

Relationship between the Expression of the Gap Junction Protein and Osteoblast Phenotype in a Human Osteoblastic Cell Line during Cell Proliferation

Hideki Chiba^{1,2,4}, Norimasa Sawada¹, Masahito Oyamada¹, Takashi Kojima¹, Shintaro Nomura³, Seiichi Ishii², and Michio Mori¹

Departments of ¹Pathology and ²Orthopedic Surgery, Sapporo Medical University School of Medicine, Sapporo 060, ³Department of Pathology, Osaka University Medical School, Suita 565

Key words: osteoblasts/differentiation/gap junctions/connexins/human

ABSTRACT. We examined i) the kinds of connexins, component proteins of gap junctions, that are expressed in osteoblasts and ii) the relationship between the expression of gap junctions and osteoblastic phenotype during cell proliferation and after the treatment with $1\alpha,25$ -dihydroxyvitamin D_3 . Human osteoblastic cells with (SV-HFO) or without (HFO) transformation by simian virus 40 and mouse osteoblast-like cells (MC3T3-E1) expressed connexin 43 (Cx43), but not Cx26 or Cx32, as revealed by Northern blot analysis and immunocytochemistry. The expression of Cx43 was significantly higher in SV-HFO cells in the confluent phase than in the proliferative phase. Similarly, the expression of alkaline phosphatase (ALP) and osteocalcin in SV-HFO cells in the confluent phase were higher than those in the proliferative phase. On the other hand, treatment of $1\alpha,25$ -dihydroxyvitamin D_3 did not change the expression of Cx43 in SV-HFO cells, but significantly induced the expression of ALP and osteocalcin. These results showed that the expression of gap junction protein in osteoblastic cells was coupled with cell differentiation in association with the expression of osteoblastic phenotype, but that the connexin expression is regulated in a way different from that of ALP and osteocalcin.

Gap junctions are intercellular channels composed of transmembrane proteins called connexins (2). It has become clear that there are several kinds of connexins, and that each of them is differently expressed in individual cell types (26). By mediating transfers of small molecules and ions between adjacent cells, gap junctions are thought to be involved in metabolic cooperation, differentiation and growth (9). Osteoblasts and osteocytes in bone matrix are connected with each other, forming a canalicular network via gap junctions (8, 11, 15, 20). It has been shown that connexin 43 (Cx43) is a major gap junction protein expressed in osteoblastic cells (6, 22, 23). However, it remains obscure what roles gap junctions play in the differentiation and proliferation of bone cells.

Recently, we have established a simian virus 40 (SV40)-transformed osteoblastic cell line, designated SV-HFO, from normal human bone. This line has char-

acteristic morphological and ultrastructural features of osteoblasts, produces alkaline phosphatase (ALP) activity and osteocalcin, and responds to $1\alpha,25$ -dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) (4). The SV-HFO cells also react to retinoic acid and transforming growth factor- β , and form mineralized nodules in the presence of β -glycerophosphate and glucocorticoids (in preparation). Thus, SV-HFO cell lines are expected to serve as a promising model for studying the differentiation and proliferation of human osteoblastic cells.

In the present study, we utilized SV-HFO cells, HFO cells and mouse osteoblast-like cells to confirm the types of connexins expressed in osteoblastic cells. We then studied the interrelation between the expression of osteoblastic features and that of connexins in SV-HFO cells during cell proliferation and after the treatment with $1,25(OH)_2D_3$.

MATERIALS AND METHODS

Cell culture. The HFO cells for primary culture were obtained at autopsy from human fetal parietal bones at the 26th week of gestation. The SV-HFO cells were established as described previously (4). The mouse osteoblast-like cells (MC3T3-E1) (25) were kindly provided by Dr. H. Kodama

⁴ To whom reprint requests/correspondence should be addressed.
Abbreviations used: Cx, connexin; SV40, simian virus 40; HFO, human fetal osteoblastic cells; SV-HFO, SV40-transformed human fetal osteoblastic cells; ALP, alkaline phosphatase; $1,25(OH)_2D_3$, $1\alpha,25$ -dihydroxyvitamin D_3 ; MC3T3-E1, mouse osteoblast-like cells; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; DIG, digoxigenin.

