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PTH AND 1,25(OH)$_2$VITAMIN D PRIMING TO GROWTH FACTORS DIFFERENTIALLY REGULATES THE OSTEOBLASTIC MARKERS IN MBA-15 CLONAL SUBPOPULATIONS

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SUMMARY: The functional modulation of enzymatic activities of alkaline phosphatase (ALK-P) and neutral endopeptidase (CD10/NEP) in MBA-15.4 and MBA-15.6 marrow stromal osteoblastic cells was studied. The hormonal effects of parathyroid hormone (PTH) and 1,25 (OH)$_2$D$_3$ combined with various growth factors (bone morphogenic protein [BMP-2 and BMP-3], TGFß and IGF-I) on these cells were monitored. The cell responses of MBA-15.4, a preosteoblastic cell, and MBA-15.6, a more mature osteoblastic cell, to the growth factors and the hormonal challenge were measured by changes of the enzymatic activities (ALK-P and CD10/NEP). The cellular response was not uniform and revealed a differential pattern.

The interactions of local and systemic factors are indisputedly important in physiological processes supporting bone remodeling. The end-product of remodeling is the maintenance of a mineralized bone matrix. Circulating hormones act on skeletal cells either directly or indirectly; their action modulates the synthesis, activation, and receptor binding of local growth factor. These changes may, in turn, stimulate or inhibit bone formation or resorption. There is evidence for direct effects of systemic hormones on the osteoblast. The modification of the hormones in response to local growth factor action may change the replication or the differentiated function of the osteoblast. Thus, the hormonal role may be to target a given growth factor to a specific cell (1,2). Skeletal effects of hormones such as PTH and 1,25-(OH)$_2$D$_3$ are known to be mediated by local growth factors (3-5). Their action has the potential of modifying bone mass either by changing the number of bone-producing cells or by acting directly on the osteoblast (1,6). Discovering the possible role of the hormones that provide tissue specificity for a given growth factor was the rationale of our experimental design in this study.

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Although the stromal compartment serves as a source for osteogenic cells (7-9), little is known regarding their growth and differentiation. The marrow stromal compartment contains osteogenic functioning cells that are closely associated with bone formation at the endosteal surface of bone. The marrow-derived osteogenic cell line, MBA-15, expresses osteoblastic-associated features in vitro and forms bone in vivo (7). Clonal lines derived from the parental MBA-15 line were presumably "arrested" at a particular stage along the osteogenic lineage (10).

The MBA-15 clonal lines differ in their morphology, proliferation rate, synthesis of extracellular matrix proteins and levels of enzyme activities (10,11). In addition, these cell lines vary in their activation of adenylate cyclase as a result of exposure to bone-seeking hormones. MBA-15.4, a fibroblast-like cell, proliferates rapidly, synthesizes equal amounts of collagen and noncollagenous proteins and responds mainly to PGE_2. MBA-15.6 has a large polygonal morphology, a limited proliferation ability, synthesizes twice the amount of noncollagenous proteins as collagen and responds strongly to PTH. These differential properties were directly reflected by the clonal cells' ability to form bone in vivo. When transplanted under a renal capsule, MBA-15.4 formed small foci of bone, while MBA-15.6 formed massive woven bone during the same period of time (10,11). We also investigated the expression of neutral endopeptidase (NEP), which is identical with that of the CALLA antigen (CD10), by the stromal osteoblasts. We had previously shown that CD10/NEP is present on marrow stromal cell subpopulations and that it is highly expressed on osteoblastic cells (12,13). CD10/NEP is an ectoenzyme present on the surface of many cell types, is widely distributed in mammalian tissue and is highly conserved in different species (14,15). This enzyme has been associated with the metabolism and regulation of a variety of peptides (16). Thus, the MBA clonal cells serve as a useful model for studying the processes involved in the differentiation of bone-forming cells.

We now focused on the effects of parathyroid hormone (PTH) and 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] on the modulation of differentiation in MBA-15.4 and MBA-15.6 cell lines. Furthermore, we examined the effects of these hormones, with or without a combination of a series of growth factors, on ALK-P and CD10/NEP activities. The response to various growth factors subsequent to priming the cultures with PTH or $1,25\text{-}(OH)_2D_3$ was also investigated.

