A Monoclonal Antibody to α-Human Atrial Natriuretic Polypeptide

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SUMMARY A monoclonal antibody to α-human atrial natriuretic polypeptide (α-hANP), KY-ANP-1, has been produced by fusion of a nonproducing mouse myeloma cell line, X63-Ag8.653, with spleen cells from BALB/c mice immunized with synthetic α-hANP conjugated to bovine thyroglobulin using the carbodiimide coupling procedure. Hybridomas were screened for antibody production by radioimmunoassay using culture media and 125I-α-hANP. They were cloned by the limiting dilution technique, expanded in culture, and injected intraperitoneally into BALB/c mice. The obtained antibody belonged to the immunoglobulin G, subclass. Analysis by a Scatchard plot revealed a high affinity for α-hANP, with an association constant of $3.1 \times 10^9$ M$^{-1}$. With this monoclonal antibody, a specific radioimmunoassay for α-hANP has been established. The antibody in mouse ascites was available for radioimmunoassay at a final dilution of 1:10. Values of IC$_{50}$ and IC$_{90}$ in this radioimmunoassay were 3 and 30 fmol/tube, respectively. The radioimmunoassay showed a cross-reactivity of 0.9% with α-rat ANP, α-hANP-(8-22) and α-ANP-(1-6) exhibited less cross-reactivity than α-rat ANP on a molar basis. There was no cross-reaction with α-ANP-(17-28). Thus, the recognized epitope must be located in the N-terminal half of the ring structure of α-hANP including Met12 residue. This radioimmunoassay could detect γ-hANP and β-hANP as well as α-hANP. The monoclonal antibody was also useful for immunohistochemical studies. ANP-positive cells were finely stained in the human atrium using the avidin-biotin-peroxidase complex technique. These results indicate that this monoclonal antibody to α-hANP will become a powerful tool for investigating the physiological and pathophysiological significance of ANP. (Hypertension 12: 117-121, 1988)

KEY WORDS atrial natriuretic factor • monoclonal antibody • radioimmunoassay • immunohistochemistry

FOLLOWING the discovery of potent diuretic, natriuretic, and vasorelaxant properties in extracts from the rat atrium by de Bold et al.,1 a family of cardiac peptides named atrial natriuretic polypeptides (ANPs), also known as atrial natriuretic factor (ANF), was isolated from human and rat atrial tissues and implicated in the control of water and electrolyte balance and blood pressure.2-5 Using a polyclonal rabbit antiserum against α-ANP-(17-28) (ANF-[115-126]), we have established a specific radioimmunoassay (RIA) for ANP that recognizes α-human ANP (α-hANP or human ANF-[99-126]) and α-rat ANP (α-rANP or rat ANF-[99-126]) equally.6 With the aid of this RIA, we have demonstrated that ANP is released from the heart and circulates in the body as a hormone7,8 and that ANP exists not only in the atrium but in the extratral tissues, including the brain,9 spinal cord,10 kidney,11 and lung.12 This antiserum against α-ANP was also used for immunohistochemical studies.13,14 Furthermore, intracerebroventricular injection of this antiserum blocked the endogenous ANP action in the brain and potentiated water intake in rats.15
Polyclonal antibodies, however, have several drawbacks in their use, such as the limited supply and the contamination with irrelevant antibodies not directed against ANP.

The monoclonal antibody technique introduced by Kohler and Milstein has now come into widespread use for the study of various substances, including biologically active peptides. The present article describes the preparation and characterization of a monoclonal antibody to α-hANP and its useful applications.

