Human C-Type Natriuretic Peptide
Characterization of the Gene and Peptide

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We isolated the human C-type natriuretic peptide gene and identified the peptide in the brain. The human C-type natriuretic peptide gene appeared to be composed of at least two exons and one intron. In the 5'-flanking region, there is an array of cis elements (an inverted CCAAT box, two GC boxes, and a cyclic AMP response element-like sequence) that is not present in upstream sequences of the atrial and brain natriuretic peptide genes. Analysis of the deduced amino acid sequence revealed that human prepro C-type natriuretic peptide comprises 126 amino acids and that the C-terminal 22-residue peptide (G-L-S-K-G-C-F-G-L-K-L-D-R-I-G-S-M-S-G-L-G-C) preceded by Lys-Lys is identical to the porcine counterpart. However, replacement of two amino acids took place in the C-terminal 53-residue sequence, corresponding to another endogenous form of the peptide. Reverse-phase high-performance liquid chromatography coupled with a radioimmunoassay for C-type natriuretic peptide demonstrated that it occurs in the human brain. C-type natriuretic peptide-like immunoreactivity was detected in discrete regions of the human brain, and its level was 10-fold higher than the atrial and brain natriuretic peptide levels, raising the possibility that C-type natriuretic peptide is the major natriuretic peptide in the human brain. (Hypertension 1992;19:809–813)

KEY WORDS • natriuretic peptides, atrial • natriuretic peptides, brain • gene • cis elements • radioimmunoassay

Identification of atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) in mammalian heart and brain has opened up the possibility of the presence of a natriuretic peptide family that is involved in the regulation of cardiovascular homeostasis.1–5 A new member of the natriuretic peptide family, designated as C-type natriuretic peptide (CNP) which has 22 amino acid residues, has recently been isolated from the porcine brain; its possible role as a neuropeptide has drawn our attention.6 Subsequently, an N-terminally elongated form with 53-amino acid residues (CNP-53) has been isolated from the porcine brain.7 These peptides, although homologous to ANP and BNP within the 17-residue ring structure formed by an intramolecular disulfide linkage, end at the second cysteine residue without the further C-terminal extension.

The amino acid sequences of mammalian ANPs are well conserved except for a single residue substitution in the ring structure,1 while a marked species difference is noted in the structure and tissue distribution of mammalian BNPs.3–5 We have recently demonstrated that ANP is produced mainly by the atrium, while BNP is produced mainly by the ventricle in rats and humans.3–5 To explore the significance of CNP in humans, it is essential to elucidate its structure and tissue distribution. In the present study, we have isolated the human CNP gene and identified the peptide in the brain.

Methods

Probe Preparation

Based on the nucleotide sequence of rat CNP complementary DNA (cDNA),5 two oligonucleotide primers for the polymerase chain reaction were generated on a model 381A DNA synthesizer (Applied Biosystems, Inc., Foster City, Calif.) (sense, 5'-ATGCACCTCTC-CCAGCTGATC-3'; antisense, 5'-TAACATCCAC-GACCGCTAT-3'). After reverse transcription of 5 μg total RNA from the rat brain by oligo(dT) priming, the resulting single-stranded cDNA was subjected to the polymerase chain reaction as described.9 A 378-bp fragment corresponding to the entire peptide coding region of rat CNP cDNA was obtained and was verified by sequencing.

Genomic DNA Blot Analysis

Human genomic DNA isolated from blood leukocytes was digested with restriction enzymes, analyzed by 0.7% agarose gel electrophoresis (10 μg per lane),8 and transferred onto a GeneScreenPlus nylon filter (Du Pont, Boston, Mass.). The filter was prehybridized at 60°C in a solution containing 50 mM Tris-HCl (pH 7.5), 1 M NaCl, 10% dextran sulfate, 1% sodium dodecyl sulfate, 200 μg/ml yeast tRNA, and 200 μg/ml salmon testis DNA. Hybridization was done in the same solution plus 32P-labeled rat CNP cDNA probe, and the filter was washed in 0.5× SSC (1× SSC is 0.16 M NaCl and 0.016 M sodium citrate) and 1% sodium dodecyl sulfate.
sulfate three times at 50°C. The blot was used to expose x-ray film for a day.

