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REVIEW

Translational research of novel hormones: lessons from animal models and rare human diseases for common human diseases

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Abstract Since the 1980s, a number of bioactive molecules, now known as cardiovascular hormones, have been isolated from the heart and blood vessels, particularly from the subset of vascular endothelial cells. The natriuretic peptide family is the prototype of the cardiovascular hormones. Over the following decade, a variety of hormones and cytokines, now known as adipokines or adipocytokines, have also been isolated from adipose tissue. Leptin is the only adipokine demonstrated to cause an obese phenotype in both animals and humans upon deletion. Thus, the past two decades have seen the identification of two important classes of bioactive molecules secreted by newly recognized endocrine cells, both of which differentiate from mesenchymal stem cells. To assess the physiological and clinical implications of these novel hormones, we have investigated their functions using animal models. We have also developed and analyzed mice overexpressing transgenic forms of these proteins and knockout mice deficient in these and related genes. Here, we demonstrate the current state of the translational research of these novel hormones, the natriuretic peptide family and leptin, and discuss how lessons learned from excellent animal models and rare human diseases can provide a better understanding of common human diseases.

Keywords Natriuretic peptide family (ANP, BNP, CNP) · Leptin · Translational research · Animal models · Genetically engineered mice

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Although a multitude of animal models have been developed to emulate various diseases, there are a few excellent animal models that mimic human disease remarkably well, such as spontaneously hypertensive rats (SHR) [1] and hereditary obese mice, ob/ob mice [2]. These models are very useful for translational research into the common human diseases, hypertension and obesity. Lessons from research on SHR, an excellent animal model for hypertension research, developed at Kyoto University led us to investigate the clinical importance of cardiovascular hormones and adipokines using appropriate animal models that mimic human diseases beyond species differences. In this review, we discuss the current state of translational research of the natriuretic peptide family and leptin and discuss the ways in which animal models and rare human diseases can educate about common human diseases.

Translational research of natriuretic peptide family

The natriuretic peptide family consists of three structurally related peptides, atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP) [3]. The biological actions of natriuretic peptides are mediated by activation of two subtypes of membranous guanylyl cyclase (GC), GC-A and GC-B, leading to

intracellular accumulation of cyclic guanine monophosphate (cGMP) [4]. The rank order of potency to induce cGMP production via GC-A is ANP ≥ BNP >> CNP, while that via GC-B is CNP > ANP ≥ BNP [5]. Thus, ANP and BNP serve as endogenous ligands for GC-A, while CNP is specific for GC-B. A third natriuretic peptide receptor with no intracellular GC domain, dubbed the clearance receptor (C-receptor), is thought to be engaged in the receptor-mediated degradation of natriuretic peptides [4]. The ANP, BNP/GC-A system plays a pivotal role in the regulation of cardiovascular homeostasis, as demonstrated by their augmentation in various pathophysiological states such as heart failure [6–10], myocardial infarction [11, 12], cardiac hypertrophy [13, 14], and hypertension [15–17]. ANP and BNP are cardiac hormones secreted primarily by the atrium and ventricle of the heart, respectively [10, 17], with strong diuretic, natriuretic, and vasodilatory activities [6, 7, 10]. ANP and BNP are used in the treatment of heart failure [18, 19] and serve as sensitive biochemical markers for heart failure and cardiac hypertrophy [8–10]. ANP infusion therapy has currently reached a greater than 30% share among drugs given for acute congestive heart failure in Japan.

CNP, the third member of natriuretic peptide family, was first purified from porcine brain [20]. While CNP is the primary natriuretic peptide in the human brain [21], it is also produced by vascular endothelial cells [22–24] and macrophages [25]. This hormone functions in the regulation of vascular endothelial function and arteriosclerosis via local effects, not by acting as a circulating hormone [26–28]. These observations indicate that CNP acts as an autocrine/paracrine regulator and as a neuropeptide [21].

The distribution of the natriuretic peptide system overlaps with the distribution of the renin–angiotensin system [21, 29–33], prompting us to examine the functional relationship of the natriuretic peptide system and the renin–angiotensin system. We demonstrated an antagonistic relationship between these two systems, both in their peripheral functions as well as their central actions [34–39]. Furthermore, the natriuretic peptide system has therapeutic implication in vascular regeneration in patients with arteriosclerosis obliterans [40].

Mice with genetic alterations in the ANP, BNP/GC-A system

Genetically engineered mice are useful tools to study the complex phenotypic effects of an altered gene in living animals. Overexpression or deficiency of each member of the natriuretic peptide family or its receptors has been generated through transgenic (Tg) or knockout (KO) technologies [41–45]. We generated Tg mice expressing BNP under the control of the serum amyloid P (SAP) component promoter, which targets hormone expression to the liver [43]. BNP-Tg mice exhibited a 100-fold increase in plasma BNP concentrations with concomitant elevations in plasma cGMP concentrations. These mice displayed significantly lower blood pressures and smaller hearts than non-Tg littermates. These results indicate that BNP functions in the long-term cardiovascular regulation and may be useful as a long-term therapeutic agent. In addition, the proteinuria and renal dysfunction observed in anti-GBM nephritis [46], the nephrosclerosis induced by subtotal nephrectomy [47], and the manifestations of diabetic nephropathy [48] were ameliorated in BNP-Tg mice compared to those in wild-type mice, indicating a possible application for the natriuretic peptide family in the treatment of renal disorders.

We also generated mice bearing a targeted disruption of the BNP gene [44]. At baseline, BNP-KO mice did not show any signs of systemic hypertension or ventricular hypertrophy; however, these animals developed multifocal fibrotic lesions within the cardiac ventricle even in the absence of additional stresses; these lesions increased in size and number in response to ventricular pressure overload, demonstrating that BNP is an antifibrotic factor acting within the ventricle of the heart as an autocrine/paracrine regulator for ventricular remodeling [44]. In addition to these cardiovascular manifestations, BNP-Tg mice exhibited marked skeletal overgrowth via endochondral bone formation [49]. Nevertheless, BNP-KO mice did not possess any skeletal abnormalities [44]. The skeletal overgrowth seen in BNP-Tg mice that express elevated plasma concentrations of BNP was similar to that seen in cartilage-specific CNP-Tg mice [49]. As the BNP/GC-A system does not have an abnormal skeletal phenotype [41, 42, 45], we postulated that the markedly increased circulating levels of BNP (100-fold greater than wild-type mice) may cross-react with GC-B to stimulate endochondral bone growth, even though the affinity of BNP for GC-B is lower than that for GC-A. This interpretation is supported by the finding that the skeletal overgrowth observed in BNP-Tg mice was not abrogated by a genetic deficiency of GC-A in BNP-Tg mice [50].

ANP transgenic mice expressing elevated levels of circulating ANP under the control of mouse transthyretin promoter [41] exhibited decreased arterial blood pressure without the induction of diuresis or natriuresis. ANP-KO mice and GC-A-KO mice displayed salt-sensitive and salt-resistant hypertension, respectively [42, 45]. Studies using GC-A-KO mice implicated the involvement of GC-A in antihypertrophic actions in the heart [51–53]. A more detailed analysis of GC-A was performed using mice bearing a conditional knockout of GC-A and indicated the importance of GC-A in vascular endothelial-cell-mediated blood pressure control [54–56].

As for the regulation of ANP and BNP gene expression, neuron-restrictive silencer elements (NRSEs) are located in the 5′-flanking region of the BNP gene and the 3′-untranslated region of the ANP gene [57]. The neuron-restrictive silencer factor (NRSF) can thus repress ANP promoter activity through binding to NRSE [58]. Studies examining dominant-negative NRSF Tg mice expressed under the control of the α-myosin heavy-chain promoter have demonstrated that NRSF plays an important role in the gene expression of both ANP and BNP and in the progression of cardiac dysfunction and lethal arrhythmia associated with heart failure [59].