**Materials and Methods**

**Preparation of Monoclonal Antibody**

Synthetic α-hANP was conjugated to bovine thyroglobulin (Sigma Chemical, St. Louis, MO, USA) using the carbodiimide procedure (Nakarai Chemicals, Kyoto, Japan) coupling procedure. Adult female BALB/c mice (Shizuoka Animal Center, Hamamatsu, Japan) were initially immunized by combined subcutaneous and intraperitoneal injections with the conjugate containing 30 μg of α-hANP emulsified in complete Freund's adjuvant (Difco Laboratories, Detroit, MI, USA). They were boosted in the same manner 3 weeks later and then bled to test for the presence of antibody by RIA with 125I-α-hANP. Five out of 10 mice gave a positive antibody response, and the two mice giving the greatest response were further boosted intravenously with the conjugate containing 50 μg of the antigen 5 weeks after the second injection. Four days later the spleens were harvested from the mice for cell fusion.

Fusion of spleen cells with a nonproducing mouse myeloma cell line, X63-Ag8.653, was performed in the ratio of 6:1 with 50% polyethylene glycol 4000 (Merck, Darmstadt, West Germany) according to the method described by Galfre et al. with slight modifications. After the fusion, cells were distributed into microwell plates and cultured in hypoxanthine-aminopterin-thymidine medium containing 15% fetal calf serum for hybridoma selection. As cells proliferated, culture media were periodically screened for their ability to bind 125I-α-hANP. Cells from the well that gave the highest antibody titer were cloned twice by the limiting dilution technique in the presence of a mouse thymocyte feeder layer. Positive clones were expanded in culture and injected intraperitoneally into BALB/c mice to produce ascitic fluid with highly concentrated antibody.

**Characterization of Monoclonal Antibody**

Isotyping of the monoclonal antibody was performed by the Ouchterlony technique (Mouse Monoclonal Typing Kit, Miles Laboratories, Elkhart, IN, USA). Binding affinity was analyzed by constructing a Scatchard plot of α-hANP with the RIA described in the next section. Epitope analysis was performed by checking cross-reactivities with several ANP-related peptides in the RIA for α-hANP.

**Radioimmunoassay**

RIA with the monoclonal antibody was performed following the polyclonal antiserum method as previously described in detail. In brief, α-hANP was radioiodinated by the chloramine-T method. The specific activity of 125I-α-hANP ranged from 400 to 800 μCi/μg. The RIA incubation mixture (500 μl/tube) consisted of 100 μl of standard α-hANP (0.8–1600 fmol/tube) or sample, 100 μl of a dilution of antibody (antisera, culture media, or ascites), 100 μl of 125I-α-hANP (approximately 10,000 cpm), and 200 μl of the standard buffer (0.1 M phosphate buffer, pH 7.0, containing 0.5% gelatin [Merck], 1 mM Na2EDTA, 0.2 mM cystine, 0.1% Triton X-100, and 0.01% merthiolate). The mixture was incubated for 24 to 48 hours at 4 °C. Bound and free ligands were separated by adding 1.0 ml of a suspension of dextran-coated charcoal consisting of 250 mg of activated charcoal (Norit SX Plus, Norit-Vereeniging N.V., Netherlands) and 25 mg of Dextran T70 (Pharmacia Fine Chemicals, Uppsala, Sweden) in 100 ml of 0.05 M phosphate buffer, pH 7.4, containing 0.01% merthiolate, and the supernatant was counted by a gamma counter (Aloka Autowell gamma system ARC-600, Tokyo, Japan). Mouse ascitic fluid was available at a final dilution of 1:10⁶ to yield a total tracer binding of about 30%.

**Measurement of α-hANP–Like Immunoreactivity in Human Atria**

The RIA with the monoclonal antibody was used to measure levels of α-hANP-like immunoreactivity (LI) in extracts from human atra. Five human right auricular tissues were obtained at cardiac surgery and stored at −70 °C until extraction. Tissues were boiled for 5 minutes in 10 volumes (2–5 ml) of 1 M acetic acid containing 20 mM HCl and then homogenized with a Polytron homogenizer (Kinematica GmbH, Lucerne, Switzerland). The homogenate was centrifuged at 18,000 g for 30 minutes at 4 °C, and the supernatant was stored at −20 °C until RIA.

