CLINICAL STUDY

Autoantibodies from patients with autoimmune thyroid disease do not interfere with the activity of the human iodide symporter gene stably transfected in CHO cells

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Abstract

Objective: The human sodium iodide symporter (hNIS) is a candidate autoantigen in autoimmune thyroid diseases. To investigate the possible existence of autoantibodies able to interfere with the biological activity of hNIS, an assay was developed using a cell line stably expressing hNIS.

Methods: hNIS complementary cDNA cloned in pcDNA3 and a neomycin resistance gene vector were co-transfected into CHO cells. After selection with geneticin, a cell line termed PA4, showing the highest level of Na125I uptake, was characterized. The time course of iodide uptake was evaluated by incubating PA4 cells with 10 μmol/l NaI and 0.1 μCi Na125I for a period up to 90 min. The accumulation of iodide increased linearly between 2 and 10 min, reaching a plateau at 45 min. The curve of iodide efflux mirrored that of iodide influx. Both perchlorate and thiocyanate inhibited iodide uptake in PA4 cells in a dose-dependent manner starting from concentrations as low as 0.01 and 0.1 μmol/l respectively and complete inhibition was obtained at concentrations of 100 μmol/l perchlorate and 1000 μmol/l thiocyanate. The sensitivity of the inhibition assay was further improved using both inhibitors after 5 min incubation and in the absence of cold NaI.

Results: Included in the study were 42 patients with Graves’ disease (25 had active hyperthyroidism, ten were euthyroid and seven had hypothyroidism); 34 patients with Hashimoto’s thyroiditis (one was euthyroid, four had subclinical hypothyroidism and 29 were overtly hypothyroid); and 19 with atrophic thyroiditis (all hypothyroid). Four out of eight whole sera from patients with Hashimoto’s thyroiditis, and 8 out of 25 whole sera from patients with Graves’ disease caused an inhibition of iodide uptake in PA4 cells greater than 20% but also in 4 out of 15 sera from normal subjects. This inhibition activity exerted by sera from patients and controls was lost after dialyzing against buffer. Accordingly, IgGs purified from sera of all patients with Graves’ disease and with Hashimoto’s thyroiditis or atrophic thyroiditis were devoid of any effect on iodide uptake.

Conclusions: In conclusion, we believe that autoantibodies able to block the function of hNIS are very rare.

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Introduction

Autoimmune thyroid diseases (ATD) are organ-specific autoimmune disorders (1) characterized by the presence in the patient’s sera of autoantibodies to thyroid-specific proteins such as thyroglobulin (Tg), thyroid peroxidase (TPO) and the thyrotropin receptor (TSHr) (1). Tg and TPO antibodies are used as clinical markers of ATD, while TSHr antibodies may either stimulate thyroid adenylate cyclase causing hyperthyroidism (TSAb) (1) or block thyroid function producing hypothyroidism (1–3).

Recently, the sodium iodide symporter (NIS) has been cloned and sequenced in the rat (4), leading to the sequencing of the human NIS (hNIS) (5). NIS, a membrane-bound glycoprotein located at the basolateral portion of the thyroid follicular cell (6), is responsible for active accumulation of iodide within the thyroid gland, a critical step in the biosynthesis of thyroid hormones (6). The protein is composed of 13 membrane-spanning segments separated by 14 extramembranous domains (6). NIS has been proposed as another candidate autoantigen in ATD (7–12) and it has been hypothesized that antibodies to hNIS may
produce a biological effect by inhibiting iodide uptake in thyroid follicular cells. Serum from a single patient with Hashimoto’s thyroiditis (HT) inhibited iodide uptake in dog thyrocyte cultures (7). IgGs from patients with Graves’ disease (GD), and more rarely from patients with HT, have been shown to bind recombinant rat NIS peptides in an ELISA assay (8). Furthermore, a few IgGs from HT patients inhibited iodide uptake in a Chinese Hamster Ovary (CHO) cell line stably expressing the rat NIS (9). Recently, Ajjan et al. (11) showed that 31% of sera from patients with GD inhibited iodide uptake in a CHO cell line stably expressing the hNIS protein. Purified IgGs from six of these GD patients also inhibited iodide uptake to a variable degree (11). The same authors described also that 22% of GD and 24% of HT sera reacted with the in vitro-translated unglycosylated $^{35}$S-hNIS protein (12). Three out of the seven sera of HT patients and 8 of the 11 sera of GD patients positive in the hNIS binding assay also inhibited iodide uptake in the bioassay using CHO cells stably expressing hNIS (12). Using a stable COS cell line expressing high levels of functional hNIS, a low prevalence of iodide uptake inhibiting activities after testing 299 sera from patients with ATD was observed by Ho et al. (13). The inhibitory activity was abolished after dialysis of the sera and could not be reproduced using purified IgGs.