(Ohu University Dental School, Fukushima, Japan). The HFO cells at passage 7 and the MC3T3-E1 cells were cultured on 100-mm culture dishes in Dulbecco's modified Eagle's medium (DMEM; Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS; JRH Bioscience, Lenexa, KS), 100 U/ml penicillin and 100 μ g/ml streptomycin (Gibco Laboratories, Grant Island, NY). The SV-HFO cells at passage 15 were cultured on 100-mm culture dishes or 12-well tissue culture plates in serum-supplemented medium as described above, or on the same dishes and plates coated with 2 μ g/cm² type I collagen (Vitrogen 100; Collagen Corp., Palo Alto, CA) in serum-free medium supplemented with 0.5% bovine serum albumin (Albumax; Gibco Laboratories) and ITS (containing 5 μ g/ml insulin, 5 μ g/ml transferrin and 5 ng/ml selenious acid; Collaborative Research, Inc., Bedford, MA). These cells were cultured at 37°C in a humidified atmosphere of 5% CO₂ in air, and the medium was renewed every 3 days. To determine the effects of 1,25(OH)₂D₃ on the synthesis of ALP and osteocalcin, SV-HFO cells cultured in serum-free medium for 3, 6, 9 or 12 days were treated with 10⁻⁷ M 1,25(OH)₂D₃ (Biomol Research Laboratories, Inc., Plymouth Meeting, PA) for the last 3 days of each culture period. To examine the effects of 1,25(OH)₂D₃ on ALP, osteocalcin and Cx43 mRNA expression, cells cultured under serum-free conditions for 3, 6 or 12 days were treated with 10⁻⁷ M 1,25(OH)₂D₃ for the last 6 hours.

Immunocytochemistry. HFO cells and MC3T3-E1 cells cultured on cover-slips in serum-supplemented medium. SV-HFO cells were cultured on cover-slips under serum-supplemented conditions or on cover-slips coated with 2 μ g/cm² type I collagen under serum-free conditions. After reaching confluence, these cells were rinsed with PBS, fixed in ice-cold acetone for 20 min at -20°C, and the presence of connexins was then examined by immunofluorescence as described previously (21). Briefly, the fixed cells were incubated with one of the following antibodies for 1 h at room temperature: rabbit polyclonal antibody against Cx26-specific peptide (amino acid residues 101-119, 1/100 dilution), rabbit anti-J-peptide antiserum against Cx32 (1/100 dilution) or rabbit polyclonal antibody against Cx43 peptides (1/100 dilution). 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 fluorescence microscope.

Growth, ALP activity and osteocalcin synthesis. To examine growth and ALP activity, the SV-HFO cells were seeded at a cell density of 5 × 10³ cells/cm² on 12-well tissue culture plates in serum-supplemented or serum-free medium as described above, and were assayed in parallel cultures. To examine osteocalcin synthesis, the cells were plated at the same density on plates only in serum-free medium to avoid contamination by serum osteocalcin. At the 1st, 3rd, 6th, 9th and 12th day after plating, the cells were dispersed by trypsinization,

and the cell number was counted in a hemocytometer using 0.15% trypan blue. Using the parallel cultures, ALP activity in cell extracts and the amount of osteocalcin secreted into the culture medium were measured by the procedure described previously (4).

Northern blot analysis. Total RNAs were isolated from cell cultures, normal rat hearts and mouse livers using the single-step thiocyanate-phenol-chloroform extraction method (5) as modified by Xie and Rothblum (27). For electrophoresis, 10 μ g of total RNAs was loaded on 1% agarose gel containing 0.5 μ g/ml ethidium bromide. Gels were capillary-blotted in 20 × saline sodium citrate (SSC) onto nylon membranes (Hybond-N; Amersham Corp., Buckinghamshire, England) and fixed by UV light.

A 0.47 kb fragment of rat osteocalcin cDNA was obtained by reverse transcription followed by polymerase chain reaction, and was subcloned into Bluescript I pKS-. The base sequence was consistent with that reported before (3).

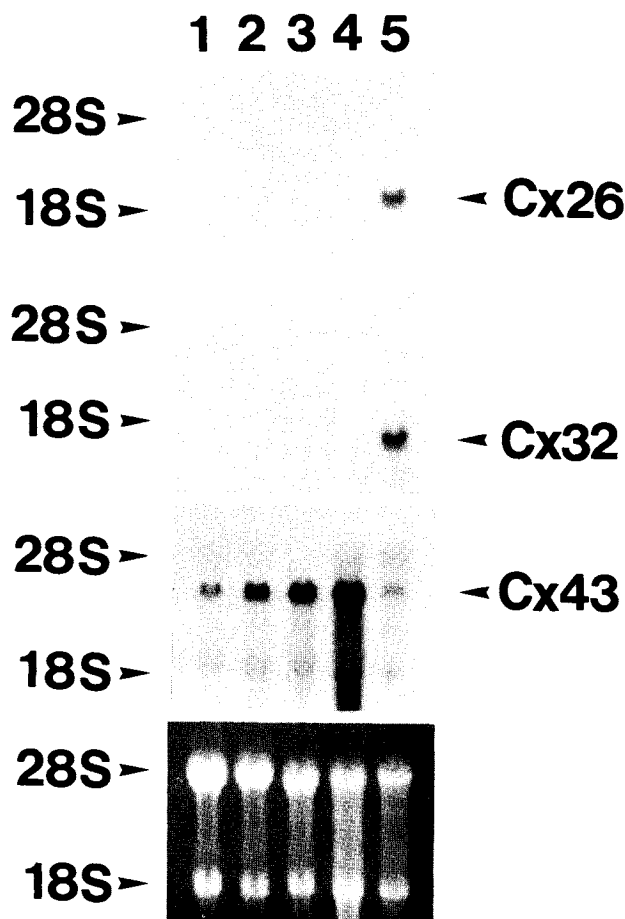


Fig. 1. Northern blot analysis of transcripts of genes encoding gap junction proteins. Lane 1, SV-HFO cells; Lane 2, HFO cells; Lane 3, MC3T3-E1 cells; Lane 4, rat hearts; Lane 5, mouse livers. The bottom panel shows the ethidium bromide stain of the filter corresponding to 28S and 18S.

