A Loss-of-Function Mutation in Natriuretic Peptide Receptor 2 (Npr2) Gene Is Responsible for Disproportionate Dwarfism in cn/cn Mouse*

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The achondroplastic mouse is a spontaneous mutant characterized by disproportionate dwarfism with short limbs and tail due to disturbed chondrogenesis during endochondral ossification. These abnormal phenotypes are controlled by an autosomal recessive gene (cn). In this study, linkage analysis using 115 affected mice of F2 progeny mapped the cn locus on an −0.8-cM region of chromosome 4, and natriuretic peptide receptor 2 (Npr2) gene was identified as the most potent candidate for the cn mutant in this region. This gene encodes a receptor for C-type natriuretic peptide (CNP) that positively regulates longitudinal bone growth by producing cGMP in response to CNP binding to the extracellular domain. Sequence analyses of the Npr2 gene in cn/cn mice revealed a T to G transversion leading to the amino acid substitution of highly conserved Leu with Arg in the guanylyl cyclase domain. In cultured chondrocytes of cn/cn mice, stimulus with CNP did not significantly increase intracellular cGMP concentration, whereas it increased in +/+ mice. Transfection of the mutant Npr2 gene into COS-7 cells also showed similar results, indicating that the missense mutation of the Npr2 gene in cn/cn mice resulted in disruption of the guanylyl cyclase activity of the receptor. We therefore concluded that the dwarf phenotype of cn/cn mouse is caused by a loss-of-function mutation of the Npr2 gene, and cn/cn mouse will be a useful model to further study the molecular mechanism regulating endochondral ossification by CNP/natriuretic peptide receptor B signal.

The skeleton of vertebrates is formed by two different processes, namely intramembranous and endochondral ossifications. The latter process leads to the development of long bones that comprise the appendicular skeleton and vertebrae. During endochondral ossification, mesenchymal cells initially differentiate into chondrocytes, and progress through proliferating, maturing, and hypertrophic stages with strict columnar alignment. Distal hypertrophic chondrocytes then undergo apoptosis and are replaced by trabecular bone. A large number of genes have been implicated in the mechanisms regulating these processes (1), and mutations of these genes often cause skeletal dysplasias with shortened extremities (2, 3).

The natriuretic peptide (NP)

1 family comprises atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP) (4), and three receptors for NPs, including natriuretic peptide receptor A (NPRA), natriuretic peptide receptor B (NPRB), and natriuretic peptide receptor C (NPRC), have been identified in mammals (5–7). NPRA and NPRB consist of extracellular ligand binding, transmembrane, protein kinase homology, and guanylyl cyclase catalytic domains. These receptors mediate ligand signals by producing an intracellular second messenger, cyclic GMP (cGMP) (2). Both ANP and BNP bind to NPRA with high affinity, and CNP prefers binding to NPRB (8). NPRC has a ligand-binding domain and a short cytoplasmic domain that lacks guanylyl cyclase activity and is considered to be involved in NP clearance (7). ANP and BNP mainly reside in the atrium and ventricle, respectively, and act as cardiac hormones that regulate central fluid volume, blood pressure, and the development of cardiovascular tissues (9). On the other hand, CNP gene (Nppc) and NPRB gene (Npr2) expressions have been detected in mice tibial growth plates (10, 11) and in the chondrogenic cell line ATDC5 (12). Since CNP stimulates the longitudinal growth of cultured fetal mouse tibias (13) and CNP-deficient mice develop dwarfism due to impaired endochondral ossification (11), the CNP/NPRB pathway has been considered to be involved in the process of endochondral ossification. However, there is no direct evidence that NPRB effects longitudinal bone growth by regulating endochondral ossification.

The achondroplastic mouse is a mutant strain with an autosomal recessive gene (cn) that arose spontaneously in the AKR/J mouse strain (14). The homozygous (cn/cn) mouse exhibits disproportionate dwarfism with short limbs, short tail, and a domed skull. These phenotypes are distinguishable from normal littermates by 7 days after birth and thereafter gradually become more prominent (15, 16). Histological analyses have revealed that the tibial epiphyseal growth plates of cn/cn mice are thinner than normal mice, and the hypertrophic chondrocyte zone is considerably narrowed (17, 18). These findings suggested that the dwarf phenotype of cn/cn mouse was caused by retarded longitudinal bone growth due to disturbed endochondral ossification. Thus, the cn/cn mouse has been believed to be a useful model for hereditary human skeletal dysplasias. However, the gene responsible for the skeletal abnormalities of the cn/cn mouse has not yet been identified. In this study, we determined the chromosomal localization of the cn locus and identified the causative mutation in the Npr2 gene.

