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Stimulation by Retinoids of the Natriuretic Peptide System of Osteoblastic MC3T3-E1 Cells

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The effects were examined of treatment with retinoids of osteoblastic MC3T3-E1 cells on the natriuretic peptide system that promotes the differentiation of osteoblastic cells. Northern blot analysis revealed high levels of mRNA for the retinoid X receptor β (RXRβ) and moderate levels of mRNAs for retinoic acid receptors α (RARα) and γ (RARγ). Exposure of MC3T3-E1 cells to 1 μM retinoid caused increases in the levels of C-type natriuretic peptide (CNP) and natriuretic peptide receptor-C (NPR-C). The activity of natriuretic peptide receptor-B (NPR-B) was unchanged after the addition of retinoid to the culture system. These results suggest that retinoids might influence the metabolism of osteoblastic cells through regulation of the natriuretic peptide system.

Atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP), and C-type natriuretic peptide (CNP) are three members of the family of natriuretic peptides. The natriuretic peptide receptor-A (NPR-A) and natriuretic peptide receptor-B (NPR-B) are both membrane guanylate cyclases and they are activated by ANP and CNP, respectively. The natriuretic peptide receptor-C (NPR-C) has almost no intracellular domain (guanylate cyclase domain) and its physiological role, apart from its role in clearance, is unclear. We reported recently that osteoblastic MC3T3-E1 cells have an autocrine CNP and NPR-B system and a large number of NPR-C (1), and that natriuretic peptides regulate the proliferation and differentiation of osteoblastic cells, as well as bone formation by these cells (1, 2). Therefore, we attempted to identify the factor(s) that regulate the natriuretic peptide system in osteoblastic cells. Retinoids have been reported to regulate the levels of natriuretic peptides and their receptors in cardiovascular systems (3, 4). Retinoids also regulate the growth of cells and tissues. The crucial roles of retinoids in controlling processes related to differentiation have also become evident from studies of a variety of systems *in vivo* and *in vitro.* In osteoblastic cells retinoids activate the expression of alkaline phosphatase (ALPase) (5-7). Therefore, we examined the effects of retinoids on the levels of components of the natriuretic peptide system in osteoblastic MC3T3-E1 cells.

In this report, we provide evidence that retinoids might promote the synthesis of CNP and NPR-C through specific receptors, such as RARα, RARγ, and RXRβ.

MATERIALS AND METHODS

Materials. All-*trans* retinoic acid and 9-*cis* retinoic acid were purchased from Sigma (St. Louis, MO, USA) and Wako Pure Chemical Co. (Osaka, Japan), respectively. cDNAs for RARs and RXRs were a generous gift from Dr. P. Chambon (IGBMC, Strasbourg, France). ^{32}P-Labeled nucleotides were from Amersham (Buckinghamshire, UK); Dulbecco's modified Eagle's medium (DMEM), α-modified minimum essential medium (α-MEM), and penicillin and

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streptomycin antibiotic mixture were from Life Technologies, Inc. (Grand Island, NY, USA); and fetal bovine serum was from JRH Biosciences (Lenexa, KS, USA).

Cell culture. MC3T3-E1 cells were a generous gift from Dr. M. Kumegawa (Meikai University, Sakado, Japan). Cells were maintained in 55-cm^2 dishes 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. After reaching confluence, the cells were detached by treatment with 0.05% trypsin, replated in either 55-cm^2 dishes or 12-well plates at a density of 1×10^4 cells/cm^2 and grown in α-MEM supplemented with 10% fetal bovine serum, 50 units/ml penicillin, 50 μg/ml streptomycin, 5 mM β-glycerophosphate, 50 μg/ml ascorbic acid, with or without 1 μM retinoid. During subculture, the medium was replaced at 4-day intervals.