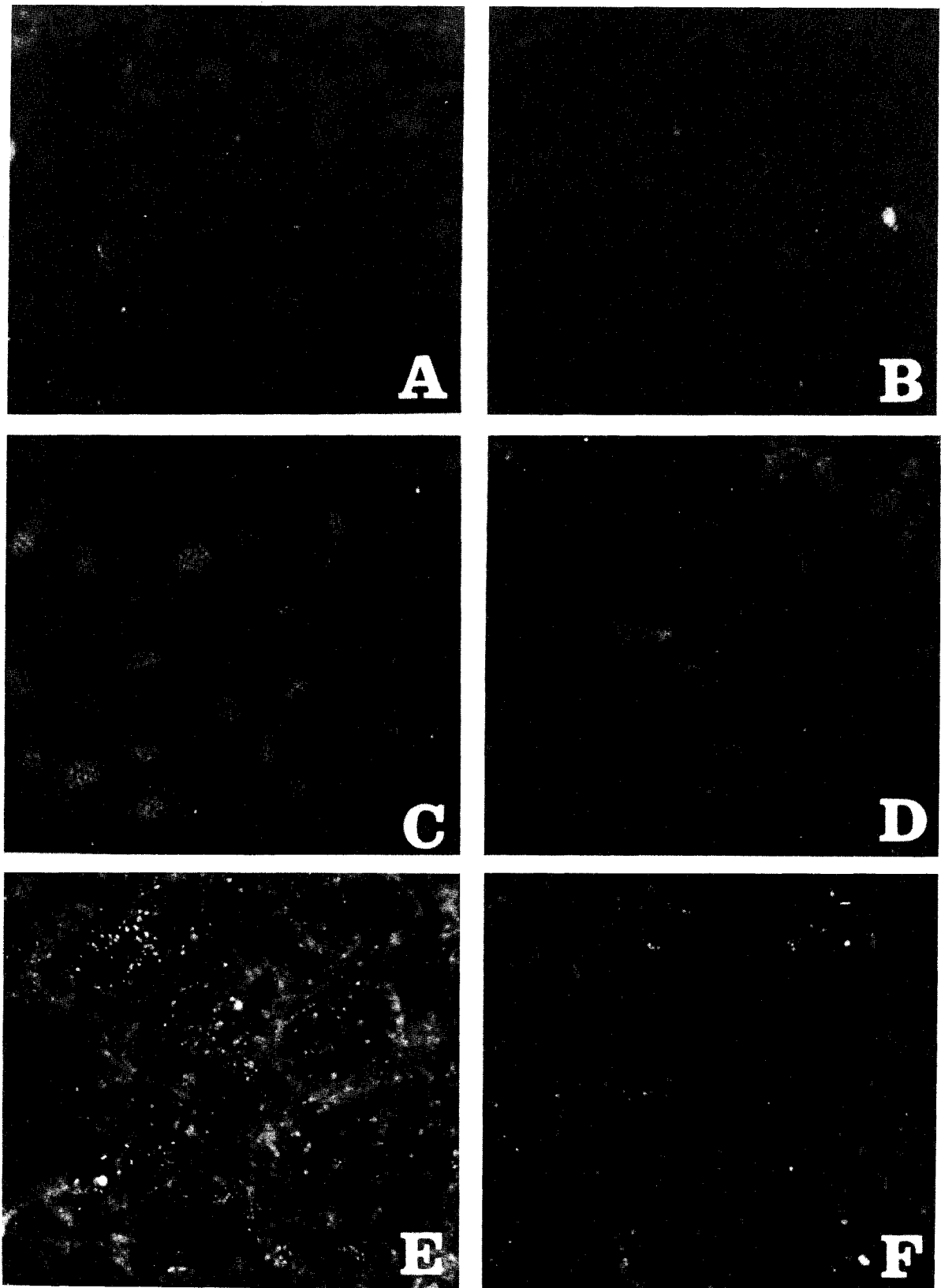


Fig. 2. Immunofluorescent localization of Cx26 (A, B), Cx32 (C, D), Cx43 (E, F) in MC3T3-E1 (A, C, E) and SV-HFO cells (B, D, F).