MATERIALS AND METHODS

Cell culture: The clonal cell line, MBA-15.4, and the MBA-15.6 subpopulations derived from a MBA-15 cell line were previously described in detail elsewhere (10). Stock cultures were maintained in growth medium DMEM containing high glucose and supplemented with 10% FCS (Beth Haemek, Israel). Cultures were passaged once weekly and medium was changed every 3 days. All cultures were incubated at 37°C in a humidified atmosphere of 10% CO_2 in air.

Experimental design: For the final experiments, cells were trypsinized and seeded at $1x10^4$ cells/ml in 24-well plates. After 24 hrs, the medium was removed and the

cells were supplemented with DMEM containing 2% charcoaled FCS (chFCS) with or without hormones. PTH was used at a concentration of 10^{-8}M and 1,25-$(OH)_2D_3$ at a concentration of 10^{-10}M. In priming experiments, cells were first exposed to the hormones for 24 hrs after which the medium was changed and the cells were or were not supplemented with the growth factors for 48 hrs as described in the following experimental design.

Treatment	Hours in culture			
	24	48	72	96
I control	10% chFCS	2% chFCS	2% chFCS	2% chFCS
II GF	10% chFCS	2% chFCS	GF	GF
III hormone	10% chFCS	hormone	2% chFCS	2% chFCS
IV hormone+GF	10% chFCS	hormone	GF	GF

Hormones and growth factors: rh-Bone morphogenic protein 2 (BMP-2) (100ng/ml) was a generous gift from the Genetic Institute, Cambridge, MA, USA. Purified osteogenin (BMP-3) (10ng/ml) (17) was kindly donated by Dr. H. Reddi, John Hopkins University, Baltimore, USA. Transforming growth factor ß (TGFß) (1ng/ml) and insulin-like growth factor (IGF-I) (10ng/ml) were purchased from Collaborative Research (Bedford, MA, USA). All growth factors were reconstituted in sterile conditions as recommended by the manufacturers; the stock was divided into small aliquots and stored at -70°C.

Enzymatic determination: Cultured cells were used for enzyme activities and protein content. ALK-P activity was determined with 2mM 4-nitrophenyl phosphate at 37°C in 0.1 M 2-amino-2-methyl-1-propanol and 2mM $MgCl_2$ (18). CD10/NEP activity was assayed by a two-stage assay at 37°C using the synthetic substrate, Suc-Ala-Ala-Leu-NH-Np, supplemented with streptomyces griseus aminopeptidase I (SGAPI) (19). Protein was measured using the Lowry and colleagues' method (20) with human serum albumin as a standard. Enzyme and protein determinations were performed in microwell plates and read by a kinetic microplate reader (Molecular Devices Corp., Palo Alto, CA, USA).

Statistical analysis: The significance of differences between experimental and control groups was analyzed by the Duncan's multiple range test.

RESULTS

Enzyme activities in response to growth factors and hormonal stimulation: MBA-15.4, a preosteoblastic cell and MBA-15.6, a more mature osteoblastic cell, were exposed to the growth factors. The responses of these cells that were challenged with BMP-2, BMP-3, TGFß and IGF-I, were not uniform and revealed a differential pattern, as measured by enzymatic ALK-P and CD10/NEP activities.

ALK-P activity in both cell lines was measured following the addition of the growth factors (Fig. 1). ALK-P levels were stimulated in response to BMP-2 and BMP-3. BMP-2 increased ALK-P levels (up to 10-fold over control) in MBA-15.4, and to a lesser extent (up to 5-fold) in MBA-15.6 cells. BMP-3 stimulated ALK-P activity (up to 2-fold) in both cells type, whereas TGFß inhibited the enzyme activity in MBA-15.6 cells, but not in MBA-15.4 cells. IGF-I had no effects on ALK-P activity in the MBA-15.6 cells but was stimulatory in the preosteoblast MBA-15.4 cells (Fig. 1). CD10/NEP activity exhibited a different pattern when these cells were introduced to the growth factors (Fig. 2). In both cell types, MBA-15.6 and MBA-15.4, the CD10/NEP activity was inhibited by BMP-2, BMP-3 and TGFß, whereas IGF-I was a potent stimulator for the MBA-15.4 cells (2 to 3-folds) but did not change the enzyme levels in MBA-15.6 cells (Fig. 2).