Genetically engineered mice of the CNP/GC-B system

We generated mice with a targeted disruption of the CNP gene; the resultant CNP-KO mice exhibited markedly short stature due to impaired bone growth [60]. Mammalian bones are formed through two different mechanisms, endochondral ossification and membranous ossification. Most mammalian bones are formed through endochondral ossification, a process during which chondrocytes in the growth plate undergo proliferation, hypertrophy, cell death, and osteoblastic replacement [61]. The short-stature phenotype of CNP-KO mice resulted from impaired bone growth through endochondral ossification [60]. CNP-Tg mice with targeted overexpression of CNP at the growth plate cartilage exhibited prominent overgrowth of those bones formed through endochondral ossification [62]. GC-B-KO mice exhibit the same short-stature phenotype as observed in CNP-KO mice [63], demonstrating that the CNP/GC-B system is a physiologically important stimulator of endochondral bone growth. Dominant-negative GC-B transgenic rats displayed blood-pressure-independent cardiac hypertrophy, suggesting evidence linking GC-B signaling to the control of cardiac growth [64].

cGMP-dependent protein kinase (cGK) has been identified as a molecule activated downstream of the natriuretic peptide family and GC system [65]. Mice depleted with the gene of one subtype of cGK, cGKII (cGKII-KO mice), exhibit a short-stature phenotype secondary to impaired endochondral bone growth [66], similar to that observed in CNP-KO mice [60]. We demonstrated that cGKII affected endochondral bone growth by functioning downstream of the CNP/GC-B system by showing that the impaired endochondral bone growth observed in cGKII-KO mice could not be rescued by targeted overexpression of CNP in the growth plate cartilage [67].

Multiple spontaneous animal models with impairments in the CNP/GC-B system have been identified [68–71]. Two strains of dwarf mice, with an autosomal recessive mutant gene, named cn/cn [68] and short-limbed dwarfism (SLW) mice [69], possess spontaneous loss-of-function mutations in the *GC-B* gene. Spontaneous mutant mice with a loss-of-function mutation in the CNP gene, named long bone abnormality (Lbab) mice, exhibit short-stature owing to their impaired endochondral bone growth [70], and this phenotype could be abrogated by targeted overexpression of CNP in the growth plate cartilage [71].

Clinical application of CNP and its analogs for skeletal dysplasia

To explore the potential applications of CNP and its analogs for clinical use, we attempted to apply the strong effect of CNP and GC-B on endochondral bone growth to skeletal dysplasia, a group of genetic disorders characterized by severely impaired bone growth [72]. Achondroplasia (Ach), the most common form of skeletal dysplasia characterized by short-limbed dwarfism, is caused by constitutive activation of fibroblast growth factor (FGF) receptor 3 [73]. The current therapy for Ach is limited to distraction osteogenesis [74], an orthopedic procedure; no efficient medical therapies have been developed as yet. We demonstrated that targeted overexpression of a CNP transgene in the growth plate cartilage of a mouse model of achondroplasia (Ach mice) rescues their impaired bone growth and short-stature phenotypes [62] (Fig. 1). To elucidate the molecular

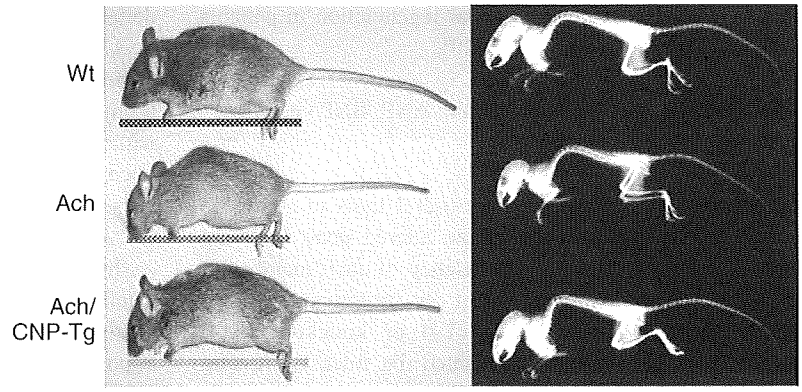

Fig. 1 Rescue of achondroplastic mice (Ach mouse) by targeted overexpression of CNP in growth plate cartilage. From *top* to *bottom* are shown the gross appearance (*left panel*) and skeletal phenotype (*right panel*, soft X-ray picture) of female wild-type mice (*Wt*), Ach mice (*Ach*), and Ach mice overexpressing CNP in the growth plate cartilage (*Ach/CNP-Tg*) at an age of 3 months

mechanism by which CNP ameliorates achondroplasia, we examined the effect of CNP on extracellular signal-regulated kinase (ERK) signaling. CNP inhibited FGF2-stimulated phosphorylation of ERK in a dose-dependent manner through cGMP activation via GC-B ligation, ultimately increasing matrix synthesis by chondrocytes [62].

We also demonstrated that systemic and continuous administration of synthetic CNP is safe and effective to reverse the impaired bone growth seen in Ach mice [75] (Fig. 2). The safety and efficacy of systemic CNP administration in preclinical studies with the observation that CNP has only a minimal effect of blood pressure in humans [76] suggest that systemic administration of CNP or CNP analogs provides a novel therapeutic strategy for the treatment of human skeletal dysplasia, including Ach.

One form of human skeletal dysplasia, acromesomelic dysplasia type Maroteaux, is caused by loss-of-function mutations in the GC-B gene [77]. This implicates the CNP/GC-B system as a physiologically important enhancer of endochondral bone growth in humans, suggesting a clinical application for CNP and CNP analogs to multiple types of human skeletal dysplasia [75].

In the near future, idiopathic short stature, a common disease of short-stature phenotype with an unknown etiology, and bone fracture, the healing of which is made through endochondral ossification, would be the next avenues to explore for a therapeutic effect of CNP treatment.

Translational research of leptin

Leptin, an adipocyte-derived hormone originally identified from hereditary obese mice (ob/ob mice) [78], plays crucial physiologic roles in the regulation of energy expenditure and food intake [79–83]. Mice [84] and rats [85, 86] bearing mutations in leptin receptors demonstrate identical phenotypes as ob/ob mice. The Koletsky rat, an obese substrain of SHR serving as a model of metabolic syndrome exhibiting both hypertension and morbid obesity, was discovered to carry an additional nonsense mutation of the leptin receptor [86].

In obese animals and subjects, plasma leptin concentrations are increased in proportion to the degree of adiposity [87–89], indicating that leptin is a satiety signal communicating the size of adipose stores to the brain [90–92] and that leptin resistance is related to obesity [87, 93–95]. Leptin deficiency in human subjects is associated with morbid obesity with insulin resistance, indicating the physiological role of leptin in both animal models and humans [96, 97]. Leptin is implicated in a number of manifestations seen in obese animal models [91, 98–101], especially obesity-related hypertension [99], abnormal reproduction [98], bone changes [100], and Cushing syndrome [102]. Leptin is also produced by human placenta [103] and choriodecidual tumors [104].

Generation of Tg mice overexpressing leptin

To explore the clinical implications of leptin *in vivo*, we generated leptin-Tg mice displaying elevated plasma leptin concentrations comparable to those seen in obese subjects [105]. A fusion gene comprised of the human SAP promoter upstream of the mouse leptin cDNA coding sequences was designed to target hormone expression to the liver [43, 106]. Overexpression of leptin in the liver resulted in the complete disappearance of both white and brown adipose tissues in mice [105]. Such a phenotype did not occur when transgene expression was targeted to adipose tissue, the endogenous site of leptin production, using adipocyte-specific promoters [107]. The hyperlepti-

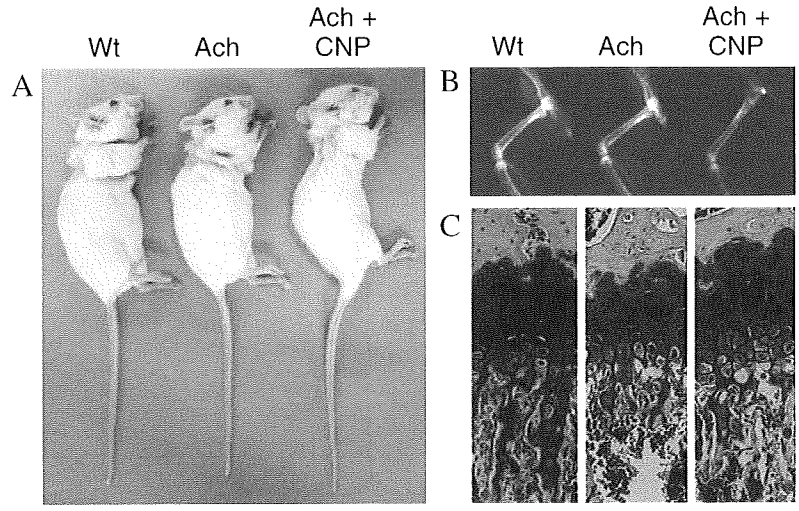

Fig. 2 Rescue of Ach mice by administration of synthetic CNP. Three-week-old female wild-type (*Wt*) or Ach mice were continuously administered CNP intravenously. The gross appearances (**a**), soft X-ray pictures of femurs (**b**), and histological pictures of tibial growth plates stained with safranin-O and hematoxylin and eosin (**c**) are shown for wild-type mice treated with vehicle (*left*), Ach mice treated with vehicle (*middle*), and Ach mice treated with 1 μg/kg per minute CNP (*right*) after a 4-week administration period. *Scale bar* in **c**, 50 μm

nemia seen in these transgenic "skinny" mice provides a unique experimental system in which the long-term effects of leptin are investigated in vivo [98–101, 105, 108, 109]. Skinny mice exhibit augmented glucose metabolism and increased insulin sensitivity of both skeletal muscle and liver [105], supporting the concept that leptin acts as an antidiabetic hormone in vivo [110–112]. These studies suggest the potential usefulness for leptin treatment of diabetes and obesity.