For the detection of ALP mRNA, membranes were prehybridized in a solution containing 50% formamide, 0.9 M NaCl, 0.1 M NaPO₄ (pH 7.4), 1% sodium dodecyl sulfate (SDS), 10 µg/ml herring sperm DNA and 5× Denhart's solution for 4 h at 42°C, and then hybridized overnight at 42°C in the same solution with a ³²P-labeled cDNA probe for human ALP obtained from the American Type Culture Collection. Next the membranes were washed twice in 2× SSC buffer containing 0.1% SDS for 5 min at room temperature and twice in 2× SSC buffer containing 1% SDS for 30 min at 68°C before exposure to film. For the detection of mRNAs of osteocalcin, Cx26, Cx32 and Cx43, digoxigenin (DIG)-labeled RNA probes were prepared from rat cDNAs (1, 19, 30) using an RNA labeling kit (Boehringer Mannheim, Mannheim, Germany), and hybridization, washing and chemiluminescent detection were carried out following the DIG luminescent protocol (10).

RESULTS

Total RNAs from postconfluent cells cultured in serum-supplemented medium and from rat heart and mouse liver tissues were examined for Cx26, Cx32 and Cx43 mRNA expressions, using specific RNA probes. As shown in Fig. 1, MC3T3-E1 cells, HFO cells, SV-HFO cells and rat heart tissue expressed Cx43 mRNA,

but not Cx26 or Cx32 mRNA. The level of Cx43 mRNA expression in MC3T3-E1 cells was higher than that in HFO cells and much higher than that in SV-HFO cells. In contrast, mouse liver tissue expressed large amounts of Cx26 and Cx32 mRNA, and a very low level of Cx43 mRNA.

Immunocytochemically, Cx43 was demonstrated as macular spots on cell membranes between adjacent MC3T3-E1 cells (Fig. 2E), HFO cells (data not shown) and SV-HFO cells (Fig. 2F). More Cx43-immunoreactive spots were observed in MC3T3-E1 and HFO cells than in SV-HFO cells. Neither Cx26 nor Cx32 was detected in those cells (Figs. 2A-2D). These data were consistent with the results obtained from Northern blot analysis.

We also examined changes in the expression of ALP, osteocalcin and Cx43 during cell growth and after the treatment with 1,25(OH)₂D₃. Since SV-HFO cells proliferated while maintaining their osteoblastic features under serum-free conditions, they were utilized for this experiment.

Figs. 3 and 4 show the growth curve of the SV-HFO cells at passage 15 and ALP activity in the parallel cultures, respectively. ALP activity in the cells increased during cell growth regardless of serum supplementation. Under serum-supplemented conditions, the level

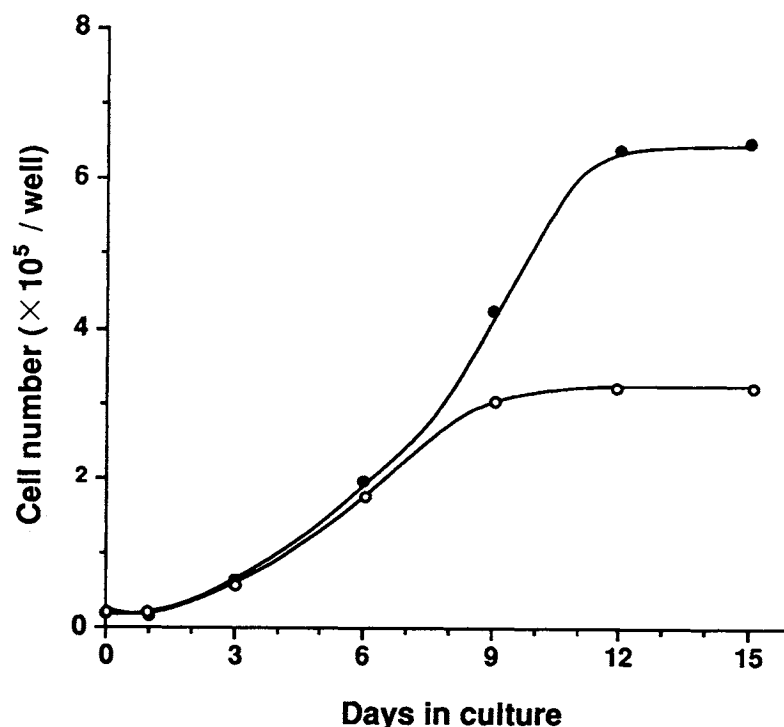


Fig. 3. Growth curve of SV-HFO cells at passage 15. The cells were seeded at a cell density of 5×10^3 cells/cm² and cultured in medium with 10% FBS on 12-well tissue culture plates (●) or serum-free medium on type I collagen at 2 µg/cm² (○). Each point shows the average cell number from four dishes.

