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Change in the Expression of C-Type Natriuretic Peptide and Its Receptor, B-Type Natriuretic Peptide Receptor, during Dedifferentiation of Chondrocytes into Fibroblast-Like Cells[1]

Hiromi Hagiwara,*,[2] Atsuto Inoue,* Mayumi Furuya,† Shoji Tanaka,† and Shigehisa Hirose†

*Research Center for Experimental Biology and †Department of Biological Sciences, Tokyo Institute of Technology, 4259 Nagatsuta-cho, Midori-ku, Yokohama 226; and ‡Suntory Institute for Biomedical Research, Shimamoto, Mishima, Osaka 618

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Chondrocytes derived from rat xiphoid cartilage dedifferentiated into fibroblast-like cells as the number of passages of the cells in culture increased. During *in vitro* dedifferentiation the growth of the cells was markedly suppressed. We had proposed previously that C-type natriuretic peptide (CNP) might be a potent antimitogenic factor for chondrocytes, and TGF-β1 induced a marked increase in CNP secretion of chondrocytes. Therefore, we investigated the expression of CNP, B-type natriuretic peptide receptor (NPR-B or GC-B), and TGF-β1 in this process. Radioimmunoassay and RNase protection analyses revealed passage-associated increase in CNP-like immunoreactivity and in levels of NPR-B mRNA, respectively. Northern blot analyses showed that the level of TGF-β1 mRNA decreased with increasing passage number. These results suggest that the expression of CNP and NPR-B might be involved in *in vitro* dedifferentiation of chondrocytes and TGF-β1 does not affect the increasing level of CNP during *in vitro* dedifferentiation.

Key words: B-type natriuretic peptide receptor, chondrocyte, C-type natriuretic peptide, dedifferentiation, proliferation.

Natriuretic peptides, including atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP), and CNP, can elicit vasorelaxant, natriuretic, and diuretic responses. The actions of natriuretic peptides at their target organs and tissues are mediated by membrane-bound receptors that are coupled to guanylate cyclase. These receptors include natriuretic peptide receptor-A (NPR-A or GC-A) and NPR-B. Guanylate cyclase directly generates the intracellular second messenger, cGMP, in response to binding of extracellular natriuretic peptide. Although NPR-A and NPR-B are structurally and functionally very similar, they have quite different ligand specificities; NPR-A binds ANP and BNP, whereas NPR-B is highly specific for CNP (*1*). CNP has been proposed to be a prototype in the family of natriuretic peptides because CNPs are highly conserved across species (*2-5*). Therefore, CNP and NPR-B seem to be particularly suitable targets for studies of novel functions of members of this family. Porcine seminal plasma contains large amounts of CNP, and NPR-B mRNA has been demonstrated in the uterus and oviduct. Thus, it seems possible that the CNP/NPR-B system might play a role in fertilization (*6*). CNP released from monocytes (*7*) has been reported to regulate the proliferation of cultured smooth muscle cells (*8*). We have also demonstrated the autocrine inhibition of chondrocyte proliferation as a result of the interaction of CNP with NPR-B on chondrocytes (*9*).

Chondrocytes belong to the family of connective-tissue cells. Differentiation of undifferentiated mesenchymal cells into chondrocytes and the dedifferentiation of chondrocytes into fibroblast-like cells are regulated by soluble factors, such as bone morphogenic proteins (BMPs) (*10*) and retinoids (*11, 12*). Culture conditions can also induce the dedifferentiation of chondrocytes *in vitro* (*13*). Chondrocytes from xiphoid cartilage dedifferentiate into fibroblast-like cells when subcultured for extended periods (*14*). However, the mechanism of such dedifferentiation has not yet been characterized.

The aim of the present study was to clarify the role of CNP in chondrocyte metabolism. We found that the expression of both CNP and NPR-B paralleled the inhibition of proliferation that accompanies the dedifferentiation of chondrocytes.