Crossbreeding of transgenic skinny mice with A-ZIP/F-1 mice, a mouse model of severe lipoatrophic diabetes

Generalized lipodystrophy, caused by a systemic deficiency of adipose tissue, is characterized by severe insulin resistance and hypertriglyceridemia [113]. A form of diabetes, called lipoatrophic diabetes, eventually develops, although the precise mechanism by which this paucity of fat results in diabetes has remained to be elucidated. Plasma leptin concentrations are markedly reduced or absent in patients with lipoatrophic diabetes and in rodent models of this disease [114–117]. Given leptin's antidiabetic action, leptin deficiency may play a role in the pathogenesis of lipoatrophic diabetes; thus, leptin may be a drug for lipoatrophic diabetes.

A mouse model of severe lipoatrophic diabetes (A-ZIP/F-1) was generated by expressing in adipose tissue a protein that inactivates basic-zipper transcription factors [116]. To assess the pathophysiological role and therapeutic potential of leptin in lipoatrophic diabetes, we crossed transgenic skinny (LepTg/+) and A-ZIP/F-1 (A-ZIPTg/+) mice to produce double transgenic mice (LepTg/+:A-ZIPTg/+) virtually lacking adipose tissue and expressing approximately tenfold higher levels of leptin than normal controls [118]. LepTg/+:A-ZIPTg/+ mice were hypophagic in comparison to A-ZIPTg/+ mice and exhibited decreased hepatic steatosis. Glucose and insulin tolerance tests displayed increased insulin sensitivity and normal glucose tolerance in LepTg/+:A-ZIPTg/+ mice, which was comparable to LepTg/+ mice. Pair-feeding experiments demonstrated that the effects of leptin were not solely due to decreased food intake. Leptin also helped to prevent diabetic nephropathy in generalized lipoatrophic diabetes mice [101]. These results demonstrate that leptin can improve insulin resistance and diabetic manifestations in a mouse model of severe systemic lipodystrophy, indicating that leptin is therapeutically useful in the treatment of lipoatrophic diabetes [118].

Leptin replacement therapy in Japanese patients with generalized lipodystrophy

We previously reported a novel homozygous mutation of *MC4R* in a Japanese woman with severe obesity (body mass index (BMI) 62 kg/m^2) [119]. *MC4R* mutations have been identified at a relatively high frequency (3–4%) in morbidly obese patients in Europe; all of the mutations reported to date occur in an autosomal-dominant fashion, with the exception of a single unique pedigree in the UK. [120, 121]. Although both parents were heterozygous for the mutation, neither exhibited such a severe obese phenotype (BMI 27 and 26 kg/m^2, respectively, which are preobese according to WHO criteria). As genetic backgrounds and lifestyles vary significantly between European and Asian countries, it is necessary to examine the effect of lifestyle on the phenotypes resulting from genetic mutations and on treatment efficacy in each country.

Four-month leptin replacement therapy has been reported to improve glucose and lipid metabolism in lipodystrophy patients in the USA [122]. To elucidate the efficacy, safety, and mechanisms underlying leptin replacement therapy in Asian patients with generalized lipodystrophy, we treated seven Japanese patients, two acquired and five congenital types, with physiological replacement dose of leptin [123, 124]. Leptin replacement therapy dramatically improved fasting glucose (mean±SE, 172±20 to120±12 mg/dl, $P<0.05$) and triglyceride (mean ± SE, 700±272 to 260±98 mg/dl, $P<0.05$) levels within 1 week. Leptin replacement reduced insulin resistance, as demonstrated by the euglycemic clamp method. Improvement of

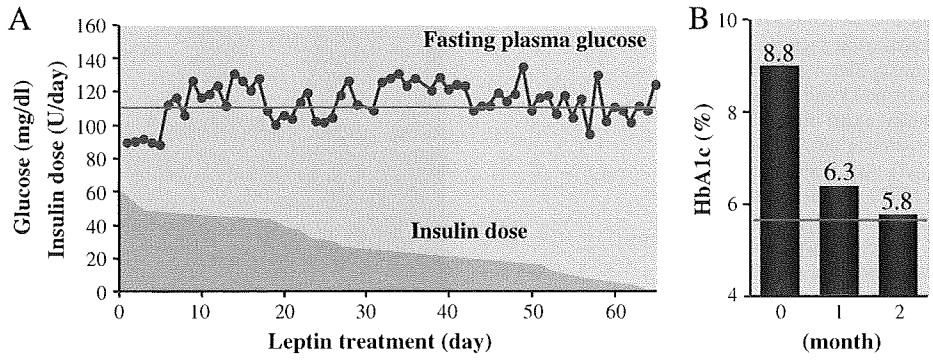

Fig. 3 **a** Daily insulin doses and fasting plasma glucose levels and **b** HbA1c levels during the first 2 months of leptin therapy in a 19-year-old male patient with congenital generalized lipodystrophy (Seipin gene mutant)

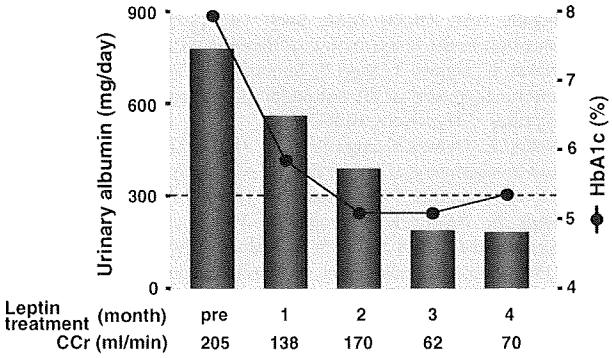

Fig. 4 Time course of daily urinary albumin secretion, creatinine clearance, and HbA1c levels during leptin treatment of a 16-year-old female patient with acquired generalized lipodystrophy

fatty liver was also confirmed by changes in computed tomography (CT) attenuation, and liver volume was calculated by CT imaging. By 4 months, six of seven patients were able to discontinue all antidiabetic drugs, including insulin (Fig. 3). The decreased fasting plasma glucose levels, triglyceride levels, and liver volumes in all seven patients were well maintained throughout the therapy period with no adverse effects. The longest period of leptin replacement therapy has now extended beyond 7 years.

Leptin treatment was also effective at combating diabetic complications. The macroalbuminuria seen in two patients regressed to microalbuminuria, while microalbuminuria in two additional patients normalized. The creatinine clearance of patients with glomerular hyperfiltration decreased with improved glucose tolerance (Fig. 4), which was consistent with previous findings in the lipoatrophic diabetes model mice [101].

We also examined the effect of leptin therapy on a 16-year-old girl with severe hypertriglyceridemia who suffered from repeated episodes of acute pancreatitis (Fig. 5). After the initiation of leptin therapy, her triglyceride levels normalized; she did not have any additional episodes of acute pancreatitis (Fig. 5). These results clearly demonstrate the safety and efficacy of the long-term leptin replacement therapy in patients with generalized lipodystrophy. While these results are impressive, it is important to remember that the efficacy of leptin replacement therapy in patients from Japan, a country in which the prevalence of obesity is relatively low, is excellent.

