Hormonal Regulation of Connexin 43 Expression and Gap Junctional Communication in Human Osteoblastic Cells

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ABSTRACT. We have recently shown that connexin 43 (Cx43), a major gap junction protein in osteoblasts, is expressed with an increase in cell density (CHIBA, H. *et al.* (1993). *Cell Struc. Funct.*, 18: 419–426). In the present study, we examined what kinds of hormones and cytokines regulate the gap junction protein in osteoblastic cells, using a human osteoblastic cell line (SV-HFO) after reaching a confluent density to avoid influence of cell proliferation. Either retinoic acid (RA) or transforming growth factor- β_1 (TGF- β_1) induced the Cx43 expression of SV-HFO cells, as revealed by Northern blot analysis and immunocytochemistry. These modulators also increased gap junctional intercellular communication, in terms of the extent of dye transfer. On the other hand, 1α , 25-dihydroxyvitamin D₃ did not influence the Cx43 expression and gap junctional intercellular communication of the cells. These results suggest that RA and TGF- β_1 might maintain bone tissue as an organized tissue *in vivo* by increasing intercellular communication of osteoblastic cells.

Gap junctions are intercellular channels, composed of transmembrane proteins called connexins (1). By allowing transfer of ions and small molecules into neighboring cells, gap junctions are considered to play important roles in cell differentiation, proliferation and tissue organization (13). Osteoblasts and osteocytes in bone matrix *in vivo* are connected with each other, forming a cell-cell network via gap junctions (8, 15, 18, 21). It has become clear that connexin 43 (Cx43) is a major gap junction protein expressed in osteoblastic cells (6, 25, 26, 27). We have recently observed that the expression of Cx43 in osteoblastic cells was induced with an increase in cell density (3). However, it remains unknown what kinds of hormones and cytokines regulate the expression of gap junction protein in osteoblastic cells.

Recently, we have established a human osteoblastic cell line from normal human bone by immortalization with simian virus 40. This cell line, designated SV-HFO, proliferates and maintains osteoblastic properties even under serum-free conditions as well as under serum-supplemented conditions (2, 3). Thus, SV-HFO cell line are expected to be a suitable model for elucidat-

ing the effects of hormones and cytokines on the phenotypic expression of human osteoblasts. In the present study, we examined the effects of $1,25(\text{OH})_2\text{D}_3$, retinoic acid and transforming growth factor- β (TGF- β), modulators of bone cells, on the Cx43 expression and gap junctional intercellular communication in SV-HFO cells.

MATERIALS AND METHODS

Cell culture. The SV-HFO cells were established as described previously (2, 3). The cells at passage 15 were seeded at a cell density of 1×10^4 cells/cm² on 100-mm culture dishes or 12-well tissue culture plates coated with $2 \mu\text{g}/\text{cm}^2$ type I collagen (Vitrogen 100; Collagen Corp., Palo Alto, CA) in serum-free Dulbecco's modified Eagle's medium (DMEM; Nissui Pharmaceutical, Tokyo, Japan) supplemented with 0.5% bovine serum albumin (Albumax; Gibco Laboratories, Grant Island, NY), ITS (containing $5 \mu\text{g}/\text{ml}$ insulin, $5 \mu\text{g}/\text{ml}$ transferrin and $5 \text{ ng}/\text{ml}$ selenious acid; Collaborative Research, Inc., Bedford, MA), $100 \text{ U}/\text{ml}$ penicillin and $100 \mu\text{g}/\text{ml}$ streptomycin (Gibco Laboratories). These cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 in air, and the medium was renewed every 3 days. To determine the effects of $1,25(\text{OH})_2\text{D}_3$ (Biomol Research Laboratories, Inc., Plymouth Meeting, PA), all-*trans* retinoic acid (RA) or TGF- β_1 (Sigma Chemical Co., St. Louis, MO) on the Cx43 expression and gap junctional intercellular communication, the cells

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Abbreviation used: Cx, connexin; HFO, human fetal osteoblastic cells; SV-HFO, SV40-transformed human fetal osteoblastic cells; $1,25(\text{OH})_2\text{D}_3$, 1α , 25-dihydroxyvitamin D₃; TGF- β , transforming growth factor- β ; RA, retinoic acid; DMEM, Dulbecco's modified Eagle's medium; DIG, digoxigenin; ALP, alkaline phosphatase.

reaching a confluent density on 35-mm or 100-mm culture dishes were treated with 10^{-7} M $1,25(\text{OH})_2\text{D}_3$, 10^{-7} M RA or 5 ng/ml TGF- β_1 for 6, 12, 24 or 48 consecutive hours. The stock solution of $1,25(\text{OH})_2\text{D}_3$ was in ethanol, and that of RA was in dimethyl sulfoxide (DMSO). Both test and control solutions contained the same concentration of ethanol or DMSO, which was less than 0.1%.