In the present paper we report the establishment of a new cell line stably transfected with hNIS and studied the activity of putative autoantibodies directed against this protein. This cell line expressed the functioning hNIS and was inhibited by both perchlorate and thiocyanate in a dose-dependent manner. Sera and IgGs from patients with GD and HT were tested to search for autoantibodies able to inhibit iodide uptake but no such activity was found.

Patients

**GD**

Diagnosis of GD was based on common clinical criteria which included hyperthyroidism as assessed by elevated serum thyroid hormone levels, undetectable TSH and high $^{131}$I thyroid uptake. At palpation, echography and scintiscan a diffuse enlargement of the thyroid gland was observed. In 30 patients a hypoechogenic pattern was demonstrated in all patients by ultrasonography. Fifty-three patients with HT or AT were included in the study. Among the 34 patients with HT, one was euthyroid (HT-E), four had subclinical hypothyroidism (HT-SH) and 29 had overt hypothyroidism (HT-H). The remaining 19 patients had AT with hypothyroidism. Eleven out of 28 patients with HT-H and 8 of 14 patients with AT had TSH binding inhibiting antibodies.

Thyroid radioiodine uptake at 24 h (normal range 30–50%) was determined in most patients (23 of 29 with HT-H and 12 of 19 with AT). Fifteen patients with HT-H showed a low iodide uptake, seven a normal uptake, while one showed an elevated uptake. All 12 patients with AT showed a low iodide uptake.

Sera were obtained before treatment in all patients with HT-E and HT-SH, while one patient with HT-H and three with AT had been receiving $l$-thyroxine for few months when the blood sample was obtained.

**Healthy controls**

Sera were also obtained from 15 healthy controls in which tests for thyroid antibodies gave negative results.

**Materials and methods**

**Laboratory investigation**

Serum FT$_4$ and FT$_3$ concentrations were measured by RIA after chromatographic separation of the free hormone (FT$_4$ RIA, FT$_3$ RIA, Lysophase; Technogenetics S.r.l., Milan, Italy). Serum TSH levels were determined by a sensitive assay (AutoDelfia hTSH Kit; Pharmacia s.p.a., Milan, Italy). TPOAb and TgAb were measured by passive agglutination (SERODIA-AMC and SERODIA-ATG, Fujirebio, Tokyo, Japan). TSHrAb were searched for using a commercial radioreceptor assay (TRAK assay; B.R.A.H.M.S., Berlin, Germany). Autoantibodies blocking the TSH-dependent production of cAMP in cell lines expressing the TSHr were measured using a previously described method (14).
**IgG preparation**

IgGs were prepared with a commercial kit according to the manufacturer’s instructions (Mab Trap G II Pharmacia Biotech, Uppsala, Sweden).

**Establishment of the CHO-K1 cell line expressing the hNIS**

The hNIS complementary DNA (cDNA) cloned into the eukaryotic expression vector pcDNA3 and encoding amino acids 1 to 612 of hNIS was cloned as described by Pohlenz et al. (15).