EXPERIMENTAL PROCEDURES

Materials—Rat CNP was purchased from the Peptide Institute, Osaka. ^{32}P-labeled nucleotides were from DuPont/NEN, Boston, MA, USA. Restriction enzymes, T7 RNA polymerase, RNase T_1, and RNase A were from Boehringer Mannheim, Mannheim, Germany; Dulbecco's modified Eagle's medium (DMEM) and penicillin/streptomycin antibiotic mixture were from Life Technologies,

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[2] To whom correspondence should be addressed.

Abbreviations: ANP, atrial natriuretic peptide; BNP, B-type natriuretic peptide; CNP, C-type natriuretic peptide; NPR-A, A-type natriuretic peptide receptor; NPR-B, B-type natriuretic peptide receptor; PBS, phosphate-buffered saline (20 mM sodium phosphate/0.13 M NaCl/1 mM EDTA, pH 7.4).

Grand Island, NY, USA; and fetal bovine serum was from Filtron, Victoria, Australia.

Isolation and Cultivation of Rat Chondrocytes—The xiphisterna of two adult Wistar rats (male; about 150 g) were aseptically separated and all adhering soft tissue was dissected away. The xiphisterna were cut into small pieces and chondrocytes were dissociated by treatment, at 37°C for 1 h, with 10 ml of DMEM that contained 1 mg/ml collagenase (150 to 250 units/mg; Wako Pure Chemical Industries, Osaka) and 1,000 protease units/ml Dispase (Godo Shusei, Tokyo). Cells were passed through a 70-μm nylon filter, pelleted by gentle centrifugation (800 × *g*), and resuspended in DMEM that contained 10% FBS. The isolated cells were plated in three 55-cm^2 dishes and grown in DMEM supplemented with 10% fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin in a humidified atmosphere of 5% CO_2 in air at 37°C. During subculture, the medium was changed every 3–4 days. The cells were cultured until they reached confluence (primary passage). Then they were detached by treatment with 0.05% trypsin and replated in three 55-cm^2 dishes or five 12-well plates. This transfer of cells to fresh dishes or plates was repeated every 3–4 days. Cells, three days after transfer, were detached from the substratum by incubation with 0.05% trypsin, and then cells were counted in a hemocytometer.

Assay of Cell Proliferation—Chondrocytes were seeded in 96-well plates at a density of 1,000 cells per well and cultured for 48 h in DMEM plus 10% FBS. Subsequently, cells were washed twice with serum-free DMEM and incubated with serum-free DMEM for 24 h. After incubation in fresh DMEM with 2% FBS and various concentrations of CNP for 18 h, cell proliferation was examined by enzyme immunoassay (5-Bromo-2′-deoxy-uridine Labeling and Detection Kit III from Boehringer Mannheim) using 5-bromo-2′-deoxyuridine (BrdU). This assay is as sensitive as assays with radioactively labeled thymidine.

Radioimmunoassay (RIA) for Quantitation of CNP—Conditioned media and cell lysates were prepared as described previously (*9*). The amounts of CNP in the conditioned medium (8 ml) and in lysates of cells from 55-cm^2 dishes were estimated as fmol/dish/48 h. RIA for CNP was performed with a specific antiserum raised against CNP by the method of Minamino *et al.* (*15*). ^{125}I[Tyr^0]-CNP was prepared by the lactoperoxidase method (*16*) and purified by reverse-phase HPLC.

Ribonuclease Protection Assay—The template cDNA for the synthesis of cRNA was prepared as a cDNA fragment encoding rat NPR-B nucleotides 648–889 (*17*). Synthesis of cRNA was carried out using an RNA transcription kit. A [^{32}P]uridine triphosphate-labeled antisense RNA (5×10^5 cpm) was transcribed from a T7 polymerase promoter and annealed with 20 μg of total RNA for 16 h at 45°C in 80% formamide, 40 mM Pipes, pH 6.4, 1 mM EDTA, and 400 mM NaCl. Nonannealing nucleic acids were digested with ribonucleases A and T_1 at final concentrations of 40 and 2 μg, respectively, in a buffer that contained 300 mM NaCl, 10 mM Tris-HCl, pH 7.4, and 5 mM EDTA at 30°C for 1 h. The protected fragments were analyzed by electrophoresis in a 5% polyacrylamide gel that contained 7 M urea and then the gel was exposed to an imaging plate. The plate was analyzed with a Fuji Film Bioimage Analyzer model Fujix BAS 2000.

