroxidase-antiperoxidase method, and stained endogenous thyroid peroxidase activity by enzyme histochemistry in thyroid sections. When cryostat thyroid sections were incubated with thyroid peroxidase activity-inhibiting immunoglobulins, immunoglobulin G deposits were seen as lines of stain on the apical border and as intracellular staining, and endogenous thyroid peroxidase activity was inhibited. In paraffin-embedded thyroid sections from 5 Hashimoto's patients and 6 Graves' patients, immunoglobulin G deposits were not found on the apical border of the follicular epithelium. In frozen thyroid sections from 22 Graves' patients, no clear deposits of immunoglobulin G on this apical border were seen. In organ-cultured thyroid slices incubated with thyroid peroxidase activity-inhibiting immunoglobulins, endogenous thyroid peroxidase activity was not inhibited. In conclusion, thyroid peroxidase activity-inhibiting immunoglobulins may reach its antigen only with difficulty. This is one of the reasons why no simple relationship is observed between thyroid peroxidase activity-inhibiting immunoglobulins and thyroid function.

Antibodies against human microsomal antigen are often present in serum of patients with autoimmune thyroid disease. The thyroid microsomal antigen recently was identified as thyroid peroxidase (TPO), which catalyzes the synthesis of thyroid hormone. This identification was based on immunological evidence (1-3), the comparison of the complementary DNA sequences for both proteins (4,5), and Western blot analysis for recombinant TPO (6). Some anti-TPO-ab inhibit the activity of TPO in vitro (7-11). These TPO activity-inhibiting immunoglobulins (TPII) seem to inhibit thyroid function in some patients with autoimmune thyroid disease, but the relationship between the TPII and thyroid function is not simple (9).

Khoury et al. (12) reported that thyroid microsomal antigen was restricted in vivo to the apical border of the follicular cells and might not be readily accessible to circulating antibodies. Nevertheless, they showed by direct immunofluorescence staining that thyroid microsomal antibodies (Mi-ab) reached and combined with the thyroid cell surface in vivo. Therefore, TPII should bind to TPO and inhibit TPO activity in vivo, inhibiting thyroid function.

To explain why the relationship between the TPII and thyroid function is not simple, we examined two possibilities: 1. TPII cannot inhibit TPO activity in vivo, even if it reaches its antigen; and 2. TPII cannot reach its antigen often enough to inhibit thyroid function in vivo.
Materials and Methods

Effects of TPII on TPO activity in cryostat sections

Human thyroid glands were obtained by surgery from 3 patients with hyperthyroid Graves' disease. These glands were immediately frozen in dry ice-acetone and stored at −80°C. In each case and in all subsequent experiments, the diagnosis was confirmed on clinical, laboratory, and histological grounds. Sera from these three patients with Graves' disease were negative for anti-microsomal antibodies; two of the three sera were negative for anti-thyroglobulin antibodies, and the other contained 1:1600 as assessed by a passive particle-agglutination test (Serodia AMC and ATG, Fuji Revio, Tokyo). IgG from an untreated patient with Hashimoto's disease and a healthy subject was purified by protein A (Pharmacia Fine Chemicals, Uppsala, Sweden) affinity chromatography. The TPII index of the patient's serum, assayed by a method previously described (9), was 0.71 (positive TPII >0.38). The patient's serum was negative for both anti-thyroglobulin antibodies and TSH-binding inhibitor immunoglobulin as assessed by a radioreceptor assay kits (Baxter Health Care Co Ltd, Tokyo). This patient was euthyroid. Serial cryostat sections, 4 and 10 μm thick, were washed three times in phosphate-buffered saline (PBS) for a total of 15 min and overlaid overnight at 4°C with 5 or 0.5 g/l IgG from this patient or a healthy subject. Sections were washed in PBS and those 4 μm thick were stained for human IgG by incubation with goat anti-human IgG conjugated with fluorescein isothiocyanate (FITC) (1:80 dilution; MBL, Nagoya, Japan) for 2 h at room temperature. One section, 4 μm thick, was incubated with mouse IgG monoclonal antibody against human thyroid microsomal antigen (1:200 dilution; kindly provided by Dr S. Hirakawa, Okayama University Medical School, Okayama, Japan) overnight at 4°C and stained for IgG by incubation with goat anti-mouse IgG conjugated with FITC (1:80 dilution; NBL, Nagoya, Japan) for 2 h at room temperature. Sections were viewed with a fluorescence microscope (Olympus, Tokyo, Japan). Sections, 10 μm thick, were stained for endogenous TPO activity by incubation with 0.05% 3,3'-diaminobenzidine tetrahydrochloride (DAB) and 0.0025% H2O2 for 30 min at room temperature. The solution of DAB was prepared by dissolving 75 mg of DAB (Wako Pure Chemical Industries, LTD, Osaka, Japan) in 150 ml of 0.05 mol/l TRIS-HCl buffer (pH 7.6). Sections were examined under a light microscope.

