CLINICAL STUDY

Sodium/iodide symporter (NIS) and pendrin are expressed differently in hot and cold nodules of thyroid toxic multinodular goiter

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Abstract

Objective: The expression of two iodide transporters, the sodium/iodide symporter (NIS) and pendrin, was analyzed in thyroid tissues of patients with toxic multinodular goiter (TMNG) and non-toxic multinodular goiter (MNG).

Methods: The levels of NIS and pendrin proteins were analyzed in total protein extracts from nodular and non-nodular tissues by Western blot.

Results: In tissue samples from TMNG, we found an increased expression of NIS (2.5-fold) in the hot nodules, and similar levels between cold nodules and non-nodular tissues. In contrast, the levels of pendrin were slightly increased in both hot and cold nodules from TMNG, and decreased (about twofold) in cold nodules from MNG. We also noticed that there was no relationship between NIS and pendrin expression.

Conclusions: Our data demonstrate that hot nodules from TMNG express a higher number of iodide transporters (mainly NIS), whereas cold nodules from TMNG, but not from MNG, show levels of the two proteins comparable with normal tissue, suggesting a role in vivo of TSH in maintaining the expression of NIS and pendrin protein in normal thyroid tissue. Finally, different mechanisms are involved in the regulation of NIS and pendrin expression.

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Introduction

Iodide uptake is the first step in thyroid hormone production by thyroid follicles. Thyroidal iodide trapping and concentration from blood are achieved by an active, energy-dependent transport process across the baso-lateral plasma membrane of the thyrocytes; iodide is then transported trans-cellularly to the apical membrane, where it is organified (1). The way to clarify the molecular mechanisms underlying iodide transport in thyroid cells has been opened up as a result of the cloning and expression studies of two proteins mainly involved in this process, namely the sodium/iodide symporter (NIS) (2, 3) and pendrin (4, 5). NIS is located in the baso-lateral plasma membrane of thyrocytes (6, 7) and catalyzes active I\(^{-}\) uptake into the cell, while pendrin, discovered as the defective protein in Pendred syndrome (PDS) (4), is a chloride/iodide transporter expressed in the apical membrane (8, 9), presumably responsible for the I\(^{-}\) accumulation in the colloid.

For years, thyroid nodules have been characterized on the basis of their ability to concentrate radioiodine, thus considered functioning or non-functioning at \(^{131}\)I-scintiscan and named ‘hot’ or ‘cold’ respectively. Many studies have shown abnormal expression of the NIS gene in various thyroid disorders (reviewed in 10, 11), especially in thyroid malignancies. In contrast, few data are available on PDS gene expression (8), or on the relationship between NIS and pendrin expression at the protein level (6–9, 12).

In this study, using Western blot analysis we examined the expression of NIS and pendrin proteins in a series of 25 thyroid nodules from ten unrelated patients with toxic multinodular goiter (TMNG), including both hot and cold nodules, in order to clarify the role of these two iodide transporters in the functional behavior of the nodular lesions. Indeed, the simultaneous presence of hot and cold nodules in the same patient makes this disease a good model for excluding interference due to genetic and environmental factors. For the first time, the protein expression...
levels were compared with the normal, non-nodular tissue of each patient. To evaluate the influence of thyroid-stimulating hormone (TSH), which is obviously suppressed in patients with TMNG, five cold nodules from patients with non-toxic multinodular goiter (MNG) who had detectable levels of TSH were also investigated.

Materials and methods

Materials

HEPES, sucrose, dithiothreitol (DTT), EDTA, phenylmethylsulfonyl fluoride (PMSF), aprotinin, leupeptin and anti-human beta-actin monoclonal antibody were obtained from Sigma-Aldrich S.r.l. (Milan, Italy); nitrocellulose membranes, horseradish peroxidase conjugated anti-rabbit or anti-mouse antibodies, autoradiography film and enhanced chemiluminescence (ECL-Plus) Western blotting detection reagents were from Amersham Pharmacia biotech (Milan, Italy); protein assay reagent, non-fat dry milk and molecular weight markers were from Bio-Rad Laboratories (Segrate, Milan, Italy).