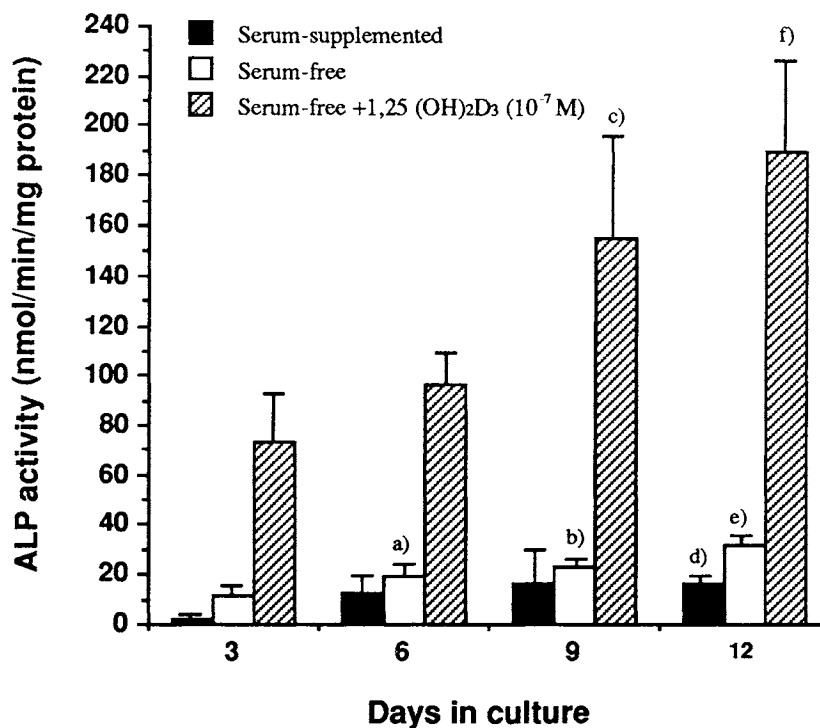


Fig. 4. Changes in alkaline phosphatase activity in the SV-HFO cells cultured under serum-supplemented and serum-free conditions with or without 1,25(OH)₂D₃. The figures are mean values \pm SD of four different wells with duplicate determinations. a), b), e) Significantly different from the value of the cells cultured for 3 days under serum-free conditions (a) $P < 0.05$, b) $P < 0.01$, e) $P < 0.001$). c) Significantly different from the value of the cells cultured for 3 days under serum-free conditions with 10⁻⁷ M 1,25(OH)₂D₃ ($P < 0.05$). d) Significantly different from the value of the cells cultured for 3 days under serum-supplemented conditions ($P < 0.001$). f) Significantly different from the values of the cells cultured for 3 days ($P < 0.01$) and 6 days ($P < 0.05$) under serum-free conditions with 10⁻⁷ M 1,25(OH)₂D₃.

of ALP in the cells cultured for 12 days was approximately 8 times higher than that of the cells grown for 3 days. Under serum-free conditions, the level of ALP at 12 days in culture was 2.6 times higher than that at 3 days. Treating the cells with 10⁻⁷ M 1,25(OH)₂D₃ for the last 3 days of each culture period significantly increased the amount of ALP, but had no effect on cell growth (data not shown).

Under serum-free conditions, culture media of SV-HFO cells at 3 and 6 days did not contain any detectable amount of osteocalcin. However, treating the cells under serum-free conditions with 10⁻⁷ M 1,25(OH)₂D₃ for the last 3 days of each culture period significantly increased the amount of osteocalcin in the medium (Fig. 5).

As shown in Fig. 6, mRNAs of ALP and Cx43 in the SV-HFO cells increased as cell density increased. The cells cultured for 12 days without 1,25(OH)₂D₃ (lane 5) expressed high levels of ALP and Cx43 mRNAs. In the cells at sparse and subconfluent densities (lanes 1 and 3), the levels of ALP and Cx43 mRNAs were very low. Osteocalcin mRNA was not detected in the cells cultured without 1,25(OH)₂D₃. MC3T3-E1 and HFO cells also exhibited similar changes of Cx43 mRNA expres-

sion during cell growth (data not shown). Treating the SV-HFO cells with 10⁻⁷ M 1,25(OH)₂D₃ for 3 days (lanes 2, 4 and 6) markedly increased ALP and osteocalcin mRNA expression and the effects of 1,25(OH)₂D₃ on the expression increased during cell growth, while expression of Cx43 mRNA was not influenced by 1,25(OH)₂D₃.

DISCUSSION

Ultrastructurally, osteoblasts and osteocytes in bone matrix are demonstrated to be connected with each other, forming a network via gap junctions (8, 15, 20). It has been recently reported that Cx43 is a major gap junction protein expressed in these cells (6, 22, 23). However, it remains unknown whether there is any relationship among gap junction formation, bone cell differentiation and proliferation.

We have confirmed in the present experiments that human and mouse osteoblastic cells *in vitro* express Cx43, but not Cx26 or Cx32. The expression of Cx43 was demonstrated by immunocytochemistry and Northern blot analysis. The amount of Cx43 expressed in SV-HFO cells, which proliferate actively (4), was lower than that