Peroxidase-antiperoxidase staining of paraffin-embedded thyroid sections

Sections of paraffin-embedded thyroid obtained from operative specimens of 6 patients with Graves' disease (4 men and 2 women) and 5 with Hashimoto's disease (5 women) were examined. The clinical data of these patients at the time of the operation are summarized in Table 1. The sections were dewaxed and stained for in vivo IgG deposits with a peroxidase-antiperoxidase (PAP)-complex kit (MILAB ICC-kit, Malmö, Sweden), according to the manufacturer's protocol.

Direct immunofluorescence staining of frozen thyroid sections

Operative specimens were obtained from 22 patients (Table 1) with Graves' disease (3 men and 19 women) and frozen in dry ice-acetone. A tonsil from a patient with a peritonsillar abscess was examined as a positive control for IgG deposits. Cryostat sections, 4 μm thick, were washed in PBS for a total of 15 min and incubated with goat anti-human IgG conjugated with FITC (1:80 dilution) for 2 h at room temperature. Sections were washed again in PBS for 15 min and examined.

Effects of TPII on TPO activity in cultured human thyroid slices

Organ culture was done as previously described (13); the IgGs from a patient positive for TPII and the healthy subject mentioned above were used. In brief, human thyroid glands were obtained by surgery from 2 patients with Graves' disease and immediately taken to our laboratory in Ham's F-10 synthetic culture medium (Flow Laboratories, McLean, VA). These 2 patients were negative for anti-microsomal antibodies and anti-thyroglobulin antibodies. Slices (0.5-1.0 mm thick, 5-25 mg) were made with a Stadie-Riggs microtome and put on grids in 750 μl of F-10 medium containing 5 g/l TPII-positive or normal IgG. After incubation in a humidified atmosphere of 5% CO2 in air for 2 h at room temperature, slices were washed three times in PBS and frozen in dry ice-acetone. Cryostat sections, 4 and 10 μm, respectively, were stained for IgG by direct immunofluorescence and endogenous TPO activity by the methods described above.

Results

Effects of TPII on TPO activity in cryostat sections

When cryostat sections from 3 Graves' thyroids were incubated with normal IgG or PBS, IgG deposits along the follicle basement membrane were seen in one thyroid from the patient with anti-thyroglobulin antibodies. In thyroids from the other two patients, without anti-thyroglobulin antibodies, no IgG deposits were seen along the follicle basement membrane, No IgG deposits on the follicular epithelial cells were seen in any of the thyroids; but in all of them endogenous TPO activity was clearly seen as brown lines on the apical border and intracellular staining of the follicular epithelial cells (Fig. 1A and B). When cryostat sections were incubated with TPII-positive IgG, IgG deposits were seen as lines of stain on the apical border and as intracel-
Effects of thyroid peroxidase activity-inhibiting immunoglobulins (TPII) on thyroid peroxidase (TPO) activity in cryostat sections. A cryostat thyroid section was incubated with normal IgG and stained for IgG by immunofluorescence (A) or endogenous TPO activity by enzyme histochemistry (B). When the cryostat section was incubated with TPII-positive IgG, IgG deposits on the follicular epithelium were seen (C), and the brown stain of endogenous TPO activity was fainter (D). Original magnification: × 200.

IgG deposits in paraffin-embedded thyroid sections
In 5 Hashimoto's thyroids, IgG on infiltrating lymphocytes was seen as brown staining (Fig. 2A); however, there were no deposits of IgG on the apical border of the follicular epithelium. In 6 Graves' thyroids, no IgG deposits were seen on the apical border of the follicular epithelium (Fig. 2B).

IgG deposits in frozen thyroid sections
Of the 22 Graves' thyroids stained by direct immunofluorescence, granular IgG deposits along the FBM were seen in 5 of the thyroids from 7 patients with anti-thyroglobulin antibodies (Fig. 3), but not in any thyroids from patients without (Table 1). No clear deposits of IgG on the apical border of the follicular epithelium were seen in any of the thyroids studied.

Effects of TPII on TPO activity in cultured human thyroid slices
In the thyroid slices cultured with normal IgG, immunofluorescence staining showed no IgG deposits, and endogenous TPO activity was clearly demonstrated (Fig. 4A and B). In the thyroid slices cultured with TPII, immunofluorescence staining showed linear deposits on the apical border of the
IgG deposits in paraffin-embedded thyroid sections. A paraffin section was stained for IgG by the peroxidase-antiperoxidase method. In Hashimoto's thyroid (A; Patient No. 1), IgG on infiltrating lymphocytes was seen as brown staining. However, deposits of IgG were not found on the apical border of the follicular epithelium in either Hashimoto's thyroid or Graves' thyroid (B; Patient No. 10). Original magnification: × 200.