Leptin therapy for more prevalent forms of diabetes

To assess the therapeutic potential for leptin treatment in insulin-deficient diabetes, we generated diabetic animals by treating wild-type and LepTg/+ mice with a relatively low dose of streptozotocin (STZ 180 g/g body weight) [125]. Plasma insulin concentrations were reduced (<0.10 ng/ml), resulting in severe hyperglycemia in both wild-type and LepTg/+ mice 2 weeks after STZ treatment. LepTg/+ mice were more sensitive to exogenously administered insulin than wild-type mice; STZ-treated LepTg/+ mice became normoglycemic at doses of insulin that did not improve the hyperglycemia in STZ-treated wild-type mice. To clarify if combination therapy with leptin and insulin is beneficial for insulin-deficient diabetes, we also examined the effect of chronic coadministration of leptin and insulin in STZ-treated wild-type mice. We demonstrated that subthreshold doses of insulin, which do not affect glucose homeostasis, are effective at improving diabetes in STZ-treated wild-type mice in combination with leptin. These results indicate that leptin therapy may be used as an adjunct for insulin therapy in insulin-deficient diabetes.

We also investigated the therapeutic usefulness of leptin in a mouse model of type 2 diabetes mellitus with increased adiposity [126], generated using a combination of a low-dose STZ (120-g/g body weight) and a high-fat diet (HFD, 45% of energy as fat; STZ/HFD). In STZ/HFD mice, continuous infusion of leptin (20-ng/g body weight per hour) reduced food intake and body weight gain and improved glucose and lipid metabolism with enhanced insulin sensitivity. Leptin therapy also decreased the triglyceride content of both the liver and skeletal muscle.

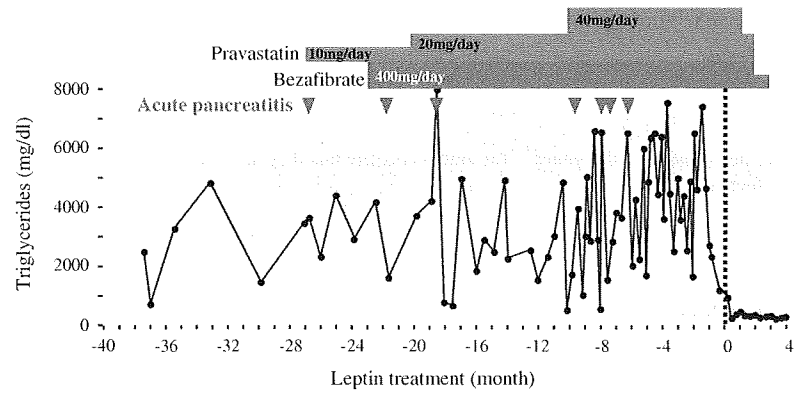

Fig. 5 Fasting serum triglyceride levels, doses of lipid-lowering drugs, and episodes of acute pancreatitis (red inverted triangle) before and after leptin therapy in a 16-year-old girl with acquired generalized lipodystrophy

These results indicate a beneficial effect of leptin therapy for type 2 diabetes mellitus with increased adiposity, which corresponds to a BMI in the range of 25–30 kg/m^2 [126].

Our previous and ongoing studies utilizing transgenic skinny mice and other animal models have demonstrated the pleiotropic actions of leptin in the regulation of energy homeostasis and food intake [98–101, 105, 108, 109] and its clinical usefulness as a therapy for multiple conditions, particularly diabetes mellitus [108, 118, 124, 125]. Tg skinny mouse may be a useful model to study the long-term effects of leptin therapy in vivo and to evaluate the clinical implications of leptin therapy.

Conclusions

Currently, the primary targets of our ongoing translational research of CNP and leptin are achondroplasia and lipoatrophic diabetes, respectively. Demonstration of the efficacy of CNP therapy for achondroplasia and leptin replacement therapy for lipoatrophic diabetes has relied heavily on basic and preclinical studies using excellent animal models. Although lipoatrophic diabetes is a rare disease in humans, the safety and efficacy of leptin replacement therapy for patients with lipoatrophic diabetes have been well established. Achondroplasia, while also a rare disease in humans, may be effectively managed with CNP therapy.

It has been possible to establish the safety and efficacy of these hormones in rare human diseases through studies that began with excellent animal models. These studies provided us with novel treatments for common human diseases, which were explored as adjacent to or in extension of these rare human diseases, as seen in the study of hypertension. Research on the SHR animal model and study of a relatively rare cause of hypertension, renovascular hypertension, led to more detailed studies on the blockade of renin–angiotensin system, bringing research forward to the current widespread field of cardiovascular disorders in translational research. These lessons teach us the importance of the breakthroughs using animal models and rare human diseases.

Conflict of interest statement The authors declare that they have no conflict of interests.

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Systemic Administration of C-Type Natriuretic Peptide as a Novel Therapeutic Strategy for Skeletal Dysplasias

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Skeletal dysplasias are a group of genetic disorders characterized by severe impairment of bone growth. Various forms of them add to produce a significant morbidity and mortality, yet no efficient drug therapy has been developed to date. We previously demonstrated that C-type natriuretic peptide (CNP), a member of the natriuretic peptide family, is a potent stimulator of endochondral bone growth. Furthermore, we exhibited that targeted overexpression of a CNP transgene in the growth plate rescued the impaired bone growth observed in a mouse model of achondroplasia (Ach), the most frequent form of human skeletal dysplasias, leading us to propose that CNP may prove to be an effective treatment for this disorder. In the present study, to elucidate whether or not the systemic administration of CNP is a novel drug therapy for skeletal dysplasias, we have investigated the effects of plasma CNP on impaired bone growth in Ach mice that specifically overexpress CNP in the liver under the control of human serum amyloid P component promoter or in those treated with a continuous CNP infusion system. Our results demonstrated that increased plasma CNP from the liver or by iv administration of synthetic CNP-22 rescued the impaired bone growth phenotype of Ach mice without significant adverse effects. These results indicate that treatment with systemic CNP is a potential therapeutic strategy for skeletal dysplasias, including Ach, in humans. (***Endocrinology*** **150: 3138–3144, 2009)**

Skeletal dysplasias are a group of genetic disorders characterized by impairment of bone growth. They comprise a diverse group of disorders that, although individually are relatively rare, together affect a large number of individuals and cause significant morbidity and mortality (1). Achondroplasia (Ach) is the most common skeletal dysplasia with a birth prevalence of approximately one of every 10,000 births (2). Recent studies in molecular genetics demonstrated that Ach is caused by constitutive active mutation of fibroblast growth factor receptor 3 (FGFR3), which results in disturbed proliferation and differentiation of growth plate chondrocytes followed by impaired endochondral bone growth (2, 3). Current therapy for Ach generally is limited to distraction osteogenesis (4), an orthopedic procedure (5). Although distraction osteogenesis provides some benefit, it is associated with a significant physical burden and time commitment from patients. As a trial for another treatment of Ach, administration of GH was performed (6) but proved to have minimal effect. New therapeutic strategies for Ach are ardently expected at present.

We previously disclosed that the C-type natriuretic peptide (CNP) and its receptor, guanylyl cyclase-B (GC-B) system is the potent stimulatory system for endochondral bone growth. Both CNP and GC-B are expressed in proliferative and pre-hypertrophic chondrocyte layers of growth plate, and mice with targeted overexpression of CNP in cartilage exhibit prominent skeletal overgrowth (7). On the contrary, mice depleted with CNP (8) or GC-B (9) are dwarf due to impaired endochondral bone growth. Furthermore, loss-of-function mutations affecting GC-B are demonstrated to cause one form of autosomal recessive human skeletal dysplasia, acromesomelic dysplasia, type Maroteaux (AMDM) (10, 11), indicating that the CNP/GC-B system is crucial for endochondral bone growth in humans as well as in mice.

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Abbreviations: AMDM, Acromesomelic dysplasia, type Maroteaux; Ach, achondroplasia; CNP, C-type natriuretic peptide; CNP-LI, CNP-like immunoreactivity; FGFR3, fibroblast growth factor receptor 3; GC-B, guanylyl cyclase-B; SAP, serum amyloid P component.

In our previous report, we demonstrated that cartilage-specific overexpression of a CNP transgene rescues the impaired endochondral bone growth of a mouse model of Ach with targeted expression of constitutive active FGFR3 in cartilage (12) (hereafter called Ach mice) by restoring the decreased matrix production in Ach growth plates through inhibition of FGFR3-mediated MAPK signaling pathway (7). To elucidate whether or not the systemic administration of CNP is a novel drug therapy for skeletal dysplasias, here we investigated the effects of plasma CNP on impaired bone growth in Ach mice that specifically overexpress CNP in the liver under the control of human serum amyloid P component (SAP) promoter or in those treated with a continuous CNP infusion system. Our results indicate that treatment with systemic CNP can be a potential therapeutic strategy for skeletal dysplasias, including Ach, in humans.