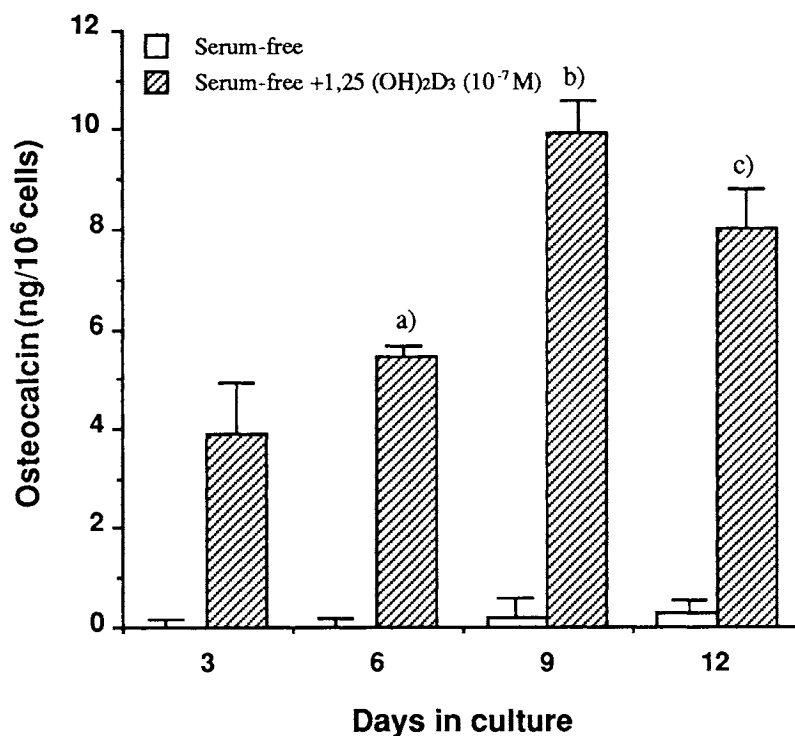


Fig. 5. Changes in the amount of osteocalcin in the SV-HFO cells cultured under serum-free conditions with or without 1,25(OH)₂D₃. The figures are mean values \pm SD of four different wells with duplicate determinations. a) Significantly different from the value of the cells cultured for 3 days under serum-free conditions with 10⁻⁷ M 1,25(OH)₂D₃ ($P < 0.05$). b) Significantly different from the values of the cells cultured for 3 days ($P < 0.001$) and 6 days ($P < 0.001$) under serum-free conditions with 10⁻⁷ M 1,25(OH)₂D₃. c) Significantly different from the values of the cells cultured for 3 days ($P < 0.001$) and 6 days ($P < 0.01$) under serum-free conditions with 10⁻⁷ M 1,25(OH)₂D₃.

expressed in HFO cells, in agreement with reports that reduction in gap junctions was more generally observed in transformed or proliferating cells than in normal

cells (14, 28). The expression of Cx43 in SV-HFO cells increased in association with the expression of ALP and osteocalcin, when the cell density reached confluency.

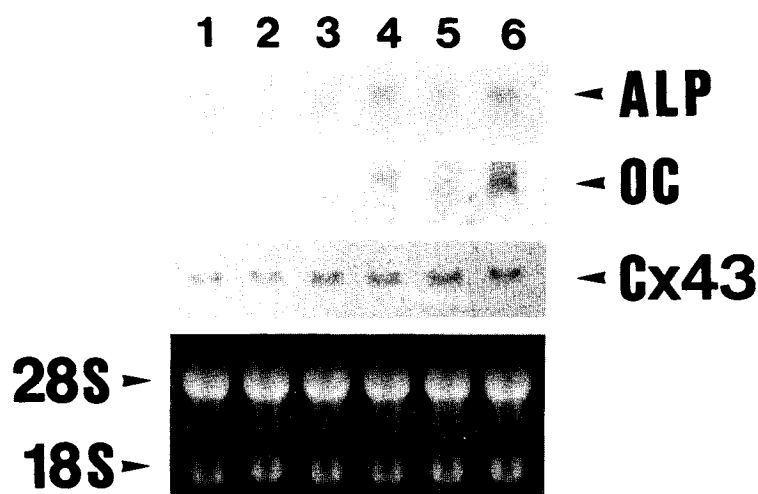


Fig. 6. Northern blot analysis of the transcripts of genes of ALP, osteocalcin and Cx43 during cell growth in the SV-HFO cells cultured without (Lanes 1, 3 and 5) or with 10⁻⁷ M 1,25(OH)₂D₃ (Lanes 2, 4 and 6). Lanes 1 and 2, 3 days; Lanes 3 and 4, 6 days; Lanes 5 and 6, 12 days, after seeding at a cell density of 5×10^3 cells/cm². The bottom panel shows the ethidium bromide stain of the filter corresponding to 28S and 18S.

Thus, gap junctions, in terms of Cx43 expression, are shown to be closely related to bone cell proliferation and differentiation.