The hNIS–pcDNA3 and a neomycin resistance gene vector (pSV2NEO) were co-transfected into CHO-K1 cells using a modified calcium phosphate method (16). Cells were plated out in 60 mm culture dishes and after 24 h were transfected with the two vectors. After 6 h incubation, cells were washed twice with Dulbecco Modified Eagle’s Medium (DMEM) and fresh medium containing 10% fetal calf serum (FCS) was added. Selection was started 48 h after transfection by addition of medium containing 400 \( \mu \text{g} / \text{ml} \) geneticin (G418 from Gibco, Paisley, Scotland, UK). Medium was changed every day for about 3 weeks. When non-transfected cells in control dishes had all died, cells were trypsinized and subjected to screening, using the Na\(^{125}\)I uptake assay. The mixed CHO-K1 cell population was subjected to limiting dilution to produce individual clonal cell lines. Briefly, serial dilutions of the transfection product were seeded into 96-well microtiter plates. Wells were screened the following day by inspection under the microscope and those containing only one cell were retained. Cells in these wells were allowed to grow to confluence, trypsinized and further amplified in 90 mm culture dishes.

**Iodide uptake assay**

One hundred thousand cells were plated in 24-well plates and cultured in DMEM containing 10% FCS. When cells reached 100% confluence, Na\(^{125}\)I uptake was determined by incubating cells at 37 °C for 45 min with 500 \( \mu \)l buffer A (Hanks’ balanced salt solution (HBSS) containing 0.5% BSA and 10 mM HEPES, pH 7.4) containing 100 000 c.p.m. of carrier-free Na\(^{125}\)I (0.1 \( \mu \)Ci) with or without NaI (5–625 \( \mu \)mol/l). After incubation, cells were quickly washed twice with 2 ml of ice-cold buffer A. Cells were then solubilized with 1 ml of 0.1 mol/l NaOH. The radioactivity from each well was counted using a \( \gamma \)-counter. Data of iodide uptake were expressed as pmol/well.

**Iodide efflux**

Iodide efflux from cells was measured as described by Weiss et al. (17). Cells were incubated with 10 \( \mu \)mol/l NaI and 100 000 c.p.m. Na\(^{125}\)I in 500 \( \mu \)l HBSS incubation buffer at 37 °C for 60 min. Cells were then washed rapidly once with 2 ml HBSS incubation buffer, the medium was removed gently (in order not to dislodge the cells) and replaced with 500 \( \mu \)l fresh non-radioactive medium every 3 min. The medium was HBSS incubation buffer with 10 \( \mu \)mol/l NaI, which was kept at 37 °C. After the last medium removal, cells were solubilized for counting along with the previously collected medium samples. The total radioactivity present at the beginning of the efflux study (100%) was calculated by adding the final radioactivity measured in the cells to the radioactivity of the media collected at different time intervals. Data of iodide efflux were expressed as pmol/well.

**Effect of sera and IgGs on NIS activity assessed by iodide uptake**

To determine the effect of sera or IgGs from patients with ATD on hNIS activity, 250 \( \mu \)l buffer A containing 50 \( \mu \)l of serum (or serum previously dialyzed against TRIS buffer) or 1 mg/ml IgG was incubated with cells for 60 min at 37 °C. After this pre-incubation, 250 \( \mu \)l buffer A containing Na\(^{125}\)I (with and without 10 \( \mu \)mol/l NaI) was added and cells were further incubated for 5 min or 45 min, followed by washing and solubilization as above. All experiments were performed in triplicate cultures. Results were expressed as percent of Na\(^{125}\)I uptake measured in parallel cultures incubated without sera or IgGs.

**Results**

**Isolation of CHO-K1 clones expressing the hNIS**

To check hNIS function, iodide uptake studies were carried out on both the transfected CHO-K1 mixed cell population and the control (wild type CHO-K1 cells). The uptake of Na\(^{125}\)I in the mixed cells was about 100-fold greater than the uptake of the control.
suggesting that a functioning NIS protein was present. Several cell clones were obtained and screened for iodide uptake (Fig. 1). A cell line termed PA_4 that showed the highest level of Na\textsuperscript{125}I uptake among all screened colonies (400-fold greater than the uptake of non-transfected CHO cells) was characterized and then used for further experiments.

