Preparation of a Monoclonal Antibody Specific for Human Stanniocalcin

Yoshio KOIDE,*[a,e] Akira IGARASHI,[d] Nobuhiro SAKUMA,[a] Kanako SATO,[a] Shinobu SHIBATA,[b] Kotaro TADA,[c] Shoichi TAKANO,[d] and Takeshi SASAKI[a]

Department of Clinical and Laboratory Medicine,[a] The Second Department of Internal Medicine,[b] and Department of Immunology,[c] Tohoku University, School of Medicine, Seiryo-machi, Aoba-ku, Sendai, Miyagi 980–8574, Japan, R & D Center, BML, Inc.,[d] Matoba, Kawagoe, Saitama 350–1101, Japan, and Tohoku College of Medical Technology and Welfare,[e] Kunimi, Aoba-ku, Sendai, Miyagi 981–8553, Japan. Received June 22, 1998; accepted August 22, 1998

Stanniocalcin (STC) is a glycoprotein hormone that was first identified in fish, where it regulates the calcium level in the body fluid. The cDNA which encodes human STC has recently been reported but the function has not been completely elucidated. We have prepared a monoclonal antibody against human STC using an analogous peptide of the putative antigenic domain in human STC; it was conjugated with keyhole limpet hemocyanin (KLH). The monoclonal antibody specifically stained the distal convoluted tubules in human kidney which is a putative target organ of STC. The ELISA was established using the monoclonal antibody and recombinant human STC as a standard antigen. The monoclonal antibody prepared in this study provides a useful tool for clinical studies of STC in human.

Key words monoclonal antibody; stanniocalcin; calcium regulating hormone

Stanniocalcin (STC) is a glycoprotein hormone that was first isolated from bony fishes. In fish, STC is synthesized and secreted by the corpuscles of Stannius, a unique endocrine gland in fish, that is associated with the surface of the kidney. The major function of fish STC is to regulate blood calcium levels by decreasing the calcium ion uptake in the gill from the aquatic environment (reviewed by Hirano,[1] and Wagner[2]). The structure of fish STCs has been elucidated by deducing from the nucleotide sequences of Australian eel[3] and coho salmon[4] of STC cDNA, and by determining the complete amino acid sequence of chum salmon STC[5]. From the characteristic analysis of fish STCs, the proteins are revealed to be homodimeric forms with molecular weights ranging from 39 to 54 kDa, and do not have sequence similarities to calcitonin, parathyroid hormone, and parathyroid hormone-related protein of mammals. So far, STC has been regarded to be a unique hormone in fish because the corpuscles of Stannius and gill do not exist in mammals.

Recently, mammalian cDNAs encoding STC were obtained from human[6,7] and mouse.[8] These amino acid sequences deduced from the cDNAs were found to have high sequence homology with fish STC. All half-Cys residues and a glycosylation consensus sequence in mammalian cDNA encoding STC were located in positions homologous to those in the fish STC. The expression of the mRNA of human (h-) STC was up-regulated by calcium ions *in vitro*.[6] h-STC has been reported to regulate calcium and phosphate ion excretion and/or reabsorption in the kidney,[9] and STC-immunoreactive cells are found in the kidney.[7,9,10] Unlike the STC of fish, the mRNA of h-STC is expressed in multiple tissues,[6,7] suggesting that h-STC may have additional functions different from fish STC. However, the functional significance of h-STC in multiple tissues has not been identified. To clarify the function of h-STC, therefore, preparation of a monoclonal antibody (MoAb) specific for h-STC is desirable.

In this study we prepared a MoAb, termed 9D12E, which recognizes h-STC using an analogous peptide which corresponds to a putative antigenic domain of h-STC as an antigen.

MATERIALS AND METHODS

Materials The synthetic 20-mer-peptide was purchased from Iwaki Glass Co, Ltd. (Tokyo, Japan). Keyhole limpet hemocyanin (KLH) and *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) were purchased from Sigma Chemicals (St. Louis, U.S.A.). Recombinant h-STC was prepared according to the method described by Olsen *et al.*[7] All other chemicals were reagent grade from commercial sources.