**Immunohistochemistry**

A tissue sample from the human right auricle was obtained at coronary bypass surgery. The tissue block was immersed in the buffered formaldehyde fixatives (4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.0) for 2 days and then rinsed with 20% sucrose solution for another 2 days. The free-floating sections (16 μm thick) were obtained by cutting with a cryostat (Histostat, AO Scientific Instruments, Buffalo, NY, USA) after the tissue block was frozen with CO2 gas. The sections were sufficiently washed with 0.1 M phosphate buffer, pH 7.4, containing 0.5% Triton X-100 and were incubated in 1% H2O2 solution for 1 hour to suppress endogenous peroxidase activity. The sections were successively incubated in 1) the monoclonal antibody (ascites diluted in 1:100 with 0.1 M phosphate buffer, pH 7.4, containing 0.5% Triton X-100) at 4 °C for 36 hours, 2) biotinyl anti-mouse...
immunoglobulin G solution at 20 °C for 120 minutes, and 3) avidin-biotin-peroxidase complex solution at 20 °C for 90 minutes. These reagents for the avidin-biotin-peroxidase complex method were purchased from Vector Laboratories, Burlingame, CA, USA (PK 4002 kit); 3,3'-diaminobenzidine was employed for coloration of peroxidase. The principle of immunohistological technique has been given elsewhere.21

**Peptides**

Synthetic α-hANP was kindly donated by Professor Hisayuki Matsuo (Miyazaki Medical College, Miyazaki, Japan); γ-hANP (human ANF-[1–126]) purified from human atria was also donated by Professor Hisayuki Matsuo. Synthetic α-rANP and three fragment peptides, α-ANP-(1–6) (ANF-[99–104]), α-hANP-(8–22) (human ANF-[106–120]), and α-ANP-(17–28), were generously supplied by Professor Yoshiaki Kiso (Kyoto Pharmaceutical University, Kyoto, Japan). Synthetic β-hANP (an antiparallel dimer of α-hANP) was provided by Dr. Ken Inouye (Shionogi Research Laboratories, Osaka, Japan). The homogeneity of peptides was confirmed by reverse-phase high performance liquid chromatography and amino acid analysis.

**Results**

**Antibody Preparation and Characterization**

After the fusion, hybridomas grew in nearly 30% (212 of 768) of the wells. ANP antibody-producing cells were recognized in about 4% (8 of 212) of these wells. After further culture and cloning, one clone that produced antibody with the strongest response was selected for expansion and characterization. The obtained monoclonal antibody belonged to the immunoglobulin G1 subclass, as determined by the Ouchterlony technique. Analysis by a Scatchard plot revealed a high affinity for α-hANP, with an association constant $(K_a)$ of $3.1 \times 10^{10} \text{M}^{-1}$ (Figure 1).

A standard curve of α-hANP and cross-reactivities of its related peptides in the RIA with the monoclonal antibody are shown in Figure 2. The RIA showed a cross-reactivity of 0.9% with α-rANP on a molar basis. α-hANP-(8–22) and α-ANP-(1–6) exhibited less molar cross-reactivity than did α-rANP (0.2 and 0.04%, respectively). There was no cross-reaction (<0.01%) with the C-terminal fragment, α-ANP-(17–28).

**Radioimmunooassay and Measurement of α-hANP-Like Immunoreactivity in Human Atria**

In the standard curve of α-hANP shown in Figure 2, the IC$_{50}$ value was 30 fmol/tube and the detection limit, in which 10% of total tracer binding was inhibited (IC$_{10}$), was 3 fmol/tube. The intra-assay and interassay coefficients of variation (for 6 fmol/tube of α-hANP) were 5.2% ($n = 6$) and 9.2% ($n = 6$), respectively. The mean recoveries for 15 and 30 fmol/tube of unlabeled α-hANP added to human atrial extracts were 104% ($n = 4$) and 107% ($n = 4$), respectively.

