Comparison of a Novel Homogeneous Cyclic Amp Assay and a Luciferase Assay for Measuring Stimulating Thyrotropin-Receptor Autoantibodies

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Keywords

Cyclic adenosine monophosphate assay · Cell-based bioassay · Graves' disease · Stimulating thyrotropin-receptor autoantibodies

Abstract

Objective: Stimulating thyrotropin-receptor antibodies (TSAb) cause Graves' disease (GD). We tested a novel homogeneous fluorescent 3',5' cyclic adenine monophosphate (cAMP) assay for the detection of TSAb in a bioassay. **Methods:** Chinese hamster ovary (CHO) cell lines expressing either a chimeric (MC4) or wild-type (WT) TSH-R were incubated with the adenyl cyclase activator forskolin, a human TSAb monoclonal antibody (M22), and with sera from GD patients. Intracellular cAMP levels were measured using a Bridge-It[®] cAMP assay, and the results were compared with a luciferase-based bioassay. **Results:** Both cell lines were stimulated with forskolin concentrations (0.006–200 μ M) in a dose-dependent manner. The linear range in the MC4 and WT cells was 0.8–25 and 3.1–50 μ M, respectively. Levels of cAMP and luciferase in forskolin-treated MC4 and WT cells were positively correlated ($r = 0.91$ and 0.84 , both $p < 0.001$). The 50% maximum stimulatory concentration of forskolin was more than 16-fold higher for the CHO-WT cells than the CHO-

MC4 cells in the cAMP assay and 4-fold higher in the luciferase assay. Incubation of both cell lines with M22 (0.006–50 ng/mL) resulted in a dose-dependent increase in cAMP levels with linear ranges for the MC4 and WT cells of 0.8–12.5 and 0.2–3.125 ng/mL, respectively. Comparison of cAMP and luciferase levels in M22-treated MC4 and WT cells also showed a positive correlation ($r = 0.88$, $p < 0.001$ and 0.75 , $p = 0.002$). A positive correlation was also noted when using patient samples ($r = 0.96$, $p < 0.001$) that were all TSH-R-Ab binding assay positive. **Conclusion:** The novel, rapid, simple-to-perform cAMP assay provides TSAb-mediated stimulatory results comparable to a luciferase-based bioassay.

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Introduction

Autoantibodies against the thyrotropin receptor (TSH-R-Ab) play an important role in the pathogenesis of autoimmune thyroid diseases [1–5]. These functional antibodies demonstrate different effects on the TSH-R and can either stimulate or block the TSH-R [6]. Stimulating TSH-R-Ab (TSAb) mediate the hyperthyroidism of Graves' disease (GD) by activating the 3',5' cyclic adenine monophosphate (cAMP) signal transduction pathway

leading to stimulation of thyroid hormone production and proliferation of thyrocytes in an unregulated manner [7–11]. In contrast, blocking TSH-R-Ab (TBAb) bind to the TSH-R and block TSH activation of the cAMP pathway which can lead to hypothyroidism [12–14]. Both TSH-R-Ab activities can be present in the same patient [15]. A number of TSH-R-Ab immunoassays have been developed, but only bioassays for TSH-R-Ab, regardless of the readout used, are able to distinguish between the functional TSH-R-Ab activities [16–18].

The second messenger cAMP is involved in different biological processes; i.e., the regulation of metabolism and transcription [19, 20]. Early studies on the serum of patients with GD revealed an increase in cAMP production using crude human thyroid-derived plasma membranes [21]. Human thyroid adenyl cyclase (AC) stimulator activity was identified by incubating human thyroid plasma membranes with TSH or IgG from patients with GD and unlabeled adenosine triphosphate [21]. Subsequent studies measured TSAb-induced AC activity using human thyroid plasma membranes and a non-hydrolyzable guanosine triphosphate analogue, guanylyl-5'-yl imidodiphosphate [22]. Historically, bioassays for TSAb measured cAMP levels in cells using radioimmunoassays and more recently non-radiometric immunoassays [23–27]. To avoid the washing steps of immunoassays and simplify the readout, bioassays employing luciferase expression have been developed for the detection of either TSAb [2, 8, 9] or TBAb [12, 13] using Chinese hamster ovary (CHO) cells expressing a chimeric human TSH-R. In addition, CHO cells expressing the wild-type (WT) TSH-R and a cAMP-responsive luciferase reporter were utilized to measure TSAb [28] and TBAb [29]. These bioassays are based on the transcription of a luciferase gene controlled by the cAMP-response element-binding protein [8, 12]. Recently, novel assays have been developed that enable single-step, homogeneous measurement of cyclic AMP levels [30], and novel biosensors have been developed that allow for real-time measurement of cAMP dynamics inside live cells [31–33]. For example, a cyclic nucleotide-gated calcium channel and aequorin was used to develop a live-cell TSAb bioassay [34].

