Monoclonal antibodies against the human sodium iodide symporter: utility for immunocytochemistry of thyroid cancer

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Abstract

The recent cloning of the thyroïdal protein that is responsible for iodide transport, the sodium iodide symporter (hNIS), has made possible studies designed to characterize its structure, function and expression in thyroïdal tissues. Using a mannose binding protein (MBP)-hNIS fusion protein as antigen, we have developed mouse monoclonal antibodies against hNIS to utilize as tools in such studies. Twenty-four clones were initially recovered which recognized the MBP-hNIS fusion protein, but only two of them were specific for hNIS while the others recognized MBP alone. Both antibodies were found to be immunoglobulin G (IgG) 1 κ (kappa). The specificity of antibodies was tested by Western blotting using membranes prepared from COS-7 cells transiently transfected with the pcDNA3 plasmid containing the full-length hNIS cDNA, or cells transfected with the pcDNA3 vector. A major band with a molecular weight (MW) of ~97 kDa, and several minor bands with MW of

~160 kDa, ~68 kDa, ~30 kDa and ~15 kDa, were detected specifically in the hNIS-transfected cells. After enzymatic deglycosylation, the major band was present at 68 kDa, as expected based upon the amino acid sequence of hNIS. Immunohistochemistry was performed with several different types of thyroid tissue and non-thyroidal tissues, using the monoclonal antibodies. Strong immunostaining was observed in Graves' tissue, with intermediate staining in papillary and follicular thyroid cancers and an absence of staining in Hürthle cell cancer. The staining was specific for the follicular epithelium and was concentrated in the basolateral portion of the cell membrane. These monoclonal hNIS antibodies should prove useful in the characterization of NIS expression in benign and malignant thyroid tissue and in studies characterizing its structure and function.

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Introduction

Recently, a new thyroid protein that is important in the physiology of thyroid hormonogenesis has been cloned and characterized, the thyroid iodide transporter (Dai *et al.* 1996). This glycoprotein, a membrane-bound structure, functions as a sodium iodide symporter (NIS), and is responsible for the thyroid gland's ability to concentrate iodide up to 40-fold with respect to plasma. Prior studies have localized it to the basolateral region of thyroid follicular cells (Carrasco 1993). The original model suggested that NIS was characterized by 12 membrane spanning segments separated by 13 extra-membranous domains (ExMD) with one of these ExMD lying at each end of the molecule. However, more recently, a modification of the original model has been proposed, containing 13 membrane spanning segments and 14 ExMDs, with the

amino terminus being extracellular and the carboxy terminus lying in the intracellular space (Levy *et al.* 1998) (Fig. 1). Through molecular cloning techniques, the amino acid sequence of the human NIS (hNIS) has been delineated (Smanik *et al.* 1996).

The importance of the characterization and study of NIS is related to its role in thyroid function, the pathogenesis of thyroid disease, and its expression or lack thereof in thyroid malignancies. It is the activity of this molecule that makes possible the most specific form of therapy for differentiated thyroid cancer, radioiodine. Expressed predominantly, although not exclusively, in thyroid tissue, it has also been suggested that NIS may represent an autoantigen in autoimmune disease of the thyroid (Morris *et al.* 1997).

In order to study the structure, function, and expression of NIS *in vitro*, antibodies with high affinity and specificity

Human Sodium/Iodide Symporter

Figure 1 Revised model of the human sodium iodide symporter (adapted from Levy *et al.* 1998). In lighter gray are the amino acid residues that comprised the hNIS portion of the fusion protein against which monoclonal antibodies were generated.

are needed as reagents. This report describes the development of such reagents and initial studies of the NIS expression in thyroid tissues.

Materials and Methods

Mannose binding protein (MBP)-hNIS fusion protein

MBP-hNIS fusion protein was generated as previously described (Jhiang *et al.* 1998). The final product consisted of the amino acid residues 468–643 of hNIS attached to MBP and it was purified using amylose resin.