Preparation of MoAb The synthetic h-STC peptide CYNRLVRSLLEADEDTVSTI (127—145), in which the 137th alanine residue was substituted with cysteine, and a cysteine residue was supplemented at the N-terminus of the peptide, was used as an antigen (Fig. 1). The synthetic peptide was conjugated to KLH through the thiol group of cysteine of the N-terminus of the peptide according to the method of Imajoh-Ohmi *et al.*[11] Briefly, 16 mg of KLH dissolved in 1 ml of 0.01 M phosphate buffer, pH 7.2, was reacted with 2.6 mg of MBS dissolved in 0.1 ml of dimethylformamide at 25 °C. After 30 min, excess MBS was removed by a Sephadex G-25 column, then the resulting MBS-reacted KLH solution (1 mg/ml, 0.6 ml) was added to the peptide solution (3 mg in 1.8 ml of 0.1 M Na_2HPO_4). The reaction mixture was stirred for 6 h at room temperature. The resulting peptide-KLH conjugate was stored at −20 °C until use.

The immunization in mouse and preparation of MoAb were performed according to conventional procedures. Female BALB/c mice (8 weeks old) were immunized intradermally with peptide-KLH conjugate (20 μg peptide/mouse) in PBS emulsified with an equal volume of Freund's complete adjuvant. After 2 and 4 weeks, mice were further immunized with 20 μg of the peptide-KLH conjugate in PBS emulsified with Freund's incomplete adjuvant. Seven days after the third immunization, the mice received a booster intraperitoneal injection of 20 μg of peptide-KLH in PBS without adjuvant. After four days the mice were sacrificed, and spleen cells were collected.

The spleen cells were fused with SP2/0-Ag14 myeloma cells in the presence of 50% polyethylene glycol 1500. The hybridomas obtained were screened for production of the an-

* To whom correspondence should be addressed.

tibody to the peptide by a non-competitive ELISA method. Hybridoma cells producing the antibody were cloned by a limiting dilution method using mouse thymocytes as feeder cells. Thus, a clonal hybridoma cell line was established. The cells were implanted intraperitoneally in BALB/c mice pretreated with 0.5 ml of pristan. Subsequently, ascites fluid containing the MoAb was obtained.

The MoAb was purified from the ascites fluid by affinity chromatography on a Protein A-Sepharose (Pharmacia LKB, Uppsula, Sweden) column following ammonium sulfate precipitation. The purified MoAb was stored at −20 °C until use.

Preparation of Polyclonal Antibody Recombinant h-STC (100 μg/ml in PBS) was emulsified with an equal volume of Freund's complete adjuvant and was injected (1 ml each) into rabbit six times at 2-week intervals. Blood was collected from the carotid artery, then the sera were separated. A polyclonal antibody was obtained from the sera using a recombinant h-STC binding Sepharose 4B according to the manufacturer's instructions. The eluate was dialyzed against PBS and stored at −20 °C until use.

Western Blotting Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) of the recombinant h-STC dissolved in sample buffer in the presence of 1% 2-mercaptoethanol was performed according to the method described by Laemmli[12] using 10% polyacrylamide gel. The apparent molecular weight of recombinant h-STC was estimated by a molecular weight marker (GibcoBRL, MD, U.S.A.). After electrophoresis, the proteins on the gel were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, MA, U.S.A.) by the method of Towbin *et al.*[13] The membrane was blocked with 1% BSA in PBS at room temperature for 1 h. After washing with PBS containing 0.1% tween 20, the membrane was incubated with the MoAb (3 μl/ml) at 4 °C overnight. Then the membrane was washed three times and subsequently incubated with horseradish peroxidase (HRP)-conjugated anti-mouse IgG (1 : 5000, Kirkegaard Lab., MA, U.S.A.) at room temperature for 1 h. Staining of the membrane was performed in diaminobentidine-hydrochloride and 0.03% H_2O_2 solution.