NP, BNP, brain NP, NPRA, NP receptor A; NPRB, NP receptor B; NPRC, NP receptor C; CNP, C-type NP; AMDM, acromesomelic dysplasia Maroteaux type.
**Materials and Methods**

Mice—Heterozygotes (cn/+) of mutant mice were obtained from the Jackson Laboratory, and the strain was maintained by sib mating of heterozygotes. JF1/Ms mice were obtained from the National Institute of Genetics, Mishima, Japan.

**Linkage Analysis**—F1 mice were generated by mating heterozygous (cn/+) mice, and the two progeny were subsequently obtained from intercrossing heterozygous (cn/+) F1 mice. Genomic DNA was extracted from mouse livers by phenol/chloroform extraction. PCR reactions for microsatellite markers proceeded as follows: 35 cycles at 94 °C for 30 s, 55–60 °C for 30 s, and 72 °C for 45 s. The primer sequences of newly generated microsatellite markers, D4Mok1 (5'-GATTGAGTTTCGACACCTCC-3') and D4Mit109 (5'-ACCTGTGCTTGGGCCTGGG-3'), were obtained from genomic sequences located at 42.2 Mb (GenBank accession number LC073290) and 43.0 Mb (GenBank accession number LC073290) on mouse chromosome 4, respectively. The reaction mixture for PCR (10 μl) contained 1× PCR buffer, 0.2 mM dNTP, 0.5 μM concentration of each primer, and 0.25 unit of TaqDNA polymerase (Amersham Biosciences). The PCR products were fractionated on 3% agarose gels and stained with ethidium bromide. The data were analyzed using Map Manager computer software.

**Detection of Mutation in Npr2 Gene**—Tibial RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The extracted RNA was then incubated with RNase-free DNase I (Takara, Ohtsu, Japan) for 1 h at 37 °C to remove contaminating genomic DNA. First strand cDNA was synthesized for reverse transcription-PCR using the Superscript preamplification system (Invitrogen) and 5'-GGAAACACAGTGACCATTGCCCATCC-3' under the following conditions: 94 °C for 2 min followed by 35 cycles of 94 °C for 30 s, 62 °C for 20 s, and 72 °C for 4 min. Amplified fragments were digested with EcoRI and NotI restriction enzyme and then ligated into pcDNA3.1 mammalian expression vector (Invitrogen). The COS-7 cells were transfected with the DNA constructs using FuGENE 6 (Roche Diagnostics), and the transfected cells were subjected to cGMP level measurement after incubation for 24 h.

**Measurement of cGMP Levels in Cultured Cells**—The cultured cells were washed twice with phosphate-buffered saline, and incubated in Dulbecco's modified Eagle's medium containing 0.1% fetal bovine serum and 0.5 mM 3-isobutyl-1-methylxanthine at 37 °C for 15 min. Then, various concentrations of CNP were added to the medium, and the incubation proceeded at 37 °C for a further 15 min. The amount of cGMP was measured using a cGMP enzyme immunoassay Biotrak system (Amersham Biosciences).

**Results**

**Mapping the cn Locus by Linkage Analysis**—To determine the chromosomal localization of the cn locus by linkage analysis, heterozygous (cn/+) mice were crossed with JF1/Ms (+/+ ) mice. Heterozygotes (cn/+) of F2 mice were intercrossed, and 483 F2 progeny were obtained, including 115 affected and 368 normal mice. The ratio of affected to normal F2 progeny did not significantly differ from the expected 1:3 distribution of recessive inheritance. Initially we examined the linkage between the cn locus and microsatellite markers on entire mouse chromosomes with ~20-cM intervals using the 115 affected mice. Significant linkage was detected between the cn locus and markers on the proximal region of chromosome 4, indicating the localization of the cn locus on this region. To narrow the critical interval of the cn locus, we further genotyped eight markers located at the proximal region of chromosome 4. As shown in Fig. 1A, no recombination was observed between the cn locus and D4Mok1, and at least one recombination was observed with the other markers. These data indicated that the cn locus was mapped on the 0.8-cM interval between D4Mit109 and D4Mit182 (Fig. 1B).

