Association of Antipituitary Antibody and Type 2 Iodothyronine Deiodinase Antibody in Patients with Autoimmune Thyroid Disease

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Abstract. Antipituitary antibody (APA) has been reported to be detected in patients with autoimmune thyroid disease. Type 2 iodothyronine deiodinase (D2) is expressed in both pituitary gland and thyroid gland. We studied the association of APA and D2 peptide antibody in patients with autoimmune thyroid disease. Rat pituitary gland homogenate and D2 peptide were used as antigens in the present study. APA and D2 peptide antibodies were measured by enzyme-linked immunosorbent assay (ELISA) in sera obtained from 42 patients with Hashimoto’s disease, 26 patients with Graves’ disease and 70 healthy control subjects. Moreover, D2 activity precipitation assay was performed in some patients with Hashimoto’s disease. APA and D2 peptide antibody were elevated in patients with Hashimoto’s disease and patients with Graves’ disease, compared with control subjects. APA was positive in 32.4% (22/68), D2 peptide antibody was positive in 26.5% (18/68) of patients with autoimmune thyroid disease. APA was positive in 31.0% (13/42) of patients with Hashimoto’s disease and 34.6% (9/26) of patients with Graves’ disease. D2 peptide antibody was positive in 26.2% (11/42) of patients with Hashimoto’s disease and 26.9% (7/26) of patients with Graves’ disease. D2 peptide antibody was correlated with APA in patients with autoimmune thyroid disease. Moreover, precipitation of D2 activity was increased in some patients with Hashimoto’s disease including a patient who also had idiopathic diabetes insipidus, and was correlated with D2 peptide antibody. These results suggest that D2 antibody may be associated with APA in patients with autoimmune thyroid disease.

Key words: Enzyme-linked immunosorbent assay, Immunoprecipitation, Graves’ disease, Hashimoto’s disease

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THE presence of antipituitary antibody (APA) in human sera was first described by Bottazo et al. in 1975 [1]. Thereafter, APA has been identified in sera of patients with pituitary disorders, including lymphocytic hypophysitis, pituitary hormone deficiency, pituitary tumors and empty sella syndrome [2–4]. These studies suggest a pathophysiological role of APA in various pituitary disorders. APA was detected by immunofluorescence (IF) method, using GH3 rat pituitary tumor cells and AtT-20 mouse pituitary tumor cells as antigens [5, 6]. Subsequently, APA has been measured by Western blot analysis [7] and enzyme-linked immunosorbent assay (ELISA) [8] using rat pituitary tissue as an antigen. Recently, human pituitary tissue has also been used as an antigen to detect APA [9].

Several attempts have been made to identify specific autoantigens for APA. Significant reduction of growth hormone (GH) values observed after mixing with APA positive serum suggests that GH in pituitary is an autoantigen for APA [3]. Recently, α-enolase [10, 11] and pituitary specific proteins, namely, pituitary gland specific factor 1a and 2 [12], have been suggested as antigens recognized by APA.

APA has also been detected in other autoimmune
endocrine disorders such as autoimmune thyroid disease including Hashimoto’s disease and Graves’ disease, suggesting a pathophysiological role for APA in those disorders [13–15]. Based on these results, one may speculate that APA may recognize a common antigen that is expressed in both pituitary gland and thyroid gland. Thyroid function is regulated by thyroid stimulating hormone (TSH) that is secreted from anterior pituitary. In patients with autoimmune thyroid disease, the presence of APA may interact with pituitary function, which may result in the development of thyroid dysfunction. It is therefore of considerable interest to study the pathophysiological role of APA in patients with autoimmune thyroid disease.

In order to bind to a nuclear receptor and exert its biological activity, thyroxine (T4), which is a major secretory product of thyroid gland, needs to be converted to 3,5,3’-triiodothyronine (T3) by selenocysteine containing oxidoreductases, namely, iodothyronine deiodinases [16]. Two types of iodothyronine deiodinase that catalyze the conversion of T4 to T3 have been identified. Type 1 iodothyronine deiodinase (D1) is present in thyroid gland, liver, kidney and many other tissues, whereas type 2 iodothyronine deiodinase (D2) is present in a limited number of tissues, including the central nervous system, pituitary gland, brown adipose tissue and pineal gland in the rat. Km of D2 is approximately 1–2 nM for T4, which is a hundred times lower than that of D1. D1, but not D2, is highly sensitive to inhibition by antithyroid drug 6-n-propylthiouracil (PTU). D1 activity is known to decrease in the hypothyroid state and is believed to have a primary role in maintaining circulating T3 levels. D2 activity, in contrast, increases in the hypothryoid state and is considered to play a critical role in providing local T3 to regulate intracellular T3 concentration. While the source of T3 mainly depends on the circulating T3 in most tissues, local intracellular conversion of T4 to T3 is an important source of intracellular T3 in certain tissues where D2 exists. In pituitary gland, the intracellular conversion of T4 to T3 by D2 plays a pivotal role in the negative feedback regulation of TSH secretion by thyroid hormones [16].

