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Autoantibody against Na^+/I^- Symporter in the Sera of Patients with Autoimmune Thyroid Disease

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Using recombinant rat Na^+/I^- symporter (NaIS) protein, we have immunochemically searched for the autoantibody in the sera from patients with autoimmune thyroid disease. We found that 22 out of 26 sera (84%) from patients with Graves' disease and 3 out of 20 sera (15%) from patients with Hashimoto's thyroiditis recognized it. By Western blot, these IgGs reacted with 80 kDa protein in FRTL-5 cell membrane, which co-migrated with the band stained by rabbit antibody toward NaIS. These results indicate that autoantibody against NaIS, newly identified antibody, frequently exists in patients with autoimmune thyroid disease, especially in Graves' disease.

Autoimmune thyroid diseases such as Hashimoto's thyroiditis and Graves' disease are the representative organ specific autoimmune disorders, in which thyroid specific proteins, e.g., thyroglobulin (Tg), thyroid peroxidase (TPO) and thyrotropin receptor (TSH-R), are the target for the autoantibody (1, 2). These antibodies are especially useful for diagnosis of the disease, but, there exists consensus that the titer of these autoantibodies is not well correlated with the clinical course of the disease (3).

Very recently, Dai et al. have cloned rat thyroid Na^+/I^- symporter cDNA and have revealed that it encodes an intrinsic membrane protein with 12 putative transmembrane domains (4). By Northern analysis, they identified the NaIS mRNA in the thyroid, but not in liver, kidney, intestine, brain or heart, suggesting that the gene is primarily expressed in thyrocytes.

Since it is likely that NaIS, thyroid specific protein, might be the target for the autoantibody in autoimmune thyroid disease, we have investigated the existence of the autoantibody against NaIS in the sera from patients with the disease.

MATERIALS AND METHODS

Cloning of rat NaIS cDNA. Total RNAs from FRTL-5 cells cultured in the presence of TSH (5) were prepared by the guanidinium/cesium chloride ultracentrifugation method and mRNAs were isolated using Oligo(dT)-Latex (Takara Shuzo, Tokyo). For polymerase chain reaction (PCR), mRNAs were transcribed into cDNAs by avian reverse transcriptase (Takara Shuzo) and then used as templates. Primers used here to obtain NaIS cDNA were as follows (A in ATG initiation codon of rat NaIS cDNA (4) is designated as +1). Sense primer: 5′-GATCGAATTCATCCTCTCCTC-ACCGATGCA (residues −29 to −9), anti-sense primer 1: 5′-CGCAGAATTCTCAGAGGTTGGTCTCCACAT (residues 1955 to 1975), anti-sense primer 2:CGCAGAATTCAGGTTGATCCGGGAATGGTT (residues 782 to 802). PCR reactions contained 10 ng of template cDNA, 1.5 U of AmpliTaq (Perkin Elmer) and 125 ng of each primer. PCR was performed for 30 cycles as following conditions; 0.5 min at 94 C, 1 min at 55 C and 2 min at 74 C. PCR products were subcloned into M13 mp18 or 19 phage and sequenced.

Preparation of NaIS fusion protein and production of its rabbit antibody. NaIS cDNA (−29 to 802 bp) was ligated into the EcoR1 site of pGEX-2T (Pharmacia) in the correct orientation and transfected into E. coli (BL21(DE3)pLysS) (Novagen). After induction with isopropyl-β-D-thio-galactopyranoside (IPTG), the fusion protein was purified using

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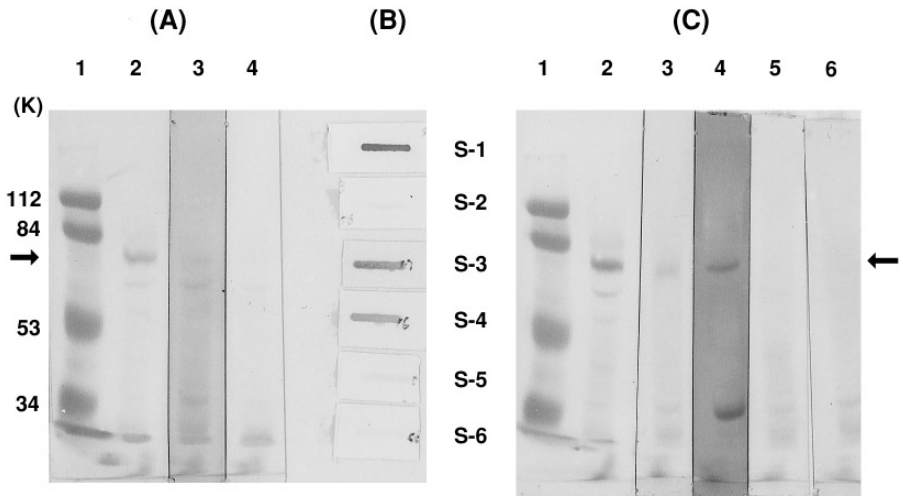