ALP activity in the SV-HFO cells increased with time in culture. Osteocalcin, a bone-specific protein produced by mature osteoblasts (13, 29), was not detected in the SV-HFO cells at sparse or subconfluent density, but was detected in the cells after they reached confluency. Treating the cells with $1,25(\text{OH})_2\text{D}_3$, a well known modulator of bone metabolism, significantly enhanced the expression of ALP and osteocalcin. This enhancement showed a further increase as cell density increased. It is known that $1,25(\text{OH})_2\text{D}_3$ are required for the binding of the vitamin D receptor complex to the vitamin D-responsive element (VDRE) (7, 12, 17, 18). Recently, Owen *et al.* (16) and Schule *et al.* (24) reported that VDREs of ALP and osteocalcin genes contain a binding site for the Jun-Fos complex (HeLa cell activating protein 1; AP-1). Thus, the binding of the Jun-Fos complex may suppress the basal and $1,25(\text{OH})_2\text{D}_3$ -induced expression of ALP and osteocalcin genes during proliferation periods. From this viewpoint, the changes in the expression of ALP and osteocalcin in SV-HFO cells during culture observed in this study may be explainable by the presence of cross-talk between the Jun-Fos complex and VDR. In contrast, Cx43 mRNA expression was not enhanced by treatment of the cells with $1,25(\text{OH})_2\text{D}_3$, suggesting that the expression of Cx43 is regulated in a way different from that of ALP and osteocalcin. To elucidate the molecular mechanism of transcriptional regulation of Cx43, sequencing of the upstream 5' flanking region of the Cx43 gene would be important.

In summary, the present study revealed that the constitutive expression of Cx43 in human osteoblastic cells was coupled with bone cell maturation in association with the expression of ALP and osteocalcin, but the connexin expression is regulated in a way different from that of ALP and osteocalcin.

Acknowledgments. We are grateful to Dr. B.J. Nicholson (State University of New York, Buffalo, NY, USA), for Cx26 cDNA, Dr. D. Paul (Harvard Medical School, Boston, MA, USA), for Cx32 and Cx43 cDNAs, Dr. Y. Shibata (Kyushu University School of Medicine, Fukuoka, Japan), for anti-Cx26 antibody, and Dr. F. Ueda (Nippon Shinyaku Co., Kyoto, Japan), for anti-Cx43 antibody. We also wish to thank Drs. Y. Oyamada and H. Isomura for their valuable advice. This work was supported by Grants-in-Aid for Scientific Research and Cancer Research from the Ministry of Education, Science and Culture, and the Ministry of Health and Welfare, Japan, and from the Hokkaido Geriatrics Research Institute.

REFERENCES

1. BEYER, E.C., PAUL, D.L., and GOODENOUGH, D.A. 1987. Connexin 43: A protein from rat heart homologous to a gap junction protein from liver. *J. Cell Biol.*, **105**: 2621–2629.
2. BEYER, E.C., PAUL, D.L., and GOODENOUGH, D.A. 1990. Connexin family of gap junction proteins. *J. Membr. Biol.*, **116**: 187–194.
3. CELESTE, A.J., ROSEN, V., BUECKER, J.L., KRIZ, R., WANG, E.A., and WOZNEY, J.M. 1986. Isolation of the human gene for bone gla protein utilizing mouse and rat cDNA clones. *The EMBO J.*, **5**: 1885–1890.
4. CHIBA, H., SAWADA, N., ONO, T., ISHII, S., and MORI, M. 1993. Establishment and characterization of an SV40-immortalized osteoblastic cell line from normal human bone. *Jpn. J. Cancer Res.*, **84**: 290–297.
5. CHOMCZYNSKI, P. and SACCHI, N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.*, **162**: 156–159.
6. CIVITELLI, R., BEYER, E.C., WARLOW, P.M., ROBERTSON, A., GEIAT, S.T., and STEINBERG, T.H. 1993. Connexin 43 mediates direct intercellular communication in human osteoblastic cell networks. *J. Clin. Invest.*, **91**: 1888–1896.
7. DEMAY, M.B., GERARDI, J.M., DELUCA, H.F., and KRONENBERG, H.M. 1990. DNA sequences in the rat osteocalcin gene that bind the $1,25$ -dihydroxyvitamin D_3 receptor and confer responsiveness to $1,25$ -dihydroxyvitamin D_3 . *Proc. Natl. Acad. Sci. USA*, **87**: 369–373.
8. DOTY, S.B. 1981. Morphological evidence of gap junctions between bone cells. *Calcif. Tissue Int.*, **33**: 509–512.
9. HERTZBERG, E. and JOHNSON, R. 1988. *Gap Junctions. Modern Cell Biology Vol. 7*. Alan R. Liss, Inc., New York.
10. HÖLTKE, H.J., SANGER, G., KESSLER, C., and SCHMITZ, G. 1992. Sensitive chemiluminescent detection of digoxigenin-labeled nucleic acids: a fast and simple protocol and its applications. *BioTechniques*, **12**: 104–113.
11. JEANSONNE, B.G., FEAGIN, F.F., MCMINN, R.W., SHOEMAKER, R.L., and REHM, W.S. 1979. Cell-to-cell communication of osteoblasts. *J. Dent. Res.*, **58**: 1415–1423.
12. KERNER, S.A., SCOTT, R.A., and PIKE, J.W. 1989. Sequence elements in human osteocalcin gene confer basal activation and inducible response to hormonal vitamin D_3 . *Proc. Natl. Acad. Sci. USA*, **86**: 4455–4459.
13. LIAN, J., STEWART, C., PUCHACZ, E., MACKOWIAK, S., SHALHOUB, V., COLLART, D., ZAMBETTI, G., and STEIN, G. 1989. Structure of the rat osteocalcin gene and regulation of vitamin D-dependent expression. *Proc. Natl. Acad. Sci. USA*, **86**: 1143–1147.
14. LOEWENSTEIN, W.R. and KANNO, Y. 1966. Intercellular communication and the control of tissue growth. Lack of communication between cancer cells. *Nature*, **209**: 1248–1249.
15. MILLER, S.C., BOWMAN, B.M., SMITH, J.M., and JEE, W.S.S. 1980. Characterization of endosteal bone-lining cells from fatty marrow bone sites in adult beagles. *Anat. Rec.*, **198**: 163–173.
16. OWEN, T.A., BORTELL, R., YOCUM, S.A., SMOCK, S.L., ZHANG, M., ABATE, C., SHALHOUB, V., ARONIN, N., WRIGHT, K.L., VAN WIJNEN, A.J., STEIN, J.L., CURRAN, T., LIAN, J.B., and STEIN, G.S. 1990. Coordinate occupancy of AP-1 sites in the vitamin D-responsive and CCAAT box elements by Fos-Jun in the osteocalcin gene: Model for phenotype suppression of transcription. *Proc. Natl. Acad. Sci. USA*, **87**: 9990–9994.
17. OZONO, K., LIAO, J., KERNER, S.A., SCOTT, R.A., and PIKE, W. 1990. The vitamin D-responsive element in the human osteocalcin gene. *J. Biol. Chem.*, **265**: 21881–21888.
18. OZONO, K., SONE, T., and PIKE, W. 1991. The genomic mechanism of action of $1,25$ -dihydroxyvitamin D_3 . *J. Bone. Miner. Res.*, **6**: 1021–1027.

