CLINICAL STUDY

Detection of autoantibodies against the pituitary-specific proteins in patients with lymphocytic hypophysitis

Susumu Tanaka, Ke-ita Tatsumi, Masahiro Kimura, Toru Takano, Yoshio Murakami1, Toshihiro Takao2, Kozo Hashimoto2, Yuzuru Kato1 and Nobuyuki Amino

Department of Laboratory Medicine, Osaka University Graduate School of Medicine D2, Suita-shi, Yama-oka 2-2, Osaka 565-0871, Japan, 1First Division, Department of Medicine, Shimane Medical University, Izumo, Japan and 2Second Department of Internal Medicine, Kochi Medical School, Kochi, Japan

(Correspondence should be addressed to Ke-ita Tatsumi; Email: tatsumi@labo.med.osaka-u.ac.jp)

Abstract

Objective: Several reports have described antipituitary antibodies by immunofluorescent or immunoblotting methods in patients with lymphocytic hypophysitis. However, with the exception of the pituitary hormones, individual antigens specific for the pituitary gland have not been studied. To understand the pathogenesis of lymphocytic hypophysitis and to diagnose this disease efficiently, we studied the presence of autoantibodies against three pituitary-specific proteins, GH and two novel pituitary-specific proteins, namely, pituitary gland specific factor 1a (PGSF1a) and PGSF2.

Design: Seventeen patients with lymphocytic hypophysitis, all of whom had pituitary enlargement (5 with lymphocytic adenohypophysitis and 12 with lymphocytic infundibuloneurohypophysitis, including 3 of the latter group proven by biopsy), and 14 patients with hypopituitarism without pituitary enlargement (10 with isolated ACTH deficiency and 4 with idiopathic TSH deficiency) were studied, and compared with 11 patients with non-functioning pituitary macroadenoma, 31 patients with other autoimmune diseases, and 36 healthy controls.

Methods: The presence of each antibody was studied by radioligand assay using recombinant human 35S-labeled protein.

Results: Three (18%) patients with lymphocytic hypophysitis having pituitary enlargement, five (36%) patients with hypopituitarism without pituitary enlargement and three (9.7%) patients with other autoimmune diseases were positive for one or more of the antibodies studied.

Conclusions: Anti-human GH, anti-PGSF1a, and anti-PGSF2 antibodies were detected in patients with lymphocytic hypophysitis and other hypopituitarism, but were not detected in patients with non-functioning pituitary macroadenoma. Detection of these antibodies may be useful for the diagnosis of lymphocytic hypophysitis.

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Introduction

Lymphocytic hypophysitis was first described in a postpartum patient in 1962 (1). Histological examination is required for the definite diagnosis of lymphocytic hypophysitis, and most pituitary glands of proven cases have been found to be enlarged, whereas a small number tended to be either atrophic or demonstrated as an empty sella (2, 3). In cases with enlarged pituitary glands, computed tomography and magnetic resonance imaging (MRI) improved the visualization of the pituitary gland, and gadolinium enhancement on MRI is helpful for obtaining a diagnosis of lymphocytic hypophysitis (4, 5). In order efficiently to distinguish lymphocytic hypophysitis from pituitary tumors and to identify the patients without enlarged pituitary glands, specific biochemical or serological markers are gaining increased attention.

Autoantibodies are useful for the diagnosis of various autoimmune diseases (6). In pituitary disorders, antipituitary antibodies have been sought to diagnose patients with lymphocytic hypophysitis. Antipituitary antibodies were originally detected by a complement fixation test in a study of Sheehan syndrome (7), followed by an immunofluorescent method using fresh-frozen human pituitary tissue (8) and animal pituitary tissue or cell lines. Recently, an immunoblotting method identified autoantigen of a 22 kDa protein and a 49 kDa protein as growth hormone (GH) and alpha-enolase respectively (9–11).

To elucidate the pathogenesis of lymphocytic hypophysitis, identification of antigens is important.
As autoantibodies against tissue-specific proteins are useful markers for various autoimmune diseases, we have recently identified two novel pituitary-specific proteins, pituitary gland specific factor 1a (PGSF1a) and PGSF2 (12), in order to assess their appropriate use as candidates for tissue-specific antigens.

To detect autoantibodies to novel proteins of which the cDNAs are available, the radioligand assay is useful (13–15). The radioligand assay involves producing 35S-labeled pituitary-specific proteins by in vitro transcription/translation and then using these proteins in the immunoprecipitation assay. The radioligand assay can detect autoantibodies directed against both linear and conformational peptides (16).