Northern blot analysis. RNA was extracted from MC3T3-E1 cells by the acid guanidinium-phenol-chloroform method (8). Total RNA (20 μg) was subjected to electrophoresis on a 1% agarose gel that contained 2.2 M formaldehyde and was then transferred to a MagnaGraph nylon membrane (Micron Separations Inc., Westborough, MA). After baking of the membrane, the RNA on the membrane was allowed to hybridize overnight with cDNAs for RARs, RXRs, or β-tubulin (the cDNAs were generous gifts from Dr. P. Chambon, IGBMC) at 42°C in 50% formaldehyde that contained 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. Each cDNA probe was radiolabeled with a Random Primer DNA Labeling Kit (Takara, Shiga, Japan). The membrane was washed twice in 1× SSC (0.15 M NaCl, 15 mM sodium citrate, pH 7.0) that contained 0.1% SDS at room temperature for 5 min each and twice in 1× SSC that contained 0.1% SDS at 55°C for 1 h each and then it was exposed to an imaging plate for 4 h. The plate was analyzed with a Bioimage Analyzer (BAS 2000; Fuji Film, Tokyo, Japan).

Radioimmunoassay (RIA) for CNP. The preparation of conditioned medium and of cell lysates and the RIA for CNP were performed as described previously (1, 2). MC3T3-E1 cells, grown in 21-cm^2 dishes for 4 days, were washed with serum-free α-MEM and subcultured in serum-free α-MEM supplemented with a retinoid at various concentrations for 48 h. The amount of CNP in the conditioned medium (3 ml) and in lysates obtained from cells in 21-cm^2 dishes was estimated as fmol/10^6 cells/24 h.

Assay of binding of ^{125}I-ANP. MC3T3-E1 cells that had been grown in 12-well plates (3.8 cm^2/well) were subcultured with serum-free α-MEM supplemented with 10^{-6} M retinoids for several hours. The cells were washed twice with ice-cold phosphate-buffered saline (pH 7.3; PBS; 20 mM phosphate, 130 mM NaCl, and 1 mM EDTA) and incubated in 0.5 ml of PBS that contained 0.2% (w/v) bovine serum albumin (fraction V; Sigma), ^{125}I-labeled ANP (920 Bq/well), and 10^{-6} M unlabeled ANP for 1 h at 4°C. After incubation, cells were dispased and collected by centrifugation. The radioactivity was measured with a gamma counter (ARC-300; Aloka, Tokyo, Japan).

Measurement of the accumulation of intracellular cGMP. Cells that had been treated with 10^{-6} M all-*trans* retinoic acid or 9-*cis* retinoic acid for 12 h or 24 h were incubated with serum-free α-MEM supplemented with 0.5 mM 3-isobutyl-1-methylxanthine at 37°C for 15 min, after two washes with serum-free α-MEM. MC3T3-E1 cells were subsequently incubated at 37°C for 15 min with 10^{-6} M CNP. After incubation, the cells were lysed by addition of 200 μl of a 0.1 N solution of HCl that contained 5 mM EDTA. The amounts of cGMP in each cell lysate were measured with a radioimmunoassay kit from Yamasa (Chiba, Japan).

RESULTS AND DISCUSSION

Identification of mRNA for RARs and RXRs in osteoblastic MC3T3-E1 cells. The biological effects of retinoids are mediated by their interactions with nuclear RARs and RXRs, which act as DNA-binding *trans*-acting transcription factors. To date, however, little information is available about RXRs in osteoblasts, as compared to the available information about RARs (5, 9). To examine the nuclear receptors for retinoic acid in MC3T3-E1 cells, we performed Northern blot analysis with specific probes. Northern blots of RNA isolated from MC3T3-E1 cells were prepared and probed with ^{32}P-labeled fragments of cDNAs for RARs and RXRs. Figure 1 shows that mRNA for RXRβ was detected at a high level and mRNAs for RARα and RARγ were detected at moderate levels. No mRNAs for RARβ, RXRα, and RXRγ were detected. In previous studies (10), we demonstrated that rat chondrocytes express mRNAs for RARα, RARγ, and RXRα. Osteoblasts and chondrocytes develop from common undifferentiated mesenchymal progenitor cells. The difference in terms of expression of subtypes of retinoid receptors between osteoblasts and chondrocytes might reflect the specific functions of the two types of cell.