Immunizations

The fusion protein was emulsified in equal ratios of Freund's complete adjuvant and 0.9% saline to a final concentration of 100 µg/ml. Five mice were immunized subcutaneously with 50 µg of the protein and boosted with an equal dose twice at 3-week intervals. Ten days after each boost, the mice were bled and their sera tested by

enzyme-linked immunosorbent assay (ELISA) for relevant antibody activity to the MBP-hNIS fusion protein. A final boost was given on week 10 by i.v. injection into a tail vein of the mouse that demonstrated the highest antibody titer of 100 µg of the MBP-hNIS fusion protein diluted in 0.9% saline, to a final volume of 1 ml.

Monoclonal antibodies (MoAbs) production

The protocol used for generation of B cell hybridomas has previously been described (De St Groth & Scheidegger 1980).

The studies and procedures were reviewed and approved by the Mayo Foundation Institutional Animal Care and Use Committee.

Enzyme-linked immunosorbent assay

Microtiter plates were coated with 10 µg/ml MBP-hNIS fusion protein or MBP only and incubated overnight at 4 °C. Non-specific binding sites were blocked with 1%

Table 1 Human NIS peptides

	Number	Sequence	Amino acids
ExMD			
10	368–388	EDLIKPRRLSLAPRKLVIISK	21
12	437–444	LPACNTPG	8
13a	468–487	PSEQTMRVLPSSAARCVALS	20
13b	483–502	CVALSVNASGLLDPALLPAN	20
13c	498–522	LLPANDSSRAPSSGMDASRPALADS	25
14a	550–569	TGPTKRSTLAPGLLWVDLAR	20
14b	565–584	WDLARQTASVAPKEEVAIDL	20
14c	580–599	VAILEDNLVKGPPEELPTGNK	20
14d	595–614	PTGNKKPPGFPLTNEEDRLF	20
14e	610–629	DRLFELGQKELEGAGSWTPC	20
14f	625–643	SWTPCVGHGGRDQQTENL	19

Underlined residues represent regions of overlap with neighboring peptides.

BSA in PBS containing 0.05%–Tween-20 (PBS-T) and incubated at 37 °C for 1 h. Mouse sera diluted in PBS-T, or hybridoma supernatant media when screening clones, were added to each well. Alkaline phosphatase goat anti-mouse (GAM; Sigma, St Louis, MO, USA) at 1:5000 dilution was used as the second stage antibody and nitrophenyl phosphate substrate in 1 mM MgCl₂, 0.5 mM ZnCl₂, pH 9.8 was used for detection. Optical density (O.D.) was read at 405 nm. Appropriate negative controls with irrelevant mouse immunoglobulin G (IgG) at identical dilutions, and positive controls were performed with each assay.

Hybridomas producing antibodies specific for hNIS rather than the MBP portion of the fusion protein were selected by differential screening. This was performed by initially selecting all positive clones obtained after the second round of subcloning and rescreening them simultaneously against both MBP-hNIS fusion protein and MBP. Only those hybridomas that tested positive against the former but negative against the latter were further subcloned.

The immunoglobulin class, subclass and light chain type of the monoclonal antibodies were determined by using a mouse hybridoma subtyping kit (Boehringer GmbH, Mannheim, Germany), according to instructions from the manufacturer.

Peptide production and epitope mapping

Peptides encompassing the amino acid sequences of the 10th, 12th, 13th and 14th ExMDs of hNIS were synthesized by solid phase synthesis and subsequently purified by HPLC on a C-18 column (Morris *et al.* 1990). The sequences were taken from Smanik *et al.* (1996). The amino acid sequences of the peptides synthesized are shown in Table 1. The purity and accuracy of the peptides

was assessed by amino acid analysis and mass spectrometry. Plates coated in triplicate with 10 µg/ml of each of the peptides described were subsequently incubated with antibody-rich ascites fluid (FP-5A and FP-13) diluted 1:5000 in 0.1% BSA-PBS-T, and ELISA was performed as described. Appropriate negative and positive controls were performed in each case.