Serial dilutions of extracts from human atria gave competition curves parallel to the standard curve for α-hANP. The level of α-hANP-LI in human atria obtained with this RIA was $169 \pm 71.5 \mu g/g$ (mean ± SE; $n = 5$), which was quite comparable to that assayed with the polyclonal antiserum. A highly significant correlation was observed between the results of the two RIAs ($r = 0.996$, $p < 0.001$). The RIA detected not only α-hANP but γ-hANP prepared from human atria on an equimolar basis.

**Figure 1.** Scatchard plot of the binding of α-hANP to the monoclonal antibody KY-ANP-I. Diluted culture supernatant containing KY-ANP-I was incubated with $^{125}$I-α-hANP (approximately 10 pM) and increasing concentrations of standard α-hANP (0–160 pM) for 48 hours at 4 °C, and the specific binding was determined as described in Materials and Methods. $B/F =$ concentration of specifically bound α-hANP; $B/F$ = bound/free ratio of the ligand.

**Figure 2.** Typical standard curve of α-hANP and cross-reactivity profiles of its related peptides in the RIA with the monoclonal antibody KY-ANP-I. $^{125}$I-α-hANP and various concentrations of standard α-hANP or related peptides were incubated with KY-ANP-I for 24 hours at 4 °C, as described in Materials and Methods.
In addition, the RIA recognized synthetic β-hANP, with a cross-reactivity of 80% on a molar basis.

Immunohistochemistry

ANP was shown on light microscopy to be a finely granular immunoreactive substance (Figure 3). These immunoreactivities were observed in the periphery of the sarcoplasm in the perinuclear region of atrial myocytes. However, no immunopositive substances were detected in the epicardium, endocardium, or any connective tissues in the human atrium. The specificity of antibody binding to atrial myocytes was verified by the inhibition of staining in the presence of excess synthetic α-hANP. Furthermore, human ventricular tissues obtained at autopsy from patients without cardiac complications were hardly stained with this monoclonal antibody.

Discussion

In the present study we have established and characterized a monoclonal antibody to α-hANP (KY-ANP-1). The antibody obtained in this study has a high affinity for α-hANP, with a $K_a$ of $3.1 \times 10^{10}$ M$^{-1}$. The $K_a$ value is comparable to those of monoclonal antibodies to human renin previously reported. Immunohistochemistry

In immunohistochemistry, ANP was shown on light microscopy to be a finely granular immunoreactive substance (Figure 3). These immunoreactivities were observed in the periphery of the sarcoplasm in the perinuclear region of atrial myocytes. However, no immunopositive substances were detected in the epicardium, endocardium, or any connective tissues in the human atrium. The specificity of antibody binding to atrial myocytes was verified by the inhibition of staining in the presence of excess synthetic α-hANP. Furthermore, human ventricular tissues obtained at autopsy from patients without cardiac complications were hardly stained with this monoclonal antibody.

Monoclonal antibodies to biologically active peptides are now commonly prepared and applied in various fields to investigate the finely organized system of the peptide concerned. Since they are homogeneous in chemical properties, including affinity and epitope specificity, and available on an unlimited scale, they are particularly useful for antigen purification, neutralization experiments, immunohistochemistry, and highly sensitive immunoassay techniques, including immunoradiometric assay and enzyme immunoassay. In the present study, the monoclonal antibody to α-hANP was applied to RIA and immunohistochemistry. In addition, applications of this specific and high affinity antibody to the neutralization experiment and the sensitive enzyme immunoassay for ANP are now in progress in our laboratory. Recently, John et al. reported a monoclonal antibody to atriopeptin, and the administration of this antibody blocked the elevation of cyclic guanosine 3',5'-monophosphate levels induced by volume expansion in rats.

In conclusion, these results indicate that the monoclonal antibody to α-hANP (KY-ANP-1) will become a powerful tool for investigating the physiological and pathophysiological significance of ANP.

Acknowledgments

We thank Mrs. Hiroko Tabata, Miss Atsuko Furu, and Miss Kazuko Horii for their secretarial assistance.

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Hypertension. 1988;12:117-121
doi: 10.1161/01.HYP.12.2.117

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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