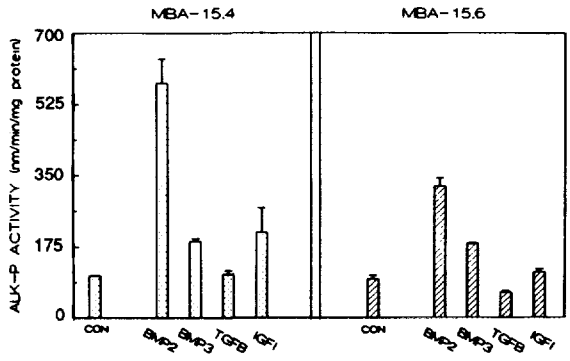

Fig. 1. MBA-15.4, a preosteoblast cell, and MBA-15.6, a mature cell of marrow stromal osteoblast, were exposed to various growth factors. Cells were challenged for 48 hrs as described in the experimental design, and ALK-P activity was determined in treated cultures and compared to untreated controls. Assays were performed in triplicate on four to six separate cultures. The results are mean ± SEM ($p<0.05$; Duncan's multiple range test).

Exposure of these cells both to PTH or $1,25(OH)_2D_3$ (Figs. 3, 4) was studied. Both hormones stimulated ALK-P activity over baseline levels in the MBA-15.4 cells' PTH (3-fold) (Fig. 3A) and $1,25(OH)_2D_3$ (2-fold) (Fig. 3C). Neither PTH (Fig. 3B) nor $1,25(OH)_2D_3$ (Fig. 3D) affected ALK-P activity in MBA-15.6. The effect of these hormones on CD10/NEP activity revealed a stimulation of the enzyme activity only following $1,25\text{-}(OH)_2D_3$ exposure in MBA-15.6 (Fig. 4D). These results suggest a differential expression of the modulation pattern of ALK-P and CD10/NEP; the response to hormonal and growth factors stimulation was reflected by the differential stage of maturation of the cells.

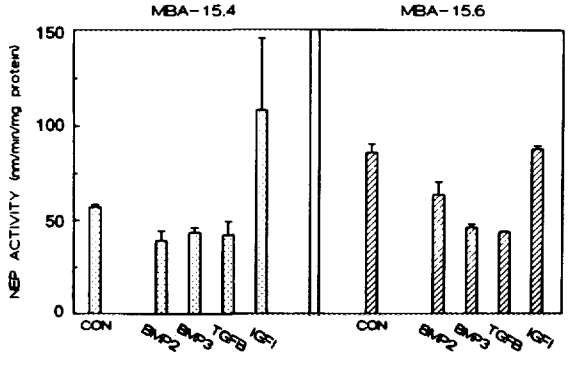

Fig. 2. MBA-15.4 and MBA-15.6 cells were exposed to various growth factors as detailed in Fig. 1. CD10/NEP activity was determined in treated cultures and compared to an untreated control. Assays were performed in triplicate on four to six separate cultures. The results are mean ± SEM ($p<0.05$; Duncan's multiple range test).