Increases by retinoids in the rate of production of CNP and in the levels of NPR-C in MC3T3-E1 cells. We reported previously that MC3T3-E1 cells produce CNP that is an

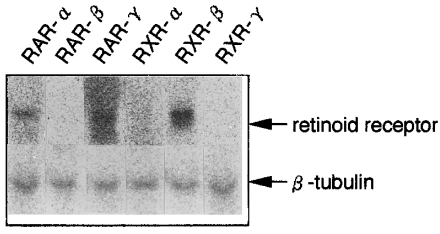

FIG. 1. Northern blot analysis of mRNAs for nuclear receptors for retinoic acid in MC3T3-E1 cells. Total RNA was isolated from osteoblastic MC3T3-E1 cells that had been subcultured for 4 days. Twenty micrograms of total RNA were subjected to electrophoresis in a 1% agarose gel and were then allowed to hybridize with ^{32}P-labeled cDNAs for human RARα, RARβ, and RARγ, and mouse RXRα, RXRβ, and RXRγ, and β-tubulin, as described under Materials and Methods.

endogenous activator of guanylate cyclase (NPR-B) and we suggested that CNP might promote the differentiation of osteoblastic cells (1). As shown in Figure 2, exposure of MC3T3-E1 cells to all-*trans* retinoic acid and 9-*cis* retinoic acid promoted the synthesis and secretion of CNP. Retinoids might regulate the synthesis of CNP in osteoblastic cells, although a binding site for a retinoid receptor has not yet been found in the promoter of the gene for CNP. It has been reported that a binding site for the receptor of 1,25-dihydroxyvitamin D_3 is located in the promoter of the rat gene for ANP and that 9-*cis* retinoic acid activates the gene for ANP (1.9-fold induction of CAT activity) (4). All-*trans* retinoic acid was also reported to suppress the phenylephrine-induced activity of the promoter of the gene for ANP in myocardial cells (3).

Figure 3A shows that retinoids enhance the binding of ANP to MC3T3-E1 cells. Their effect appears to be specific for NPR-C. The activity of NPR-B (production of cGMP upon stimulation by CNP) was unchanged by the addition of retinoids to the culture system, as shown in Figure 3B. The role of an elevated level of NPR-C is unclear. The gene for NPR-C has been cloned from bovine (11), mouse and human (12). Various putative regulatory elements were found in the mouse gene (cAMP-response element; binding sites for AP-1, AP-2, SP-1, and NF-κB, and a shear stress-responsive element) and in the bovine gene (binding sites for AP-2, SP-1, and NF-κB, and an IFβ silencer element). There is one putative binding site for a retinoid receptor (AGGTCA) in the promoter region of the

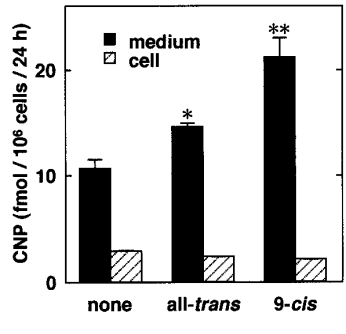

FIG. 2. Retinoid-induced production of CNP in MC3T3-E1 cells. CNP-like immunoreactivity was determined in culture media and lysates of MC3T3-E1 cells. Cells were grown in 21-cm^2 dishes, washed twice with serum-free α-MEM, and incubated with 3 ml of serum-free α-MEM for 48 h without or with the indicated retinoid. Cell lysates obtained from these cells and conditioned media (3 ml) were subjected to radioimmunoassays for CNP, and amounts were estimated as fmol/10^6 cells/24 h. Four dishes were used per group, and results are shown as means ± SD. *$p<0.005$ *vs.* none; **$p<0.002$ *vs.* none.