**Iodide kinetic characteristics of the PA_4 clone**

The time course of iodide uptake was evaluated by incubating PA_4 cells with 10 \textmu mol/l NaI for a period up to 90 min. The maximum Na\textsuperscript{125}I uptake of PA_4 cells, when plated in 24-well plates, was between 30 000 and 40 000 c.p.m./well. Non-transfected CHO cells had an uptake of 100 c.p.m./well.

The accumulation of iodide in PA_4 cells increased linearly between 2 and 10 min, reaching a plateau at 45 min (Fig. 2). The curve of iodide efflux measured after 60 min incubation mirrored that of iodide influx (Fig. 2), indicating that equilibrium was established with a velocity of iodide transport of 90 pmol/min per well in both directions. The velocity of iodide uptake increased with increasing concentrations of extracellular iodide following the Michaelis–Menten equation (Fig. 3). Fig. 3 also shows the kinetic parameters of iodide uptake, as examined by Lineweaver–Burk analysis. The value for \(K_m\) was 11.5 \textmu mol/l and that of maximum velocity was 113 pmol/min per well. Both perchlorate and thiocyanate inhibited iodide uptake in PA_4 cells in a dose-dependent manner (Fig. 4a and b); when incubated for 45 min in the presence of 10 \textmu mol/l NaI inhibition started from concentrations as low as 0.01 and 0.1 \textmu mol/l respectively and complete inhibition was obtained at concentrations of 100 \textmu mol/l perchlorate and 1000 \textmu mol/l thiocyanate. Inhibition of iodide uptake was more evident at 5 min than at 45 min incubation (Table 1). The concentration producing 50% inhibition was 0.5 vs 2.8 \textmu mol/l (5 min vs 45 min respectively) for perchlorate, and 6.2 vs 10.3 \textmu mol/l (5 min vs 45 min respectively) for thiocyanate. To increase the sensitivity of the inhibition assay we tested both inhibitors in the presence of Na\textsuperscript{125}I with no cold NaI added. Under these conditions the lowest concentration of inhibitor that produced a significant inhibition of iodide uptake was 10 times lower for both perchlorate and thiocyanate (Table 1).

**Effects of patients' sera and IgGs on iodide uptake**

**Effects of sera** We started to study the effect of only eight whole sera from the 53 patients with HT and 25
whole sera from the 42 patients with GD on Na$^{125}$I uptake activity of PA$_4$ cells. Four out of eight sera of patients with HT, and 8 out of 25 sera of patients with GD caused an inhibition of iodide uptake greater than 20% (Fig. 5). A similar inhibiting effect was produced by 4 out of 15 sera obtained from normal subjects. After dialyzing against the buffer, all these sera lost their inhibiting activity on iodide uptake (Fig. 6). After the results obtained with this first series of normal sera tested we decided to use directly purified IgGs for further experiments.

Effects of IgGs

IgGs were prepared from 42 sera of patients with GD and 53 sera of patients with HT or AT. The iodide uptake inhibition assay was performed both in the presence of 10 $\mu$mol/l NaI and in its absence, after 5 and 45 min incubation. None of the IgGs was

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Figure 5 The inhibition of iodide uptake by normal sera, GD and HT or AT patients’ sera. Results are the mean of triplicate cultures \(\pm\) S.E.s (S.E.s are so small that they fall within the symbols) and are expressed as % of Na$^{125}$I uptake in parallel cultures incubated with buffer alone without sera.

Figure 6 The inhibition of iodide uptake by one normal serum, one serum from a GD patient and one serum from a HT patient after incubation with different dilutions of the sera (white columns) or different dilutions of sera after dialysis against buffer (stippled columns). Results are calculated as in Fig. 5.
able to inhibit iodide uptake in the various experimental conditions (Fig. 7).