Northern blot analysis. Total RNAs were isolated from cell cultures using the single-step thiocyanate-phenol-chloroform extraction method (5) as modified by Xie and Rothblum (30). For electrophoresis, 10 μg of total RNAs was loaded on 1% agarose gel containing 0.5 $\mu\text{g}/\text{ml}$ ethidium bromide. Gels were capillary-blotted in $20\times$ saline sodium citrate (SSC) onto nylon membranes (Hybond-N; Amersham Corp., Buckinghamshire, England) and fixed by UV light.

For the detection of Cx43 mRNA, digoxigenin (DIG)-labeled RNA probes were prepared from rat cDNA using an RNA labeling kit (Boehringer Mannheim, Mannheim, Germany), and hybridization, washing and chemiluminescent detection were carried out following the DIG luminescent protocol (14).

Immunocytochemistry. SV-HFO cells were cultured on cover-slips coated with 2 $\mu\text{g}/\text{cm}^2$ type I collagen under the serum-free condition. After reaching confluence, the cells were incubated in the presence or absence of 10^{-7} M $1,25(\text{OH})_2\text{D}_3$, 10^{-7} M RA or 5 ng/ml TGF- β_1 for 24 hours. These cells were then rinsed with phosphate-buffer saline (PBS), fixed in acetone for 20 min at -20°C , and the presence of Cx43 was investigated by the procedure described before (3, 23). Briefly, the fixed cells were incubated with rabbit polyclonal antibody against Cx43 peptides (1/100 dilution) for 1 h at room temperature. After washing with PBS, the cells were reacted with swine fluorescein-conjugated anti-rabbit immunoglobulin (1/100 dilution; DAKO, Copenhagen, Denmark) for 1 h, rinsed with PBS, mounted with Mowiol 4-88 polyvinyl alcohol-based medium (Hoechst, Frankfurt, Germany), and examined under a fluorescent microscope.

Analysis of gap-junctional intercellular communication. Gap junctional intercellular communication was assayed using the scrape-loading/dye transfer technique of El-Fouly

et al. (9). In brief, the cells were rinsed with PBS, scrape-loaded using a razor blade, and supplied with a 0.05% solution of the gap junction-permeable dye, Lucifer yellow (Polysciences, Inc., Warrington, PA), and the gap junction-impermeable dye, rhodamine dextran (Molecular Probes, Inc., Eugene, OR), in PBS. After leaving these cells in dye solution for 2 min at room temperature, the cells were rinsed with PBS several times, and examined under a fluorescent microscope. The extent of gap-junctional intercellular communication was calculated as the total number of Lucifer yellow-labeled cells per 350 μm field/ the number of rhodamine dextran-labeled cells in the same field (19). Measurement were from 10 consecutive 350 μm fields of at least 3 petri dishes per condition.

RESULTS

To minimize the influence of cell proliferation on the Cx43 expression (3), the cells, after reaching a confluent cell density, were used. In addition, to exclude the effect of serum on the expression, the cells were cultured under the serum-free conditions.

The expression of Cx43 mRNA was significantly induced by 10^{-7} M RA time-dependently for at least 24 hours, with the maximal increase at 6 hours. Cx43 mRNA expression was also enhanced at 6, 12 and 24 hours after treatment with 5 ng/ml TGF- β_1 (Figs. 1 and 2). The effects of RA and TGF- β_1 on Cx43 mRNA expression were also dose-dependent (data not shown). In contrast, the levels of Cx43 mRNA expression did not change during $1,25(\text{OH})_2\text{D}_3$ treatment (Figs. 1 and 2).

Immunocytochemically, Cx43 was demonstrated as macular spots on cell membranes between adjacent cells. Immunoreactive spots were not detected in cells incubated with preimmune serum (data not shown). Either 10^{-7} M RA or 5 ng/ml TGF- β_1 significantly induced Cx43-positive spots in SV-HFO cells (Fig. 3). On the other hand, $1,25(\text{OH})_2\text{D}_3$ did not influence the number of Cx43-positive spots.

Figure 4 shows fluorescent photomicrographs demonstrating the intercellular transfer of Lucifer yellow dye

Fig. 1. Northern blot analysis of the transcript of Cx43 gene (3.0 kb) after treating confluent SV-HFO cells with 10^{-7} M $1,25(\text{OH})_2\text{D}_3$, 10^{-7} M RA, 5 ng/ml TGF- β_1 , or vehicle only. The bottom panel shows the ethidium bromide stain of the filter corresponding to 28S ribosomal RNA.

