A sensitive and practical bioassay for thyrotropin using cultured FRTL-5 cells: assessment of bioactivity for serum TSH in patients with chronic renal failure

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Abstract. A sensitive bioassay for TSH employing a practical extraction method was developed, and the bioactivities in patients with chronic renal failure receiving hemodialysis were compared with those in normal subjects. Serum samples were obtained from 12 normal subjects and 12 patients with chronic renal failure receiving hemodialysis. TSH was extracted from the serum using anti-human TSH monoclonal antibody coated tubes, followed by elution with 2.0 mol/l guanidine-HCl solution (pH 3.2). After the eluate had been dialyzed against phosphate buffered saline (pH 7.4) and again against TRIS-HCl solution (pH 7.4) and then lyophilized, it was reconstituted with hypotonic Hanks’ solution. Bioassay for TSH was performed by measuring the levels of cAMP released into the medium from cultured FRTL-5 cells incubated with the extract. The mean immunoreactive recovery rates of TSH from the serum in normal subjects and patients with chronic renal failure were about 42% (± 6) and 40% (± 2), respectively. The present bioassay was sufficiently sensitive to detect a serum TSH level of 1.0 mU/l when 3.0 ml of serum was used. Extracts from standard sera at concentrations ranging from 1.0 to 10 mU/l added to the culture medium caused significant linear increases in cAMP production. Based on analysis of covariance the regression line between the immunoreactivities and bioactivities of serum TSH in patients with chronic renal failure (y = 0.90x + 0.3, r = 0.92) was not significantly different from that in normal subjects (y = 1.04x + 0.1, r = 0.93). These results suggest that the present bioassay for TSH is sensitive and practical, and that the bioactivity of TSH in patients with chronic renal failure is similar to that in normal subjects.

Serum concentrations of T₄ and T₃ are often decreased in patients with severe nonthyroidal illnesses, and serum free T₄ concentrations are usually low normal or somewhat decreased (1,2). Serum TSH concentrations, however, are usually within the normal range and occasionally diminished or elevated, the response to TRH being blunted (2,3). In a recent study, Lee et al. (4) demonstrated that serum TSH in patients with nonthyroidal illnesses had altered glycosylation, which might be associated with a reduced biological activity. However, to our knowledge, there is no direct evidence of a reduced biological activity of serum TSH in such patients.

In the present study, a sensitive bioassay for serum TSH employing a practical extraction method was developed, and the biological activity of serum TSH was determined in patients with chronic renal failure receiving hemodialysis as a group with nonthyroidal illnesses.

Subjects and Methods

Subjects
The study groups included 12 patients (8 men and 4 women) with chronic renal failure receiving hemodialysis, aged 20 to 72 years, and 12 normal subjects (10 men and 2 women), aged 27 to 65 years.

The patients with chronic renal failure had received
regular 4-h hemodialysis twice or three times a week for from three to twelve months prior to the study. They were infused with about 4000 units of heparin on each hemodialysis, and had not received glucocorticoids or dopamine before the study. Sample sera from the patients were obtained just before regular hemodialysis, and at least 44 h after hemodialysis.

In the patients with chronic renal failure, serum $T_4$ levels, measured by the commercial RIA kit, ranged from 0.52 to 1.28 nmol/l, and were all below the normal levels (normal range: 1.32 to 2.52). Serum $T_4$ levels, measured by the RIA kit, were widely scattered from 34.2 to 107.8 nmol/l, and 5 patients had low $T_4$ levels (normal range: 59.1 to 176.1). There was no significant relationship between concentrations of $T_4$ or $T_3$ and the duration of the dialysis in this study.

Methods

Extraction of serum TSH was performed by employing a single anti-human TSH β-subunit monoclonal antibody coated on the plastic tube used in the RIA-gnost® hTSH kit of an immunoradiometric assay (IRMA) kit (Hoechst Japan, Tokyo, Japan). TSH free serum was obtained from Hoechst Japan, which was utilized in the RIA-gnost hTSH kit.

Characterization of the monoclonal antibody used in extraction of serum TSH. This antibody recognized only one TSH epitope and is not considered to be related to changes in TSH glycosylation (5–7). The percent cross-reactivity was lower than 0.01%. It was based on the unit ratio of the anti-TSH antibody for FSH and LH, calculated as the percentage of observed concentration of TSH/concentration of cross-reactant. The affinity of the antibody was strong enough for the concentration of TSH in the serum, which was incubated with the tube about 2 h, to be less than 10% of that before the incubation when serum TSH was from 1.0 to 20 mU/l.