**Detection of Mutation in Npr2 Gene**—By comparison of the linkage map with the mouse genome sequence, two genes, Npr2 and Rnor, which are functionally related to the phenotypes of cn/cn mice, were found in the critical region of the cn locus (Fig. 1B). Mutations in human othologues of Npr2 and Rnor genes have been found in patients with acromesomelic dysplasia Maroteaux type (20) and cartilage-hair hypoplasia (21), respectively. These human disorders are characterized by skeletal dysplasias with disproportionate short stature. Thus, we considered that these genes are the most likely candidate genes for the cn locus. To identify the causative mutation in these genes,
we amplified and sequenced the entire coding region of these genes and compared the cn/en and +/+ mice. The nucleotide sequence of the Rmrp gene showed no causative mutation, and the Rmrp gene was therefore excluded from the candidates. However, sequence analysis of the Npr2 cDNA fragment revealed a T to G transversion leading to an amino acid substitution of Leu to Arg at codon 885 in cn/cn mouse (Fig. 2A). We confirmed this mutation by sequencing exon 18 of Npr2 genomic DNA in cn/cn mice (data not shown). To confirm whether the T to G substitution is unique to the cn mutant allele, PCR fragments including exon 18 of the Npr2 gene were amplified from five inbred strains of mice and digested by the AflIII restriction enzyme, for which a recognition site is generated by the T to G substitution. Consequently, a 348-bp fragment amplified from the cn mutant allele was digested with AflIII, whereas the fragments from +/+ and AKR/J, the original strain of cn/cn mouse, as well as other four strains, were not digested (Fig. 2C). These results indicated that the substitution of T to G in the Npr2 gene is specific to the cn mutant allele.

Effect of the Missense Mutation on Guanylyl Cyclase Activity of Npr2—The Npr2 gene encodes a NPRB, which mediates CNP signals via cGMP production by its intracellular guanylyl cyclase domain. Since Leu at codon 885 on the guanylyl cyclase domain of NPRB is highly conserved among various species (Fig. 2B), this amino acid residue seemed critical for the domain function. To examine the effect of the amino acid substitution of Npr2 on guanylyl cyclase activity, we measured the amount of cGMP produced by CNP stimulation in the chondrocytes obtained from the costal cartilage of cn/cn and +/+ mice. The levels of cGMP in the chondrocytes of +/+ mice increased dose-dependently in response to CNP stimulus, whereas the cGMP levels did not significantly change in cn/cn mice (Fig. 3A). Furthermore, we investigated the guanylyl cyclase activity in COS-7 cells transfected with the expression vector containing the mutant or wild-type Npr2 gene. Intracellular cGMP levels obviously increased in COS-7 cells transfected with the wild-type gene after exposure to $10^{-6}$ M CNP, but no significant change was observed in COS-7 cells transfected with a mutant gene (Fig. 3B). Western blotting indicated equivalent amounts of Npr2 proteins in both cells (data not shown). These results demonstrated that the missense mutation in the Npr2 gene impaired the guanylyl cyclase activity of NPRB.

**DISCUSSION**

In this study, we precisely mapped the cn locus on chromosome 4 and identified a missense mutation in the Npr2 gene. This mutation leads to the substitution of highly conserved hydrophobic residues (Leu) with the hydrophilic residue (Arg) in the guanylyl cyclase domain of Npr2. We further confirmed that cGMP was not produced in response to CNP stimulus in cells expressing the mutant Npr2 gene. Therefore, it is likely that the guanylyl cyclase activity of Npr2 in cn/cn mouse was disrupted by amino acid substitution in the highly conserved region of the guanylyl cyclase domain. Several lines of evidence have suggested that the Npr2 gene plays a critical role in regulating endochondral ossification. 1) The Npr2 gene is predominantly expressed in the proliferating and prehypertrophic chondrocytes of the epiphyseal growth plate (10, 11); 2) cyclic GMP, as well as CNP, stimulates longitudinal bone growth (22), and mice with a targeted disruption of CNP or cGMP-dependent protein kinase type II, a downstream mediator of cGMP, develop dwarfism due to impaired endochondral ossification (11, 23, 24); 3) mutations in the NPR2 gene have been identified in patients with Acromesomelic dysplasia Maroteaux type, a hereditary human disorder characterized by disproportionate dwarfism (20). We therefore concluded that the retarded longitudinal bone growth of cn/cn mouse resulted from cGMP deficiency in the chondrocytes during endochondral ossification caused by a loss-of-function mutation in the Npr2 gene.

The cn/cn mice exhibit disproportionate dwarfism with short limbs and tail, and the prominent abnormality during endochondral ossification consists of decreased numbers of proliferating and hypertrophic chondrocytes in a narrowed epiphyseal growth plate (17, 18, 25, 26). These skeletal phenotypes of cn/cn mice are morphologically and histologically very similar to those of CNP-deficient mice (11). In addition, decreased
levels of cGMP are detected in both the tailbone of CNP-deficient mice and in cells expressing the mutated Npr2 gene (11). Although CNP not only binds to NPRB with high affinity but also binds NPRA with low affinity (8, 27), NPRA-deficient mice show no skeletal abnormality (29). Taken together, these findings indicate that the NPRB/cGMP pathway selectively mediates the CNP signal to promote longitudinal bone growth by regulating endochondral ossification.