IgGs were also tested in the same experimental conditions (after 5 and 45 min incubation, both in the presence and in the absence of NaI) with another clone designated PA14. This clone had an iodide uptake activity that was tenfold lower than PA4 cells, the reduced iodide uptake of the PA14 probably being due to a lower level of expression of a functioning hNIS protein on the cell surface. Again, none of these IgGs was able to inhibit iodide uptake in PA14 cell line (data not shown).

Discussion

We established new cell lines stably expressing hNIS and used them to search for autoantibodies able to inhibit the iodide uptake. Given that thyroid-specific proteins such as Tg, TPO and the TSHr act as autoantigens in ATD (1), it is conceivable that hNIS could also act as an autoantigen. Differences in the structural characteristics and in the exposure to immunocompetent cells of these proteins might be responsible for a different ability to trigger autoimmunity. Tg is a glycosylated iodoprotein that accumulates in the follicular lumen of thyroid follicles and small amounts of this protein are detected in serum (1). TPO is a glycosylated hemoprotein mainly bound to the apical membrane of thyroid follicular cells (1). Both Tg and TPO are expressed on the surface of thyroid follicular cells. The TSH receptor is a membrane-bound protein with a large extracellular portion (18). NIS is a membrane-bound glycoprotein located at the basolateral portion of the thyroid follicular cell (6) with a very short extracellular domain. Patients with HT or AT often show a low radiiodine uptake (1–3), probably due to thyroid gland atrophy or by lymphocytic infiltration. On the other hand, the possibility of autoantibodies inhibiting the Na\textsuperscript{125}I symporter activity has been hypothesized to have a role in reducing the iodide uptake in patients with hypothyroid ATD. However, it has been shown that the amounts of NIS mRNA and protein are increased in thyroid tissue from Graves’ patients (19). It has also been demonstrated that the TSH/cAMP pathway up-regulates NIS gene expression and NIS protein abundance in thyroid follicular cells in primary culture (19). These data are in agreement with the elevated levels of 131I uptake in patients with GD. It is commonly believed that the increased NIS expression in GD is due to increased cAMP accumulation induced by TSHr antibodies.

After cloning the rat NIS cDNA, Endo et al. (8) used the recombinant protein blotted on nitrocellulose sheets to detect antibodies against NIS in sera of patients with ATD. Eighty-four percent of sera from patients with GD and 15% of sera from patients with HT recognized the recombinant protein. The corresponding patient’s IgGs also stained the recombinant NIS in Western blot (8). Morris et al. (10) reported that 24 out of 27 IgGs from patients with GD, 17 out of 27 IgGs from patients with HT and 7 out of 20 IgGs from normal subjects were able to bind to rat recombinant NIS peptides in an ELISA system. However, antibodies able to recognize linear epitopes may be devoid of a functional activity (1). One out of 144 sera from patients with HT inhibited iodide uptake in dog thyrocytes cultures (7). In another study, Endo et al. (9) showed that 4 out of 34 IgGs from patients with HT inhibited \textsuperscript{125}I uptake in cells stably transfected with the rNIS (9). Two of these IgGs had a weak inhibitory activity (20% and 15%), while the other two IgGs gave a more pronounced inhibition of \textsuperscript{125}I uptake ranging from 40 to 60% (9). Aikan et al. (11) found that 30% of sera from patients with GD inhibited iodide uptake in CHO cells stably expressing hNIS protein. The inhibitory activity was also demonstrated using six IgGs. In a subsequent study (12) the same authors described that 22% of GD and 24% of HT sera reacted with the in vitro-translated unglycosylated \textsuperscript{35}S-hNIS protein. Three of the seven sera from HT patients and 7 of 11 sera from GD patients which were positive in the hNIS binding assay also inhibited iodide uptake in the bioassay (12).