Materials and Methods

Mice

Ach mice (FVB background) were created as reported previously (12), whereas the methods used to generate SAP-CNP-Tg mice (C57BL/6J background) will be reported in detail elsewhere (Kake T., H. Kitamura, Y. Adachi, T. Yoshiaki, T. Tachibe, Y. Kawase, K. Jishage, A. Yasoda, M. Mukoyama, and K. Nakao, submitted for publication). Ach mice and SAP-CNP-Tg mice were crossed to generate double-transgenic Ach/SAP-CNP-Tg mice; female F1 progeny were used for the analyses. ICR mice were purchased from Shimizu Experimental Supplies (Kyoto, Japan). Animal care and all experiments were conducted in accordance with the institutional guidelines of Kyoto University Graduate School of Medicine.

Measurement of plasma CNP concentrations

Plasma CNP-22 concentrations were measured using liquid chromatography-mass spectrometry (13). Because the lower limit of detection of liquid chromatography-mass spectrometry was 0.2 ng/ml plasma CNP-22, RIAs for CNP were performed (14) when CNP concentrations were less than 0.2 ng/ml. The cross-reactivity of CNP-53 in the RIA was about 30% on a molar basis.

Administration of CNP to mice

CNP-22 was purchased from the Peptide Institute (Minoh, Japan) and continuously infused into mice via the jugular vein using a mouse continuous infusion system (Instech Laboratories, Plymouth Meeting, PA) equipped with a syringe pump (Harvard Apparatus, Holliston, MA). Female ICR mice or Ach mice (3 wk old) were treated with vehicle or CNP at the indicated doses for 3 or 4 wk.

Skeletal analysis and histology

Skeletal analysis was performed as previously described (15). Briefly, mice were subjected to soft x-ray analysis (30 kVp, 5 mA for 1 min; Softron Type SRO-M5; Softron, Tokyo, Japan), and the lengths of the bones were measured on the soft x-ray film. To evaluate the bone mineral density at the midshaft of the femora, femora of ICR mice at the end of the treatment period were subjected to peripheral quantitative computed tomography using an XCT Research SA instrument (Stratec Medizintechnik GmbH, Pforzheim, Germany), as previously reported (7). For histological analysis, bones were fixed in 10% formalin in 0.01 M PBS (pH 7.4), decalcified in 5% formic acid, and embedded in paraffin. Five-micrometer-thick sections were sliced and stained with safranin-O, hematoxylin, and eosin. Immunohistochemical studies were performed by using rabbit anti-type II collagen antibody (LSL, Tokyo, Japan), rabbit anti-type X collagen antibody (LSL), goat anti-PTH/PTHrP receptor antibody (Santa Cruz Biotechnology, Santa Cruz, CA), goat anti-Indian hedgehog antibody (Santa Cruz Biotechnology), or goat anti-Runx2 antibody (Santa Cruz Biotechnology), and the methods will be described in detail elsewhere (Kake T., H. Kitamura, Y. Adachi, T. Yoshiaki, T. Tachibe, Y. Kawase, K. Jishage, A. Yasoda, M. Mukoyama, and K. Nakao, submitted for publication).

Statistical analysis

Data are expressed as means ± SEM or SD. The statistical significance of differences between mean values was assessed using Student's *t* test.

Results

Rescue of impaired bone growth of Ach mice by blood-borne CNP from a CNP transgene under the control of SAP promoter

To confirm whether or not blood-borne CNP effectively stimulates endochondral bone growth in mice, we developed transgenic mice in which CNP was overexpressed in the liver under the control of human SAP promoter; compared with wild-type mice, these mice showed increased concentrations of plasma CNP-like immunoreactivity (CNP-LI) (Kake T., H. Kitamura, Y. Adachi, T. Yoshiaki, T. Tachibe, Y. Kawase, K. Jishage, A. Yasoda, M. Mukoyama, and K. Nakao, submitted for publication). Two transgenic mouse lines showed phenotypes similar to those of transgenic mice that specifically overproduce CNP in the growth plate; the mouse line with the milder phenotypes was used as the SAP-CNP transgenic mice (SAP-CNP-Tg mouse) in the present study. The plasma CNP-LI concentration was 7.5 pg/ml in SAP-CNP-Tg mice, whereas it was less than 4 pg/ml in wild-type mice. In SAP-CNP-Tg mice, no significant effects were observed for hemodynamic parameters, including systolic blood pressure [104.7 ± 2.0 and 107.2 ± 2.0 (mean ± SD) mm Hg in SAP-CNP-Tg and wild-type mice, respectively], or for blood biochemical parameters, including electrolyte concentrations (Table 1). SAP-CNP-Tg mice exhibited skeletal overgrowth, and at the age of 10 wk, each bone formed through endochondral ossification was longer and its growth plate was wider in SAP-CNP-Tg mice than in their wild-type littermates. Nevertheless, immunohistochemical analyses of tibial growth plates from 10-wk-old mice revealed that the expression patterns and intensities of chondrocyte differentiation markers including type II and X collagens, PTH/PTHrP receptor, Indian hedgehog, and Runx2 are not changed in the SAP-CNP-Tg growth plate compared with those in the wild-type growth plate (Kake T., H. Kitamura, Y. Adachi, T. Yoshiaki, T. Tachibe, Y. Kawase, K. Jishage, A. Yasoda, M. Mukoyama, and K. Nakao, submitted for publication).

Ach mice crossed with SAP-CNP-Tg mice (double-transgenic Ach/SAP-CNP-Tg mice) showed no marked difference in body length at birth compared with Ach mice, probably because the SAP-CNP transgene was first expressed after birth as previously reported (16). Nevertheless, at the age of 2 wk, Ach/SAP-CNP-Tg mice were longer than their Ach littermates and were similar in length to their wild-type littermates after 6 wk of age (Fig. 1, A and B). Soft x-ray analysis demonstrated that the impaired growth of bones formed via endochondral ossification, such as the humerus, radius, ulna, femur, and tibia, was rescued

TABLE 1. Biochemical parameters of SAP-CNP-Tg mice

	TP (g/dl)	Alb (g/dl)	AST (IU/liter)	ALT (IU/liter)	Al-P (IU/liter)	F-Cho (mg/dl)	T-Cho (mg/dl)	TG (mg/dl)	Glu (mg/dl)	Ca (mg/dl)	BUN (mg/dl)	IP (mg/dl)	CRE (mg/dl)	Na (mEq/liter)	K (mEq/liter)	Cl (mEq/liter)
SAP-CNP-Tg																
Mean	4.96	2.95	39.08	5.29	482.57[a]	17.36	65	15.64	242.3	3.15	24.56	7.55	0.31	147.24	5.68	105.31
SD	0.3	0.17	7.1	1.59	271.92	5.62	12.64	8.82	49.62	1.67	1.66	1.44	0.09	2.06	1.16	2.07
Wild type																
Mean	5.06	2.96	39.71	6.14	210.14	19.71	69.21	20.79	259.5	3.52	22.6	7.32	0.34	146.79	5.25	105.64
SD	0.28	0.32	7.92	1.83	106.49	3.1	10.18	9.5	33.59	1.57	4.78	1.36	0.02	1.84	1.31	2.45

For SAP-CNP-Tg and wild-type mice, n = 8 each. TP, Total protein; Alb, albumin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; Al-P, alkaline phosphatase; F-Cho, free cholesterol; T-Cho, total cholesterol; TG, triglyceride; Glu, glucose; BUN, blood urea nitrogen; IP, inorganic phosphorus; CRE, creatinine.

[a] $P < 0.01$, significant difference against wild type (unpaired *t* test).