Patients

A total of 25 nodules from ten unrelated patients with thyroid TMNG were studied. Five nodular tissues from another five patients with MNG and non-nodular tissues from two patients with Graves’ disease were also examined. Non-nodular, normal tissue from each patient was also investigated. All patients lived in an area of moderate–severe iodine deficiency. Informed consent was obtained from all patients. A map of the nodules, as they appeared at scintiscan and ultrasound examination, was provided for the surgeon, so that nodules defined by scintiscan could be unequivocally identified post-operatively. Tissue specimens, obtained at the time of surgery, were frozen in liquid nitrogen. Toxic multinodular goiter was diagnosed on the basis of clinical thyrotoxicosis, elevated serum levels of free thyroid hormones, and undetectable TSH levels, associated with a multinodular goiter presenting predominant $^{131}$I uptake in one (or more) nodule(s) as shown by $^{131}$I scanning. The thyroid nodules were classified as hot or cold according to the scintigraphic examination. All nodules were histologically classified as adenomas according to the WHO recommendations (13). The clinical features of the patients are shown in Table 1. This study was approved by the local ethical committee.

Protein extraction and Western blot

Total proteins were extracted from thyroid tissues as follows. Frozen tissue was thawed and homogenized in 1 ml of buffer containing 250 mmol/l sucrose,
10 mmol/l HEPES-KOH (pH 7.5), 1 mmol/l EDTA, 1 mmol/l PMSF, 10 μg/ml leupeptin, 10 μg/ml apro-
tinin. The homogenate was centrifuged at 14 000 g
(4 °C for 15 min) and the supernatant (which con-
tained the whole cell lysate) was quantified spectro-
photometrically using the Bradford method.
Fifty micrograms of protein were loaded onto a
4–20% gradient SDS polyacrylamide gel and subjected
to electrophoresis at a constant voltage (120 V).
Electroblotting to a Hybond-P ECL nitrocellulose
membrane was performed for 2 h at 125 mA using a
Mini Trans blot electroblotting system. Blocking was
done using TTBS/milk (TBS, 1% Tween 20 and 5%
non-fat dry milk) for 2 h at room temperature. The
membrane was then incubated with a 1/1000 dilution
of affinity purified rabbit anti-NIS (6) or 1/1500
dilution of anti-pendrin (8) polyclonal antibody or a
1/5000 dilution of mouse monoclonal anti-human
beta-actin antibody, overnight at 4 °C in TTBS/milk.
After one 15-min and two 5-min washes in TTBS, the
membrane was incubated with a 1/2000 dilution of a
horseradish peroxidase conjugated anti-rabbit or anti-
mouse antibody in TTBS/milk. After one 15-min and
two 5-min washes in TTBS, the protein was visualized
by an enhanced chemiluminescence Western blot
detection system. Quantification was achieved by
densitometric scanning. The same procedure was
repeated after pre-incubating the antibody overnight
at the same dilutions with 50 μg of peptide hNIS 615–
643 (6), or hPDS 765–780 (8). The anti-NIS and anti-
pendrin antibodies used in this study recognized NIS
and pendrin proteins at the baso-lateral and apical
thyrocyte plasma membrane respectively, and no signal
in the cytosol and/or nucleus was detectable in
immunohistochemical analysis (6, 8).

**Statistical methods**

The expression levels of NIS and pendrin detected in the
different nodular tissues were compared with those in
normal thyroid tissues. The significance of differences
between group means was analyzed by Student’s
(paired) t-test. A level of P < 0.05 was considered
statistically significant. The relationships between NIS
and pendrin expression were studied using the Pearson
correlation coefficient. All calculated probability values
were two-tailed and a P value of <0.05 was considered
significant.

**Results**

**Detection of NIS protein**

Western blot analysis was carried out with protein
extracts from hot and cold nodules and the perinodular
tissue of thyroid toxic multinodular goiter. In all the
samples, and in the Graves’ thyroid tissues used as
positive controls, anti-NIS antibody did recognize the
NIS protein as a major band that migrated with the
78 kDa molecular weight marker (Fig. 1). Pre-incuba-
tion of the same antibody with 50 μg of peptide hNIS
615–643 (see Materials and methods) determined the
disappearance of the signal, demonstrating the speci-
ficity of the band detected (data not shown). The levels
of NIS protein were variable among hot nodules, but
they were always higher than in the corresponding
normal tissues, with an average increase of approxi-
mately 2.5-fold. In contrast, only slight and not
significant differences were detected between cold
nodules and perinodular specimens of the same
patients (Fig. 2 and Table 2).

