NOTE

Application of Improved Coupling Assay Method for Peroxidase of Diseased Thyroids: Report of Three Cases

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Abstract. Recently we have developed an assay method for peroxidase-catalyzed coupling of iodothyronine residues of thyroglobulin, which is applicable to human diseased thyroid tissues. In the present study, the assay method as well as usual peroxidase assay methods were applied to thyroids of three patients (No. 1: familial goiter with impaired thyroglobulin synthesis, No. 2: mild chronic thyroiditis, No. 3: dyshormonogenetic goiter) who showed organification of iodine with high TSH levels and low thyroid hormone levels in sera. In general, these patients showed relatively high activities measured by guaiacol oxidation assay, iodide oxidation and coupling assay compared with those of control thyroids. Iodothyronine content in thyroglobulin was very low except thyroxine in No. 2. These results indicate that factors other than peroxidase may be responsible for the cause of the hypothyroid state. The coupling assay method used here is therefore useful for the detection of the 'coupling defect' in patients in a hypothyroid state.

IT IS WELL established that thyroid peroxidase (TPO) plays an important role in the biosynthesis of thyroid hormones by catalyzing the oxidation of iodide, the iodination of tyrosyl residues of thyroglobulin and the coupling of the iodothyrosyl residues [1]. Thus, there are many papers showing that impaired peroxidase in thyroids is related to some thyroid diseases [2, 3]. The assay for peroxidase activity in various thyroid diseases has been performed by a number of investigators, but determination of the coupling activity of the enzyme has scarcely been touched on. This is due to difficulty of the assay for coupling activity with the amount of thyroid tissues available [4]. So far, several clinical papers have reported patients who were considered to be suffering from a coupling defect [5–7]. However, the defect was not demonstrated since no attempt was made to measure coupling activity in these diseased thyroid tissues.

Recently, we have developed an assay method which is suitable for human thyroid tissues [4]. In the present study, this method was applied to measure the coupling activity of TPO from the thyroids of three patients. Although these three patients were all differently diagnosed, they had in common low levels of thyroid hormones in sera, and it was necessary to test the coupling activity as well as guaiacol and iodide oxidation.

Materials and Methods

Case reports

The first patient (No. 1) is a 33-yr old female who had a large goiter. Her personal history and clinical data were already given [8] and will be described here briefly. At the time of admission she was clinically hypothyroid: TSH, 57 μIU/ml...
(normal<9); thyroxine (T₄), 1.0 μg/100 ml (normal 5–13); 3,5,3'-triiodothyronine (T₃), 1.8 ng/ml (normal 1.0–2.0); and free T₄, 0.2 ng/100 ml (normal 0.8–2.4); free T₃, 7.4 pg/ml (2.5–6.0). However, iodine organification seemed normal since the thyroidal ¹²³I uptake was 59.8% and 54.5% before and after perchlorate administration. The ⁹⁹mTc scintigram showed increased uptake with several cold spots. The patient finally received total thyroidectomy. Histological examination showed that the thyroid consisted of microfollicular adenoma and cystic adenoma. She was diagnosed at that time as having familial goiter with impaired Tg synthesis. Genetic studies performed recently revealed that the Tg mRNA from the excised thyroid gland had about 200 nucleotides missing, probably the site corresponding to exon 4 of the Tg gene (Ieiri and Vassart et al., unpublished data).

The second patient (No. 2) a 14-yr old female was admitted to the clinic of Nippon Medical School because of a mild goiter with tenderness. She is one of twins and was born at 36 weeks of gestation, weighing 1450 g. Her mother and the twin sister had had Hashimoto's disease. The patient had febrile convulsion at the age of 1 and started to take anti-convulsant medication at the age of 5 when EEG showed an epileptic pattern. At the age of 14 she suffered from mild swelling in the region of the thyroid gland with tenderness and pain on swallowing, revealing hypothyroidism: serum TSH, 170 μU/ml; T₄, 3.2 μg/100 ml and T₃, 0.9 ng/ml. At the time of admission, she was clinically euthyroid: T₄, 8.5 μg/100 ml; T₃, 1.2 ng/100 ml; free T₄, 1.3 ng/100 ml, and serum TSH, 4.5 μU/ml, rising to 44 μU/ml, 15 min after TRH injection (200 μg iv). ¹²³I-Thyroid scintigraphy showed high density of both lobes and enlargement of the right lobe. Radioactive iodine uptake was much higher than the normal level, being 47.6% in 3 h and 88% in 24 h. Perchlorate administration showed no discharge of inorganic iodine. Anti-TSH receptor antibody test and TGHA were negative, but MCHA was 400. The histological examination showed the infiltration of a few lymphocytes into the interstitial tissues and no germinal center. Hyperplasia with high columnar epithelium was seen infrequently. It seemed to be almost normal or mild chronic thyroiditis.