After the molecular cloning of D2 cDNA [17], we and other investigators reported that D2 mRNA was unexpectedly detected in human thyroid gland [18, 19] and other human tissues [20–22], suggesting previously unrecognized roles of D2 in those tissues. Recently, we have reported that D2 is also expressed in AtT-20 mouse pituitary tumor cells as well as GH3 rat pituitary tumor cells [23], which have been used to detect APA by IF method [5, 6]. Thus, D2 is present in human thyroid gland as well as pituitary gland including GH3 rat pituitary tumor cells and AtT-20 mouse pituitary tumor cells. D2 is a microsomal protein, and APA [13–15], as well as antimicrosomal antibody [24], has been detected in patients with autoimmune thyroid disease. Therefore, we hypothesize that D2 may be one of the candidates for antigens recognized by APA.

In the present study, we have attempted to examine the presence of anti-D2 antibody in sera of patients with autoimmune thyroid disease and studied the correlation between APA and anti-D2 antibody.

**Materials and Methods**

**Subjects**

Control sera were collected from 70 healthy volunteers (27 males and 43 females, aged 19 to 60 years). Serum samples were obtained from 42 patients with Hashimoto’s disease (7 males and 35 females, aged 6 to 83 years) and 26 patients with Graves’ disease (6 males and 20 females, aged 11 to 74 years). Diagnosis of Hashimoto’s disease was made on the basis of a diffusely enlarged firm thyroid gland and elevated titers of antimicrosomal antibody and/or antithyroglobulin antibody. Diagnosis of Graves’ disease was made on the basis of clinical hyperthyroidism with a diffusely enlarged thyroid gland, elevated serum free T4 and free T3 concentrations, suppressed TSH concentrations and increased levels of TSH receptor antibody (TRAb).

**Preparation of rat pituitary antigens**

Rat pituitary glands were obtained from Rockland Immunochemicals (Gilbertsville, PA) and 10 pituitary glands were homogenized in 3 ml of homogenizing buffer (0.25 M sucrose, 0.1 mM EDTA, 3 mM Tris-HCl buffer, pH 7.4) by a Polytron homogenizer (Brinkmann Instruments, Westbury, NY) and centrifuged at 10,000 × g at 4°C for 10 min. The resultant supernatant was used as the source of pituitary antigen for APA assay [8].

**Preparation of D2 peptide antigen**

D2 peptides corresponding to amino acid 132 to 152...
(TCPFSTQLPAFRKLVEEFSS) of human D2 [20, 25] including a catalytically active domain of the enzyme and amino acid 90 to 104 (SSTEGGDNSNGTQE) [20] were synthesized at the Peptide Institute, Osaka, Japan. A catalytically active domain of D2 contains selenocysteine, and is conserved among different species. Homology of rat and human D2 amino acid sequence in amino acid 132–152 is 90.5% and that in amino acid 90–104 is 26.7%. Selenocysteine was substituted with cysteine to synthesize D2 peptide (132–152).

**ELISA for APA and D2 peptide antibody**

APA and D2 peptide antibody were measured by ELISA as previously described [8]. Microtiter plates from Nunc-Immuno Module (Nunc A/S, Roskilde, Denmark) were coated with 100 μl of rat pituitary antigen (supernatant diluted to 25 μg/ml in 0.05 M sodium bicarbonate buffer, pH 9.6) or 100 μl of D2 peptide (132–152) or D2 peptide (90–104) (adjusted to 50 μg/ml in 0.05 M sodium bicarbonate buffer, pH 9.6) in each well by incubation overnight at 4°C. The plates were washed with 0.05% PBS-Tween 20 and blocked with equal volume of 100 μM potassium phosphate buffer containing 1 mM EDTA and 40 mM dithiothreitol (pH 7.0), centrifuged at 3,000 rpm at 4°C for 20 min and the supernatant was used for the assay. One hundred and twenty-five μl of pituitary homogenate was mixed with 10 μl of 0.25 mg IgG and incubated for 2 h at 4°C. One hundred μl samples were subsequently mixed with 100 μl of 0.4 mg IgG-SORB (protein A fixed to inactivated *Staphylococcus aureus*, Enzyme Center, Malden, MA) in 100 mM potassium phosphate buffer containing 1 mM EDTA (pH 7.0) to bind IgG and incubated for 15 min at 4°C. Subsequently, the samples were centrifuged for 5 min at 3,000 rpm at 4°C to precipitate IgG-SORB bound IgG. One hundred and twenty μl of supernatant was mixed with equal volume of 100 mM potassium phosphate buffer containing 1 mM EDTA and 40 mM dithiothreitol (pH 7.0), and used for the measurement of D2 activity.