Here, we searched for autoantibodies to human GH (hGH), PGSF1a, and PGSF2 by radioligand assay in order to support the diagnosis of lymphocytic hypophysitis and other pituitary disorders.

Subjects and methods

Subjects

Serum samples were obtained from 17 patients with lymphocytic hypophysitis having pituitary enlargement (5 patients with lymphocytic adenohypophysitis and 12 patients with lymphocytic infundibuloneurohypophysitis (17), including 3 patients from the latter group with a proven diagnosis by biopsy), 14 patients with hypopituitarism without pituitary enlargement (10 patients with isolated adrenocorticotropin (ACTH) deficiency and 4 patients with idiopathic thyrotropin (TSH) deficiency), 11 patients with non-functioning pituitary macroadenoma, 31 patients with other autoimmune diseases (10 patients with Graves’ disease, 10 patients with Hashimoto’s thyroiditis, 7 patients with systemic lupus erythematosus (SLE), and 4 patients with mixed connective tissue disease), and 36 healthy controls.

Lymphocytic adenohypophysitis was suspected by pituitary dysfunction associated with an intrasellar mass which demonstrated gadolinium enhancement on MRI. Lymphocytic infundibuloneurohypophysitis was diagnosed by the presence of central diabetes insipidus, with swelling of the posterior pituitary or pituitary stalk on MRI. We obtained informed consent from all patients.

The mean ages and sex distribution are summarized in Table 1. The mean ages of the groups of patients were not significantly different from the mean age of the healthy controls.

Radioligand assay

Autoantibodies to hGH, PGSF1a, and PGSF2 were determined by radioligand assay according to previously described procedures (15). Briefly, cDNAs for each antigen were amplified from human pituitary gland cDNA by PCR using the following primer pairs containing either an EcoRI or a XhoI site: hGH 5’-GAATTCATGGCTACAGGCTCCCGGACGTC and 5’-CCGCTGAGTTTATTATTAGGACAAGGCTGGT; PGSF1a 5’-GAATTCCATGCACGGAATTAGGACAAGGCTGGT and 5’-CCGCTGAGTTTATTATTAGGACAAGGCTGGT; PGSF2 5’-GAATTCATGCACGGAATTAGGACAAGGCTGGT and 5’-CCGCTGAGTTTATTATTAGGACAAGGCTGGT. After amplification, the PCR products were digested with EcoRI and XhoI and ligated into the pET 28a (+) expression vector (Novagen, Madison, WI, USA). Antigens were translated in vitro using the TNT Quick Coupled Transcription/Translation System (Promega, Madison, WI, USA) and [35S]methionine (Amersham Pharmacia Biotech, Arlington Heights, IL, USA) according to the manufacturer’s instructions. The products were applied to a NAP-5 column (Amersham Pharmacia Biotech) and 100 μl fractions were collected. Collected fractions were analyzed by SDS-PAGE (15% polyacrylamide gel), and bands of 23 kDa, 16 kDa, and 27 kDa were detected by autoradiography corresponding to in vitro expression of hGH, PGSF1a, and PGSF2. The fractions

Table 1 Clinical characteristics of the patients and healthy controls.

<table>
<thead>
<tr>
<th>Subjects</th>
<th>n</th>
<th>Age (years) Mean±S.D.</th>
<th>Sex, no. male/ no. female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocytic hypophysitis with pituitary enlargement</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymphocytic adenohypophysitis (suspected by MRI)</td>
<td>5</td>
<td>31–77 51.3±22.9</td>
<td>1/4</td>
</tr>
<tr>
<td>Lymphocytic infundibuloneurohypophysitis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proven by biopsy</td>
<td>3</td>
<td>33–60 47.3±13.5</td>
<td>2/1</td>
</tr>
<tr>
<td>Suspected by MRI</td>
<td>9</td>
<td>14–62 43.3±17.1</td>
<td>5/4</td>
</tr>
<tr>
<td>Hypopituitarism without pituitary enlargement</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isolated ACTH deficiency</td>
<td>10</td>
<td>20–81 54.3±16.9</td>
<td>7/3</td>
</tr>
<tr>
<td>Idiopathic TSH deficiency</td>
<td>4</td>
<td>27–70 56.3±20.3</td>
<td>1/3</td>
</tr>
<tr>
<td>Non-functioning pituitary adenoma</td>
<td>11</td>
<td>36–75 53.4±11.8</td>
<td>1/10</td>
</tr>
<tr>
<td>Other autoimmune diseases</td>
<td>31</td>
<td>13–76 43.6±13.8</td>
<td>8/23</td>
</tr>
<tr>
<td>Healthy controls</td>
<td>36</td>
<td>24–74 49.9±13.8</td>
<td>18/18</td>
</tr>
</tbody>
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containing each of the $^{35}$S-labeled-antigens were diluted with reaction buffer (NaCl 150 mmol/l, Tris 50 mmol/l, pH 7.4. Tween-20 1 ml/l, BSA 4 g/l, and NaN$_3$ 1 g/l) and were stored at $-80^\circ$C until use.