Library Screening
A human genomic DNA library (Clontech Inc., Mountain View, Calif.), propagated in bacteriophage αEMBL-3 vector, was plated onto Escherichia coli host strain LE392 and transferred onto the nylon filters. Hybridization and autoradiography were done as described above. Approximately 10^6 clones were screened, and nine positive signals were obtained. DNA from one clone (AHCNP141) harbored an approximately 15-kb human CNP gene fragment, and an approximately 3.0-kb BamHI-SalI fragment of this clone was further analyzed.

DNA Sequence Determination
All DNA sequences were determined using the dideoxy chain termination method.

Tissues and Extraction Procedure
A brain sample was obtained from a patient without neurological complications at autopsy, and tissue extraction was performed as described.5-7 Informed consent was obtained from the patient's family. This study was approved by the ethical committee on human research of Kyoto University (No. 61-9).

Radioimmunoassays
The radioimmunoassays (RIAs) for ANP, human BNP, and CNP were carried out as we previously reported.5,10 The cross-reactivities of human BNP and CNP in the RIA for ANP were less than 0.01%, and those of ANP and CNP in the RIA for human BNP were less than 0.01% and less than 1%, respectively.

Reverse-Phase High-Performance Liquid Chromatography
Reverse-phase high-performance liquid chromatography (HPLC) was carried out on a Nucleosil 5C18 column (4.6x150 mm, Macherey-Nagel, Duren, FRG) eluted with a linear gradient of acetonitrile from 20% to 40% in 0.1% trifluoroacetic acid.

Results
Structure of the Human C-Type Natriuretic Peptide Gene
Southern blot analysis of human genomic DNA with the rat CNP cDNA probe always identified a single hybridizing band after digestion with restriction enzymes (Figure 1), suggesting that a single copy of the CNP gene is present in the human genome. To further isolate the human CNP gene, a human genomic library was screened. Figure 2 shows the restriction enzyme map of the 2,917-bp BamHI-SalI fragment of AHCNP141 (panel A) and its complete nucleotide and deduced amino acid sequences (panel B). The human CNP gene appeared to be organized into at least two exons and one intron. Location of the intron was defined by comparison of this genomic sequence with the rat CNP cDNA sequence.8 Splicing donor (AG/GT) and acceptor (AG/G) consensus sequences were located at the putative exon/intron borders.11 A TATAAA sequence (TATA box) occurred at nucleotide positions 134-139, and an inverted CCAAT box, two GC boxes, and a cyclic AMP response element-like sequence12 were tandemly located in the 5'-flanking region of the gene (Figure 2). No typical polyadenylation signal (AATAAA) was found in the 3'-untranslated region sequenced. A potential initiation methionine codon (ATG) occurred at nucleotide positions 310-312, representing a reasonable translation initiation site.13 The first exon contained the 5'-untranslated region, the putative signal peptide (the first hydrophobic 23 amino acids),14 and the sequence of the first seven amino acids of the prohormone. The second exon had the remainder of the prohormone from GTC, a valine codon to TAG, a termination codon, and extended to the 3'-untranslated region. Inspection of the predicted amino acid sequence revealed that human preproCNP comprises 126 amino acids and that the C-terminal 22-residue peptide (G-L-S-K-G-C-F-G-L-K-L-D-R-I-G-S-M-S-G-L-G-C) preceded by Lys^-Lys is identical to porcine CNP.6 However, replacement of two amino acids (Gln 67 and Ala 78) took place in the C-terminal 53-residue sequence of human preproCNP, corresponding to porcine CNP-53.7

Identification of C-Type Natriuretic Peptide in Human Brain
To further identify the peptide in the brain, human brain extract was subjected to reversed-phase HPLC. As shown in Figure 3, CNP-like immunoreactivity was composed of several peaks. One peak eluting at the retention time of 48 minutes comigrated with synthetic
CNP, and another higher peak was detected at the retention time of 66 minutes. Table 1 shows CNP-, ANP-, and BNP-like immunoreactivity levels simultaneously determined in discrete regions of the human brain. CNP-like immunoreactivity was detected throughout the brain with its high levels in the hypothalamus, midbrain, thalamus, and medulla oblongata.

CNP-like immunoreactivity levels were approximately one order of magnitude higher than ANP- and BNP-like immunoreactivity levels.