D2 activity was measured as described previously [27]. Samples were incubated in a total volume of 50 μl with 2 nM of $[^{125}\text{I}]_{3}^{T_{4}}$, which was purified on the day of experiment, in 100 mM potassium phosphate buffer containing 1 mM EDTA and 20 mM dithiothreitol (pH 7.0) in the presence of 1 mM PTU at 37°C for 1 h. The reaction was terminated by the addition of 100 μl of 2% BSA and 800 μl of 10% trichloroacetic acid. The released $^{125}\text{I}$ was separated by column chromatography using AG 50W-X2 resin and counted. The protein concentration was determined by Bradford’s method using BSA as a standard [28]. The deiodinating activity was calculated as fmoles of I− released/mg protein/h after multiplying by a factor of 2 to correct the random labeling in the equivalent 3’ and 5’ positions. Precipitation of D2 activity was calculated as $\left(1 - \text{D2 activity in IgG precipitated sample/} \text{D2 activity in control sample without IgG} \right) \times 100$%.
Statistical analysis

Results are expressed as mean ± SD. Statistical difference was calculated by Student’s t test. A value of p<0.05 was accepted as indicating statistical significance.

Results

ELISA of APA and D2 peptide antibody in sera of control subjects

APA and D2 peptide antibody were measured by ELISA in sera of 70 control subjects who did not have goiter, antimicrosomal antibody, or antithyroglobulin antibody. Fig. 1A shows the distribution of APA in control subjects. APA values were 0.99 ± 0.50 (mean ± SD). When the cut-off value of APA was determined as 2.0 (mean + 2SD), APA was positive in two of 70 control subjects. Fig. 1B shows the distribution of D2 peptide (132–152) antibody in control subjects. D2 peptide antibody values in sera from control subjects were 0.90 ± 0.42 (mean ± SD). When the cut-off value of D2 peptide antibody was determined as 1.74 (mean + 2SD), D2 peptide antibody was positive in three of 70 control subjects. None of the control subjects showed positive results for both APA and D2 peptide antibody.

APA in patients with autoimmune thyroid disease

APA was measured by ELISA in serum samples of patients with Hashimoto’s disease or Graves’ disease. As shown in Fig. 2, APA was significantly higher in patients with Hashimoto’s disease (1.92 ± 1.09, mean ± SD) and in patients with Graves’ disease (1.58 ± 0.98) compared with control subjects (0.99 ± 0.50) (p<0.001), in agreement with previous observations [8, 13, 15]. When the cut-off value of APA was determined as 2.0, APA was positive in 22 of 68 (32.4%) patients with autoimmune thyroid disease, 13 of 42 (31.0%) patients with Hashimoto’s disease and 9 of 26 (34.6%) patients with Graves’ disease as shown in Table 1.

D2 peptide antibody in patients with autoimmune thyroid disease

D2 peptide antibody was measured by ELISA in serum samples of patients with Hashimoto’s disease or Graves’ disease. As shown in Fig. 3, D2 peptide antibody was significantly higher in patients with Hashimoto’s disease (2.09 ± 3.18, mean ± SD) and in patients with Graves’ disease (1.32 ± 0.71) compared with control subjects (0.90 ± 0.42) (p<0.001). When the cut-off value of D2 peptide antibody was determined as 1.74, D2 peptide antibody was positive in 18 of 68 (26.5%) patients with autoimmune thyroid disease, 11 of 42 (26.2%) patients with Hashimoto’s disease and 7 of 26 (26.9%) patients with Graves’ disease as shown in Table 1.
Correlation between APA and D2 peptide antibody in patients with autoimmune thyroid disease

The correlation between APA and D2 peptide antibody in patients with autoimmune thyroid disease was studied. As shown in Fig. 4, a relatively weak but positive correlation was observed between APA and D2 peptide antibody (r = 0.331, p<0.01). Patients who showed positive results for both APA and D2 peptide antibody were 9 of 68 (13.2%) patients with autoimmune thyroid disease, 6 of 42 (14.3%) patients with Hashimoto’s disease, and 3 of 26 (11.5%) patients with Graves’ disease as shown in Table 1.