A patient’s serum sample (diluted 1:12.5) and radiolabeled-human antigen were incubated overnight at 4°C. The labeled antigen–antibody complex was transferred onto a 96-well filtration plate (Multiscreen HVPP, 0.45 μm, Millipore Corp., Bedford, MA, USA), precipitated with Protein G-Sepharose 4 Fast Flow (Amersham Pharmacia Biotech), and blocked with blocking buffer (NaCl 150 mmol/l, Tris 50 mmol/l, pH 7.4, Tween-20 1 ml/l, BSA 30 g/l, and NaN$_3$ 1 g/l). The labeled antigen–antibody–Protein G-Sepharose complex was washed 10 times with washing buffer (NaCl 150 mmol/l, Tris 50 mmol/l, pH 7.4, Tween-20 10 ml/l) using a 96-well filtration system. The plate was then dried and OptiPhase SuperMix (Perkin Elmer Life Science, Boston, MA, USA) was added to each well. The quantity of precipitated, $^{35}$S-labeled-antigen was counted using a 1450 MicroBeta TriLux apparatus (Perkin Elmer Life Science). All samples were assayed in duplicate. Antibody levels were expressed as indexes, which were calculated as follows:

\[
\text{Antibody index} (U) = \frac{\text{counts per minute (c.p.m.) of the unknown serum}}{\text{c.p.m. of the pooled serum of healthy controls}}
\]

**Inhibition experiment**

To confirm the antibody specificity, an inhibition study was performed with positive and negative sera for anti-hGH antibodies obtained from patients with pituitary disorders. A patient’s serum sample and radiolabeled hGH were incubated together with 1 μg recombinant hGH (gift from NIHON Chemical Research Corp., Ashiya, Japan) or ovalbumin (Sigma Chemical Co., St Louis, MO, USA) overnight at 4°C, and the radioligand assay was carried out as described above.

**Statistical analysis**

Pearson’s test was used to analyze the correlation coefficient. A difference at $P < 0.05$ was considered to be significant.

**Results**

**Dilution curve**

Sera obtained from two patients with lymphocytic hypophysitis, one patient with isolated ACTH deficiency, and one patient with idiopathic TSH deficiency, all of which were positive for anti-hGH antibodies, were diluted in reaction buffer. Significant binding for all positive sera for anti-hGH antibodies were observed at dilutions of 1:12.5 (Fig. 1). Significant binding for PGSF1a and PGSF2 was also observed at dilutions of 1:12.5 (data not shown). Therefore, we decided to use a dilution of 1:12.5 in the subsequent experiments.

**Precision**

The within-run coefficient of variation (CV) was 3.6% ($n = 10$) for sera with a low anti-hGH antibody concentration (mean index, 1.29 U) and 4.9% ($n = 10$) for sera with a high anti-hGH antibody concentration (mean index, 5.87 U). The between-run CV was 5.2% ($n = 6$) for sera with a low anti-hGH antibody concentration and 4.6% ($n = 6$) for sera with a high anti-hGH antibody concentration. The within-run CV was 3.1% ($n = 6$, mean index, 1.58 U) and the between-run CV was 9.2% ($n = 6$) for sera with anti-PGSF1a antibodies.

**Figure 1** Dilution curves of anti-hGH antibody-positive and -negative sera obtained from patients with pituitary disorders. The anti-hGH antibody indexes of four anti-hGH antibody-positive (●) and two anti-hGH antibody-negative (○) sera are plotted as a function of serum dilution.
The within-run CV was 2.6% \((n = 6, \text{mean index, } 1.15 \text{U})\) and the between-run CV was 9.5\% \((n = 6)\) for sera with anti-PGSF2 antibodies.

**Inhibition study**

To exclude the possibility of non-specific reactions in the radioligand assay method, an inhibition study was performed. The anti-hGH antibody indexes containing positive antibodies were markedly decreased when reacted with recombinant hGH, whereas they were unchanged when reacted with ovalbumin as a control (Fig. 2).