Extraction of TSH. Extraction of serum TSH was performed by employing the anti-human TSH β-subunit monoclonal antibody coated on the plastic tube used in the RIA-gnost hTSH kit. 3.0 ml of serum or human TSH (from pituitary: Sigma Chemical Co, St. Louis, MO) dissolved in 3.0 ml of TSH free serum (standard serum) was divided into three aliquots and each aliquot (1.0 ml) was applied to an antibody-coated tube. The tubes were kept overnight at room temperature. After decantation of the serum, the tubes were washed twice with 1.0 ml of 0.9% saline solution. The TSH bound to the antibody was eluted by adding 0.4 ml of 2.0 mol/l guanidine-HCl solution (pH 3.2) to the tubes. The eluates from three aliquots were collected and immediately dialyzed against 0.01 mol/l phosphate buffer containing 0.15 mol/l saline (pH 7.4) for 4 h at 4°C, and again against 0.02 mol/l TRIS-HCl buffer (pH 7.4) overnight at 4°C. (8). After the dialysates had been lyophilized, they were reconstituted with 1.3 ml of hypotonic Hanks’ solution containing 1.5% bovine serum albumin, 0.02 mol/l HEPES, and 0.5 mmol/l 3-isobutyl-1-methylxanthine (9). The reconstituted samples (the extracts) were stored at −20°C before use and the assay was performed within 2 weeks.

Bioassay for TSH. The bioassay for TSH was performed by measuring the levels of cAMP released into the medium from cultured rat thyroid (FRTL-5) cells (10), incubated with the extracts. The cloned FRTL-5 cells were allowed to grow in Coon’s modified Ham’s F-12 medium supplemented with 5% calf serum with 6-hormone (6H) mixture consisting of bovine insulin, hydrocortisone, transferrin, somatostatin, glycyL-L-histidyl-L-lysine acteate and bovine TSH until the cells were confluent in plastic culture dishes (Beckton-Dickinson & Co, Lincoln Park, NJ). They were then allowed to remain in the same medium without bovine TSH (5H) for the next 5–10 days. After subsequent digestion with Hanks’ solution containing collagenase, trypsin, and chicken serum, the dispersed cells were suspended in the 5H medium, adjusting the cell count to (8–10) × 10^4 cells in 0.35 ml of the medium. Then, 0.35 ml of the suspension was poured to each of the 24 wells in tissue culture dishes (Beckton-Dickinson & Co). After overnight incubation at 37°C, the supernatant was removed and 0.3 ml of the extract was added to the wells, followed by incubation for 2 h. The levels of extracellular cAMP were measured with a commercial RIA kit (Yamasashi Shoyu, Chiba, Japan). All measurements were run in duplicate. The bioactivity of TSH was expressed as the standard TSH equivalent (mU/l) calculated from the levels of cAMP.

Measurement of TSH immunoreactivity. As well as with the RIA-gnost hTSH kit, the immunoreactivity for TSH of the extract and serum was measured with the Magic TSH kit of an IRMA kit (Corning, Chiba, Japan), in which anti-human TSH polyclonal antibody different from the monoclonal antibody used in the extraction method was employed. The percent cross-reactivity of the antibody of the Magic TSH kit for FSH and LH was lower than 0.1%, and the affinity of the antibody was as good as that of the RIA-gnost hTSH kit.

Statistical analysis

Between-group comparisons of mean values and differences in regression lines were performed by the unpaired Student’s $t$-test and analysis of covariance, respectively. Correlation coefficients were calculated by the standard technique.

Results

There was a significant linear relation between serum TSH concentrations, measured by the Magic TSH kit, and those, measured by the RIA-gnost hTSH kit, in normal subjects ($y = 0.76x + 0.3$, where $x$ is the concentration of TSH measured by the Magic TSH kit, and $y$ is the concentration of TSH measured by the RIA-gnost hTSH kit.)
r = 0.98, P < 0.01) and in patients with chronic renal failure receiving hemodialysis (y = 0.84x + 0.1, r = 0.99, P < 0.01). Similarly, TSH concentrations of the extract, measured by both IRMA kits, correlated well in normal subjects (y = 0.84x + 0.2, r = 0.99, P < 0.01) and in patients with chronic renal failure receiving hemodialysis (y = 0.85x + 0.2, r = 0.99, P < 0.01). Furthermore, the mean immunoreactive recovery rate measured by the Magic TSH kit, and that measured by the Diagnost hTSH kit were not significantly different in the two populations (42 ± 6% and 46 ± 7% in normal subjects, and 40 ± 2% and 42 ± 4% in patients with chronic renal failure, respectively; mean ± SEM). Therefore, only the values measured by the Magic TSH kit are shown for TSH immunoreactivity in the following results.

**Extraction of TSH**

About 70% of serum TSH bound to the antibody was eluted by adding guanidine-HCl solution, and about 60% of the eluted TSH was recovered after dialysis and lyophilization, as measured by the immunoassay. The immunoreactive recovery rates of TSH from the serum in normal subjects and in patients with chronic renal failure ranged from 35 to 48% and from 37 to 43%, averaging 42% (± 6) and 40% (± 2), respectively. Those of human pituitary TSH (standard serum) averaged 43% (± 3), ranging from 40 to 46%. The mean recovery rates were not significantly different for the three types of TSH. In normal subjects, a significant linear relation was observed between the TSH values, as determined by the immunoassay, before and after extraction (y = 0.48x - 0.2, r = 0.99, P < 0.01). Similarly, a direct relation was observed in patients with chronic renal failure (y = 0.42x, r = 0.99, P < 0.01).