Although APA was correlated with D2 peptide antibody, APA was not correlated with titers of antimitochondrial antibodies (r = 0.040, p>0.05) or antithyroglobulin antibodies (r = −0.073, p>0.05) in patients with Hashimoto’s disease, and TRAb (r = −0.229, p>0.05) in patients with Graves’ disease, in agreement with previous observation [15]. In addition, D2 peptide antibody was not correlated with titers of antimitochondrial antibodies (r = 0.173, p>0.05) or antithyroglobulin antibodies (r = 0.317, p>0.05) in patients with Hashimoto’s disease, and TRAb (r = −0.293, p>0.05) in patients with Graves’ disease.

Table 1. Number of patients who showed positive results for APA and D2 peptide antibody

<table>
<thead>
<tr>
<th></th>
<th>Hashimoto’s disease (n=42 total)</th>
<th>Graves’ disease (n=26 total)</th>
<th>Autoimmune thyroid disease (n=68 total)</th>
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<tbody>
<tr>
<td>APA</td>
<td>13 (31.0%)</td>
<td>9 (34.6%)</td>
<td>22 (32.4%)</td>
</tr>
<tr>
<td>D2 peptide Ab</td>
<td>11 (26.2%)</td>
<td>7 (26.9%)</td>
<td>18 (26.5%)</td>
</tr>
<tr>
<td>both Abs</td>
<td>6 (14.3%)</td>
<td>3 (11.5%)</td>
<td>9 (13.2%)</td>
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Fig. 2. APA in control subjects and patients with autoimmune thyroid disease. APA was measured in serum samples of 70 control subjects, 42 patients with Hashimoto’s disease and 26 patients with Graves’ disease by ELISA as described in Materials and Methods. *p<0.01.

Fig. 3. D2 peptide antibody in control subjects and patients with autoimmune thyroid disease. D2 peptide (132–152) antibody was measured in serum samples of 70 control subjects, 42 patients with Hashimoto’s disease and 26 patients with Graves’ disease by ELISA as described in Materials and Methods. *p<0.01.

Fig. 4. Correlation between APA and D2 peptide antibody in patients with autoimmune thyroid disease. APA and D2 peptide (132–152) antibody was measured in 42 patients with Hashimoto’s disease and 26 patients with Graves’ disease by ELISA as described in Materials and Methods.
Precipitation of D2 activity by IgG of patients with Hashimoto’s disease

Among the patients studied, the patient with Hashimoto’s disease who had the highest D2 peptide antibody of 20.87 (indicated as an open circle in Figs. 3 and 4) also showed a positive APA of 4.3. It was of considerable interest that this patient also had idiopathic diabetes insipidus. As demonstrated in Table 2, precipitation of D2 activity by IgG was studied in six patients with Hashimoto’s disease including this patient (designated as patient 6). Precipitation of D2 activity by IgG of six control subjects was 23.29 ± 2.64% (mean ± SD), and four of six patients with Hashimoto’s disease showed precipitation of D2 activity higher than 28.57% (mean + 2SD of six control subjects) as shown in Table 2. Precipitation of D2 activity in the patient with Hashimoto’s disease and idiopathic diabetes insipidus (patient 6) was markedly increased to 61.3%. There was a significant positive correlation between D2 peptide antibody and precipitation of D2 activity in these patients and control subjects (n = 12, r = 0.792, p<0.01).

Discussion

In the present study, APA measured by ELISA was significantly elevated in patients with autoimmune thyroid disease, such as Hashimoto’s disease and Graves’ disease, in agreement with previous observations [8, 13, 15]. APA was positive in 32.4% of patients with autoimmune thyroid disease, 31.0% of patients with Hashimoto’s disease and 34.6% of patients with Graves’ disease.

APA has been measured by different techniques including IF method using GH3 rat pituitary tumor cells and AtT-20 mouse pituitary tumor cells [5, 6], Western blot analysis [7] and ELISA [8]. APA has been identified in patients with pituitary disorders, such as lymphocytic hypophysitis, pituitary hormone deficiency, pituitary tumors and empty sella syndrome [2–4]. APA has also been detected in other autoimmune endocrine disorders such as autoimmune thyroid disease including Hashimoto’s disease and Graves’ disease [13–15]. These previous observations suggest a pathophysiological role for APA in those endocrine disorders. It was reported that APA was not correlated with thyroid autoantibodies [15]. In agreement with previous observation, APA was not significantly correlated with antimicrosomal antibody, antithyroglobulin antibody or TRAb in patients with autoimmune thyroid disease in the present results. In the present study, rat pituitary was used to detect APA because of the limitation of use of human pituitary tissue, although human pituitary tissue was a preferable antigen to detect APA in human sera [9].