**Antipituitary antibodies in pituitary disorders**

Using this radioligand assay, we examined serum samples obtained from patients with pituitary disorders and compared the results with those of other autoimmune diseases and healthy controls (Fig. 3). An antibody index greater than the cut-off value was considered to be positive. The cut-off values (anti-hGH antibodies 1.32 U; anti-PGSF1a antibodies 1.35 U; anti-PGSF2 antibodies 1.31 U) were calculated as the mean + 3 S.D. in healthy controls.

As shown in Fig. 3, three (18\%) patients with lymphocytic hypophysitis having pituitary enlargement, five (36\%) patients with hypopituitarism without pituitary enlargement and three (9.7\%) patients with other autoimmune diseases were positive for one or more of the antibodies studied. Anti-hGH antibodies were found in 20.0\% (1/5), 33.3\% (1/3), 10\% (1/10), 25\% (1/4), and 6.5\% (2/31) of patients with lymphocytic adenohypophysitis, lymphocytic infundibuloneurohypophysitis (biopsy-proven), isolated ACTH deficiency, idiopathic TSH deficiency, and other autoimmune diseases (Hashimoto’s thyroiditis and mixed connective tissue disease) respectively. Anti-PGSF1a antibodies were found in 33.3\% (1/3), 20.0\% (2/10), and 3.2\% (1/31) of patients with lymphocytic infundibuloneurohypophysitis (biopsy-proven), isolated ACTH deficiency, and other autoimmune disease (SLE) respectively. Anti-PGSF2 antibodies were found in 20.0\% (1/5), 11.1\% (1/9), 10.0\% (1/10), 50.0\% (2/4), and 6.5\% (2/31) of patients with lymphocytic adenohypophysitis, lymphocytic infundibuloneurohypophysitis (suspected by MRI), isolated ACTH deficiency, idiopathic TSH deficiency, and other autoimmune diseases (Hashimoto’s thyroiditis and SLE) respectively. Data from patients with pituitary diseases positive for these autoantibodies are summarized in Table 2.

**Relationship between anti-hGH antibody index and serum hGH concentration**

To determine whether or not serum GH concentrations were affected by anti-hGH antibody titers, the anti-hGH antibody index was compared with serum GH concentration. There was no relation between the two (Fig. 4).
Figure 3 Anti-hGH, anti-PGSF1a, and anti-PGSF2 antibody indexes in sera obtained from patients with pituitary disorders. (A) Anti-hGH antibody indexes are from sera of patients with lymphocytic adenohypophysitis \((n = 5)\), lymphocytic infundibuloneurohypophysitis proven by biopsy \((n = 3)\) and those suspected by MRI \((n = 9)\), isolated ACTH deficiency \((n = 10)\), idiopathic TSH deficiency \((n = 4)\), non-functioning pituitary macroadenoma \((n = 11)\), other autoimmune diseases \((n = 31)\), and sera obtained from healthy subjects \((n = 36)\) studied by the radioligand assay. Anti-hGH antibody indexes shown for each sample are the mean of at least two experiments. The horizontal dotted line shows the cut-off value of 1.32 U. (B) Anti-PGSF1a antibody indexes are shown as for the above mentioned patients. The horizontal dotted line shows the cut-off value of 1.35 U. (C) Anti-PGSF2 antibody indexes are shown as for the above mentioned patients. The horizontal dotted line shows the cut-off value of 1.31 U.
anti-PGSF1α antibody ($r = 0.752$, $P < 0.0001$, Fig. 5A) and with that of anti-PGSF2 antibody ($r = 0.640$, $P = 0.0003$, Fig. 5B). No correlation was found between the index of anti-PGSF1α antibody and that of anti-PGSF2 antibody (Fig. 5C).

**Discussion**

We detected autoantibodies against pituitary-specific antigens using a radioligand assay without using pituitary tissues, and quantitated these antibodies so as to help elucidate the pathogenesis of lymphocytic hypophysitis and to diagnose lymphocytic hypophysitis without aggressive surgery. Numerous studies have sought to identify antipituitary antibodies that could be used as noninvasive serological markers. Recently, an immunoblotting method was reported to diagnose lymphocytic hypophysitis efficiently (10, 18, 19). The method is capable of detecting multiple autoantigens at once. However, this method is qualitative and the size of the detected antigens and the frequency of antipituitary antibodies varies among reports, and is therefore difficult to standardize. The properties of the particular human pituitary tissues used could have exerted an influence on the overall results of this method. In contrast, the radioligand assay has advantages over immunoblotting. The radioligand assay has the merit of avoiding extraction and denaturation of proteins and of being totally objective. Moreover, the radioligand assay allows detection of high-affinity
autoantibodies directed against both linear and conformational peptides of diverse autoantigens.