In the present study the potential biological effect of autoantibodies from patients with GD or HT on iodide transport was searched for using two new cell lines stably expressing a functioning hNIS protein. Our cell clone (PA4) accumulated 400-fold more Na\textsuperscript{125}I than cells transfected with the vector alone. This uptake had a similar time course compared with cell clones expressing the rat NIS or FRTL-5 thyroid cells (20). Half-maximal levels were reached after 5–10 min and the plateau after 30 min. Similarly, the Na\textsuperscript{125}I uptake was inhibited by both perchlorate and thiocyanate in a dose-dependent manner. The curve of iodide efflux mirrored that of iodide influx, indicating that equilibrium was established with the same velocity of iodide

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transport in both directions. All these features indicate that the PA4 line expresses a functioning hNIS and can be used as a tool to detect thyroid autoantibodies that interfere with hNIS function.

When some sera from patients with HT or GD were tested, no stimulation of iodide uptake was found, while 50% of whole sera from patients with HT or AT and 32% of whole sera from patients with GD caused an inhibition of Na\(^{125}\)I uptake (Fig. 5). However, an inhibition was also produced by a few normal sera (Fig. 5). In keeping with previous results (9), sera from either ATD patients or controls lost their inhibitory activity on Na\(^{125}\)I uptake after dialyzing against the buffer (Fig. 6). This indicates that sera may contain dialyzable inhibitor(s) of NIS activity different from IgGs. Therefore, the use of purified IgGs is necessary to accurately evaluate the antibody activity.

We then tested purified IgGs from ATD patients following the experimental procedure reported in previous papers (11, 12, 17). None of our IgGs was able to interfere with iodide uptake. Our results are in contrast with the observation of Ajjan et al. (11). The discrepancy between our and previous studies is unlikely to be explained by differences in the kinetic characteristics of the stable cell lines expressing the NIS protein. Indeed, the time course of iodide uptake, and the iodide efflux properties of the two cell lines were similar. Both perchlorate and thiocyanate inhibited iodide uptake in the two cell lines, starting from concentrations as low as 0.01 and 0.1 \(\mu\)mol/l respectively. The 50% inhibition was between 0.1 and 1 \(\mu\)mol/l for perchlorate in the cell line described by Ajjan and 2.8 \(\mu\)mol/l in our bioassay. The concentration of thiocyanate producing a 50% inhibition of iodide uptake was similar in our cell line compared with that of Ajjan et al. (11). Furthermore, to increase the sensitivity of our assay we modified the experimental conditions by testing the inhibitors during the initial phase of iodide accumulation (when no significant efflux of iodide occurs) and in the absence of cold NaI (which reduces the uptake of Na\(^{125}\)I). Under these conditions both perchlorate and thiocyanate exhibited a greater inhibitory activity and lower concentrations of both inhibitors could be detected (Table 1). Even in these experimental conditions none of the IgGs from patients and controls were able to inhibit iodide uptake. The main difference between the two cell lines is that we used a stable cell line with a high iodide uptake with respect to the cell line used by the group of Ajjan et al. (11). Therefore we tested our IgGs using a cell clone (PA14) having a lower iodide uptake. Also using these cells none of our IgGs inhibited iodide uptake. In another paper Ajjan et al. (12) established a new cell line with a higher iodide uptake. Using these cells the few IgGs tested from patients with HT and GD were able to inhibit iodide uptake (12). Only four IgGs out of 34 showed a relative potent inhibitory activity on iodide uptake by the cells in the study of Endo et al. (9). In this paper the kinetic characteristics of the cell line are not described and only IgGs from patients with HT were used. Recently, using a stable COS cell line expressing high levels of functional NIS, a low prevalence of iodide uptake inhibiting activities after testing 299 sera from patients with ATD was observed by Ho et al. (13). The inhibitory activity was abolished after dialysis of the sera and could not be reproduced using purified IgGs. Besides, three anti-hNIS monoclonal antibodies were obtained by genetic immunization by Pohlenz et al. (21). None of these antibodies were able to inhibit iodide uptake by COS cells stably expressing hNIS. This last observation does not imply that other antibodies could not be able to interfere with NIS activity.

In conclusion, our results do not confirm previous studies reporting the occurrence of iodide uptake inhibitory IgGs in patients with ATD. This discrepancy can not be attributed to a lower sensitivity of our assay and we conclude that IgGs able to block the iodide transporter are very rare.

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