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C-Type Natriuretic Peptide as an Autocrine/Paracrine Regulator of Osteoblast

Evidence for Possible Presence of Bone Natriuretic Peptide System

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C-type natriuretic peptide (CNP) is a local regulator in the brain and vascular wall. We present data to demonstrate the production and action of CNP in the osteoblast. CNP increased cGMP production, far more potently than atrial natriuretic peptide (ANP) in an osteoblastic cell line, MC3T3-E1. Since ANP and CNP are the ligands for two particulate guanylate cyclases, guanylate cyclase-A (GC-A) and guanylate cyclase-B (GC-B), respectively, these results reveal the expression of GC-B in MC3T3-E1. In addition, CNP mRNA and CNP-like immunoreactivity were detected in cell extracts from MC3T3-E1 and its culture medium, respectively. Both CNP and 8-bromo cGMP dose-dependently decreased [^{3}H]thymidine uptake, without affecting alkaline phosphatase activity. These results indicate that CNP is a novel autocrine/paracrine regulator of osteoblast and suggest the presence of "bone natriuretic peptide system."

Bone is composed of heterogeneous population of skeletal lineage cells, i.e. osteoblast, osteocyte, osteoclast, and chondrocyte. Moreover non-osteogenic cells such as adipocyte, vascular endothelial cells, and stromal cells are abundantly present in the bone marrow, and are also considered to be involved in the regulation of bone function (1). For example, the stromal cells can support the osteoclast formation from the hematopoietic stem cells. The close interaction between the bone and the vasculature has been well recognized. That is, bone contains abundant vasculature in it, and the vascular invasion is necessary for the enchondral bone formation to occur (1). However little is known about the precise role of the vasculature in the bone formation. Therefore, we have made a hypothesis that vasoactive peptides derived from the blood vessel have roles in the bone formation.

Natriuretic peptide family consists of atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP). ANP and BNP, which are abundantly expressed in the atrium and ventricle, respectively, mainly act as cardiac hormones. In contrast, CNP is widely distributed in the brain and its role as a neurotransmitter or neuromodulator has been suggested (2–6). In addition to its action in the brain (4–6), CNP also works as an autocrine/paracrine regulator in peripheral tissues. We have previously demonstrated the production of CNP by the vascular endothelial cells and the presence of receptors specific for natriuretic peptides in vascular smooth muscle cells. These data indicate the important local roles of CNP in vascular function and led us to propose the notion of "vascular natriuretic peptide system" (7,10).

Two types of natriuretic peptide receptors are cloned and sequenced (5–6). Biologically active receptors are particulate guanylate cyclases; i.e. particulate guanylate cyclase A (GC-A) and particulate guanylate cyclase B (GC-B). The other type of receptor, clearance receptor, is not coupled with guanylate cyclase and is considered to be engaged in the metabolic clearance of natriuretic peptides. The rank order of the affinity of natriuretic peptides for these receptors are

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ANP≥BNP⋙CNP for GC-A, CNP>ANP≥BNP for GC-B and ANP>CNP>BNP for the clearance receptor (9).

In the present study, we have examined the possible role of CNP as an autocrine/paracrine regulator in the bone.

MATERIALS AND METHODS

Cell culture. Mouse osteoblastic cell line, MC3T3-E1 was cultured in α-Modified Eargle's Minimal Essential Medium (α-MEM) with 3% fetal calf serum (FCS) in a humidified atmosphere containing 5% CO_2. Experiments were done at confluency.

cGMP response to natriuretic peptides. Mouse ANP and CNP were purchased from Peptide Institute (Osaka, Japan). Mouse BNP was synthesized by the solid phase method (7). MC3T3-E1 cells in 24-well plates, were washed with α-MEM containing 0.1% bovine serum albumin (BSA) and 25mM Hepes, pH 7.4 and exposed to various concentrations of mouse ANP, BNP or CNP at 37°C for 30 min in the presence of 0.5 mM 3-isobutyl-1-methylxanthine. Intracellular cGMP concentration was assayed by radioimmunoassay (RIA) as described previously (8) and corrected by the cell number.

Binding assay for CNP. MC3T3-E1 cells in 24-well plate were washed with Hank's balanced salt solution containing 0.1% BSA and incubated with [^{125}I]Tyr^{0}-mouse CNP in the presence of various concentrations of unlabeled CNP at 4°C for 2 h (9). After the incubation, cells were washed and solubilized with 0.5N NaOH and were counted for the radioactivity by the γ-counter.