**Bioassay of TSH**

Addition of extracts from the standard serum (1.0 to 10 mU/l) into the culture medium resulted in significant increases in the levels of cAMP released from the cultured FRTL-5 cells (Fig. 1). The levels of cAMP in the medium incubated with extracts from standard serum with 1.0 mU/l were significantly higher than those with no TSH, as measured by triplicate determinations (Fig. 1). Therefore, the minimum detectable concentration of TSH in the standard serum was considered to be about 1.0 mU/l. Serial dilutions of the sera, obtained from normal subjects, yielded curves parallel to the standard curve (Fig. 2). Addition of 5 and 10 mU/l of standard TSH to two sera of normal subjects gave 119 and 91% of the calculated TSH equivalent.
in the present assay. The intra-assay coefficients of variation ranged from 10.8 to 16.5%, with a mean of 14.2%. The inter-assay coefficients of variation ranged from 8.3 to 30.1%, with a mean of 18.0%.

**Bioactivity of TSH**

Fig. 3A shows a significant linear relation between the serum TSH values, measured by the immunnoassay, and the bioactivities of the TSH, measured by the present assay, in 12 normal subjects ($y = 1.04x + 0.1$, $r = 0.93$, $P < 0.01$). Similarly, a close relation between the serum TSH values and the bioactivities of the TSH was evident in 12 patients with chronic renal failure ($y = 0.90x + 0.3$, $r = 0.92$, $P < 0.01$) (Fig. 3B). The regression line between the immunoreactivities and the bioactivities of the serum TSH in the patients with chronic renal failure was not significantly different from that in the normal subjects on the basis of analysis of covariance.

**Discussion**

It is well known that bioassays for TSH have limited measurement sensitivity and precision (11). Although the cytochemical bioassay is sufficiently sensitive for measuring normal or even suppressed levels of TSH in serum (12), the procedure is rather difficult to perform and the equipment required for the assay is available only in a limited number of laboratories.

Dahlberg et al. (13) developed a bioassay based on cAMP production using FRTL-5 rat thyroid cells in which normal serum TSH levels could be detected. More recently, Nissim et al. (14) reported a sensitive bioassay involving iodine uptake in FRTL-5 cells. They employed an immunofinity chromatography extraction method, in which the antibody to the α-subunit of chorionic gonadotropin was coupled and packed in the column, for partial purification of serum TSH. Up to 400 times purification of the extracts was achieved, as evaluated from the serum TSH levels, and interfering substance(s) in the serum may therefore also have been concentrated by their method. The advantages of the present method are that the extraction is specific for human TSH, the procedure requires no high degree concentration of the serum, and the extraction procedure is simple to perform.

The monoclonal antibody recognizes only one
epitope as antigen. This epitope is not considered to be affected by changes in TSH glycosylation, since no changes have been reported in TSH immunoreactivity caused by changes in TSH glycosylation (5–7). If the antibody used in the present extraction method binds to deglycosylated TSH and glycosylated TSH differently, and if the amount of deglycosylated TSH in patients with nonthyroidal illnesses is different from that in normal subjects, then the mean immunoreactive recovery rate in these patients would be different from that in normal subjects. However, the mean recovery rate in the patients with chronic renal failure was similar to that in normal subjects, measured both by the RIA-ghost employing a monoclonal antibody and by the Magic TSH employing a polyclonal antibody. Therefore, the monoclonal antibody is likely binding to deglycosylated TSH as well as glycosylated TSH.

The anti-TSH monoclonal antibody employed in this extraction was highly specific for human TSH. An elution procedure with guanidine-HCl, immediately followed by dialysis against phosphate buffered saline and TRIS-HCl solution, and by lyophilization, had been shown previously not to affect the biological activity of human TSH (8). The mean immunoreactive recovery rates of serum TSH were somewhat low in both normal subjects and patients with chronic renal failure. However, the mean immunoreactive recovery rate of human pituitary TSH was similar to that of serum TSH in normal subjects and in patients with chronic renal failure, though TSH is a heterogeneous molecule in its physicochemical characteristics (15,16). Therefore, the bioactivities determined in the present bioassay were considered to reflect the bioactivities of whole serum TSH.

In patients with severe nonthyroidal illnesses the TSH concentrations are usually within the normal range despite low serum thyroid hormone concentrations (2). In the present study, the bioactivities of TSH in patients with chronic renal failure were not reduced as compared with those in normal subjects, suggesting that reduced biological activities of TSH are not responsible for decreased thyroid hormone concentrations in these patients.

The present bioassay for TSH is thus sensitive and practical, and the bioactivities of TSH in patients with chronic renal failure are considered to be similar to those in normal subjects.

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