19. PAUL, D.L. 1986. Molecular cloning of cDNA for rat liver gap junction protein. *J. Cell Biol.*, **103**: 123–134.
20. PLAUMBO, C., PALAZZINI, S., and MAROTTI, G. 1990. Morphological study of intercellular junctions during osteocyte differentiation. *Bone*, **11**: 401–406.
21. SAKAMOTO, H., OYAMADA, M., ENOMOTO, K., and MORI, M. 1992. Differential changes in expression of gap junction proteins connexin 26 and 32 during hepatocarcinogenesis in rats. *Jap. J. Cancer Res.*, **83**: 1210–1215.
22. SCHILLER, P.C., MEHTA, P.P., ROSS, B.A., and HOWARD, G.A. 1992. Hormonal regulation of intercellular communication: parathyroid hormone increases connexin 43 gene expression and gap-junctional communication in osteoblastic cells. *Mol. Endocrinol.*, **6**: 1433–1440.
23. SCHIRRMACHER, K., SCHMITZ, I., WINTERHAGER, E., TRAUB, O., BRUMMER, F., JONES, D., and BINGMANN, D. 1992. Characterization of gap junctions between osteoblast-like cells in culture. *Calcif. Tissue Int.*, **51**: 285–290.
24. SCHÜLE, R., UMESONO, K., MANGELSDORF, D.J., BOLADO, J., PIKE, J.W., and EVANS, R.M. 1990. Jun-Fos and receptors for vitamin A and D recognize a common response element in the human osteocalcin gene. *Cell*, **61**: 497–504.
25. SUDO, H., KODAMA, H., AMAGAI, Y., YAMAMOTO, S., and KASAI, S. 1983. In vitro differentiation and calcification in a new clonal osteogenic cell line derived from newborn mouse calvaria. *J. Cell Biol.*, **96**: 191–198.
26. WILLECKE, K., HENNEMANN, H., DAHL, E., JUNGBLUTH, S., and HEYNES, R. 1991. The diversity of connexin genes encoding gap junctional proteins. *Eur. J. Cell Biol.*, **56**: 1–7.
27. XIE, W. and ROTHBLUM, L.I. 1991. Rapid, small-scale RNA isolation from tissue culture cells. *BioTechniques*, **11**: 325–327.
28. YAMASAKI, H., ENOMOTO, T., SHIBA, Y., KANNO, Y., and KAKUNAGA, T. 1985. Intercellular communication capacity as a possible determinant of transformation sensitivity of BALB/c 3T3 clonal cells. *Cancer Res.*, **45**: 637–641.
29. YOON, K., RUTLEDGE, S.J.C., BUENAGA, R.F., and RODAN, G.A. 1988. Characterization of the rat osteocalcin gene: stimulation of promoter activity by 1,25-dihydroxyvitamin D₃. *Biochemistry*, **27**: 8521–8526.
30. ZHANG, J.T. and NICHOLSON, B.J. 1989. Sequence and tissue distribution of a second protein of hepatic gap junctions, Cx26, as deduced from its cDNA. *J. Cell Biol.*, **109**: 3391–3401.

(Received for publication, October 15, 1993
and in revised form November 29, 1993)