RNA extraction, reverse transcription-polymerase chain reaction (*RT-PCR*) *coupled with Southern blot analysis.* Total RNA was extracted by guanidinium thiocyanate-CsCl method. First strand cDNA was synthesized from each 2.5 μg of total RNA by reverse transcriptase (Superscript, GIBCO BRL) with oligo(dT) primer (Promega). The primers and PCR conditions were previously described (10,11). The PCR products were transferred to nylon membrane and hybridized with ^{32}P-labeled oligo CNP probe (10).

[^{3}*H*]*Thymidine uptake.* At confluency, the cells in 24-well plate were incubated in the starvation medium (α-MEM containing 10 mM Hepes, pH 7.2, 0.1% FCS) for 24 h, followed by incubation in the medium containing various concentrations of ANP or CNP for 36 h. Cells were then labeled with 0.35 μCi/well of [methyl-^{3}H]thymidine for 4 h. The acid-insoluble precipitate was dissolved by 1N NaOH, neutralized with HCl and counted for radioactivity. Data are expressed as percentages of the control values.

Alkaline phosphatase (*ALP*) *activity.* ALP activity was measured using p-nitrophenyl phosphate as a substrate. Cells in 96 well plate were cultured in starvation medium for 24 h and exposed to medium containing the various concentrations of ANP or CNP for 48 h. After washing with ice-cold PBS, cells were solubilized with 0.5% Triton X and measured for ALP activity. One unit of activity was defined as one micromole of p-nitrophenol liberation per min at 22°C. The activity was corrected for cell number.

Radioimmunoassay (*RIA*) *for CNP.* Cells were cultured in 10 cm dish with 5 ml of α-MEM with 0.5% FCS for 24 or 48 h in the presence or absence of transforming growth factor-β_1 (TGF-β_1; R and D Systems, Minneapolis, MN). The CNP-like immunoreactivity (CNP-LI) concentration in the medium was measured by RIA after extraction and concentration by Sep-Pak C_{18} (12).

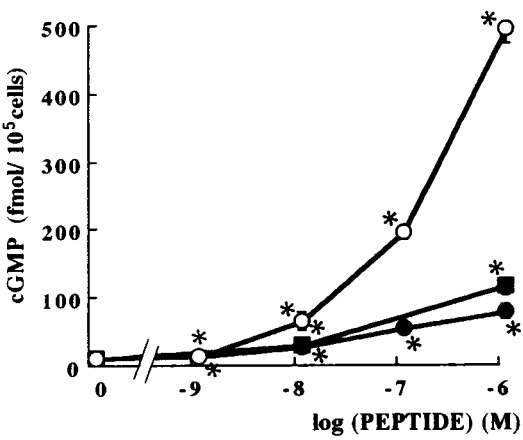

FIG. 1. Cyclic GMP response to natriuretic peptides. MC3T3-E1 cells at confluency were exposed to ANP (●), BNP (■), and CNP (□) for 30 min. Intracellular cGMP contents were measured by RIA and corrected by the cell number. Data are mean ± SD from quadricate determinations. Asterisk (*) shows the statistical difference from the basal value ($P<0.05$).

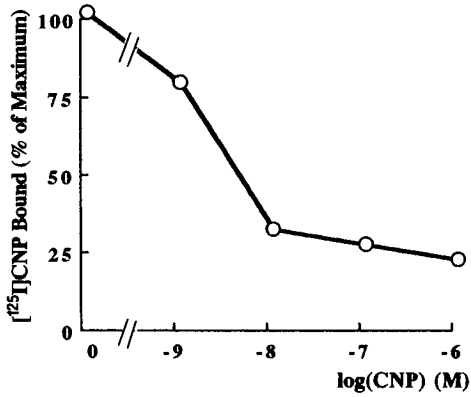

FIG. 2. Binding assay for CNP. After the incubations with [^{125}I]Tyr^{0}-mouse CNP in the absence or presence of various concentrations of unlabeled CNP at 4°C for 2 h, cells were washed and solubilized with 0.5N NaOH and counted for the radioactivity. Data are mean from triplicate determinations.

Statistical analysis. Statistical analysis was done by Bonferroni's methods (13). Significant difference was defined as $P<0.05$.

RESULTS

cGMP response to natriuretic peptides. At confluency, ANP, BNP and CNP dose-dependently stimulated cGMP production in MC3T3-E1 cells (Fig. 1). The cGMP production by CNP was at least one order of magnitude greater than those by ANP and BNP.

Binding assay. Radiolabeled CNP was bound to MC3T3-E1 cells, which was dose-dependently displaced by unlabeled CNP (Fig. 2). The half maximal binding was observed at approximately 3×10^{-9} M of CNP.