The aim of this study was to directly compare a novel homogeneous fluorescent assay for cAMP with luciferase for measuring TSAb using CHO cells engineered to express either a WT-TSHR or a chimeric TSHR (MC4) [8, 35, 36]. Levels of cAMP and luciferase were measured following treatment of cells with a nonspecific cAMP inducer, a monoclonal TSAb, and sera from hyperthyroid patients containing TSAb.

Methods

Subjects

A total of 33 untreated hyperthyroid patients with GD were enrolled. GD was defined as biochemical hyperthyroidism with positive thyroid-binding inhibitory immunoglobulins (TBII; Cobas e411 analyzer; Roche Diagnostics, Mannheim, Germany) and an enlarged gland with a typical “thyroid storm pattern” on Doppler ultrasound.

Dilutions of Forskolin, M22 MAb Bovine TSH, and Patient Serum

A stock solution of forskolin (10 mg, Cat # F6886; Sigma-Aldrich, St. Louis, MO, USA) was prepared at 20 mM in dimethylsulfoxide (AppliChem GmbH, Darmstadt, Germany) and for M22 MAb (4 µg of IgG with 2.5 mg of BSA/vial, freeze-dried; Kronus, Star, ID, USA) at 2 µg/mL in phosphate-buffered saline. Both forskolin and M22 MAb were prepared as two-fold dilutions in reaction buffer (RB; Thyretain, Quidel, CA, USA). Bovine TSH (10 IU/vial; Sigma-Aldrich) concentrations were diluted 1:11 in normal serum. All serum samples from untreated patients with GD were diluted 1:11 in RB.

Genetically Engineered Cell Lines

CHO cell lines express either a chimeric (MC4) or WT human TSH-R, as described previously [2]. Both cell lines also contain a firefly luciferase gene under the control of the glycoprotein hormone alpha subunit promoter that contains two binding sites for cAMP-response element-binding protein.

Bioassay for TSAb

TSAb were measured using a luciferase-based bioassay that utilizes CHO-MC4 cells (Thyretain) according to the manufacturer's instructions. Briefly, CHO-MC4 or CHO-WT cells were seeded and grown to confluent cell monolayers in 96-well plates for 15–18 h at 37 °C, 5% CO₂. Specimens and three assay controls (positive, reference, and normal) were diluted 1:11 in RB. Therefore, 300 µL RB and 30 µL of the sample were mixed, and 100 µL were added to the cell monolayer and each plate was incubated for 3 h at 37 °C, 5% CO₂. Each sample was measured in duplicate. Subsequently, the cells were lysed with lysis buffer containing the substrate for the luciferase. The relative light unit (RLU) values were quantified in the luminometer (Tecan Infinite M200; Tecan, Crailsheim, Germany).

Measurement of cAMP

Intracellular cAMP levels were measured with a homogeneous, fluorescent assay that is based on binding of cAMP to a DNA-binding protein (Bridge-It® cAMP designer fluorescence assay, Cat # 122934; Medimotics LLC, St. Louis, MO, USA) [30]. The assay principle is based on two DNA fragments, each containing half of the cAMP-dependent DNA-binding protein (CAP) binding site each of which is labeled with a fluorescence donor and a quencher, respectively. The presence of cAMP induces conformation changes of CAP and greatly increases its binding affinity to the annealed DNA fragments. The annealing of the two DNA fragments brings the probe and quencher into close proximity, resulting in fluorescence quenching. Thus, cAMP levels are inversely proportional to the level of fluorescence.

When measuring cAMP in the TSAb bioassay, several steps of the bioassay protocol were modified. Both, CHO-MC4 and CHO-WT cells were seeded and grown for 17 h at 37°C, 5% CO₂. Before addition of samples, each well was pre-filled with 100 µL of 3-isobutyl-1-methylxanthine (IBMX, final concentration in the well 0.75 mM) solution prepared in RB (Thyretain). Forskolin and M22 samples (100 µL per well) were added in duplicate, and the plate was incubated for 3 h (forskolin), 30 min (M22) at 37°C, 5% CO₂. All patient serum samples were diluted 1:11 in RB (30 µL serum plus 300 µL RB) and measured in the CHO-MC4 cell line. Subsequently, 100 µL of the cAMP designer assay solution was added according to the manufacturer's instructions (Mediomics LLC). The plate was covered with tinfoil and incubated for further 30 min on an orbital shaker at room temperature. The solutions were transferred to a whole black 96-well plate, and the absolute fluorescence (excitation ~485 nm, emission ~540 nm) values were quantified in a fluorescence plate reader (Tecan Infinite M200). Each sample was measured in duplicate and independently repeated at least three times. The relative fluorescence (RF) values were calculated based on the raw fluorescence data as follows: RF = (F0 - F)/F0. F0 = fluorescent intensity of the blank or buffer control, F = fluorescent intensity of the sample. The occurrence of fluorescence quenching leads to a reduction of the fluorescence intensity.