The third patient (No. 3) is a 9-yr old male who had a mild goiter with a solitary nodule (4.0 × 3.0 cm) in the right lobe of the thyroid. He was of short stature (−2.25 S.D. from the mean), and his mental development was within the normal range. There is no family history of thyroid disease. At the time of admission he was latent hypothyroid: TSH, 13μU/ml; T₄, 5.0 μg/100 ml; T₃, 2.2 ng/ml; free T₄, 0.79 ng/100 ml; and free T₃, 5.1 pg/ml. Serum TSH (17 μU/ml) rose to 76 μU/ml, 15 min after TRH injection (5 μg/kg, iv). The ⁹⁹mTc scintigram showed a markedly increased uptake with a cold spot in the right lobe. However iodine organification seemed normal since the thyroidal ¹³¹I uptake was 26% and 38% before and after perchlorate administration. The patient underwent right hemithyroidectomy. A specimen of the left lobe was taken for biopsy. Histological examination of the nodule showed a dominantly trabecular/microfollicular pattern with scanty colloid. These features are compatible to those of follicular adenoma (embryonal type). The dominant pattern of the specimen of the left lobe was also microfollicular/simple follicular. These results suggest that in this thyroid there is dysshormogenic goiter rather than an adenoma.

Materials
The thyroids obtained at surgery were frozen immediately after surgery and stored at −70°C until use. Human thyroglobulin (Tg) used for the coupling assay was, starting from pooled Graves' thyroid tissues, purified by ammonium sulfate fractionation and DEAE-cellulose chromatography [9]. The Tg preparation which had 8.8 iodine atoms, 0.52 mol T₄ and 0.1 mol T₃ per molecule was then treated with chloramine T to have 41 iodine atoms per molecule as described previously [4].

Assay for guaiacol oxidation, iodide oxidation and coupling activities of human thyroids
Homogenization of thyroid tissues and assay for peroxidase activity were performed as described previously [10]. Briefly, human thyroid tissues (0.5–1 g) were homogenized with 20 mM Tris-HCl buffer, pH 7.4, containing 0.25 M sucrose, 40 mM NaCl, 10 mM MgCl₂, 100 mM KCl, and centrifuged at 700 × g for 10 min. The supernatant were further centrifuged at 105,000 × g for 60 min, and the pellets were suspended in the same buffer. For assay for guaiacol oxidation activity and iodide oxidation activity, the mitochondria-
microsomal fraction, after treatment with 0.7% sodium cholate at 4°C for 60 min, was used for the reaction mixtures described previously [10]. One guaiacol unit (GU) and iodide unit (IU) were defined as the enzyme amounts which produce one absorbance unit at 470 nm and 350 nm, respectively, per second under the conditions specified.

Coupling activity of TPO of human thyroids was measured essentially by the method of Ohmori et al. [4]. The mitochondrion-microsome suspension mentioned above was incubated with trypsin (50 BAEE unit/mg protein) plus sodium cholate (0.7% v/v) at 37°C for 60 min. After the incubation, trypsin inhibitor (1.5 mg/mg protein) was added to the mixture, followed by chilling (This sentence was erroneously omitted from the previous paper, [4]). The reaction mixture contained, in a total 0.5 ml of 50 mM sodium phosphate buffer, pH 7.4, chemically iodinated Tg (2 mg/ml), glucose (15 mM), diiodotyrosine (50 mM), glucose oxidase (0.05 µg/ml) and a suitable amount of the solubilized mitochondria-microsomes and incubated at 37°C for 15 min. Reaction was stopped by adding methyl mercaptoimidazole (2.5 mM) and the mixture was incubated with Pronase (3 mg/ml) at 37°C for 20 h for hydrolysis of Tg. Liberated iodothyronines were extracted with ethylacetate under acidic conditions and determined by reversed-phase HPLC as described previously [4]. One unit of coupling activity (CU) was defined as the formation of one iodothyronine residue per mol of Tg per min under the conditions.

Purification of Tg and determination of T₄, T₃ in Tg molecules

Preparation of Tg from thyroids of patients Nos. 1–3 was performed according to the method of Ui and Tarutani [9] with some modifications. Tg in cytosol from thyroids was precipitated with 1.75 M ammonium sulfate and further purified by DEAE-cellulose chromatography. Tg was hydrolyzed with Pronase, and the iodothyronines extracted were analyzed by reversed-phase HPLC [4].

Determination of protein and DNA

Protein and DNA were determined by the method of Lowry et al., [11] and Burton [12], respectively.

Results

The guaiacol oxidation and iodide oxidation activities of TPO in lesion portions from patients Nos. 1–3 and in the adjacent control portion from No. 3 were measured by the 'ordinary assay method' [10] and tabulated in Table 1. The values for protein-based specific activities obtained by guaiacol assay were comparable to those of normal human thyroids (30.0±5.7 mGU/mg protein (n=10), [10] except in the lesion of No. 2 and the adjacent control portion of No. 3 which had considerably low activities. The protein-based specific activities of lesions of these patients measured by iodide assay were 3–9 times higher than those of normal human thyroids (6.6±2.8 mIU/mg protein) (n=10) [10]. The table also shows activities per g tissue, which had a similar tendency in these tissues.

