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THE NON-OSTEOGENIC MOUSE PLURIPOTENT CELL LINE, C3H10T1/2, IS INDUCED TO DIFFERENTIATE INTO OSTEOBLASTIC CELLS BY RECOMBINANT HUMAN BONE MORPHOGENETIC PROTEIN-2

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SUMMARY: The possibility that the non-osteogenic mouse pluripotent cell line, C3H10T1/2 (10T1/2), could be induced to differentiate into osteogenic cells by various hormones and cytokines was examined *in vitro*. Of a number of agents tested, recombinant human bone morphogenetic protein-2 (rhBMP-2) and retinoic acid induced alkaline phosphatase (ALP) activity in 10T1/2 cells. rhBMP-2 also induced mRNA expression of ALP in the cells. Dexamethasone, 1α,25-dihydroxyvitamin D_3, transforming growth factor-β1 and insulin-like growth factor-I did not stimulate ALP activity. Treatment with rhBMP-2 greatly induced cAMP production in response to parathyroid hormone in 10T1/2 cells. No ALP activity was induced in NIH3T3 fibroblasts treated with rhBMP-2 or retinoic acid. These results indicate that 10T1/2 cells have a potential to differentiate into osteogenic cells under the control of BMP-2.

It is believed that osteoblasts are developed from undifferentiated mesenchymal progenitor cells, which can also differentiate into other specialized connective tissue cells such as chondrocytes, muscle cells and adipocytes (1,2). Several osteoblast-like cells have been used to study the mechanism of osteoblast differentiation in *in vitro* systems. But, such osteoblast-like cells are inappropriate to explore the events occurring in the early stage of osteoblast differentiation from undifferentiated mesenchymal progenitor cells. To understand precisely the mechanism of osteoblast differentiation, an appropriate *in vitro* system is required, which reflects the early stage of differentiation from uncommitted mesenchymal cells into osteoblasts.

The mouse fibroblastic cell line, C3H10T1/2 clone 8 (10T1/2) established from an early mouse embryo (3), has the pluripotent activity to differentiate into

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myotubes, adipocytes and chondrocytes when treated with 5-azacytidine (4). This cell line has been used to study the mechanism of the differentiation from undifferentiated mesenchymal cells into myotubes and adipocytes (5,6). However, the possibility that 10T1/2 cells could be induced to differentiate into osteoblasts has not been reported.

Differentiation of osteoblasts is regulated by various hormones and cytokines. Recently, we found that recombinant human bone morphogenetic protein-2 (rhBMP-2), a new member of the transforming growth factor-β (TGF-β) superfamily (7), has the bioactivity not only of inducing ectopic bone formation *in vivo* (8) but also of stimulating osteoblast differentiation of immature osteoblast-like cells *in vitro* (submitted for publication). It is also reported that retinoic acid stimulates osteoblast differentiation in immature osteoblast-like cells (9,10). This prompted us to examine the effects of various hormones and cytokines to induce osteoblastic properties in non-osteogenic 10T1/2 cells. We report here that treatment with rhBMP-2 induces 10T1/2 cells to differentiate into osteoblastic cells.

MATERIALS AND METHODS

Hormones and Cytokines: Retinoic acid and dexamethasone were purchased from Sigma (St. Louis, MO); 1α,25-dihydroxyvitamin D_3 [1α,25(OH)$_2$D3] from Philips-Duphar (Amsterdam, The Netherlands); human platelet-derived transforming growth factor-β1 (TGF-β1) from R & D Systems, Inc. (Minneapolis, MN); recombinant human insulin-like growth factor-I (IGF-I) from KabiGen (Stockholm, Sweden). Bioactive rhBMP-2 was produced by Chinese hamster ovary cells and purified as described previously (8).

Cell Culture: The mouse clonal cell line, C3H10T1/2 clone 8 (10T1/2) obtained from the RIKEN Cell Bank (Tsukuba Science City, Japan) was maintained in Eagle's basal medium (GIBCO, Grand Island, NY) containing 10% heat-inactivated fetal bovine serum (FBS) (GIBCO). NIH3T3 fibroblasts were maintained in Dulbecco's modified minimum essential medium supplemented with 10% FBS. All cells were inoculated in 24- or 48-well tissue culture plates at 1.25×10^4 cells per cm^2. Sixteen hr after the inoculation, the culture media were changed to fresh media containing various hormones and cytokines.

Alkaline Phosphatase (ALP) Activity: The ALP activity of the cell lysates was assayed using *p*-nitrophenyl phosphate as a substrate (11). The protein concentration was determined by the BCA protein assay method (Pierce, Rockford, IL).