Northern Blot Analysis—Total RNA (20 μg) was subjected to electrophoresis on a 1% agarose gel containing 2.2 M formaldehyde and transferred to a MagnaGraph nylon membrane (Micron Separations, Westborough, MA, USA). The membrane was hybridized with a cDNA probe for TGF-β1 (gift from Dr. Harold L. Moses, Vanderbilt University, Nashville, TN, USA) at 42°C for 16 h in 50% formaldehyde containing 5 × SSPE (1 × SSPE is 0.15 M NaCl, 15 mM NaH_2PO_4, pH 7.0, 1 mM EDTA), 2 × Denhardt's solution (0.1% each of bovine serum albumin, polyvinylpyrrolidone, and Ficoll), 1% SDS, and 100 μg/ml herring sperm DNA. The cDNA probe was radiolabeled

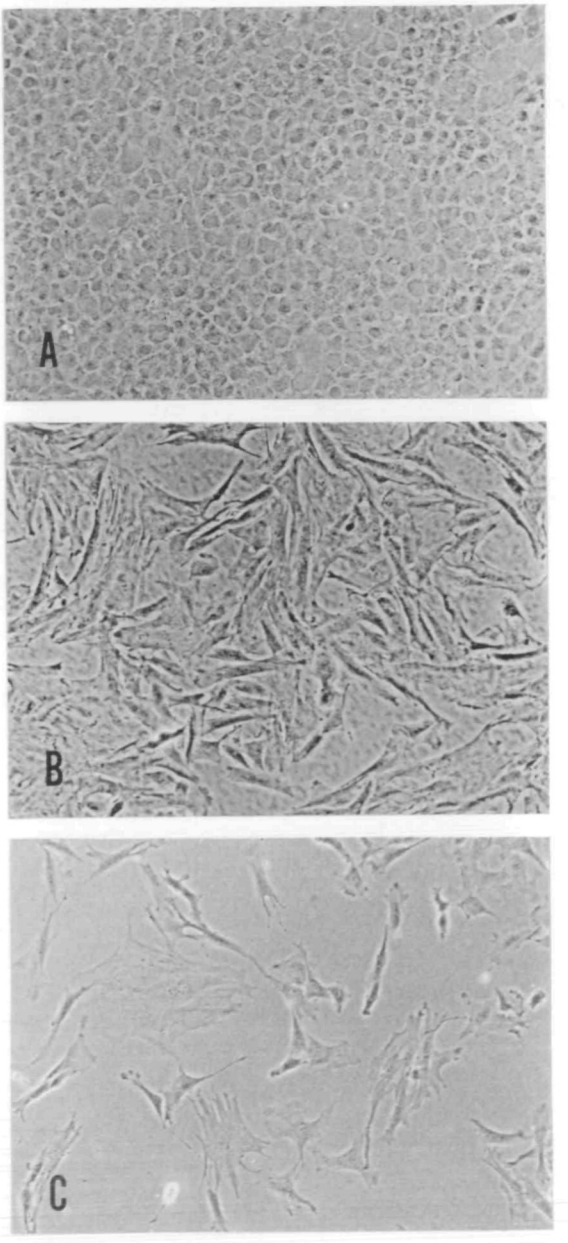

Fig. 1. **Phase-contrast micrographs of cultured chondrocytes from rat xiphoid cartilage.** A: Cultured cells at primary passage (×100). B and C: The cells after 5 and 14 passages (×100), respectively Notice fibroblastic appearance as the number of passages in culture increases. The cultivation was performed as described under "EXPERIMENTAL PROCEDURES."

with a Random Primer DNA Labeling Kit (Takara, Shiga). The membrane was washed in 1×SSC (0.15 M NaCl, 15 mM sodium citrate, pH 7.0) containing 0.1% SDS at room temperature for 5 min and in 1×SSC containing 0.1% SDS at 55°C for 1 h, and exposed to an imaging plate for 4 h. The plate was analyzed with a Bioimage Analyzer (BAS2000; Fuji Film, Tokyo).