The values for the coupling activities of TPO in microsomes from the patients are also presented in Table 1. These values are markedly higher than those for porcine thyroids (1.82 CU/mg protein) [14] and those of adjacent control portions of an adenomatous goiter and Plummer disease (0.71 and 0.72 CU/mg protein, respectively, unpublished data).

The Tg of these patients was purified from their thyroids and subjected for determination of the amount of iodothyronines by HPLC as described previously [4]. As shown in Table 2, the lesion portions of No. 3 and No 1 had only a very small amount of iodothyronines or none at all in Tg in contrast to that of No. 2 which had almost the normal value. It is noteworthy that iodothyronines in control portion adjacent to No. 3 are rather low.

Discussion

Patient No. 1 with a large goiter was apparently in a hypothyroid state. As described in the previous paper [8], the major protein in the soluble fraction from the thyroid was an albumin-like protein and the minor components were immunoreactive Tg proteins, whose molecular size was smaller than 19S, and to a lesser degree 19S Tg. Most iodine atoms were incorporated into the albumin, and Tg was also slightly iodinated. The present study showed that the Tg contained practically no T₃ and only a very small amount of T₄ in the molecule. It was not determined whether
Table 1. Peroxidase activity and coupling activity of TPO of patients' thyroids.

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Sampling Portion</th>
<th>Guaiacol assay</th>
<th>Iodide assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(mGU/mg protein)</td>
<td>(mGU/g tissue)</td>
</tr>
<tr>
<td>1</td>
<td>Lesion</td>
<td>29.9</td>
<td>128.4</td>
</tr>
<tr>
<td>2</td>
<td>Lesion</td>
<td>11.6</td>
<td>106.2</td>
</tr>
<tr>
<td>3</td>
<td>Lesion</td>
<td>46.5</td>
<td>363.5</td>
</tr>
<tr>
<td></td>
<td>Adjacent control</td>
<td>5.2</td>
<td>73.8</td>
</tr>
</tbody>
</table>

Table 2. Content of iodothyronines in thyroglobulin from patients

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Sampling Portion</th>
<th>Iodothyronines (mol/mol Tg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>T$_3$</td>
</tr>
<tr>
<td>1</td>
<td>Lesion</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>Lesion</td>
<td>0.22</td>
</tr>
<tr>
<td>3</td>
<td>Lesion</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Adjacent control</td>
<td>0.036</td>
</tr>
</tbody>
</table>

the iodinated albumin contained iodothyronines, but the results in an earlier paper suggest that this may be the case [13]. The measurable amounts of T$_4$ and T$_3$ in serum may be explained by iodination of these proteins catalyzed by TPO which was found in the thyroid at a higher level than control. However, the amount of immunoreactive Tg protein in lesion portion was extremely low, and the thyroid hormone formation on albumin-like proteins is not efficient [14]. Thus, the hypothyroidism of the patient was considered to be caused by impaired Tg synthesis in spite of the normal level of TPO, iodine uptake and hydrogen-peroxide generating system.

Patient No. 2 had also a small goiter and was diagnosed as mild chronic thyroiditis. The thyroid function tended to fluctuate between a hypothyroid state and near euthyroid state. Iodine uptake observed in the almost euthyroid state reached a level of 48% at 3 h and 88% at 24 h, and the iodine atoms incorporated were not discharged following administration of perchlorate. These results raised the possibility that the hypothyroidism was caused by some failure in the coupling activity of iodothyrosine residues. However, the value for the TPO coupling activity of the patient was much higher than the values of normal tissues. Another cause of the disease should therefore be sought.

Patient No. 3 had a mild goiter with a solitary nodule and was in a latent hypothyroid state. Yet iodine uptake was enhanced and perchlorate discharge test was negative. A failure in coupling reaction was therefore suspected. However, the peroxidase activity and coupling in the lesion portion of the thyroid tissue obtained by partial thyroidectomy were rather high (Table 1), while no iodothyronine was found in the Tg of the patient (Table 2). On the other hand, the peroxidase activity and iodothyronine content of thyroglobulin in adjacent control portion of the tissue were of normal or lower levels. These results for patient No. 3 indicate that the hypothyroid state might be caused by factors other than a defect in peroxidase. Since the iodothyronine content even in the Tg of the normal portion was very low, one would expect an abnormal molecular structure of Tg or a defect in the H$_2$O$_2$-generating system etc. The paucity of the Tg, however, hampered further examination.

These three patients which were diagnosed
different are common in that they showed marked iodine organification with low levels of thyroid hormones in the sera. In such patients, a coupling defect is usually suspected as one of the causes. However, no direct evidence was ever offered because of the lack of an appropriate assay system. The assay method used here was found to be suitable for TPO from human abnormal thyroid tissue [4] and previously applied to thyroids with Graves’ disease [15]. The present studies are its second application to abnormal human thyroids. The values for coupling activity in the thyroids of two patients (Nos. 1 and 2) were not greatly different from those of the adjacent normal portion of adenomatous goiters, and may be regarded as the standard values for normal human thyroids in finding abnormal coupling activity in patients in the future.

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References