Fig. 2. Northern blot analysis of the transcript of Cx43 gene 6 hours after treating confluent SV-HFO cells with 10^{-7} M $1,25(\text{OH})_2\text{D}_3$, 10^{-7} M RA, a combination of the two, 0.1% EtOH or 0.1% DMSO. The bottom panel shows the ethidium bromide stain of the filter corresponding to 28 S ribosomal RNA.

from scraped-loaded SV-HFO cells to contiguous cells. In control culture, a little transfer of Lucifer yellow dye is detectable, indicating that only limited gap-junctional intercellular communication are present. Treating the cells with 10^{-7} M RA or 5 ng/ml TGF- β_1 for 6, 12 and 24 hours significantly increased the extent of Lucifer yellow transfer in terms of the gap-junctional intercellular

communication, while it did not influence the extent of rhodamine dextran transfer. The number of coupled cells increased 2.2-fold ($P < 0.001$) or 2.6-fold ($P < 0.001$) after 12 hours exposure to 10^{-7} M RA or 5 ng/ml TGF- β_1 . In contrast, $1,25(\text{OH})_2\text{D}_3$ did not change the extent of dye transfer. These data were consistent with the results obtained from Northern blot analysis and immunocytochemistry.

DISCUSSION

The SV-HFO cell line is a new model for studying the differentiation and proliferation of human osteoblastic cells. The SV-HFO cells were immunocytochemically positive for vimentin but negative for keratin and epithelial membrane antigen, which suggested that they were of mesenchymal origin. Phase-contrast microscopic and electron microscopic observations supported the mesenchymal nature of these cells. Osteoblastic nature was indicated by evidence showing that the cells produce ALP and osteocalcin and respond to $1,25(\text{OH})_2\text{D}_3$ (2). We have also observed that the cells have a potential to form mineralized tissues *in vitro* (in preparation).

Gap junctions are implicated in metabolic cooperation, differentiation and growth of cells (13). It has been shown that Cx43 is a major gap junction protein in osteoblasts, and that the Cx43 expression is correlated with the extent of intercellular communication (6, 25, 26, 27). We have recently found that the Cx43 expression in osteoblasts is associated with an increase in cell density (3). However, the mechanism regulating Cx43 expression in osteoblasts remains unknown, except for the ob-

Fig. 3. Immunofluorescent staining of Cx43 in SV-HFO cells cultured in the absence (A) or presence of 10^{-7} M RA (B) and 5 ng/ml TGF- β_1 for 24 hours (C).

Fig. 4. Fluorescent photomicrographs demonstrating the intercellular transfer of Lucifer yellow dye via gap junctions in SV-HFO cells cultured in the absence (A) or presence of 10^{-7} M RA (B) and 5 ng/ml TGF- β_1 for 12 hours (C).

servation that parathyroid hormone enhances Cx43 expression in osteoblastic cells (25).

In this study, we have shown that Cx43 expression and gap junctional communication in SV-HFO cells is enhanced by RA treatment, but not by $1,25(\text{OH})_2\text{D}_3$. Gap junctional intercellular communication and the expression of connexins are shown to be altered by retinoids (12, 17, 20, 22, 24, 29). To achieve function, RA and $1,25(\text{OH})_2\text{D}_3$ are required to bind to their receptors complex to responsive elements (7, 10, 11, 16, 31). These findings suggest the possibility that RA functions as a crucial modulator of Cx expression via an RA-responsive element located at the upstream 5' flanking region of the Cx gene, whereas the gene lacks a vitamin D-responsive element.

We have shown that TGF- β_1 up-regulates Cx43 expression and gap junctional communication in SV-HFO cells. This is in contrast to previous findings which showed that canalicular cell process formation of mouse osteoblast-like cells on the reconstituted basement membrane (Matrigel) is blocked by TGF- β , and recovered by treatment with antibodies against TGF- β (28). Although the reason for the inconsistency is not clear, it may come from differences in culture conditions, in particular extracellular matrix.

The expression of alkaline phosphatase (ALP) in SV-HFO cells was significantly induced by $1,25(\text{OH})_2\text{D}_3$, and suppressed by RA and TGF- β_1 . The expression of osteocalcin, a bone-specific protein, was up-regulated by treating the cells with $1,25(\text{OH})_2\text{D}_3$ and RA, while down-regulated by TGF- β_1 (4). On the other hand, we have observed that the mineralization of SV-HFO cells was markedly reduced by treating these cells with RA or TGF- β_1 (unpublished data). These findings mean that there might be no direct relationship between gap junctional intercellular communication and osteoblast differentiation. To clarify the role of gap junctional communication during osteoblast differentiation, more detailed experiments are required.

In conclusion, we have demonstrated in the present study that RA and TGF- β_1 regulate the Cx43 expression and gap junctional communication of a human osteoblastic cell line.

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