ELISA A 96-well plate (No. 2595, Costar, MA, U.S.A.) was incubated at 4 °C overnight with 50 μl of the MoAb (1 μg/ml) in 0.05 M bicarbonate buffer (pH 9.5). A plate coated with the antibody was then incubated with 1% BSA in PBS for 1 h at room temperature. 50 μl of recombinant h-STC serially diluted with 1% BSA was added to the well and incubated at 37 °C for 1 h. After three washings with PBS, 50 μl of anti-recombinant STC polyclonal antibody (2 μg/ml) was added and incubated at 37 °C for 1 h. After washing, 50 μl of 1 : 5000 diluted goat anti-rabbit IgG conjugated with HRP (Kirkegaard Lab., MA, U.S.A.) was added and incubated at 37 °C for 1 h. After washing, 50 μl of *o*-phenylendiamine solution was added and incubated for 30 min at room temperature. The peroxidase reaction was stopped by 2*N*-H_2SO_4, and the absorbance of 492 nm was measured by a plate reader.

Immunohistochemistry Human kidney tissues obtained from the Department of Pathology, Tohoku University Hospital, were immediately fixed in 4% paraformaldehyde for 2 h at room temperature. They were dehydrated in a series of increasing concentrations of ethanol and embedded in paraffin. The specimens were sectioned at 2 μm and then mounted on glass slides.

Immunohistochemical staining was performed using the MoAb, and a commercially-available avidin-biotin-peroxidase system (Vectastain ABC-mouse IgG kit). Briefly, after deparaffinization of the section, endogenous peroxidase was blocked by treatment with 0.3% H_2O_2 in methanol for 30 min. After blocking of non specific protein binding with 10% normal goat serum in PBS for 30 min, the MoAb (3 μl) was added to a final concentration of 3 μg/ml and incubated overnight at room temperature. Biotinylated anti-mouse IgG was then added, and the avidin-biotin-peroxidase reaction was initiated. The enzyme activity was developed with di-

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                          -30       -20       -10        1        10        20        30
                           +         +         +         +         +         +         +
1, Salmon STC                                                FSPNSPSDVARCLNGALDVGCGTFACLENS
2, Mouse STC          MLQNSAVILALVISAAATHEAEQNDSVSPRKSRVAAQNSAEVVRCLNSALQVGCGAFACLENS
3, Human STC          MLQNSAVLLVLVISASATHEAEQNDSVSPRKSRVAAQNSAEVVRCLNSALQVGCGAFACLENS

                          40        50        60        70        80        90       100
                           +         +         +         +         +         +         +
1, Salmon             TCDTDGMHDICQLFFHTAATFNTQGKTFVKESLRCIANGVTSKVFQTIRRCGVFQRMISEVQEECYSRLD
2, Mouse              TCDTDGMYDICKSFLYSAAKFDTQGKAFVKESLKCIANGITSKVFLAIRRCSTFQRMIAEVQEDCYSKLN
3, Human              TCDTDGMYDICKSFLYSAAKFDTQGKAFVKESLKCIANGVTSKVFLAIRRCSTFQRMIAEVQEECYSKLN

                         110       120       130       140       150       160       170
                           +         +         +         +         +         +         +
1, Salmon             ICGVARSNPEAIGEVVQVPAHFPNRYYSTLLQSLLACDEETVAVVRAGLVARLGPDMETLFQLLQNKHCP
2, Mouse              VCSIAKRNPEAITEVIQLPNHFSNRYYNRLVRSLLECDEDTVSTIRDSLMEKIGPNMASLFHILQTDHCA
3, Human              VCSIAKRNPEAITEVVQLPNHFSNRYYNRLVRSLLECDEDTVSTIRDSLMEKIGPNMASLFHILQTDHCA
   Peptide as antigen                          CYNRLVRSLLEADEDTVSTI

                         180       190       200       210
                           +         +         +         +
1, Salmon             QGSNQGPNS
2, Mouse              QTHPRADFNRRRTNEPQKLKVLLRNLRGTGDSPSHIKRTSQESA
3, Human              QTHPRADFNRRRTNEPQKLKVLLRNLRGEEDSPSHIKRTSHESA
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Fig. 1. Comparison of Amino Acid Sequences of Stanniocalcin and a Peptide Used in the Preparation of MoAb as an Antigen

The sequences are taken from salmon,[5] mouse,[8] and human.[6]

aminobenzidine tetrahydrochloride as the substrate, and the slides were finally counterstained in methyl green solution. As a control, a section was stained with the same MoAb preabsorbed with recombinant STC (30 μg/ml).