**Detection of pendrin protein**

By using a polyclonal anti-pendrin antibody (8) in
immunoblot experiments, a major band of approxi-
mately 95 kDa was detected in the protein extracts of
thyroid tissues from patients with Graves’ disease and
TMNG (Fig. 3). Also in this case, the specificity was
demonstrated by the absence of the band when
incubating the rabbit anti-PDS immune serum with
an excess amount of hPDS 765–780 peptide (data not
shown). Again, variability among samples was
detected, with a slight increase of the pendrin in both
cold and hot nodules as compared with the non-
nodular tissues (Fig. 2 and Table 2).

Western blot analysis in cold nodules from patients
with MNG and detectable levels of TSH showed a
decrease in both NIS and pendrin expression as
compared with the normal tissues (Fig. 4 and
Table 2).
Figure 3

Western blot analysis under reduced conditions of protein extracts from a Graves' tissue (positive control), NIH 3T3 fibroblasts (negative control) and nodular (hot and cold) and non-nodular tissues from two patients with TMNG, using a polyclonal anti-pendrin antibody (upper panel) and a monoclonal anti-human beta-actin antibody (lower panel). A representative of three separate experiments is shown. Lane 1 = negative control; lanes 2, 3, 4 = non-nodular (normal), hypofunctioning (cold) nodule and hyperfunctioning (hot) nodule from patient with TMNG (no. 3 in Table 1); lanes 5, 6, 7 = non-nodular (normal), hypofunctioning (cold) nodule and hyperfunctioning (hot) nodule from patient with TMNG (no. 10 in Table 1); lane 8 = positive control.

Table 2

<table>
<thead>
<tr>
<th></th>
<th>NIS protein</th>
<th>Pendrin protein</th>
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<tbody>
<tr>
<td><strong>TMNG</strong></td>
<td></td>
<td></td>
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<tr>
<td>Hot nodular tissues (n = 12)</td>
<td>240.0±97.2*</td>
<td>155.4±59.1*</td>
</tr>
<tr>
<td>Cold nodular tissues (n = 13)</td>
<td>116±36.7</td>
<td>170.0±46.4*</td>
</tr>
<tr>
<td><strong>MNG</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cold nodular tissues (n = 5)</td>
<td>66.1±16.2*</td>
<td>76.9±18.3</td>
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</tbody>
</table>

Statistical analysis was performed using the paired Student's t-test: *P < 0.05.
Correlation of NIS and pendrin expression in each patient with TMNG

By analyzing the data from each patient, the expression of the two iodide transporters did not correlate in either hot or cold nodules (data not shown). Thus, as shown in Fig. 2, in the hot nodules with the maximum increase of NIS expression (more than two times over the control, nos 4, 7 and 9), pendrin expression was not increased to the same extent. Similarly, the cold nodules with the lowest levels of NIS (nos 3 and 9), showed increased pendrin expression.

Discussion

In iodine-deficient areas, TMNG has a high prevalence and is responsible for a high percentage of cases of thyrotoxicosis. Histologically, they are characterized by the presence of multiple hyperplastic nodules and well encapsulated adenomas, with at least one nodule characterized by TSH-independent growth, iodide uptake and function, coexisting with one or more ‘cold’ nodules. The resulting phenotype is represented by a large spectrum of clinical manifestations of thyrotoxicosis (14).

The pathogenesis of the hot nodules of TMNG does not seem to differ from that of toxic adenomas, being related to the constitutive activation of the cAMP pathway due to mutations in the TSH receptor gene. Although, as in toxic adenomas, unequal prevalence in different studies and the large heterogeneity in the phenotypes challenge this theory (15–17). However, the role of cAMP in inducing at least an increase in iodide uptake, partially due to the stimulation of NIS gene and protein expression, is suggested by several in vitro studies in both rat and human cells (18–21). In contrast, molecular alterations that lead to the development of cold nodules remain unclear. Except for the reduced/lost ability to concentrate radioiodine, cold nodules, even in a given patient, may differ for clonality, growth rate, TSH dependence and expression of tissue-specific markers (22). In TMNG, nodules showing different behaviours regarding iodide trapping are present in a given patient. For this reason, this disease represents a unique and enviable in vivo model to study the molecular mechanisms underlying this process, limiting the interferences of both genetic and environmental factors.