Statistical Analysis

Data were presented as mean ± standard error (SEM). Experimental data were statistically analyzed with SPSS version 23 (SPSS, Chicago, IL, USA) and Graph Pad Prism software, Inc. (version 5.04), San Diego, CA, USA. Correlations were verified with either Spearman's or Pearson's test. A result was significant when $p < 0.05$.

Results

The demographic and serological data of all 33 untreated patients with Graves' hyperthyroidism are shown in Table 1.

cAMP and Luciferase Levels following Nonspecific Stimulation with Forskolin

Intracellular levels of cAMP and luciferase in CHO-MC4 and CHO-WT cells were measured following incubation with forskolin, an adenyl cyclase stimulator, at concentrations from 0.006 up to 200 µM (online suppl. Fig. 1; for all online suppl. material, see www.karger.com/doi/10.1159/000504509). In the Bridge-It assay, the linear range was from 0.8 to 25 µM for the CHO-MC4 cells and from 3.1 to 50 µM for the CHO-WT cells (online suppl. Fig. 1a). In the luciferase assay, the linear range was from 0.09 to 6.25 µM with the CHO-MC4 cells and from 0.19 to 3.125 µM for the CHO-WT cells (online suppl. Fig. 1b). There was a high correlation between the two assays for both cell lines (CHO-MC4: Spearman's $r = 0.91$, $p < 0.001$ and CHO-WT: Spearman's $r = 0.84$, $p <$

Table 1. Demographic and serological data of untreated patients with GD

N	33
Gender (F/M)	24/9
Age, years	59±3
TSH (0.27–4.2 µIU/mL)	0.00±0.01
fT3 (3.1–6.8 pmol/L)	14.9±1.3
fT4 (12–22 pmol/L)	27±1.9
TBII (<1.75 IU/L)	37±53
TSAb luciferase bioassay (RLU)	8,068±1,700
cAMP Bridge-It assay (RF%)	74±2

Number (N) and mean ± SEM values are shown. RF% = percentage of relative fluorescence.

0.001). The 50% maximum stimulatory concentration of forskolin was more than 16-fold higher for the CHO-WT cells than the CHO-MC4 cells in the cAMP assay and 4-fold higher in the luciferase assay.

Dose-Response Curve after Incubation with Bovine TSH

Different concentrations of bovine TSH between 3.125 and 3,000 mIU/L were measured in both assays (online suppl. Fig. 2a, b).

Dose-Response Curve after Incubation with the Specific Stimulator M22 MAb

Intracellular levels of cAMP and luciferase in both CHO cell lines were measured following incubation of the cells with the human TSH-R-stimulating MAb M22 at concentrations from 0.006 to 50 ng/mL (Fig. 1). In the cAMP assay, the linear ranges for the CHO-MC4 and CHO-WT cells were from 0.8 to 12.5 ng/mL and from 0.2 to 3.125 ng/mL, respectively (Fig. 1a). Similar dose-response curves for both CHO cell lines after M22 stimulation were seen with the luciferase bioassay (Fig. 1b). There was a high correlation in the CHO-MC4 cells between the cAMP assay and the luciferase assay (Spearman's $r = 0.88$, $p < 0.001$); however, the correlation between the assays was somewhat lower with the CHO-WT cell line (Spearman's $r = 0.75$, $p = 0.002$). Higher levels of both cAMP and luciferase were induced in the CHO-MC4 cells compared to the CHO-WT cells.

Measurement of cAMP and Luciferase Levels after Incubation with Sera from GD Patients

All 33 polyclonal serum samples from untreated hyperthyroid patients with GD were measured in both the

Fig. 1. a Dose-response curve of M22 MAb in the CHO-MC4 and CHO-WT cells measured in the cAMP assay. On the x-axis, the M22 MAb concentrations (0.006–50 ng/mL) are presented in a logarithmic scale, and on the y-axis, there is RF%. The outlined circles represent the data for the CHO-MC4 cells, and the black triangles present those for the CHO-WT cells. The induction time was 30 min. All M22 MAb concentrations were measured in duplicate, and values are shown as mean $\pm$ SEM. **b** Dose-response

curve of M22 MAb in CHO-MC4 and CHO-WT cells measured in the luciferase bioassay. On the x-axis, the M22 MAb concentrations (0.006–50 ng/mL) are presented in a logarithmic scale, and on the y-axis, there are the RLU values. The outlined circles represent the data for the CHO-MC4 cells, and the black triangles represent those for the CHO-WT cells. The induction time was 3 h. All M22 MAb concentrations were measured in duplicate, and values are shown as mean $\pm$ SEM.