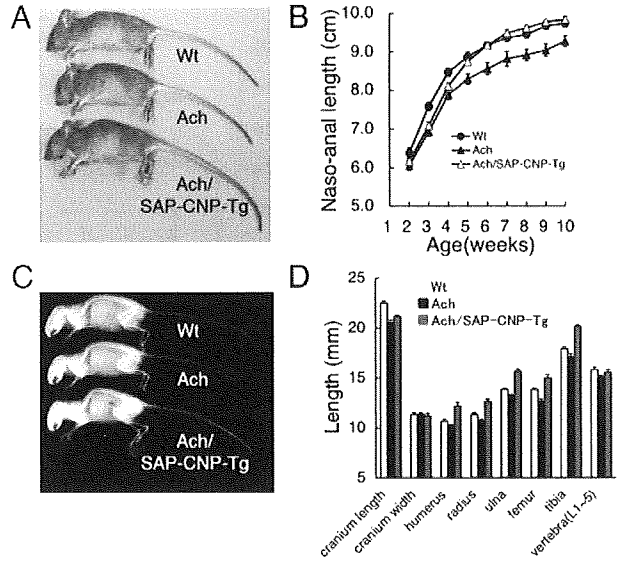

FIG. 1. Crossing Ach mice with SAP-CNP-Tg mice rescued the Ach skeletal phenotype. A, Gross appearances of 10-wk-old wild-type (Wt), Ach, and Ach/SAP-CNP-Tg mice; B, growth curves of Wt, Ach, and Ach/SAP-CNP-Tg mice from 2–10 wk after birth (●, Wt mice; ▲, Ach mice; △, Ach/SAP-CNP-Tg mice); C, soft x-ray picture of 10-wk-old Wt, Ach, and Ach/SAP-CNP-Tg mice; D, bone lengths of mice at the age of 10 wk measured on soft x-ray films (*white bars*, Wt mice; *black bars*, Ach mice; *gray bars*, Ach/SAP-CNP-Tg mice).

in Ach/SAP-CNP-Tg mice; indeed, these bones were longer in Ach/SAP-CNP-Tg mice than in wild-type mice (Fig. 1, C and D). As for cranium, the shortness of longitudinal length in Ach mice was not recovered in Ach/SAP-CNP-Tg mice. The width, of which the growth is dependent on membranous ossification, did not differ among the three genotypes (Fig. 1D). Histological analysis revealed that the narrowed growth plate observed in Ach mice was not found in Ach/SAP-CNP-Tg mice (Fig. 2). Chondrocytes in the growth plate, and in particular hypertrophic chondrocytes, were smaller in Ach mice than in wild-type mice, whereas in Ach/SAP-CNP-Tg mice, they were larger than in wild-

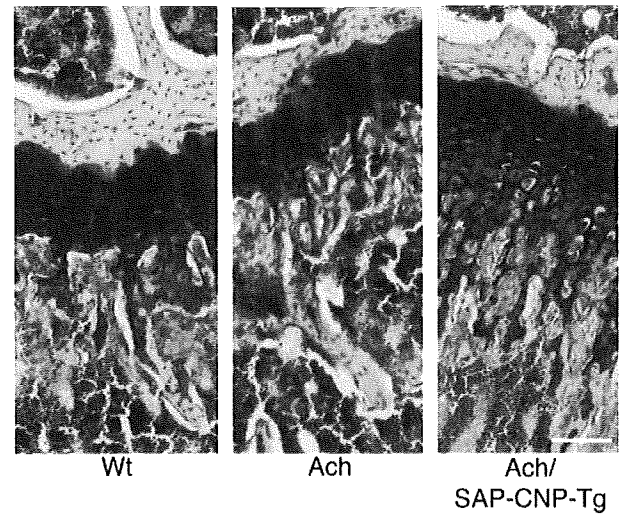

FIG. 2. Histological analysis of tibial growth plates from 4-month-old wild-type (Wt), Ach, and Ach/SAP-CNP-Tg mice. Samples were stained with safranin-O, hematoxylin, and eosin. *Scale bar*, 100 μm.

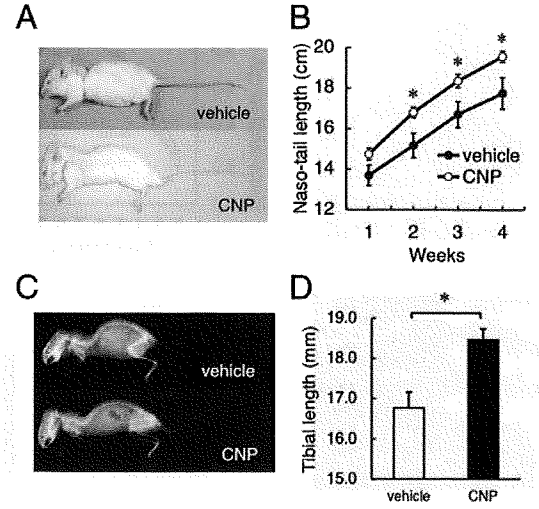

FIG. 3. Effects of iv administration of synthetic CNP-22 on bone growth. Continuous administration of vehicle or CNP-22 to female ICR mice was performed for 4 wk beginning 3 wk after birth. A, Gross appearances of vehicle-treated (*upper panel*) or CNP-22-treated at a dose of 5 μg/kg · min (*lower panel*) ICR mice at the end of the 4-wk administration period beginning at 3 wk of age. B, Growth curves showing the naso-tail length during the administration of vehicle (●, n = 4) or 1 μg/kg · min CNP-22 (○, n = 4–5). *, $P < 0.05$. C, Soft x-ray examination of mice treated with vehicle (*upper panel*) or CNP-22 at a dose of 5 μg/kg · min (*lower panel*). D, Tibial lengths of ICR mice treated with vehicle (*white bar*) or 1 μg/kg · min CNP (*black bar*) for 4 wk. n = 4 (vehicle-treated group), and n = 3 (CNP-treated group). *, $P < 0.05$.

type mice. These results strongly indicate that CNP produced in the liver was able to affect the chondrocytes in the growth plate.

Effects of iv administration of CNP on endochondral bone growth of wild-type mice

Next we examined the effects of systemic CNP administration on bone growth in wild-type mice. The administration of synthetic CNP-22 to 3-wk-old mice via the jugular vein using a continuous infusion system equipped with a syringe pump resulted in a dose-dependent elevation of the plasma CNP-22 concentration. Plasma concentrations of CNP-22 measured using liquid chromatography-mass spectrometry (13) were 5.0 ± 0.3 and 29.3 ± 5.0 (mean ± SD) ng/ml for infusion rates of 0.1 and 1.0 μg/kg mouse body weight per minute, respectively, whereas the concentration was less than 0.2 ng/ml in vehicle-administered mice. Wild-type mice treated with CNP-22 at a dose of 1 μg/kg · min from the age of 3 wk were obviously longer than vehicle-treated mice after 1 wk iv CNP-22 treatment and were significantly elongated after the 4-wk administration period (Fig. 2, A and B). The naso-anal and naso-tail lengths of mice administered 1 μg/kg · min of CNP-22 were 12 and 10% longer, respectively, than those of vehicle-administered mice at the end of the 4-wk administration period (Fig. 2B). The body weights were not changed between the two groups (data not shown). Soft x-ray analysis revealed skeletal overgrowth in the CNP-22-administered mice (Fig. 3, C and D). Bone mineral density at the midshaft of the femur was not substantially different between the two groups [463 ± 30 and 527 ± 81 mg/ml^3 (mean ± SD) in groups administered vehicle and CNP-22, respectively; n = 4 for each group]. The thicknesses of the growth plates of the long bones

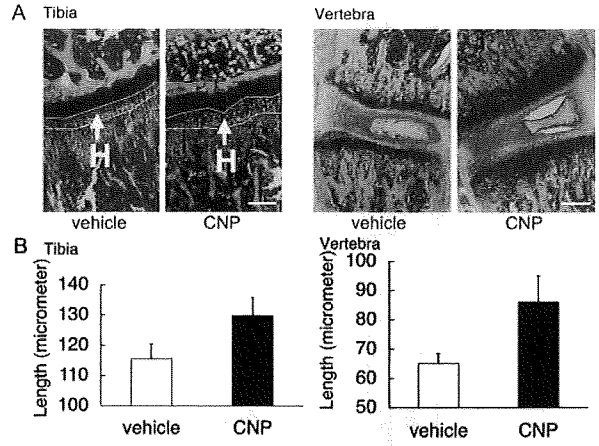

FIG. 4. Effects of systemic administration of CNP-22 on the growth plates of ICR mice. A, Histological pictures of the tibial (*left two panels*) and the vertebral (*right two panels*) growth plates of ICR mice administered vehicle (*left panel* in each group) or 1 μg/kg · min CNP (*right panel* in each group) for 4 wk and stained with safranin-O, hematoxylin, and eosin. Areas in the tibial growth plate between the *yellow lines* (denoted with an H) represent hypertrophic chondrocyte layers. *Scale bar*, 100 μm. B, Lengths of tibial (*left panel*) or vertebral (*right panel*) growth plates of ICR mice treated with vehicle (*white bars*) or 1 μg/kg · min CNP (*black bars*) for 4 wk, measured on histological pictures.