Functional expression of hNIS in COS-7 cells

COS-7 cells were grown in Dulbecco's modified Eagle's medium (Gibco-BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum, 1% glutamine and 1% penicillin/streptomycin. Fresh media were replaced every other day until the cells reached approximately 75% confluency, at which time they were subcultured into 12-well plates at a density of ~100 000 cells/well. These cells were transfected by the lipofectamine technique (Gibco-BRL) with a pcDNA3 plasmid containing the gene for the full-length hNIS (amino acids 1–643) or the pcDNA3 vector only. Forty-eight hours after transfection, iodide uptake was assessed as previously described (Weiss *et al.* 1984). hNIS-transfected cells were incubated with various dilutions (1:10–1:1000) of the monoclonal antibodies for 1 h at 37 °C, in order to assess their effect on NIS activity.

Preparation of membranes

Membranes from COS-7 cells were prepared by a modification of a previously described procedure (Kaminsky *et al.* 1994). In brief, the transfected cells were washed with PBS, harvested and resuspended in ice-cold 250 mM sucrose, 10 mM Hepes pH 7.5, 1 mM EDTA, containing aprotinin (10 µg/ml), leupeptin (10 µg/ml) and 1 mM phenylmethylsulfonyl fluoride. The samples were centrifuged twice at 500 × *g* for 15 min at 4 °C. Na₂CO₃ (100 mM) was added to the resulting supernatant and shaken for 45 min at 4 °C, and then centrifuged at 100 000 × *g* for 15 min. The pellet obtained was resuspended in 500 µl of a buffer containing 250 mM sucrose, 10 mM Hepes pH 7.5, 1 mM MgCl₂ and aliquoted for future use. Protein content was quantitated using the Bio-Rad DC protein assay (BioRad, Hercules, CA, USA).

Western blot analyses

Membrane samples were prepared for electrophoresis by addition of NuPAGE SDS sample buffer with or without reducing agent, (NuPAGE; Novex, San Diego, CA, USA). The samples were then incubated at 70 °C in a water bath for 10 min and 2 µg membrane protein from cells transfected with FL-hNIS or control vector were loaded onto a 4–12% acrylamide gradient gel. After electrophoresis, the proteins were transferred to nitrocellulose membranes by electroblotting at 25 mV for 1 h.

Nonspecific binding sites were blocked with 5% milk in 20 mM Tris pH 7.4, 137 mM NaCl, 0.1% Tween-20 (TBST) for 1 h. The membranes were then incubated for 2 h at room temperature with the antibody preparations diluted 1:5000 with TBST. After this, they were washed 3 times with TBST and incubated for 1 h with peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratory, West Grove, PA, USA) at a 1:5000 dilution. After washing with the same buffer 4 times, the proteins were visualized with chemiluminescent detection reagents (ECL; Amersham, Arlington Heights, IL, USA).

Deglycosylation of membrane proteins was carried out with N-glycosidase F (New England Biolabs, Beverly, MA, USA) according to the manufacturer's protocol, as previously described (Jhiang *et al.* 1998).

Immunostaining

Paraffin-embedded sections of normal thyroid gland, Graves' disease thyroid gland, papillary, follicular and Hürthle cell thyroid cancers, as well as some non-thyroidal tissues (skin, adipose tissue) were stained by the immunoperoxidase method. The tissue sections were obtained from the archives of our Pathology department and deparaffinized by serial passages in xylene and graded alcohol, and endogenous peroxidase activity was blocked by incubation of slides in a 50% by volume solution of 3% H₂O₂/methanol for 10 min. Subsequently, the slides were subjected to antigen retrieval with 10 mM citrate buffer steam for 30 min and then rinsed for 2 min in tap water. Non-specific binding sites were blocked by applying 5% normal goat serum (NGS) diluted in PBS-T and incubated at room temperature for 30 min. Primary antibody (FP-13) diluted 1:1500 in 1% NGS-PBS-T was added and slides incubated for 1 h at room temperature. After this, they were rinsed in tap water for 2 min and PBS-T for another 2 min and subsequently incubated for 20 min with biotin-SP-conjugated goat-anti-mouse IgG at a 1:200 dilution in 1% NGS-PBS-T. After rinsing again as described above, the slides were incubated for 20 min in peroxidase-conjugated streptavidin (DAKO, Copenhagen, Denmark) at a 1:300 dilution in the same buffer, rinsed and then incubated for 2 min in sodium acetate solution (pH 5.2) followed by 15 min in 3-amino-9-ethyl-carbazole (AEC) substrate solution (Sigma). This solution was prepared by dissolving one 20 mg AEC substrate tablet in 5 ml dimethyl formamide, adding 95 ml of a sodium acetate solution, pH 5.2 and 1 ml 3% hydrogen peroxide. The solution was filtered prior to use. Finally, the slides were rinsed with tap water for 1 min, counterstained with hematoxylin and then rinsed under running tap water for 5 min. They were mounted using a glycerol gelatine previously warmed in a water bath.