Detection of CNP mRNA by RT-PCR coupled with Southern blot. CNP mRNA expression was studied by RT-PCR coupled with Southern blot analysis in MC3T3-E1 and brain as a positive control, where CNP mRNA is known to be abundantly expressed (4,10). A single band with a predicted size of 384 bp was detected in MC3T3-E1 and brain (Fig. 3).

CNP production. The CNP-LI concentration in the medium was less than 0.12 f mol/ml under the basal condition. However, after the exposure of the cells to 100 pM of TGF-β_1 for 24 or 48 hours, CNP-LI was detected in the culture medium (Table 1).

DNA synthesis and ALP activity. DNA synthesis as evaluated by [^{3}H]thymidine uptake, was significantly and dose-dependently suppressed by 10^{-9} M and higher concentrations of CNP. It was significantly suppressed by ANP only at 10^{-6} M (Fig. 4A). The inhibitory action on [^{3}H]thymidine uptake was also observed by 8-bromo cGMP in a dose-dependent manner (Fig. 4B). The confluent MC3T3-E1 cells expressed constantly high ALP activity, which was not significantly affected by CNP, ANP (Fig. 5A) or 8-bromo cGMP (Fig. 5B).

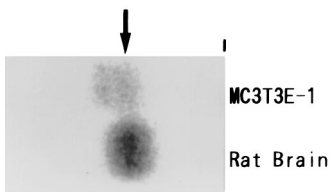

FIG. 3. RT-PCR coupled with Southern blot analysis for CNP. The first strand cDNA was synthesized from 2.5 μg of total RNA with reverse transcriptase with oligo(dT) primer. PCR products were transferred to nylon membrane and hybridized with ^{32}P-labeled oligo CNP probe.

TABLE 1
CNP-like Immunoreactivity in the Culture Medium

Incubation	24 hours	48 hours
Control	<0.12 f mol/ml	<0.12 f mol/ml
TGF-β	0.26	0.31

Note. Cells were cultured with or without TGF-β_1 for 24 or 48 h. CNP-like immunoreactivity (CNP-LI) concentrations in the media were measured by RIA after extraction and concentration by Sep-Pak C_{18}. Data are the mean of the duplicate determinations.

DISCUSSION

In the present study, we demonstrate the CNP production and expression of GC-B, specific for CNP in MC3T3-E1, which is an osteoblastic cell line derived from normal mouse calvaria and retains much of the normal osteoblastic phenotypes (14). CNP was much more potent than ANP and BNP in cGMP production, and CNP bound to the MC3T3-E1 cells with a high affinity. These observations are compatible with the expression of GC-B in MC3T3-E1. RT-PCR coupled with Southern blot analysis revealed CNP mRNA with the size comparable to that in the brain. Although little amount of CNP was produced under the basal culture conditions, CNP production was augmented by TGF-β_1, which is known to potently enhance the CNP production from the vascular endothelial cell (16). In the bone, TGF-β_1 produced by the osteoblast, stimulates the matrix protein synthesis, and is considered to be involved in bone formation and fracture repair (15). Therefore, the potentiation of CNP production by TGF-β_1 in MC3T3-E1 is likely to suggest the implication of CNP in the bone remodeling.

We have also studied the roles of CNP in MC3T3-E1. CNP dose-dependently decreased DNA synthesis, which was mimicked by 8-bromo cGMP. In contrast, ANP had little effects. ALP activity, which is a marker for osteoblast differentiation, was not significantly affected by CNP, ANP or 8-bromo cGMP. These results, together with the above data, indicate that CNP has growth-inhibitory effect on MC3T3-E1 cells via GC-B.

There are few reports on the roles of natriuretic peptides in the bone. Vargas et. al. reported that