The follicular epithelium only in marginal follicles that had been cut, not in unbroken follicles (Fig. 4C). There was no difference in endogenous TPO activity compared with the slices cultured with normal IgG (Fig. 4D). In marginal cut follicles to which TPII bound, we could not clearly see whether TPO activity on the apical border was inhibited, because intracellular staining of endogenous TPO activity was strong and diffuse.

Discussion

To understand the lack of a simple relationship between the TPII value and thyroid function, we examined two possibilities. First, can TPII inhibit the activity of TPO, an enzyme existing in its native form in the tissue? TPII inhibits the activity of TPO prepared by solubilization of the 105 000 × g pellet of thyroid homogenate with sodium deoxycholate and trypsin (9). In this study, TPII bound to TPO and inhibited TPO activity when cryostat thyroid sections were incubated with TPII. Therefore, if TPII has access to TPO in vivo, TPII should inhibit TPO activity.

Second, does TPII have access to TPO often enough to inhibit thyroid function in vivo? IgG deposits on the apical border of the follicular epithelium, probably where TPO catalyzes thyroid hormone formation, were not found in paraffin-embedded thyroid sections of patients with either Graves' or Hashimoto's disease by PAP staining, or in frozen thyroid sections from patients with Graves' disease by direct immunofluorescence staining. Thyroid microsomal antibodies have been thought to participate in thyroid cell damage by mediating complement-dependent antibody-mediated cytotoxicity (14) or antibody-dependent cell-mediated cytotoxicity (15). Follicular epithelial cells to which TPII binds may be destroyed by these mechanisms, and together with them any possible IgG deposits. So we examined the effects of TPII on TPO activity using organ-cultured thyroid slices. Such culture is an ideal method to observe the accessibility and the effects of TPII in vitro, because the structure of the follicles is conserved. Yamashita et al. (16) reported that Graves' IgG
Table 1.
Presence of IgG deposits in thyroid tissue in relation to clinical and serological features in 33 patients with autoimmune thyroid disease.

<table>
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<tr>
<th>No.</th>
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<th>Mi-ab (× 100)</th>
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Tg-ab: anti-thyroglobulin antibodies; Mi-ab: anti-microsomal antibodies; AB: apical border of follicular epithelial cells; FBM: follicle basement membrane; HD: Hashimoto's disease; GD: Graves' disease; -: negative; +: positive; ++: strongly positive.

stimulates the release of T₃ and the production of thyroidal cAMP from organ-cultured Graves' thyroid slices. Therefore, IgG should enter the cultured thyroid slices. No IgG deposits on the apical border of complete follicles were found and TPO activity was not inhibited when cultured thyroid slices were used. Therefore, TPII seemed not to reach TPO under these conditions. Khoury et al. (12) reported that thyroid microsomal antibodies reached and combined with the thyroid cell surface in vivo, as seen with direct immunofluorescence staining of frozen thyroid sections. In our immunofluorescence study, we could not find any in vivo IgG deposits on the apical border of the follicular epithelial cells, even when we changed the dilution of goat anti-human IgG conjugated with FITC to up to 1:10 or prolonged the incubation time to overnight (data not shown). So far, in vivo IgG deposits in the thyroids from patients with autoimmune thyroid disease have been studied by direct immunofluorescence staining in about six reports (12,17-20). There have been no reports of IgG deposits on the apical border of the follicular epithelium except by Khoury et al. (12).

In our direct immunofluorescence study, granular IgG deposits along the FBM were seen in 5 of 7 patients with anti-thyroglobulin antibodies. These IgG deposits may be immune complexes of thyroglobulin and anti-thyroglobulin antibodies, because thyroglobulin is released from the thyroid and so is readily accessible to circulating antibodies. Hanafusa et al. (21) reported that reconstituted thyroid follicles reverse their polarity in the presence of high concentrations of fetal calf serum, allowing the microvillus portion of the cell to be externalized. Therefore, IgG deposits along the follicle basement membrane also may be immune complexes of anti-TPO antibodies and TPO that exist on the externalized microvilli. However, we think they are not, because the finding of these IgG deposits was correlated with the finding of anti-thyroglobulin, not anti-microsomal antibodies. This finding may also suggest that reversal of thyroid epithelial polarity is an uncommon phenomenon in vivo.

In our previous report (9), thyroid function was lower in patients with Hashimoto's disease who had TPII than in those who did not. Therefore, the significance of anti-TPO antibodies may only be one expression of the immune reactivity in autoimmune thyroid disease. However, anti-TPO antibodies may work on follicles already broken by some other mechanisms.

In Graves' disease, it is believed that TSH receptor antibodies stimulates the synthesis of thyroid
hormone. We analysed the relationship among thyroid function, TPII and TSH-binding inhibitor immunoglobulin in patients with Graves' disease of the previous report (9) using multiple regression analysis. TPII had not significant effect on thyroid function (data not shown).

In conclusion, TPII may reach its antigen only with difficulty. This is one of the reasons why no simple relationship is observed between the TPII value and thyroid function.

References


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