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Concentration of mRNA for the Natriuretic Peptide Receptor-C in Hypertrophic Chondrocytes of the Fetal Mouse Tibia[1]

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The roles of natriuretic peptides in cardiovascular homeostasis have been well characterized. A recent study revealed that mice lacking natriuretic peptide receptor-C (NPR-C) exhibit skeletal-overgrowth. We, therefore, performed *in situ* hybridization with riboprobes to determine the localization of mRNAs for receptors for natriuretic peptides in the growth plate of the fetal mouse tibia The amount of mRNA for NPR-A was below the detectable level in the growth plate. The mRNA for NPR-B was detected predominantly in proliferating chondrocytes. By contrast, high levels of mRNA for NPR-C were found in hypertrophic chondrocyies. In other regions of the growth plate, the levels of mRNA for NPR-C were very low. The patterns of expression of mRNAs for NPR-B and NPR-C, namely, subtype switching during differentiation from proliferating chondrocytes to hypertrophic chondrocytes, suggest that these receptors might be involved in the growth and differentiation of the growth plate during fetal development in the mouse.

Key words: differentiation, *in situ* hybridization, hypertrophic chondrocyte, mouse tibia, natriuretic peptide receptor C.

There are three known natriuretic peptide isoforms atrial natriuretic peptide (ANP); brain natriuretic peptide (BNP); and C-type natriuretic peptide (CNP) (*1*, *2*). ANP and BNP appear to be responsible for systemic blood pressure and body fluid homeostasis. CNP acts as a neuropeptide as well as a local factor. Three types of receptor for natriuretic peptides have been defined by molecular cloning and expression: natriuretic peptide receptor-A (NPR-A or GC-A); natriuretic peptide receptor-B (NPR-B or GC-B); and natriuretic peptide receptor-C (NPR-C). NPR-A and NPR-B, which are specific for ANP and CNP, respectively, belong to the membrane-bound guanylate cyclase family (*1*). By contrast, NPR-C has a short cytoplasmic tail and lacks guanylate cyclase activity (*1*). NPR-C exhibits only weak selectivity for the various natriuretic peptides and accounts for the majority of NPRs in most target tissues.

The formation of bone occurs through two different processes, namely, endochondral and membranous ossification. Longitudinal bone growth is determined by the endochondral ossification in the cartilaginous growth plate. Endochondral ossification is a major mode of bone formation that occurs as chondrocytes undergo proliferation, hypertrophy, cell death, and osteoblastic replacement. Studies *in vitro* have indicated that the natriuretic peptide system acts on the proliferation and differentiation of bone cells such as chondrocytes (*3*), osteoblasts (*4*, *5*), and osteoclasts (*6*), although mechanisms underlying such involvement remain unclear. Furthermore, recent evidence *in vivo* suggests that the natriuretic peptide system might be essential for bone metabolism: mice lacking NPR-C (*7*) and overexpressing BNP (*8*) exhibit a skeletal overgrowth phenotype. It is surprisingly that NPR-C, that has been thought to be a clearance receptor (*9*), is involved in the formation of bone. However, no reports have shown the localization of NPRs on the cartilaginous growth plate. To further understand the mechanism of action of the natriuretic peptide system in bone, we demonstrated, by means of *in situ* hybridization (*10*), the localization of mRNAs for NPRs in the growth plate of the fetal mouse tibia. Sense and antisense ^{35}S-labeled cRNAs were synthesized from the following cDNAs or genomic DNAs. cDNAs for rat NPR-A (nucleotides 1011–1185) (*11*), human NPR-B (nucleotides 245–1651) (*12*) and rat NPR-C (nucleotides 1–1641) (*13*) were prepared by means of the polymerase chain reaction. Genomic DNAs for mouse type X collagen (*14*) and H4 histone (*15*) were obtained from Dr. Bjorn Olsen (Harvard Medical School, Boston, MA) and Dr. Gary Stein (University of Massachusetts, School of Medicine, Worcester, MA), respectively.

Figure 1 shows typical examples of the detection of mRNAs for NPR-B and NPR-C in tibias from fetal mice (E16.0). To define the regions of expression of mRNAs for NPRs, we compared the localization of these mRNAs with those of mRNAs for specific molecular markers of chondrocytes, namely, H4 histone, which is specific for proliferating

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Abbreviations: ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; CNP, C-type natriuretic peptide; NPR-A or GC-A, natriuretic peptide receptor-A; NPR-B or GC-B, natriuretic peptide receptor-B; NPR-C, natriuretic peptide receptor-C.