Fig. 2. Correlation of results of the luciferase and cAMP TSAb bioassays using serum from 33 untreated patients with GD. The Bridge-It cAMP assay results are shown on the x-axis as RF% values, and the luciferase results are shown on the y-axis as RLU values. In both assays, CHO-MC4 cells were used. All serum samples were also TSH-R-Ab binding assay positive.

cAMP Bridge-It assay and in the luciferase bioassay using CHO-MC4 cells (Fig. 2). The percentage of RF (RF%) and RLU obtained by the cAMP Bridge assay and the luciferase bioassay correlated positively ($r = 0.96$, $p < 0.001$). All

serum samples were positive when measured with a TSH-R-Ab binding immunoassay (TBII, Cobas e411; Roche). Further 50 GD samples were tested with both cAMP and luciferase assays and yielded similar results. All samples were also measured with the Cobas binding assay and were TBII positive

Discussion

In the present work, in a cell-based bioassay for TSAb, a novel, simple-to-perform, homogeneous, fluorescence-based cAMP assay was evaluated and compared to a luciferase assay using both polyclonal TSHR-Ab sera from hyperthyroid GD patients as well as monoclonal TSHR-Ab. This novel Bridge-It cAMP assay offers several advantages; i.e., ease-of-use and a wide linear range. Most importantly, the assay is homogeneous in that there are no washing steps. This greatly simplifies the assay protocol compared to most cAMP assays that involved the use of radioisotopes and/or immunoassays [21, 22, 37, 38].

The performance of the cAMP assay was first evaluated using an activator of AC, forskolin, which nonspecifically raises intracellular cAMP levels. As measured by the Bridge-It cAMP assay, forskolin increased cAMP levels in both CHO cell lines in a dose-dependent manner. In the CHO-MC4 cell line that is used in the FDA-cleared

Thyretain luciferase-based bioassay, the 50% maximum concentration of forskolin was much lower compared to the CHO-WT cell line. This is an interesting result because forskolin acts independently of the TSH-R and, therefore, this result cannot be attributed to differences in the structure of the two receptors or the number of receptors per cell. In contrast, studies with the stimulating human monoclonal antibody (M22) [39] allow for comparison of the signal transduction of the two TSH-R. When stimulating with M22 MAb, the CHO-MC4 cells produced higher levels of cAMP and exhibited a broader linear range compared to the CHO-WT cells. Furthermore, differences were also detected in the dose-response curves in the cAMP assay and the luciferase bioassay after incubation of the CHO-MC4 cells with M22 MAb. As previously reported [12, 13], the CHO-MC4 cells were more sensitive at detecting M22 MAb in the luciferase bioassay compared to the CHO-WT cells, and this was also the case with the cAMP assay. Based on this enhanced sensitivity and lower EC values [12], the chimeric cell line has been used since then to further develop and optimize the luciferase cell-based bioassay. Indeed, the obtained data with the novel cAMP assay confirm our experience and results with both cell lines and suggest that the basis for these observed differences is probably not related to the chimeric TSH-R alone.

When measuring polyclonal patient sera, the data obtained in the two assays expressed as RF% and RLU showed a high correlation. The Bridge-It cAMP assay directly measures the amount of existing cAMP after cell lysis. In contrast, the luciferase bioassay measures cAMP-induced expression of the luciferase reporter gene that needs a longer induction time to initiate the gene transcription and translation. The advantage of the more time-consuming luciferase assay is that the signal is amplified, which may enable greater sensitivity compared to the cAMP assay. There has been concern, however, that

the luciferase readout, being an indirect measure of cAMP induction, might not correlate with direct measurement of cAMP when detecting TSAb. The positive correlation between the two assays with high *r* values of forskolin and M22 for both CHO cell lines demonstrates that this concern is not justified. Nevertheless, the data of the present study indicates that the homogeneous cAMP assay is an acceptable alternative for the detection of TSAb and allows the quantitative measurement of M22 MAb TSAb activity. Currently, a prospective study of serum samples from patients with untreated GD with different titers of TSAb is ongoing.

In conclusion, measurement of TSAb in a cell-based bioassay using a novel, homogeneous cAMP assay provides equivalent TSAb measurements compared to a luciferase-based bioassay.

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Statement of Ethics

This study was consistent with the Declaration of Helsinki, and the written informed consent was approved by the local ethics committee. This research complies with the guidelines for human studies.

Disclosure Statement

T.D., M.K., and C.W. have nothing to disclose. Y.-H.C. is the President of Mediomics, USA. P.D.O. and G.J.K. consult for Mediomics and Quidel, USA, and the JGU laboratory has received research-associated funding from Quidel, USA.

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