Although the phenotypes of cn/cn and CNP-deficient mice are similar in terms of gross appearance and in the histological features of tibial growth plate, the survival rates of these mice are quite different. The mortality of CNP-deficient mice is 70% at 100 days of age (11), while that of the cn/cn mice used in this study was less than 10% (data not shown). CNP functions not only in endochondral ossification but also in cardiovascular regulation and vascular tone (30). In addition, CNP has been detected in various tissues such as the brain, spinal cord, heart, kidney, lung, thymus, liver, stomach, uterus, and ovary (31). Thus, the CNP signal, possibly mediated by other receptor(s) including NPRA, might play a role in these tissues other than skeletal tissues. Another possible explanation for this discrepancy in mortality is their distinct genetic backgrounds. Lane and Dickie (14) described that most cn/cn mice used in their study died before 3 months of age. The cn/cn mice used in their study had an Akrd/ background, while the cn/cn mice used in this study had a mixed background of C57BL/6J, C3H/HeDiSn, and LG/J (14, 25). Since it is unclear whether cn/cn and the CNP-deficient mice died from the same causes, further studies on the cause of death of cn/cn and the CNP-deficient mice with an identical genetic background would provide a novel function of CNP and/or NPRB in tissues other than in bone growth.

Acrornosomal dysplasia Maroteaux type (AMDM) is an autosomal recessive human disorder characterized by disproportionate short stature and shortening of the extremities (32), but its exact etiology remains unclear because of the lack of a suitable animal model for this disorder. Mutations in the Npr2 gene have recently been identified in affected families, and declined guanylyl cyclase activities of NPRB of mutant alleles have also been observed (20). With respect to the symptoms of AMDM, the disproportionately short limbs become more apparent during childhood than during the postnatal and neonatal periods (33). The skeletal abnormalities of cn/cn mice also became progressively more obvious with age (15, 16). Therefore, cn/cn mouse is a useful model for investigating the pathogenesis and therapeutic approaches of AMDM.

It is apparent that both CNP and cGMP signals play important roles in the regulation of endochondral ossification. However, the precise role of the signal pathway through NPRB during endochondral ossification remains unclear. The cn/cn mouse is the first animal model with a mutated Npr2 gene and can be used to evaluate the physiological function of NPRB. In cn/cn mice, the proliferative activities of proliferating chondrocytes in the epiphyseal growth plate are significantly lower than in control mice (25, 26). The numbers of proliferating and hypertrophic chondrocytes are also severely reduced in cn/cn mice (17, 18). The signal pathway through NPRB therefore appears to positively affect chondrocyte proliferation and differentiation. However, further studies are needed to elucidate how this pathway regulates chondrocyte proliferation and differentiation. Fibroblast growth factor 3 (FGFR3) is a one of the most important factors that negatively regulate the proliferation and differentiation of chondrocytes (34). Mice expressing constitutively activated Fgfr3 histologically exhibit very similar chondrocytic abnormalities in the epiphyseal growth plates to those of cn/cn mice (34). Furthermore, CNP transgenic mice rescue the achondroplastic phenotype of Fgfr3 mutant mice by inhibiting the MAPK pathway through FGFR3, and new therapeutic approaches via the activation of CNP/NPRB have been suggested for the treatment of human achondroplasia (28). Taken together, CNP signals mediated by NPRB might regulate chondrocyte proliferation and differentiation during endochondral ossification through modification of the signal from FGFR3. Further studies using cn/cn mice, as well as CNP-deficient mice, will help to clarify the role of the CNP/NPRB/cGMP pathway in chondrocytes during endochondral ossification and to develop novel therapeutic approaches for human skeletal dysplasias.

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FIG. 3. Effect of amino acid substitution on guanylyl cyclase activity of NPRB. A, amount of cGMP in cultured chondrocytes of cn/cn and +/+ mice. The levels of cGMP in the chondrocytes of +/+ mice increased dose-dependently in response to CNP stimulus, while no significant increase of intracellular cGMP level was observed in cn/cn mice. The data are the mean ± S.E. B, amount of cGMP in COS-7 cells transfected with a mutated or normal Npr2 gene. The COS-7 cells transfected with the mutated Npr2 gene (R885) showed an undetectable level of cGMP after 10^{-6} M CNP stimulus, whereas a remarkable increase of cGMP production was observed in COS-7 cells transfected with the wild-type gene (L885). The data are the mean ± S.E. of 10 wells.
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