and vertebrae in the CNP-22-administered mice were greater than that in the vehicle-administered mice (Fig. 4A). The thicknesses of the tibial and vertebral growth plates in CNP-22-administered mice were 31 and 32% greater, respectively, than those in the vehicle-administered mice (Fig. 4B). Among the

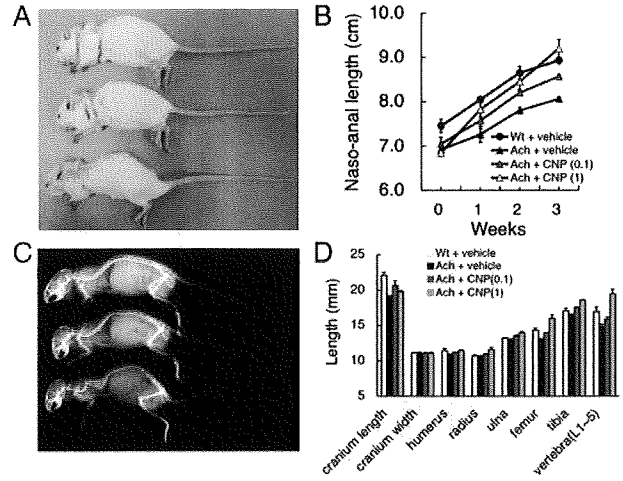

FIG. 5. Rescue of the Ach skeletal phenotype by CNP-22. Continuous iv administration was performed in 3-wk-old female wild-type (Wt) or Ach mice. A, Gross appearances of vehicle-treated Wt (*upper panel*), Ach (*middle panel*), or 1 μg/kg · min CNP-22 administered Ach (*lower panel*) mice at the end of the 4-wk administration period beginning at 3 wk of age. B, The dose-dependent effect of CNP-22 on the growth of Ach mice. *Black circles*, *black triangles*, *gray triangles*, and *white triangles* represent the naso-anal lengths of Wt mice treated with vehicle, Ach mice treated with vehicle, Ach mice treated with 0.1 μg/kg · min CNP-22, and Ach mice treated with 1 μg/kg · min CNP-22, respectively. The week after the commencement of treatment is shown on the x-axis. C, Soft x-ray analysis of Wt mice treated with vehicle, Ach mice treated with vehicle, and Ach mice treated with 1 μg/kg · min CNP-22 (from *top* to *bottom*) at the end of the 4-wk administration period. D, Bone lengths of Wt or Ach mice administered vehicle or CNP-22 for 4 wk. *White bars*, Wt mice treated with vehicle; *black bars*, Ach mice treated with vehicle; *dark gray bars*, Ach mice treated with 0.1 μg/kg · min CNP-22; *light gray bars*, Ach mice treated with 1 μg/kg · min CNP-22.

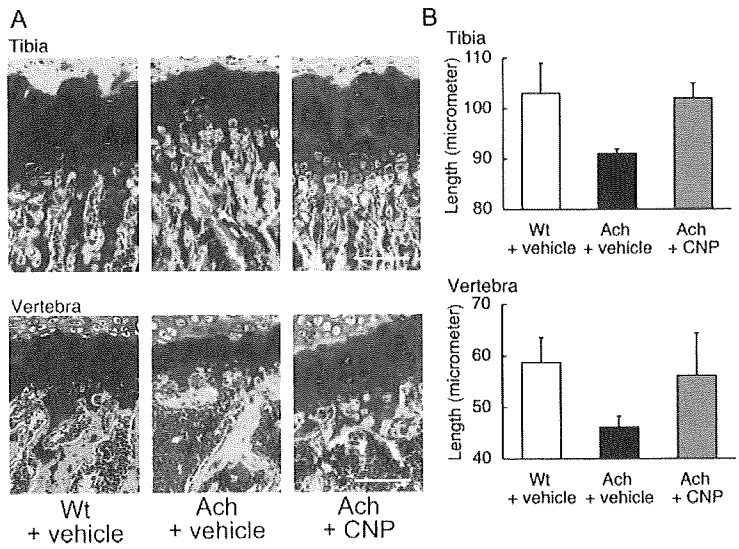

FIG. 6. Effects of systemic administration of CNP-22 on the growth plates of Ach mice. A, Histological pictures of the tibial (*upper panel*) and vertebral (*lower panel*) growth plates of mice treated with vehicle or CNP-22 for 4 wk. Samples were stained with safranin-O, hematoxylin, and eosin. From *left* to *right*, growth plate of a wild-type (Wt) mouse treated with vehicle, that of an Ach mouse treated with vehicle, and that of an Ach mouse treated with 1 μg/kg · min CNP-22. *Scale bar*, 50 μm. B, Lengths of tibial (*upper panel*) and vertebral (*lower panel*) growth plates of mice treated with vehicle or CNP-22 for 4 wk, measured on histological pictures. *White bars*, lengths of growth plates of Wt mice treated with vehicle; *black bars*, those of Ach mice treated with vehicle; *gray bars*, those of Ach mice treated with 1 μg/kg · min CNP.

growth plate layers, the hypertrophic chondrocyte layer was markedly thickened in response to CNP-22 (Fig. 4A).

We also investigated the effects of sc administration of CNP-22 using the same continuous infusion system; administration of similar doses, however, did not produce significant effects (data not shown).

Rescue of impaired bone growth of Ach mice by systemic administration of CNP

Based on the pilot study of CNP-22 in wild-type mice, CNP-22 was administered iv to 3-wk-old Ach mice. CNP-22 administration resulted in dose-dependent growth in Ach mice (Fig. 5, A and B). At the end of the 3-wk administration period, iv administration of CNP-22 at a dose of 0.1 μg/kg · min rescued 58% of the shortened naso-anal length phenotype of Ach mice, whereas a dose of 1 μg/kg · min resulted in mice that were longer than wild-type controls (Fig. 5, A and B). Soft x-ray analysis revealed promoted skeletal growth of Ach mice administered CNP at the dose of 1 μg/kg · min (Fig. 5C). Radius, ulna, femur, and tibia bones of Ach mice treated with 0.1 μg/kg · min CNP-22 were similar to those of vehicle-treated wild-type mice, whereas CNP-22 administration at a dose of 1 μg/kg · min resulted in longer bones than those from wild-type mice (Fig. 5D). The width of cranium, of which the growth is dependent on membranous ossification, was not changed between all groups (Fig. 5D). In histology, both the proliferative and hypertrophic chondrocyte layers in the tibial and vertebral growth plates were narrow in Ach mice, whereas they were comparable to those of wild-type mice after the administration of CNP at a dose of 1 μg/kg · min for 3 wk (Fig. 6). Hypertrophic chondrocytes were smaller in Ach mice than in wild-type mice, whereas after CNP-22 administration, they were similar in size to the hypertrophic chondrocytes of wild-type mice (Fig. 6A).

Discussion

The present study demonstrates that systemic administration of CNP is a novel therapeutic strategy for skeletal dysplasias including Ach. Previously, we exhibited that the CNP/GC-B system is a potent stimulatory system of endochondral bone growth in the growth plate; CNP and GC-B are expressed mainly in the pre-hypertrophic chondrocyte layer of the growth plate (8), and mice with targeted overexpression of CNP in the growth plate exhibit prominent skeletal overgrowth (7, 17), whereas mice depleted with CNP or GC-B exhibit short stature owing to their impaired bone growth (8, 9). We started the translational research of the growth-promoting effect of the CNP/GC-B system on bones into skeletal dysplasias, congenital disorders characterized by severe impairment of bone growth. In our previous report, we exhibited that targeted overexpression of CNP in the growth plate of Ach mice could rescue their impaired bone growth, demonstrating that CNP may be an effective treatment for this disorder (7). In the present study, we have investigated whether or not systemic administration of CNP could be a drug therapy for skeletal dysplasias. We exhibited that blood-borne CNP from a CNP transgene specifically expressed in the liver or by continuous iv administration could recover the shortness and the impaired bone growth observed in Ach mice. We also verified the safety of circulating CNP whose plasma concentration affects bone growth; blood pressure, electrolytes, biochemical markers, and metabolic parameters were not significantly changed in SAP-CNP-Tg and wild-type mice. These results demonstrate that systemic administration of CNP is a possible drug therapy for Ach. Because current therapy for Ach generally is limited to distraction osteogenesis (4), an orthopedic procedure (5), and the benefit of distraction osteogenesis is limited, systemic administration of CNP can be a prominent therapeutic strategy for skeletal dysplasias including Ach.