FIG. 1. Immunodetection of NaIS by rabbit antibody and patients' sera. (A) Western blot analysis of NaIS by rabbit antibody to NaIS. Membrane fraction (10 μg of protein) from FRTL-5 cells, cultured in the presence of TSH, was electrophoresed in 10% polyacrylamide-0.1% SDS and transferred to nitrocellulose filter. The filters were incubated with rabbit antiserum (1:500) (lane 2), absorbed serum (lane 3) and preimmune serum (1:500) (lane 4), followed by further staining with peroxidase conjugated anti-rabbit IgG (Jackson Immunoresearch) (1:1000). Lane 1: prestained M.W. marker (BioRad). (B) Immunodetection of recombinant NaIS by rabbit antibody and patients' sera. Recombinant NaIS (50 ng) was slot blotted onto nitrocellulose sheets. S1 and 2 were stained with rabbit antibody (1:500) and preimmune serum (1:500), respectively. S3 and 4 were stained with the sera from Graves' patients 1 and 2 (1:500), respectively, and S5 and 6 were by normal subjects (1:500). (C) Western blot analysis of NaIS by Graves' IgGs. FRTL-5 cell membrane on the filter was incubated with rabbit antibody (lane 2), Graves' IgGs from patients 1 and 2 (lanes 3 and 4) and normal subjects (lanes 5 and 6), and then stained with peroxidase conjugated anti-human IgG (Jackson Immunoresearch, 1:1000). Lane 1: M.W. marker.

Glutathione Sepharose 4B. This glutathione S-transferase (GST)/N-terminal portion of NaIS fusion protein (1 mg) was emulsified with Freund's complete adjuvant and injected into rabbits every 2 weeks. For obtaining NaIS whole molecule, we ligated the cDNA (−29 to 1975 bp) into pTrcHis-A (Invitrogen) and also transfected into E. coli (BL21(DE3)pLysS). Histidine tagged NaIS protein was solubilized with 6 M guanidine and was affinity purified using HisBond column (Invitrogen).

Immunostaining of NaIS protein. For Western blotting, membrane fraction was prepared from FRTL-5 cells in the buffer containing 10 mM Tris-HCl, 5 mM NaCl, 1 mM phenylmethylsulfonyl fluoride and 50 μg/ml of leupeptin (pH 7.4) (6). They were separated in 0.1% SDS-10% polyacrylamide gel and transferred to nitrocellulose filter. For detection of GST/N-terminal NaIS fusion protein or histidine tagged NaIS whole protein, these recombinant proteins (50 ng) were slot blotted on nitrocellulose sheets. Immunostaining of these NaIS proteins was performed as described previously (6).

Patients' sera. Diagnosis of Graves' disease was made by conventional criteria (diffuse goiter, hyperthyroidism and exophthalmos) and elevated TSH-binding inhibitor immunoglobulin (TBII) activity. Diagnosis of Hashimoto's thyroidits was made on the basis of elevated anti-Tg or anti-TPO antibody activity in patients with a hard diffuse goiter without TBII activity, and additional evidence by fine needle aspiration.

RESULT AND DISCUSSION

At first, we amplified rat NaIS cDNA (−29 to 802 bp) from mRNA of FRTL-5 cells, cultured rat thyroid epithelial cells (5), by polymerase chain reaction methods. After sequencing, we ligated it into pGEX-2T (Pharmacia). Glutathione S-transferase (GST)/N-terminal portion of NaIS (amino acid No.1 to 231) fusion protein, purified by Glutathione Sepharose 4B, was injected into rabbits, and 6 weeks later, we obtained the antisera against it. The antibody recognized rat NaIS as 80 kDa protein in FRTL-5 cell membrane, the molecular size of which well coincides with that reported by Carrasco et al. (personal communication). The positive