RESULTS AND DISCUSSION

Polyclonal antibodies against h-STC,[7] as well as those against fish-STC[14—16] have been prepared through immunization with recombinant STC protein. There are, however, no reports for obtaining MoAb specific for h-STC. The reason for this can be attributed to the identical sequence of the deduced mature protein of h-STC and that of mouse-STC[8] (Fig. 1). To obtain the MoAb, we used an analogous peptide corresponding to a possible antigenic domain of h-STC as an antigen. The domain was screened by employing hydrophilicity, secondary structure, and homology search analysis. The fragment consisting of amino acid residues from 127 to 145 of the deduced amino acid sequence of h-STC, which has high hydrophilic property with no homology to any published sequences in human proteins, was chosen as a possible antigenic domain.

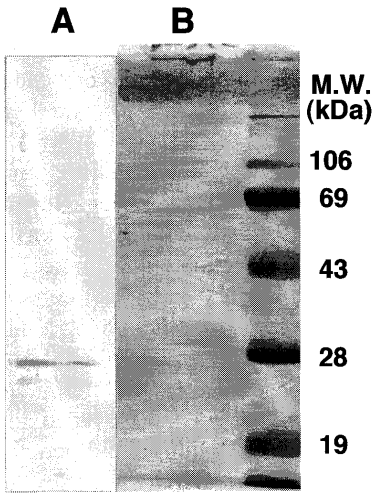

Fig. 2. Western Blotting of Recombinant h-STC

Electrophoresis was carried out on 1 μg of sample after reduction by 2-mercaptoethanol. A, Proteins are stained with MoAb (3 μg/ml); B, stained with Coomassie blue. The apparent molecular weights of the marker proteins are given on the right.

The synthetic h-STC peptide CYNRLVRSLLEADEDTVSTI, which was substituted with an amino acid residue (A for C137 of h-STC), was used to prepare the MoAb as an antigen. One hybridoma clone, termed 9E12D, which produces monoclonal anti-peptide antibody was prepared by fusing mouse myeloma cells with spleen cells from mice immunized with the peptide. The isotype of the MoAb was IgG_{2b}, which was determined by a mouse monoclonal sub-isotyping kit. The reactivity of the MoAb with h-STC was examined in the three ways: Western blotting, ELISA, and immunohistochemistry. In Western blotting, the MoAb reacted with the protein band (M.W. of 25 kDa) of recombinant h-STC (Fig. 2). As shown in Fig. 3, a sandwich ELISA was developed by combining the MoAb and anti-recombinant h-STC polyclonal antibody (rabbit). The recombinant h-STC was measured in a dose-dependent manner. The minimum measurable concentration of h-STC was 100 ng/ml by this assay system. Next, to examine whether the MoAb recognize the native STC of human origin, the binding ability of the MoAb was checked by immunohistochemical staining on human kidney section. As shown in Fig. 4, the MoAb stained the distal convoluted tubules specifically, and did not stain other cells such as glomeruli, loops of Henle, smooth muscle cells, or hepatocytes. The staining reaction in the proximal tubules was abolished following pretreatment with recombinant h-STC. These results suggested that the MoAb prepared against the peptide recognized native h-STC in human kidney specifically. Further immunohistochemical study using the MoAb is in progress to localize the other tissues that expressed STC mRNA.