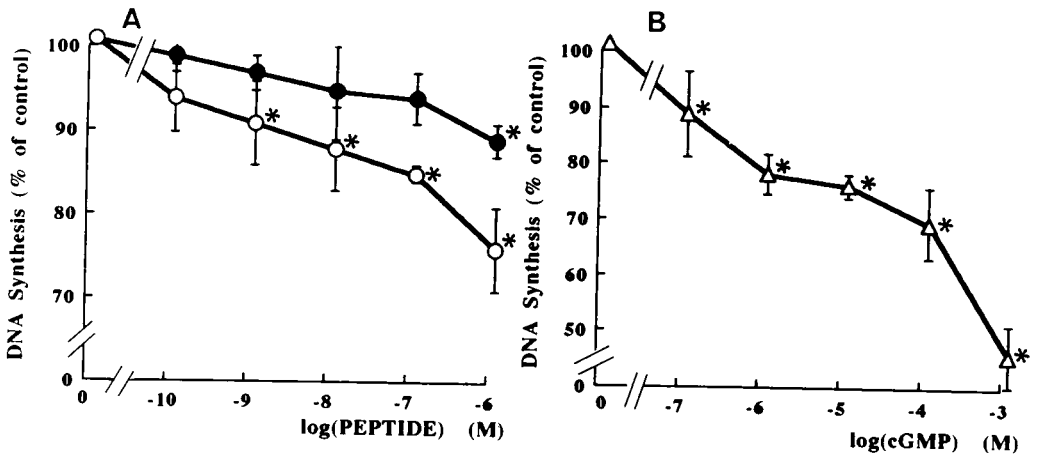

FIG. 4. Effects of mouse ANP (1–28) (●), CNP (1–22) (○) (A) and 8-bromo cGMP (△) (B) on [^{3}H]thymidine uptake. Cells were cultured in the starvation medium for 24 hours, in the medium containing the test substances for another 36 hours, and were labeled with [methyl-^{3}H]thymidine for 4 h. The acid insoluble fraction was counted for radioactivity. Data are expressed as the percentage of the control values and are mean ± SD from quadricate determinations. Asterisk (*) denotes the statistical difference from the control value ($P<0.01$).

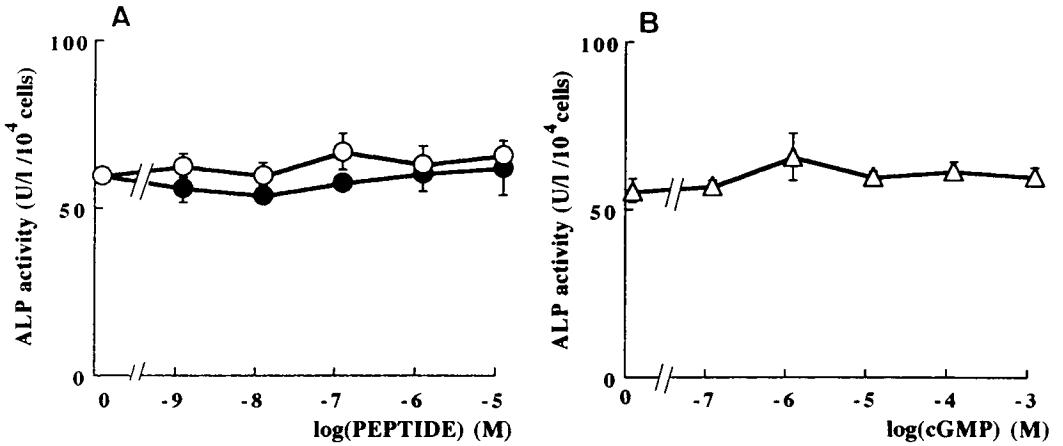

FIG. 5. Effects of mouse ANP (1–28) (●), CNP (1–22) (○) (A) and 8-bromo cGMP (△) (B) on ALP activity. Cells were cultured in the starvation medium for 24 h and exposed to medium containing the test substances for 48 h. Cells were solubilized with 0.5% Triton X and measured for ALP activity using p-nitrophenyl phosphate as a substrate. One unit of activity was defined as one micromole of p-nitrophenol liberation per min at 22°C. The activity was corrected for cell number.

ANP has no major direct effects on the bone formation or resorption (17). However it is obvious that the roles of natriuretic peptides in the bone must be re-evaluated because our results clearly show that CNP is far more potent than ANP in the osteoblastic cells.

Recently Hagiwara et. al. reported the CNP production, the presence of GC-B and growth-inhibitory action of CNP in cultured chondrocyte from rat xiphisterna (18). More recently, Holiday et. al. reported the CNP production and GC-B expression in the $1,25(OH)_2$ D_3-treated mouse bone marrow culture, which is one of the standard methods to generate osteoclast (19). Moreover they have reported that CNP increases the bone-resorbing activity of the osteoclast. However, the cell type responsible for CNP production in their system, remains to be determined, since their bone marrow culture system contains heterogeneous population of cells, such as osteoblast, stromal cells and vascular endothelial cells as well as osteoclast. In this study, we have demonstrated the implication of CNP as an autocrine/paracrine regulator in the osteoblastic cell line for the first time. It is possible, however, that the CNP action in the bone is also exerted by CNP released from the vascular endothelial cells, which are abundantly present in the bone marrow and are the rich sources for CNP production (7,10). These results indicate that CNP is an important local regulator in various types of cells of the bone and cartilage, such as the osteoblast, chondrocyte and osteoclast.

In summary the present study indicates an autocrine/paracrine function of CNP in the osteoblast and the possible presence of “bone natriuretic peptide system”.

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