Review of the charts of the patients whose slides are shown revealed that none of the patients with thyroid

Figure 2 Mouse antibody activity against MBP-hNIS fusion protein measured as optical density (O.D.) at 405 nm of mouse antibody titers after the second bleed, at 1:5000 dilution in ELISA. Means $\pm$ S.E. are shown.

cancer had received treatment with either thyroid hormone or radioiodine prior to the surgery. The patient with Graves' disease had received treatment with methimazole (15 mg/day) and Lugol solution for 2 weeks prior to the surgery.

Results

Mouse antibody titers

Ten days after the first boost, all mice had developed antibody titers, detectable at 1:10 000 dilution of the serum. After the second bleeding, antibody titers had increased even further. The mouse with the highest titers against MBP-hNIS fusion protein had detectable antibody at 1:50 000 dilution which was 3.6 times the O.D. of the negative control at the same dilution (Fig. 2).

Production of monoclonal antibodies and isotyping

After fusion and subcloning, twenty-four hybridoma clones were initially identified which recognized the MBP-hNIS fusion protein by ELISA. After differential screening, 2 clones were recovered which recognized MBP-hNIS fusion protein but not MBP (MoAbs FP-5A and FP-13). These clones were specific for hNIS and, on isotyping, were found to be IgG 1 κ (kappa).

Epitope mapping

The two antibodies obtained, when tested by ELISA against each of the peptides initially described, were found

Figure 3 Epitope mapping of antibodies against hNIS: ELISA assay activity of monoclonal and polyclonal anti-MBP-hNIS fusion protein antibodies against hNIS peptides 10 through 14, MBP and MBP-hNIS fusion protein. Both monoclonal antibodies (FP-5A and FP-13) recognized the carboxy-terminus sequence of hNIS (peptide 14f), and the fusion protein, although FP-5A also showed low-level activity against MBP. Polyclonal antibodies showed low-level activity against peptides 13c, 14c and 14f and strong activity against the fusion protein and MBP. Means $\pm$ s.e. are shown.

to recognize only the peptide containing the amino acid sequence 625–643 (14f) in the carboxy terminus of hNIS (Fig. 3). Antibody FP-5A also showed some level of activity against MBP, although this activity was much lower than that against the MBP-hNIS fusion protein (Fig. 3).

Iodide uptake

COS-7 cells transiently transfected with the FL-hNIS cDNA clone in the pcDNA3 vector accumulated 23·45-fold more Na^{125}I than cells transfected with the pcDNA3 vector only (Fig. 4) and this uptake was inhibited in the presence of 10 μM perchlorate, a known competitive inhibitor of thyroid iodine uptake (Taurog 1996).

Western blotting analysis

Western blotting was performed to assess recognition of the recombinant hNIS protein, in COS-7 membrane preparations, by the monoclonal antibodies. The hNIS proteins were detected by monoclonal antibodies against the MBP-hNIS fusion protein as a major band with a

Figure 4 Functional expression of hNIS in COS-7 cells. hNIS-transfected COS-7 cells showed a 23-fold increase in iodide uptake compared with COS-7 cells transfected with control vector. This uptake was inhibited in the presence of perchlorate (ClO_4), a known competitive inhibitor of iodide uptake.