Discussion

In the present study, we isolated the human CNP gene and identified the peptide in the brain. Nucleotide se-
quency analysis revealed that the human CNP gene consists of at least two exons and one intron and that human preproCNP, lacking the C-terminal extension from the ring structure, is encoded by two coding regions. No typical polyadenylation signal was found in the 3' untranslated region sequenced, suggesting that the human CNP gene would have a long 3'-untranslated region and/or that there would be another intron in this region. As reported previously, the ANP and BNP genes are organized into three exons and two introns and the third exon contains some of the coding region for the C-terminal extension.15 16 Whether the CNP gene has the third exon or not must await further investigation. Isolation and sequence determination of human CNP cDNA are necessary to elucidate the complete structure of the gene.

In the 5'-flanking region of the gene, there is an array of cis elements (an inverted CCAAT box, two GC boxes, and a cyclic AMP response element-like sequence) that is not present in upstream sequences of the ANP and BNP genes.15 16 We have recently demonstrated that ANP is synthesized and secreted mainly by the atrium, whereas BNP is synthesized and secreted mainly by the ventricle in rats and humans.3 5 However, no significant amount of CNP-like immunoreactivity was detected in the human heart and plasma (data not shown). Differences observed among promoter sequences of the ANP,15 BNP,16 and CNP genes suggest that gene expressions of three members of the natriuretic peptide family are regulated differently.

Analysis of the amino acid sequence deduced from the human CNP gene has revealed that human preproCNP comprises 126 amino acids. The first 23-residue sequence of human preproCNP has been regarded as the signal peptide.14 Therefore, human proCNP would be generated after cleavage between Ala1-Lys and Lys4. The C-terminal 22-residue peptide preceded by a typical processing signal (Lys40-Lys1) is identical to porcine CNP.6 Using reverse-phase HPLC coupled with an RIA for CNP, we have demonstrated that CNP occurs in the human brain. Recently, CNP-53 has been isolated from the porcine brain as the major endogenous form of CNP.7 The highest peak in our reverse-phase HPLC presumably represents human CNP-53, because the reverse-phase HPLC profile in the present study is consistent with previous reports on porcine CNP and CNP-53.6 7 Although examined in only one case, the CNP-like immunoreactivity level is at least one order of magnitude higher than ANP- and BNP-like immunoreactivity levels, raising the possibility that CNP is the major natriuretic peptide in the human brain.

Recently, Tawaragi et al17 have cloned and sequenced the gene for porcine CNP. Nucleotide sequences of human and porcine CNP genes are highly conserved (93%) over the coding region. Differences between human and porcine preproCNP amino acid sequences occur at five positions, which results in a 96% amino acid sequence homology.

During the course of this study, Tawaragi et al18 have reported isolation of the human CNP gene.

Acknowledgment

The nucleotide sequence reported in this paper has been submitted to the GenBank Data Bank with accession number D90337.

References

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Table 1. C-Type Natriuretic Peptide-, Atrial Natriuretic Peptide-, and Brain Natriuretic Peptide-Like Immunoreactivity Levels in Discrete Regions of Human Brain

<table>
<thead>
<tr>
<th>Brain regions</th>
<th>CNP-LI</th>
<th>ANP-LI</th>
<th>BNP-LI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerebral cortex</td>
<td>0.61</td>
<td>&lt;0.07</td>
<td>&lt;0.06</td>
</tr>
<tr>
<td>Thalamus</td>
<td>2.90</td>
<td>&lt;0.07</td>
<td>&lt;0.06</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>4.02</td>
<td>0.13</td>
<td>&lt;0.06</td>
</tr>
<tr>
<td>Midbrain</td>
<td>3.28</td>
<td>&lt;0.07</td>
<td>&lt;0.06</td>
</tr>
<tr>
<td>Pons</td>
<td>&lt;0.40</td>
<td>0.15</td>
<td>&lt;0.06</td>
</tr>
<tr>
<td>Medulla oblongata</td>
<td>2.78</td>
<td>0.08</td>
<td>&lt;0.06</td>
</tr>
<tr>
<td>Cerebellum (cortex)</td>
<td>0.85</td>
<td>&lt;0.07</td>
<td>&lt;0.06</td>
</tr>
</tbody>
</table>

Values are in picomoles per gram wet tissue. CNP-LI, C-type natriuretic peptide-like immunoreactivity; ANP-LI, atrial natriuretic peptide-like immunoreactivity; BNP-LI, brain natriuretic peptide-like immunoreactivity.
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