Alterations in the expression of the NIS gene have already been demonstrated in different thyroid pathologies, with increased levels in Graves’ disease and toxic adenomas, and decreased or undetectable levels in benign and malignant cold nodules (reviewed in 10, 11, 23), including metastases of differentiated thyroid carcinomas (24), with a high variability among different samples (25, 26). Since an important autoregulatory effect of thyroid function is played by the intra-thyroidal iodide after its organification (27), apical transport to the follicular lumen should also be considered. The recent characterization of the genetic alteration causing Pendred syndrome, a hereditary disease characterized by neurosensorial deafness and goiter, allowed for the identification of pendrin, a chloride/iodide transporter mainly expressed in the thyroid and involved in iodide transport at the apical membrane of the thyrocytes (4, 5). Modifications in the PDS gene transcript have been demonstrated in some thyroid diseases, with the most relevant reductions detected in thyroid carcinomas (8).

Our data show that, when compared with the non-nodular tissues, the highest levels of NIS expression were detected in the hot nodules, confirming our previous data obtained in isolated toxic adenomas, both at the protein level with immunohistochemical analysis (6) and at the mRNA level with RT-PCR (26, 28). As expected, inter-individual variability was also detected among the samples, as well as an absence of relationship with the degree of thyrotoxicosis and other clinical parameters. In cold nodules, NIS expression was similar to the non-nodular tissue; this finding may result from

Figure 4 (A) Western blot analysis under reduced conditions of protein extracts from nodular and non-nodular tissues from four patients with MNG, using anti-NIS (upper panel), anti-pendrin (middle panel) and anti-beta actin (lower panel) antibodies. A representative of three separate experiments is shown. N = normal thyroid tissue; C = hypofunctioning (cold) nodule. (B) Levels of NIS and pendrin protein in hypofunctioning nodules of patients with MNG. Values are calculated as in Fig. 2. Statistical analysis was performed as described in Materials and methods.
decreased NIS expression in normal tissue, owing to TSH suppression.

Inter-individual heterogeneity is also present in the expression of pendrin in TMNG tissues. However, it was slightly increased in hot nodules (~1.5-fold) and, surprisingly, to the same extent in cold nodules, as compared with normal non-nodular tissue. Again, this finding may be related to decreased pendrin expression in normal tissue, due to TSH suppression. Indeed, these data suggest a complex regulatory mechanism of pendrin expression, in which both the intranodular iodide and the TSH/cAMP pathway may play a role. The inhibitory effect of intranodular iodide is only partially compensated by the putative increase of cAMP production occurring in hot nodules, and is also subjected to regulation by TSH: it may not be accidental that the smaller differences between nodular and normal tissues were documented in the samples with the higher levels of serum TSH. Moreover, reduced pendrin expression was detected in our pilot series of cold nodules from patients with MNG, in the presence of normal serum TSH levels. The role of TSH in regulating the expression of pendrin in vivo, suggested by our data, is in contrast to some in vitro data obtained in rat FRTL5 cells, where TSH was not able to stimulate pendrin gene expression (9), but is in agreement with some findings obtained in human thyroid cell lines in vitro (F Arturi, D Russo, S Filetti, M Schlumberger & JM Bidart, unpublished observations).

Finally, no relationship was found between the expression levels of NIS and pendrin, supporting the concept of different regulatory pathways for the expression of these two iodide transporters, which present various degrees of alteration in thyroid nodular disease, as already hypothesized (25). However, a functional defect of the apical membrane of the thyrocytes, as well as failing iodide transport, have already been described in a human multinodular goiter (29).

In conclusion, our study demonstrates that hot nodules from TMNG express higher numbers of iodide protein transporters (mainly NIS), consistent with their accelerated rate of iodine utilization for thyroid hormone synthesis. Conversely, cold nodules from TMNG but not from MNG show a similar level of the two proteins when compared with the non-nodular tissue. Moreover, our findings suggest an in vivo role for TSH in maintaining the expression of NIS and PDS and the presence of different regulatory mechanisms of NIS and pendrin expression. Further studies will clarify whether a relationship exists between the level of the iodide transporters and their function at the thyrocyte surface.

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