Figure 5 Western blot of hNIS using transfected COS-7 cell membranes. SDS-PAGE was performed under reduced (left) and non-reduced (right) conditions. Note absence of band in cells transfected with control vector incubated with antibody (FP 13 or FP-5A). Also note similar migration of the 97 kDa band and differences between the 30 kDa and 15 kDa bands under both conditions.

molecular weight (MW) of ~ 97 kDa, and several minor bands with MW of ~ 160 kDa, ~ 68 kDa, ~ 30 kDa and ~ 15 kDa, all of them specific for the hNIS-transfected cells as compared with cells transfected with control vector (Fig. 5). The MW of hNIS in membrane fractions subjected to enzymatic deglycosylation was ~ 68 kDa, which was as expected based upon the amino acid sequence of hNIS (Fig. 6, left). After preincubation of the antibody with peptide 14f, the 97 kDa and 68 kDa bands disappeared (Fig. 6, right).

Immunostaining of thyroid and non-thyroidal tissues

Thyroid tissue from patients with Graves' disease incubated with monoclonal antibody against hNIS showed the most intense immunostaining, which was fairly homogeneous, present in almost all follicles and well

localized to the basolateral portion of the follicular cell membrane (Fig. 7A–B). No staining was observed in Graves' tissue incubated with irrelevant antibody (Fig. 7C). Hürthle cell cancers, which are recognized for their common lack of ability to concentrate iodine (Carcangiu *et al.* 1991, Caplan *et al.* 1994), did not show staining (Fig. 7D). Intermediate staining was observed in papillary and follicular thyroid cancers and, in these, the staining pattern was distinct from that observed in Graves' tissue, being more diffuse, patchy, and less well localized to the basolateral region (Fig. 7E–G). Immunocytochemistry of normal thyroid tissue showed patchy areas of intense staining, mostly in a follicular pattern alternating with other follicles with minimal or no staining (Fig. 7H). The staining was, in all cases, specific for the follicular epithelium (Fig. 7A–J). After preabsorption of the antibody with the peptide 14f, no immunostaining was

Figure 6 Western blot of hNIS using transfected COS-7 cell membranes before and after overnight preincubation with glycanase (left) and before and after preincubation with free peptide (14f) (right). Note the disappearance of the 97 kDa band and persistence of the 68 kDa band in the presence of glycanase (left); also note the disappearance of the 97 kDa and 68 kDa bands after preincubation with free peptide.

observed (Fig. 7I-J). Non-thyroidal tissues (skin, adipose tissue) incubated with these antibodies did not show immunostaining (Fig. 7K-L).

Discussion

We have generated monoclonal antibodies against the human sodium iodide symporter that are capable of recognizing this protein by ELISA, Western blotting and immunohistochemistry with a high degree of specificity. Western blotting of COS-7 membranes transfected with hNIS showed a major band with a MW ~97 kDa and several other minor bands with MW of ~160 kDa,

~68 kDa, ~30 kDa and ~15 kDa. These minor bands most likely represent degradation fragments of the protein although further study is required to assess this fully. However, it is also possible that the ~68 kDa band seen in the hNIS-transfected cells represents non-glycosylated hNIS. The MW of the major band of hNIS observed under reduced and non-reduced conditions did not differ (Fig. 5). These results are in agreement with those of Jhiang *et al.* (1998) using polyclonal antibodies against hNIS. However, significant differences were observed in the intensity of the 30 kDa and the 15 kDa bands when SDS-PAGE was performed under reduced vs non-reduced conditions. Under non-reduced conditions the 30 kDa band was more intense than the 15 kDa band, while the opposite was true when SDS-PAGE was