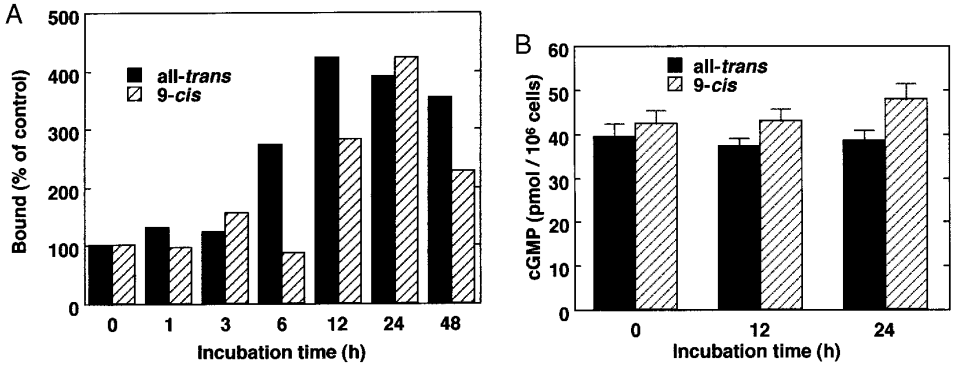

FIG. 3. Stimulatory effects of retinoids onbinding of ^{125}I-ANP to cells (A) and the formation of intracellular cGMP in response to CNP (B). Cells in 12-well plates were cultured for three days with α-MEM that contained 10% fetal bovine serum, 5 mM β-glycerophosphate, and 50 μg/ml ascorbic acid. They were washed twice with serum-free DMEM and incubated with serum-free DMEM with 1 μM retinoid for 12 h or 24 h. (A) The cells were incubated at 4°C for 1 h with ^{125}I-ANP (920 Bq) in the presence or in the absence of unlabeled 1 μM ANP. Subsequent steps for quantitating the binding of the radiolabeled ligand are described in Materials and Methods. (B) Cells treated with a retinoid were exposed to 1 μM CNP for 15 min at 37°C in the presence of 0.5 mM 3-isobutyl-1-methylxanthine and then intracellular levels of cGMP were determined as described in Materials and Methods. Values represent the means ± SD of results from three wells and data are typical of results from three separate experiments.

mouse gene for NPR-C (12). However, it is unclear as yet whether this site controls the expression of NPR-C. There are multiple binding sites for AP-2 in the 5′-flanking regions of the bovine and mouse genes for NPR-C (11, 12). Recently, it was reported that binding of the transcription factor AP-2 to the consensus binding site was enhanced in retinoic acid-treated cells (13). Therefore, retinoids might play a key role in AP-2-dependent activation in the promoter region of the gene for NPR-C.

In the present study, we showed that retinoids increased the levels of components of the natriuretic peptide system, which included CNP and NPR-C, in osteoblastic MC3T3-E1 cells. These results suggest that retinoids might be involved in the metabolism of osteoblastic cells through regulation of the natriuretic peptide system.

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REFERENCES

1. Inoue, A., Hiruma, Y., Hirose, S., Yamaguchi, A., Furuya, M., Tanaka, S., and Hagiwara, H. (1996) *Biochem. Biophys. Res. Commun.* **221,** 703–707.
2. Hagiwara, H., Inoue, A., Yamaguchi, A., Yokose, S., Furuya, M., Tanaka, S., and Hirose, S. (1996) *Am. J. Physiol.* **270,** C1311–C1318.
3. Zhou, M. D., Sucov, H. M., Evans, R. M., and Chien, K. R. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92,** 7391–7395.
4. Kahlen, J. P., and Carlberg, C. (1996) *Biochem. Biophys. Res. Commun.* **218,** 882–886.
5. Nakayama, Y., Takahashi, K., Noji, S., Muto, K., Nishijima, K., and Taniguchi, S. (1990) *FEBS Lett.* **261,** 93–96.
6. Katagiri, T., Yamaguchi, A., Ikeda, T., Yoshiki, S., Wozney, J. M., Rosen, V., Wang, E. A., Tanaka, H., Omura, S., and Suda, T. (1990) *Biochem. Biophys. Res. Commun.* **172,** 295–299.
7. Rodan, S. B., Wesolowski, G., Hilton, D. J., Nicola, N. A., and Rodan, G. A. (1990) *Endocrinology* **127,** 1602–1608.

8. Chomczynski, P., and Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159.
9. Noji, S., Yamaai, T., Koyama, E., Nohno, T., and Taniguchi, S. (1989) *FEBS Lett.* **257,** 93–96.
10. Hagiwara, H., Inoue, A., Nakajo, S., Nakaya, K., Kojima, S., and Hirose, S. (1996) *Biochem. Biophys. Res. Commun.* **222,** 220–224.
11. Saheki, T., Mizuno, T., Iwata, T., Saito, Y., Nagasawa, T., Uchida Mizuno, K., Ito, F., Ito, T., Hagiwara, H., and Hirose, S. (1991) *J. Biol. Chem.* **266,** 11122–11125.
12. Yanaka, N., Kotera, J., Taguchi, I., Sugiura, M., Kawashima, K., and Omori, K. (1996) *Eur. J. Biochem.* **237,** 25–34.
13. Dyck, J. R., and Fliegel, L. (1995) *J. Biol. Chem.* **270,** 10420–10427.