Several studies were undertaken to identify responsible autoantigens for APA. To date, GH [3], α-enolase [10, 11] and pituitary specific proteins [12] have been suggested as candidates for antigens recognized by APA.

D2 is a selenodeiodinase, which has low Km for T$_4$, increases in the hypothyroid state and is considered to play a critical role in providing local T$_3$ to regulate intracellular T$_3$ concentration [16]. In the rat, D2 is expressed in the central nervous system, pituitary gland, brown adipose tissue, and pineal gland, but not in thyroid gland. In pituitary gland, intracellular production of T$_3$ from T$_4$ by D2 plays a pivotal role in suppressing TSH secretion by thyroid hormones. We and other investigators have demonstrated that D2 is unexpectedly
expressed in human thyroid gland [18, 19], and we have recently reported that D2 is expressed in AtT-20 mouse pituitary tumor cells as well as GH3 rat pituitary tumor cells [23], which have been used to detect APA by IF method. D2 is thus highly expressed in pituitary and human thyroid gland. D2 is a microsomal protein, and APA [13–15], as well as antimicrosomal antibody [24], has been detected in autoimmune thyroid disease. We therefore speculate that D2 may be another candidate for the antigen recognized by APA.

We have attempted to detect anti-D2 antibody using D2 peptide corresponding to amino acid 132–152 in patients with autoimmune thyroid disease [20]. Amino acid 132–152 is a selenocysteine containing catalytically active domain of D2, and is conserved among different species. The homology of rat and human D2 amino acid sequence in amino acid 132–152 is 90.5%. Autoantibody to D2 peptide has been reported to be increased in patients with Graves’ ophthalmopathy [25]. In the present study, D2 peptide antibody was elevated in patients with Hashimoto’s disease and patients with Graves’ disease, compared with control subjects. D2 peptide antibody was positive in 26.5% of patients with autoimmune thyroid disease, 26.2% of patients with Hashimoto’s disease and 26.9% of patients with Graves’ disease. D2 peptide antibody was not correlated with thyroid related autoantibodies, such as antimicrosomal antibody, antithyroglobulin antibody or TRAb in patients with autoimmune thyroid disease.

It is noteworthy that a relatively weak but positive correlation was observed between APA and D2 peptide antibody in patients with autoimmune thyroid disease in the present study. Moreover, patients who showed positive results for both APA and D2 peptide antibody were 9 of 68 (13.2%) patients with autoimmune thyroid disease, 6 of 42 (14.3%) patients with Hashimoto’s disease, and 3 of 26 (11.5%) patients with Graves’ disease. These results suggest the pathophysiological relationship between APA and D2 peptide antibody in patients with autoimmune thyroid disease. It is of interest that the patient with Hashimoto’s disease and idiopathic diabetes insipidus showed the highest D2 peptide antibody and a positive APA, suggesting that D2 may be a common autoantigen in thyroid gland for Hashimoto’s disease and in pituitary gland for idiopathic diabetes insipidus. To support this concept, D2 activity was reported to be present in the posterior lobe as well as in the anterior lobe of pituitary gland [29].

D2 activity precipitation assay was performed in the present study to measure antibody that could bind D2 enzyme. Precipitation of D2 activity was strikingly elevated in the patient with Hashimoto’s disease and idiopathic diabetes insipidus, and there was a positive correlation between precipitation of D2 activity and D2 peptide antibody in patients with Hashimoto’s disease and control subjects in the present study.

The presence of D2 peptide antibody in sera of patients with other disorders including pituitary disorders, that are known to have APA, remains to be elucidated in further studies. Although APA did not alter in the clinical course of autoimmune thyroid disease [15], it is of interest to investigate whether D2 peptide antibody changes in the clinical course of the disease.

In summary, the present study demonstrated that D2 peptide antibody was elevated in patients with autoimmune thyroid disease, and that a weak but significantly positive correlation was observed between APA and D2 peptide antibody in patients with autoimmune thyroid disease. These results suggest that D2 antibody may be associated with APA in patients with autoimmune thyroid disease.

Acknowledgments

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