RESULTS AND DISCUSSION

Chondrocytes were isolated from the rat xiphoid cartilage and maintained in DMEM in the presence of 10% fetal bovine serum. Cells were subcultured as described in "EXPERIMENTAL PROCEDURES." Chondrocytes in culture showed polygonal morphology at early passages (Fig. 1A). No fibroblast-like cells could be observed in any culture. However, the typical polygonal shape of chondrocytes began to change to fibroblast-like morphology after five passages (Fig. 1B) and completely changed at 14 passages (Fig. 1C). After five passages, the growth rate of chondrocytes gradually decreased, as visualized by light microscopy. We counted the cells in the cultures after different numbers of passages. As shown in Fig. 2, cell numbers decreased markedly with increasing passage number. These results suggest the presence of a potent antimito-

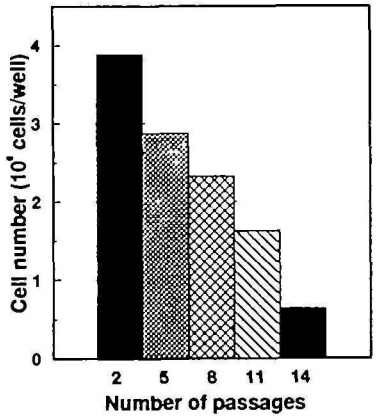

Fig 2 **The decrease of the number of cultured chondrocytes with increasing passage number.** Chondrocytes in a 55-cm² dish were detached by treatment with 0.05% trypsin and then replated in five 12-well plates This transfer step was repeated every 3-4 days. Cells were counted in a hemocytometer after dispersion of the cells from plates (3 days after transfer) with a 0.05% solution of trypsin

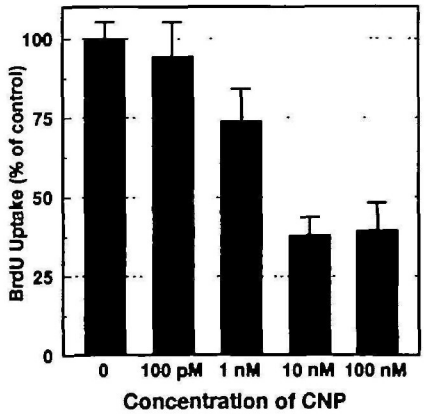

Fig. 3. **Effects of CNP on the proliferation of chondrocytes.** The inhibitory effects of CNP on the BrdU incorporation of rat chondrocytes (after three passages) were examined. Values are represented as "% of control." The control value refers to the extent of incorporation of BrdU by cells in DMEM that contained 2% fetal bovine serum without CNP. Cells and samples were prepared as described in "EXPERIMENTAL PROCEDURES."

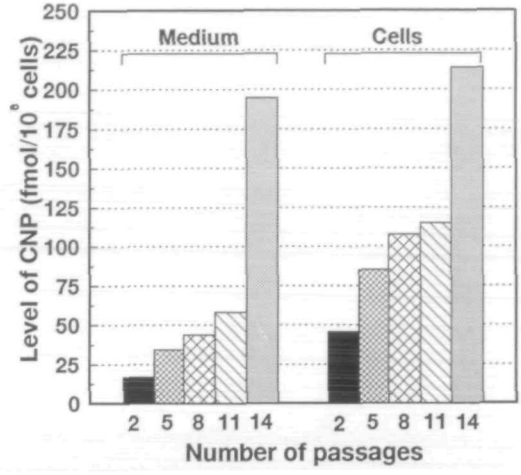

Fig. 4. **Production of CNP with increases in passage number.** CNP-like immunoreactivity was determined in the culture medium and in lysates of rat chondrocytes after 2, 5, 8, 11, and 14 passages. Cells were grown in 55-cm² dishes, washed twice with serum-free DMEM, and incubated with 8 ml of serum-free DMEM for 48 h Lysates of these cells and the conditioned medium (8 ml) were subjected to the radioimmunoassay for CNP and the amounts of CNP were estimated as fmol/dish/48 h. Four dishes were used per group.