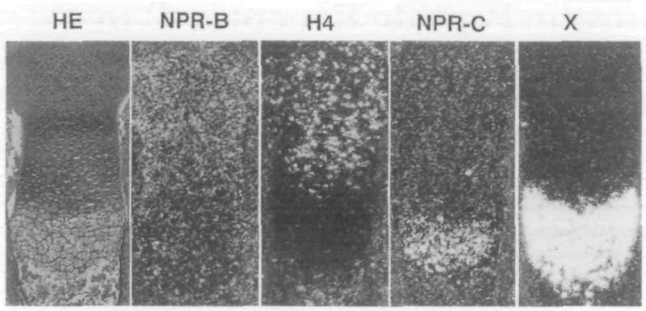

Fig. 1. **Expression of mRNAs for NPR-B and NPR-C in the mouse embryo tibia on day 16.** Pregnant ICR mice (SPF/VAF Crj: CD-1; Charles River Japan, Kanagawa) were killed by rapid cervical dislocation, and 16-day-old (E16.0) fetuses were removed from the uteri. *In situ* hybridization was performed on serial sections with ^{35}S-labeled cRNA probes that were specific for mRNAs for NPR-B, NPR-C, H4 histone (H4), and type X collagen (X). Sections were stained, after hybridization, with hematoxylin and eosin (HE staining). Bright-field (left panel) and dark-field views are shown. Bars = 100 μm.

chondrocytes, and type X collagen, which is specific for hypertrophic chondrocytes. The amount of mRNA for NPR-A was below the detectable level in the tibias (data not shown). We found moderate expression of mRNAs for NPR-B in the growth plates of tibia. The expression of NPR-B mRNA was higher in the region of proliferating chondrocytes and the signals overlapped those corresponding to the mRNA for H4 histone. The expression of mRNA for NPR-B was under the detection level in prehypertrophic and hypertrophic chondrocytes. By contrast, high-level expression of mRNA for NPR-C was detected in hypertrophic chondrocytes. On comparing the localization of mRNA for NPR-C with that for type X collagen, we found that there is less expression of mRNA for NPR-C in the region of prehypertrophic chondrocytes.

We found in this study that chondrocytes that expressed the mRNAs for NPR-B and NPR-C had distinct respective phenotypes. mRNA for NPR-B was present in the region of proliferating chondrocytes. The CNP and NPR-B system has been reported to be involved in both membranous (*5*) and endochondral ossification (*16*). Yasoda *et al.* (*16*) reported that the addition of CNP or an analog (8-bromo-cGMP) of its second messenger to the culture medium of tibias from fetal mice increased the heights of zones of proliferating and hypertrophic chondrocytes. Mice that lack type II cGMP–dependent protein kinase undergo abnormal endochondral ossification (*17*). These various observations together suggest that cGMP produced by NPR-B in response to CNP in the region of proliferating chondrocytes might act to stimulate the growth and differentiation of chondrocytes.

By contrast, in the present study, high levels of mRNA for NPR-C were found in hypertrophic chondrocytes of the fetal mouse growth plate. We previously showed high-density localization of NPR-C in chondrocytes of eel gill cartilage (*18*). NPR-C expression has also been suggested to be associated with chondrogenic differentiation, as judged using mouse chondrogenic cell line ATDC5 (*19*). Recent studies *in vivo* demonstrated that NPR-C–deficient mice (*8*) and mice homozygous for mutations in the *Npr3* gene encoding NPR-C (*20*) exhibit a skeletal overgrowth phenotype. NPR-C, which does not have a guanylate cyclase domain, has been thought to be involved in the clearance of ligands (*9*). NPR-C might neutralize the effects of CNP by removing it from the environment (the zone of hypertrophic chondrocytes). However, there is some evidence that NPR-C associates with G proteins (*21*, *22*) and regulates the proliferation of cells (*23*, *24*). NPR-C has been reported to activate endothelial nitric oxide synthase through G proteins in smooth muscle cells (*22*). The potential direct effects of NPR-C on bone metabolism remain to be characterized in detail. Our finding should stimulate further studies on the physiological significance of NPR-C, which was previously consider to be merely a clearance receptor.

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