Previously, anti-hGH antibodies have been reported by ELISA and an immunoblotting method in patients with various pituitary disorders (10, 20, 21), and anti-rat GH antibodies were detected in a rat model of an experimental hypophysitis in which rats were immunized with pituitary homogenates (22). These studies and the availability of purified hGH prompted us to investigate anti-hGH antibodies in order to evaluate the radioligand assay for detecting antipituitary antibodies. Anti-hGH antibodies were detected in four patients with pituitary disorders (Table 2; patients 1, 2, 4, and 7); an inhibition study using recombinant hGH confirmed that the current method specifically detected anti-hGH antibodies (Fig. 2). Using the radioligand assay, we demonstrated the presence of anti-hGH antibodies in patients with lymphocytic hypophysitis and in patients with hypopituitarism without pituitary enlargement.

In order to investigate autoantibodies to novel pituitary-specific proteins, we used PGSF1α and PGSF2 (12) as candidates. These proteins were recently identified as transcripts frequently expressed in the pituitary gland. PGSF1α is an entirely novel protein of 128 amino acids. PGSF2 is a splice variant of the immunoglobulin (Ig)-like domain containing 1 (IGDC1) (23), consisting of an N-terminal signal peptide, two Ig-like domains and a C-terminal transmembrane domain, and is a human counterpart for the recently identified rat inhibin binding protein-short isoform (InhBP-S) (24). In humans and rats, PGSF2/InhBP-S and IGDC1/InhBP-long isoform are expressed specifically in tissues with secretory granules.

Anti-PGSF1α antibodies and anti-PGSF2 antibodies were detected in three and five patients with lymphocytic hypophysitis and other hypopituitarism respectively (Table 2). As all patients with non-functioning pituitary macroadenoma were negative for anti-hGH, -PGSF1α, or -PGSF2 antibodies, detection of these antibodies may be useful for the differential diagnosis between lymphocytic hypophysitis and non-functioning pituitary macroadenoma.

It remains unclear whether the antibodies we detected are the cause or the result of lymphocytic hypophysitis. If these antibodies are the result, not the cause, of pituitary damage, one might expect a similar or higher frequency of antibodies in patients with pituitary tumors compared with those in patients with other pituitary disease since the degree of pituitary

Figure 5 Correlation between each of the antibody indexes (n = 31). (A) Anti-hGH antibody index vs anti-PGSF1α antibody index: r = 0.752, P < 0.0001. (B) Anti-hGH antibody index vs anti-PGSF2 antibody index: r = 0.640, P = 0.0003. (C) Anti-PGSF1α antibody index vs anti-PGSF2 antibody index.
destruction and antigen leakage from the pituitary might well be more extensive in the former. Thus our results suggest that the antibodies we detected might be the cause of lymphocytic hypophysitis.

Anti-PGSF1a antibodies and anti-PGSF2 antibodies did not appear together in patients with pituitary diseases, although all four patients positive for anti-hGH antibodies did carry either anti-PGSF1a antibodies or anti-PGSF2 antibodies. The existence of one or more autoantibodies also suggests that these patients harbor an autoimmune etiology. The independent appearance of anti-PGSF1a antibodies or anti-PGSF2 antibodies indicates that these autoantibodies may differently play a large role in the pathogenesis of the disease, functioning like GAD65 for insulin-dependent diabetes mellitus (25).

In the present study, patients with lymphocytic hypophysitis and those with hypopituitarism without pituitary enlargement were positive for one or more of the antibodies studied. In previous reports, in most of the cases of lymphocytic hypophysitis proven by histology or autopsy, the pituitaries were enlarged, whereas normal-sized or atrophied pituitaries and empty sella have also been observed (2); the latter patients are thought to suffer from a later stage of lymphocytic hypophysitis (3). Thus, most patients with hypopituitarism without pituitary enlargement, including isolated ACTH deficiency, might actually be at a late stage of lymphocytic hypophysitis.

Using a radioligand assay, we have identified autoantibodies to hGH and two novel pituitary-specific proteins in patients with lymphocytic hypophysitis, isolated ACTH deficiency, and idiopathic TSH deficiency. The radioligand assay effectively evaluates the existence of autoantibodies to other antigens expressed in the pituitary gland, and may therefore lead to an elucidation of the pathogenic antigens associated with lymphocytic hypophysitis.

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