Northern Blot Analysis: Total RNA was isolated by the method using guanidine thiocyanate-cesium chloride (12). Ten micrograms of the RNA were separated in a 1.5% agarose-formaldehyde gel, then transferred onto a Hybond-N membrane (Amersham, UK). The membrane was hybridized with a [^{32}P]-labeled cDNA probe of rat ALP (13) (a gift from Dr. M. Noda, Merck Sharp and Dohme Res. Labs., NJ) in Rapid Hybridization Buffer (Amersham).

cAMP Response to Parathyroid Hormone (PTH): After cells were preincubated for 20 min in culture media containing 0.5% bovine serum albumin and 1 mM 3-isobutyl-1-methylxanthine, they were incubated for 8 min with 200 ng/ml of human PTH(1-34) (provided by Dr. M. Hori, Toyo Jozo Co., Shizuoka, Japan) dissolved in the same media. The content of cAMP in the cell layer was determined with a radioimmunoassay kit (Yamasa Co., Chiba, Japan).

RESULTS

Figure 1 shows the effect of various hormones and cytokines in inducing ALP activity in 10T1/2 cells. Untreated 10T1/2 cells showed a very low level of ALP activity. Of the hormones and cytokines tested, 1 μg/ml of rhBMP-2 and 10^{-6} M retinoic acid significantly induced ALP activity in 10T1/2 cells. The other hormones and cytokines had no effect.

Treating 10T1/2 cells with rhBMP-2 at concentrations higher than 100 ng/ml increased ALP activity dose-dependently (Fig. 2). Neither rhBMP-2 nor retinoic acid induced ALP activity in NIH3T3 cells at any concentrations tested (control; 2.6 ± 0.1, 1 μg/ml of rhBMP-2; 2.4 ± 0.2, 10^{-6} M retinoic acid; 2.1 ± 0.1 nmol/min/mg of protein).

Figure 3 shows northern blot analysis of the mRNA expression in 10T1/2 cells. The untreated 10T1/2 cells expressed no detectable mRNA of ALP, but the treatment with rhBMP-2 at 1 μg/ml induced the steady state level of the mRNA expression of ALP.

Untreated 10T1/2 cells produced a small amount of cAMP in response to PTH, but the treatment with rhBMP-2 dose-dependently increased the PTH-dependent cAMP production (Table 1). The maximal increase observed was 12.8-fold at 1 μg/ml of rhBMP-2. No alcian blue- and toluidine blue-positive chondrocytes, multinucleated myotubes, and oil red O-positive adipocytes appeared after the treatment with rhBMP-2 or retinoic acid at any concentrations tested in 10T1/2 cells (data not shown).

DISCUSSION

This is the first demonstration that the non-osteogenic pluripotent mouse embryonic cell line, 10T1/2, has a potential to differentiate into osteoblastic cells. It is known that both ALP activity and PTH response appear in osteoblast and chondroblast differentiation. In the cultures of 10T1/2 cells, no alcian blue- and

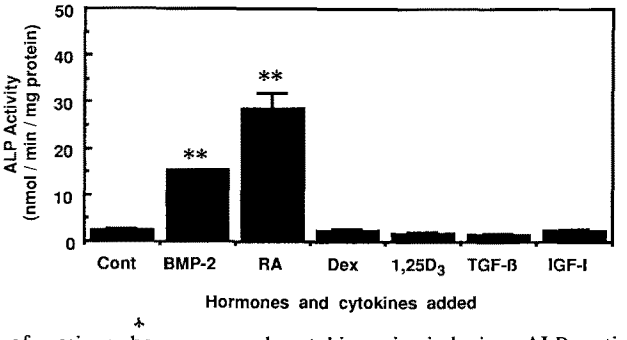

Fig. 1. Effect of various hormones and cytokines in inducing ALP activity in 10T1/2 cells. Cells were cultured for 3 days with 1 μg/ml of rhBMP-2, 10^{-6} M retinoic acid (RA), 10^{-7} M dexamethasone (Dex), 2 x 10^{-8} M $1\alpha,25(OH)_2D_3$ ($1,25D_3$), 1 ng/ml of TGF-β1 or 40 ng/ml of IGF-I. ALP activity was measured as described in MATERIALS AND METHODS. Data are means ± SEM of three wells. Significantly different from the control culture: ** $p < 0.001$.