The physiological activities of STC in mammals have been studied. Recombinant h-STC has a regulatory effect on calcium and phosphate metabolism in rat kidney and intestine *in vitro*[17,18] which was similar to the function of fish STC. However, h-STC differ from the fish-STC in terms of mRNA expression. Fish STC mRNA is expressed only in the corpuscles of Stannius, while h-STC mRNA seem to be expressed in multiple tissues.[6,7] These findings suggest that h-STC may exert additional influence on other tissues. On the other hand,

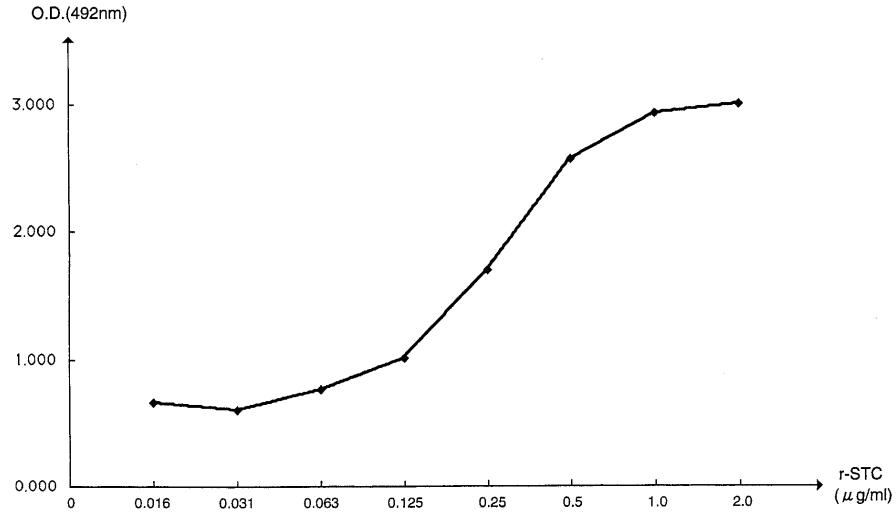

Fig. 3. Dose–Response Curve of Recombinant h-STC Using the Sandwich ELISA as Described in "Materials and Methods"

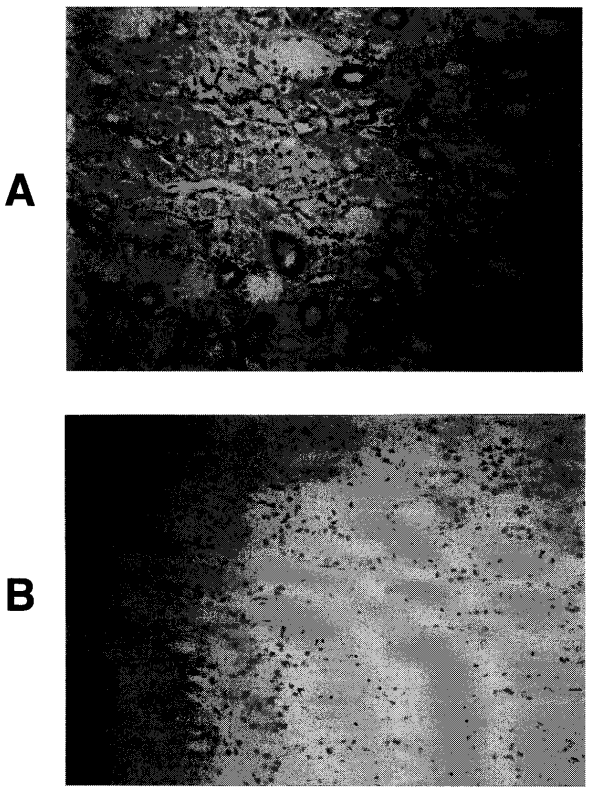

Fig. 4. Immunostaining of Human Kidney

A, stained with the MoAb (3 μg/ml); B, stained with the same MoAb preabsorbed with recombinant h-STC (30 μg/ml).

before the discovery of h-STC, several researchers reported that salmon STC and its fragment peptide have effects on the metabolism of mammalian bone[19—21]; the question of whether h-STC has the same function remains to be studied.

To date, the physiological functions of h-STC are not completely elucidated. The MoAb for specific h-STC prepared in this study would be a useful tool not only for understanding the roles of h-STC in human but also for investigation of the clinical significance of STC using diagnostic procedures.