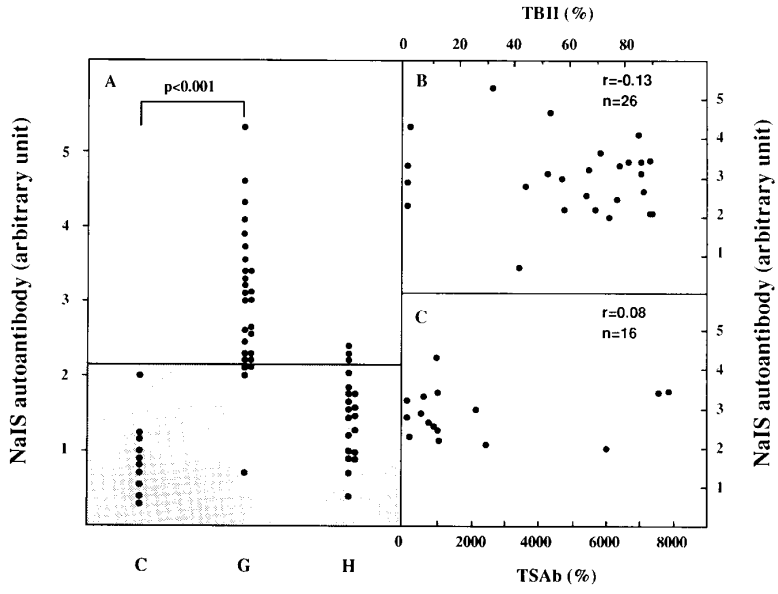

FIG. 2. Incidence of autoantibody against NaIS in Graves' disease and Hashimoto's thyroiditis (A). The mean ± 2SD of the staining intensity of normal subjects (n = 10) is accounted for normal range. Statistical difference was calculated with Mann-Whitney test. (B) Correlation of NaIS autoantibody with TBII activity of Graves' IgG. TBII activity of the patients' IgGs was measured by TSH-R. Assay Kit from Ortho-Clinical Diagnostic K.K. (C) Correlation of NaIS autoantibody with TSAb activity of Graves' IgG. TSAb activity was measured using porcine thyrocytes as described previously (7).

staining was absorbed by the addition of the antigen to the serum and no band was visible with pre-immune serum (Fig. 1A). Next, we ligated rat NaIS cDNA (−29 to 1975 bp, full coding sequence) to pTrcHis-A (Invitrogen), and obtained histidine tagged NaIS protein using Metal Affinity Chromatography. This recombinant NaIS protein was clearly stained by rabbit antibody toward NaIS (Fig. 1B).

Using this recombinant protein slot blotted on nitrocellulose sheets, we have attempted to detect the autoantibody against NaIS in the sera from patients with autoimmune thyroid disease by staining with peroxidase conjugated anti-human IgG (Fig. 1B). Fig. 2A represents the binding intensity of patients' sera to the protein measured by reflection type of densitometer. The recombinant protein was significantly detected by the sera of patients with Graves' disease (n=26) as compared to those of control subjects (n=10) (Graves' group 3.0 ± 0.95 vs control group 1.0 ± 0.56; $p<0.001$). When the mean ± 2SD of the control values is accounted for the normal range, we found that 22 out of 26 Graves' sera were positive (84%). We also studied whether the sera from patients with Hashimoto's thyroiditis react with NaIS protein or not. No statistical significance was observed in difference between binding signal to the protein by Hashimoto's sera (1.47 ± 0.49) and control sera. However, 3 out of 20 sera were also found to be positive (15%) (Fig. 2A). High incidence of NaIS autoantibody in Graves' disease and relatively low incidence in Hashimoto's thyroiditis was reminiscent of that of TSH-R autoantibody in Graves' disease. So, we further studied the correlation between the autoantibody to NaIS and to TSH-R in Graves' patients (Fig. 2B and C). No correlation was found between the staining intensity of NaIS and TSH-binding inhibitor immunoglobulin (TBII) activity (2) and thyroid-stimulating antibody (TSAb) activity (7). We also found no relation between the intensity and the titer of anti-Tg antibody or anti-TPO antibody in the sera (data not shown).

To confirm that these positive sera truly react with NaIS protein, we performed Western blot analysis (6) using patient's IgG, which strongly stained recombinant NaIS. These Graves' sera clearly stained 80 kDa protein in FRTL-5 cell membrane, which co-migrated with the band recognized by rabbit antibody toward NaIS (Fig. 1C).

We reported here the autoantibody against NaIS protein, newly identified antibody, in autoimmune thyroid disease. It is of particular interest that the incidence is extremely high in Graves' disease, and the value is almost comparable to that of TSH-R autoantibody in the disease (1-3). Although the function of this autoantibody remains to be elucidated, it seems possible that the autoantibody might affect iodide influx into the thyrocytes and influence the thyroid function of patients with autoimmune thyroid disease.

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