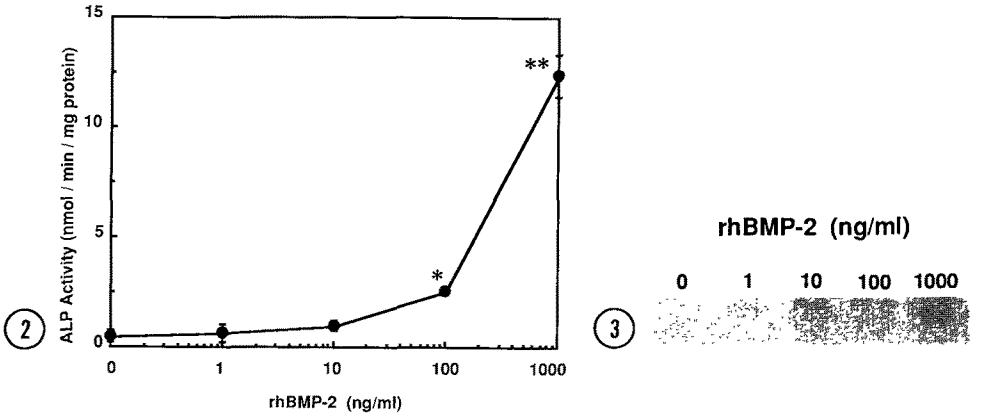

Fig. 2. Dose-dependent rhBMP-2 stimulation of ALP activity in 10T1/2 cells. Cells were treated for 3 days with graded concentrations of rhBMP-2, then the ALP activity was determined as described in MATERIALS AND METHODS. Data are means ± SEM of three wells. Significantly different from the culture without rhBMP-2: * $p < 0.01$, ** $p < 0.001$.

Fig. 3. Northern blot analysis of the mRNA expression of ALP in 10T1/2 cells. Ten micrograms of the total RNA isolated from 10T1/2 cells cultured for 3 days with various concentrations of rhBMP-2 were applied. Northern blot analysis was performed as described in MATERIALS AND METHODS.

toluidine blue-positive cells appeared after the treatment with rhBMP-2. Apparently 10T1/2 cells do not differentiate into chondrocytes when treated with rhBMP-2.

All the hormones and cytokines tested in the present study have been reported to affect the ALP activity and/or PTH response in some osteoblast-like cells (9,10,14-16). Recombinant hBMP-2 stimulated these osteoblastic properties in rat clonal immature osteoblast-like cells, ROB-C26 (17), and the well characterized osteoblast-like MC3T3-E1 cells (unpublished data). Considering all of the effects of rhBMP-2 in these cells and in 10T1/2 cells, it appears that rhBMP-2 induces osteogenic differentiation of not only committed osteoblasts but also non-osteogenic mesenchymal progenitor cells. Retinoic acid is reported to induce osteogenic

Table 1. Effect of rhBMP-2 on cAMP production in response to PTH in 10T1/2 cells

rhBMP-2 added (ng/ml)	Amounts of cAMP produced (pmol/well)		
	PTH (-)	PTH (+)	PTH (+) / PTH(-) ratio
0	3.6 ± 0.3	4.5 ± 0.5	1.3
1	3.7 ± 0.1	4.0 ± 0.5	1.1
10	3.8 ± 0.2	4.7 ± 0.3	1.2
100	3.5 ± 0.3	21.6 ± 1.4**	6.1
1000	3.5 ± 0.2	44.4 ± 3.8*	12.8

Cells were cultured for 3 days with graded concentrations of rhBMP-2. The amount of cAMP was determined as described in MATERIALS AND METHODS. Data are means ± SEM of three wells. Significantly different from each control level (without PTH): *$p<0.01$, **$p<0.001$.

differentiation in immature osteoblast-like cells (9,10). In the present study, we demonstrated that retinoic acid has a strong potency to induce ALP activity in 10T1/2 cells. But the treatment of 10T1/2 cells with 10^{-6} M retinoic acid did not appreciably stimulate cAMP production in response to PTH (PTH (+)/PTH (-) ratio, 1.0 for 3 days and 2.3 for 7 days). Apparently retinoic acid alone is not enough to induce differentiation of 10T1/2 cells into osteoblastic cells. Additional factor(s) may be required. The other hormones and cytokines did not stimulate osteogenic differentiation in 10T1/2 cells. These agents may modulate osteoblast differentiation only in committed osteoblasts.

BMP-2 is highly homologous to decapentaplegic (dpp) and Vg1, which are involved in the developmental process of lower animals (7). Retinoic acid also acts as a natural morphogen during the process of pattern formation in developing chick limb buds (18). This may indicate that BMP-2 and retinoic acid are involved in the commitment of undifferentiated mesenchymal cells into osteogenic cells.

In conclusion, 10T1/2 cells have the potential to differentiate into osteogenic cells under the control of BMP-2. This cell line may provide a suitable *in vitro* model for studying the mechanism of osteoblast differentiation of undifferentiated mesenchymal progenitor cells.

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