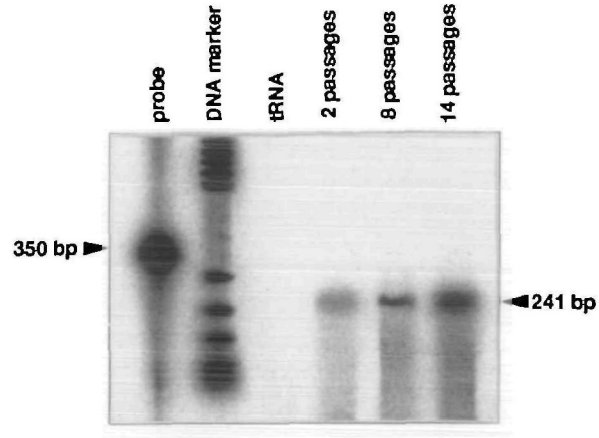

Fig. 5 **Levels of NPR-B mRNA in cultured chondrocytes after different numbers of passages, as determined by RNase protection analysis.** Total RNA (20 μg) isolated from cultured chondrocytes after 2, 8, and 14 passages was annealed with a 350-bp ^{32}P-cRNA probe (arrowhead). The RNA was then digested with RNases A and T_1, and the protected fragments (241 bp; arrowhead) were analyzed by electrophoresis and fluorography as described in "EXPERIMENTAL PROCEDURES." The intensity in units of photo-stimulated luminescence, PSL of each band was analyzed with a Bioimage Analyzer. After 2 passages, it was 1,303; after 8 passages, 2,195; and after 14 passages, 3,927. PSL is a unit that is based on the radioactivity detected by the analyzer (*18*).

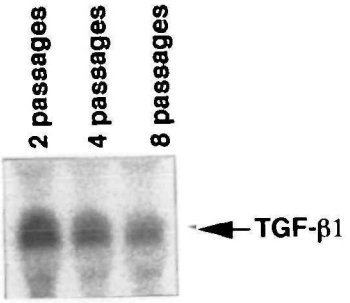

Fig 6. **Northern blot analyses of TGF-β1 mRNA in cultured chondrocytes with increasing passage number.** Total RNA (20 μg) isolated from cultured chondrocytes were subjected to electrophoresis in an agarose gel and hybridized with ^{32}P-labeled mouse TGF-β1 mRNA as described in "EXPERIMENTAL PROCEDURES."

genic factor(s) whose level increases with continued subculture.

We previously reported that CNP inhibited mitogenesis in chondrocytes *via* NPR-B (*9*). Figure 3 shows a typical example of the significant inhibition of the mitogenic activity of chondrocytes by CNP. The inhibitory effect of CNP on chondrocyte mitogenesis is dose-dependent. Therefore, we attempted to examine the levels of expression of CNP and NPR-B in cells at different numbers of passages during dedifferentiation of chondrocytes into fibroblast-like cells. We performed a radioimmunoassay using an antiserum against CNP and an RNase protection analysis using a cDNA fragment that encoded part of rat NPR-B. Figure 4 shows the levels of CNP-like immunoreactivity in the conditioned medium and in the cells with increasing passage number. The increase in passage number was accompanied with large increases in the levels of CNP in both the conditioned medium and the cells. RNase protection analysis revealed at least three-fold higher levels of NPR-B mRNA after 14 passages than after 2 passages (Fig. 5). These results are in good agreement with the results related to proliferation of cells (Fig. 2).

To see whether the expression of TGF-β1 in cultured chondrocytes was induced with increasing passage number, Northern blot analyses were carried out. As shown in Fig. 6, TGF-β1 message levels markedly decreased with increase in passage number. These results indicate that TGF-β1 is not involved in the induction of the expression of CNP and NPR-B during *in vitro* dedifferentiation of chondrocytes.

In summary, we have demonstrated that the elevated levels of CNP and NPR-B observed after culture *in vitro* of the cells for long periods might modulate the proliferation and might be involved in the maintenance of metabolism of cartilage *via* regulation of the proliferation of chondrocytes. We next need to elucidate the mechanisms of regulation of the expression of genes for CNP and NPR-B during dedifferentiation of chondrocytes into fibroblast-like cells.

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