Figure 7 Immunostaining of thyroid tissue with monoclonal antibodies against MBP-hNIS fusion protein. (A-C) Graves' tissue: (B) note strong staining in the basolateral portion of the follicular cell membrane, (C) Graves' tissue incubated with irrelevant antibody (mouse IgG), (D) Hürthle cell cancer; note absence of staining. (E-F) Follicular thyroid cancer: (F) note the patchy and poorly localized staining pattern in malignant cells. (G) Papillary thyroid cancer. (H) Normal thyroid tissue. (I) Graves' tissue before and (J) after preabsorption with free peptide 14f. (K-L) Non-thyroidal tissues with no immunostaining: (K) skin, (L) adipose tissue.

performed after incubating the membranes with the reducing agent. This difference suggests the presence of disulfide bonds in the 30 kDa fragment which are at least partially eliminated in the reduction process, although reduction was probably incomplete since the 30 kDa band faded but did not completely disappear. It has previously been demonstrated that hNIS is a glycoprotein. Its expected MW in the non-glycosylated form is ~68 kDa, similar to that found in non-glycosylated COS-7 cells transfected with hNIS. We found the MW of glycosylated hNIS in our transfected COS-7 cells to be ~97 kDa, slightly higher than that previously reported (Jhiang *et al.* 1998). This difference is most likely due to different conditions of gel electrophoresis or minor differences in the quality or quantity of glycosylation achieved in our system. Our results with the deglycosylated membranes of transfected COS-7 cells are consistent with the expected MW of hNIS.

Neither of the antibodies inhibited iodide uptake in transfected COS-7 cells. This is probably due to the fact that they recognize an epitope which is localized in the intracellular side of the membrane. Antibodies raised against extracellular domains would be helpful in clarifying this question.

The results of the analysis of the activity of our monoclonal antibodies against the different peptides, MBP and the fusion protein, demonstrate the specificity of our antibodies against hNIS. The polyclonal antibodies previously raised against the same MBP-hNIS fusion protein (Jhiang *et al.* 1998) were analyzed simultaneously with our monoclonal antibodies and found to have a lower level of activity against three different peptides (13c, 14c and 14f) of hNIS, although they also recognized MBP to a much higher degree (Fig. 3). However, the MoAbs were more specific against the 14f peptide of hNIS, although FP-5A displayed low level activity against MBP as well, albeit to a much lesser degree than either the polyclonal antibodies or its activity against the fusion protein. FP-13 had no activity against MBP. Mammalian cells, on the other hand, do not express bacterial MBP and therefore the activity of the MoAb FP-5A or the polyclonal antibodies against this molecule did not interfere with their ability to recognize hNIS or its peptides by either ELISA or Western blotting analysis.

The results of immunohistochemistry also show that our antibodies are capable of recognizing hNIS with high specificity. The ability of these antibodies to stain different types of thyroid tissue with varying intensity is probably related to the degree of expression of this protein in the different tissues. This is supported by the demonstration of very strong staining in several Graves' disease tissues, the variable although often lower and more patchy staining in certain thyroid cancers, and the lack of significant staining in Hürthle cell cancer tissues, which are notorious for their frequent lack of ability to concentrate radioactive iodine (Carcangiu *et al.* 1991, Caplan *et al.* 1994). The specificity

of our antibodies for NIS is demonstrated by their ability to recognize only follicular rather than stromal cells, and by the finding that the strongest immunostaining is observed in the basolateral aspect of the follicular cell membrane (Fig. 7A-B), where the iodide symporter has been localized (Carrasco 1993). The lack of immunostaining of non-thyroidal tissues (such as skin and adipose tissue) when incubated with these antibodies also supports their specificity for hNIS.

In summary, we have generated monoclonal antibodies against a fusion protein containing the last extracellular loop and the carboxy terminus of hNIS, which are capable of recognizing this protein with high affinity and specificity and are useful for immunostaining of thyroid tissue. Because the degree of immunostaining probably correlates with the expression of this protein in different thyroid tissues, these antibodies may be useful in predicting the ability of thyroid cancers to concentrate iodine. They may thus prove to be important tools in evaluating the role of NIS expression in the biological behavior of thyroid malignancies.

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