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REFERENCES

1) Hirano T., "Vertebrate Endocrinology: Fundamentals and Biomedical Implications," Vol. 3, eds. by Pang P. K. T., Schreibman M. P., Academic Press, San Diego, 1989, pp. 139—169.
2) Wagner G. F., *Fish Physiol.*, **13**, 273—306 (1994).
3) Butkus A., Roche P. J., Fernley R. T., Haralambidis J., Penschow J. D., Ryan G. B., Trahair J. F., Tregear G. W., Coughlin J. P., *Mol. Cell. Endocrinol.*, **54**, 123—134 (1987).
4) Wagner G. F., Dimattia G. E., Davie J. R., Copp D. H., Friesen H. G., *Mol. Cell. Endocrinol.*, **90**, 7—15 (1992).
5) Yamashita K., Koide Y., Itoh H., Kawada N., Kawauchi H., *Mol. Cell. Endocrinol.*, **112**, 159—167 (1995).
6) Chang A. C. M., Janosi J., Hulsbeek M., de Jong D., Jeffrey K. J., Noble J. R., Reddel R. R., *Mol. Cell. Endocrinol.*, **112**, 241—247(1995).
7) Olsen H. S., Cepeda M. A., Zhang Q-Q., Rosen C. A., Vozzolo B. L., Wagner F., *Proc. Natl. Acad. Sci. U.S.A.*, **93**, 1792—1796 (1996).
8) Chang A. C. M., Dunham M. A., Jeffrey K. J., Reddel R. R., *Mol. Cell. Endocrinol.*, **124**, 185—187(1996).
9) Wagner G. F., Guiraudon C. C., Milliken C., Copp D. H., *Proc. Natl. Acad. Sci. U.S.A.*, **92**, 1871—1875 (1995).
10) Haddad M., Roder S., Olsen H. S., Wagner G. F., *Endocrinology*, **137**, 2113—2117 (1996).
11) Imajoh-Ohmi S., Tsujimura K., Inagaki M.,(eds) "Protocol for Experiments of Anti-peptide Antibodies" Shujunsha, Tokyo, 1994, 48—52 (in Japanese).
12) Laemmli U. K., *Nature* (London), **227**, 680—685 (1970).
13) Towbin H., Staehelin T., Gordon J., *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350—4354 (1979).
14) Kaneko T., Fraser R.A., Labedz T., Harvey S., Lafeber F. G. J. G., Pang P. K. T., *Gen. Comp. Endocrinol.*, **69**, 238—245 (1988).
15) Gellersen B., Wagner G. F. Copp D. H., Friesen H. G., *Endocrinology*, **123**, 913—921 (1988).
16) Sundell K., Bjornsson B. Th., Itoh H., Kawauchi H., *J. Comp. Physiol. B.*, **162**, 489—495 (1992).
17) Wagner G. F., Vozzolo B. L., Jaworski E., Haddad M., Kline R. L., Olsen H. S., Rosen C. A., Davidson M. B., Renfro J. L., *J. Bone Mineral. Res.*, **12**, 167—171 (1997).
18) Madsen K. L., Tavernini M. M., Yachimec C., Mendrick D. L., Alfonso P. J., Buergin M., Olsen H. S., Antonaccio M. J., Thomson A. B. R., Fedorak R. N., *Am. J. Physiol.*, **274**, G96—G102 (1998).
19) Lafeber F. P. J. G., Hermman-Erlee M. P. M., Frisk G., Wendelaar-Bonga S. E., *J. Exp. Biol.*, **143**, 165—175 (1989).
20) Stern P. H., Shankar G., Fargher R. C., Copp D. H., Milliken C. E., Sato K. J., Goltzman D., Herrmann-Erlee M. P. M., *J. Bone Mineral. Res.*, **6**, 1153—1159 (1991).
21) Yoshiko Y., Kosugi T., Koide Y., *Biochim. Biophys. Acta*, **1311**, 143—149 (1995).