As for the method of systemic administration of CNP, we also investigated the effects of sc administration of CNP-22 using the same continuous infusion system; administration of similar doses, however, did not produce significant effects. This finding could be a result of degradation of CNP-22 by neutral endopeptidase, which reportedly is abundantly expressed in sc tissues of mice (18). Future studies are necessary to evaluate neutral endopeptidase in sc tissues of humans other than mice.

The results showed that a higher plasma CNP-22 concentration was required to rescue the Ach phenotype in the mice with the infusion pump than in the Ach/SAP-CNP-Tg mice. In addition to CNP-22, CNP-53 is an endogenous form of CNP that has a longer biological half-life than CNP-22 (19). The degree of cross-reactivity of CNP-53 in the RIA for CNP is about 30% on a molar basis, indicating that the plasma of the transgenic mice

may contain CNP-53 and/or pro-CNP, a precursor of CNP that shows little cross-reactivity in the RIA. Further studies are necessary to elucidate the molecular forms of the CNP-LI proteins secreted from the liver in SAP-CNP-Tg mice. Another reason for the differences between the SAP-CNP-Tg mice and the infusion pump model may be that the increased levels of circulating CNP are present earlier during the development of the transgenic model, *i.e.* just after birth in Ach/SAP-CNP-Tg mice, compared with 3 wk of age for mice with the CNP infusion pump.

Safety analysis of the systemic administration of CNP-22 showed no change in systolic blood pressure. This is consistent with the result of our previous report demonstrating that systemically administered CNP in humans did not produce significant effects on hemodynamic parameters, including blood pressure (20). In addition, no adverse effects on bone mineral density or blood biochemistry, including electrolyte concentrations, were observed, indicating that chronic CNP treatment is safe. Nevertheless, safety issues with CNP need further study, because only short-term potential toxicity has been examined in the current study.

The clinical significance of CNP and its receptor, GC-B, in endochondral bone growth has been established in humans, because loss-of-function mutations in the human GC-B gene cause AMDM, a form of skeletal dysplasia (10, 11); the skeletal phenotypes similar to those of patients suffering from AMDM are also observed in GC-B knockout (9) and GC-B mutant (21, 22) mice. Among human skeletal dysplasias, AMDM is likely to be resistant to treatment with CNP. On the other hand, because spontaneous mutations in the mouse CNP gene (23, 24) are known to result in phenotypes identical to those observed in CNP knockout mice (8) and CNP mutant mice are rescued by targeted overexpression of CNP in their cartilage (25), patients with loss-of-function mutations in the CNP gene, which have not been reported to date, will likely be very sensitive to CNP administration. In addition, because administration of CNP could successfully stimulate the endochondral bone growth of wild-type mice, CNP would be potentially used for skeletal dysplasias other than achondroplasia or the putative form of skeletal dysplasia caused by loss-of-function mutations in the CNP gene. Recent progress in molecular genetics has identified mutations in various genes as the causes of skeletal dysplasias (1); therefore, in case we try to use CNP for the treatment of one form of skeletal dysplasia caused by mutations in a certain gene, we might better predict the therapeutic effects by investigating the molecular interactions between the gene product and CNP in endochondral ossification.

In conclusion, we have demonstrated the efficacy and safety of iv administration of CNP-22 for impaired endochondral bone growth in Ach mice. These results suggest that systemic administration of CNP or CNP analogs provides a novel therapeutic strategy for human skeletal dysplasias, including Ach.

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A mouse model of ghrelinoma exhibited activated growth hormone-insulin-like growth factor I axis and glucose intolerance

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Iwakura H, Ariyasu H, Li Y, Kanamoto N, Bando M, Yamada G, Hosoda H, Hosoda K, Shimatsu A, Nakao K, Kangawa K, Akamizu T. A mouse model of ghrelinoma exhibited activated growth hormone-insulin-like growth factor I axis and glucose intolerance. *Am J Physiol Endocrinol Metab* 297: E802–E811, 2009. First published July 14, 2009; doi:10.1152/ajpendo.00205.2009.—Ghrelin is a stomach-derived peptide that has growth hormone-stimulating and orexigenic activities. Although there have been several reports of ghrelinoma cases, only a few cases have elevated circulating ghrelin levels, hampering the investigation of pathophysiological features of ghrelinoma and chronic effects of ghrelin excess. Furthermore, standard transgenic technique has resulted in desacyl ghrelin production only because of the limited tissue expression of ghrelin *O*-acyltransferase, which mediates acylation of ghrelin. Accordingly, we attempted to create ghrelin promoter SV40 T-antigen transgenic (GP-Tag Tg) mice, in which ghrelin-producing cells continued to proliferate and finally developed into ghrelinoma. Adult GP-Tag Tg mice showed elevated plasma ghrelin levels with preserved physiological regulation. Adult GP-Tag Tg mice with increased plasma ghrelin levels exhibited elevated IGF-I levels despite poor nutrition. Although basal growth hormone levels were not changed, those after growth hormone-releasing hormone injection tended to be higher. These results indicate that chronic elevation of ghrelin activates GH-IGF-I axis. In addition, GP-Tag Tg mice demonstrated glucose intolerance. Insulin secretion by glucose tolerance tests was significantly attenuated in GP-Tag Tg, whereas insulin sensitivity determined by insulin tolerance tests was preserved, indicating that chronic elevation of ghrelin suppresses insulin secretion and leads to glucose intorelance. Thus, we successfully generated a Tg model of ghrelinoma, which is a good tool to investigate chronic effects of ghrelin excess. Moreover, their characteristic features could be a hint on ghrelinoma.

ghrelin; glucose metabolism

GHRELIN is a stomach-derived 28-amino acid (AA) peptide hormone with octanoyl modification of third Ser residue, which is essential for its binding to growth hormone (GH) secretagogue receptor (GHS-R) (20). There have been several reports regarding ghrelin-producing tumors (9, 17, 36, 37). As far as we know, only two cases have elevated plasma ghrelin level (9, 36). However, the ghrelin-producing cells in the stomach, known as X/A-like cells, account for about 20% of the endocrine cell population in the oxyntic glands (10). It may be reasonable to estimate that far more ghrelinoma cases have been overlooked and diagnosed as nonfunctioning tumors. Hormone-producing tumors demonstrate their characteristic symptoms by chronic effects of each hormone, which may be a key symptom to making a correct diagnosis. Conversely, the characteristic symptom often tells us the chronic effects of each responsible hormone. Acute effects of ghrelin have been studied extensively by many researchers, and a wide variety of acute effects of ghrelin have been discovered, such as the regulation of growth hormone (GH) release, food intake, gastric acid secretion, gastric motility, blood pressure, and cardiac output (23, 25, 26, 31, 33, 34). However, chronic effects of ghrelin have not been fully understood.

To understand the chronic effects of ghrelin, genetically engineered mouse models would be useful. Several groups, including ours, have developed transgenic animals in which ghrelin transgenes are driven by several different promoters (2, 4, 18, 29, 38, 41). All of these animals except for one line created by Reed et al. (29) using the neuron-specific enolase (NSE) promoter and another line recently reported by Bewick et al. (5) using the bacterial artificial chromosome produced only desacyl ghrelin rather than acylated ghrelin. Until the recent identification of ghrelin *O*-acyltransferase (GOAT), which mediates ghrelin octanoylation (40), it had been unclear how acylation of ghrelin takes place. GOAT is expressed mainly in stomach and intestine, and a small amount of GOAT is also present in pancreas (12). This limited expression area of GOAT made it impossible to create ghrelin-overproducing transgenic animals by standard procedures. When we started this study, GOAT had not yet been identified. Accordingly, we choose an approach in which an increase in the number of ghrelin-producing cells in mice would result in increased levels of circulating ghrelin. By taking this approach, we successfully obtained ghrelin promoter-SV40 T-antigen transgenic (GP-Tag Tg) mice. In these mice, ghrelin concentration elevates with age in concordance with the proliferation of ghrelin cells. The aim of this study was to elucidate the pathophysiological features of ghrelinoma and the chronic effects of ghrelin elevation.

MATERIALS AND METHODS

Animals. Two types of fusion genes comprising the 5′-flanking region of human ghrelin gene (4,085 or 1,479 bp) (19) and SV40 T-antigen were designed (Fig. 1*A*). The purified fragments (10 μg/ml) were microinjected into the pronucleus of fertilized C57/B6 mouse (SLC, Shizuoka, Japan) eggs. The viable eggs were transferred into the oviducts of pseudopregnant female ICR mice (SLC) by using standard techniques. Transgenic founder mice were identified by

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