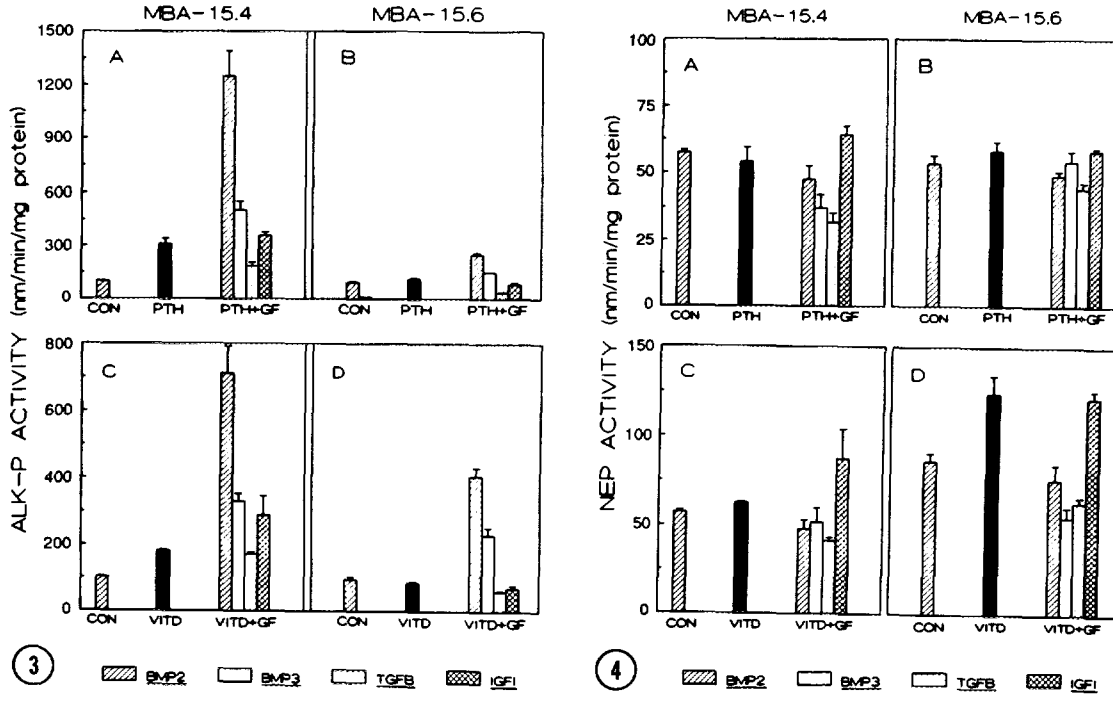

Fig. 3. ALK-P activity in clonal subpopulations, MBA-15.4 and MBA-15.6, exposed to the hormones PTH (10^{-8}M) (A,B) or $1,25(OH)_2D_3$ (10^{-10}M) (C,D). Following 24 hrs of exposure, the cells were challenged with or without various growth factors for an additional 48 hrs as described in the experimental design. CON - basal levels, PTH (A,B) and VITD (C,D) treatment. Growth factors were challenged for an additional 48 hrs. Assays were performed in triplicate on four to six separate cultures. The results are mean ± SEM ($p<0.05$; Duncan's multiple range test).

Fig. 4. CD10/NEP activity in clonal subpopulations, MBA-15.4 and MBA-15.6, exposed to hormones PTH (A,B) or $1,25(OH)_2D_3$ (C,D) described (in Materials and Methods and legend for Fig. 3). Assays were performed in triplicate on four to six separate cultures. The results are mean ± SEM ($p<0.05$; Duncan's multiple range test).

Effect of growth factors on cells primed by systemic hormones: ALK-P activity in cells exposed to $1,25(OH)_2D_3$ or PTH was increased in MBA-15.4 or unchanged when monitored in MBA-15.6 cells (Fig. 3). We then examined the effects of growth factors challenge on the clonal (MBA-15.4 and MBA-15.6) cells following a 24-hr treatment with either PTH or $1,25(OH)_2D_3$ (Fig. 3). ALK-P activity was monitored with an additive increase over hormonal modulation in MBA-15.4 cells followed by BMP-2 (4-fold) and BMP-3 (1.8 fold) (Fig. 3A, C). IGF-I did not change PTH stimulation (Fig. 3A) but increased the $1,25(OH)_2D_3$ elevation of ALK-P (Fig. 3C). TGFß reversed the PTH elevation (Fig. 3A) and did not alter the $1,25(OH)_2D_3$ stimulation (Fig. 3C). Pretreatment of MBA-15.6 with PTH did not alter the ALK-P activity in the presence of growth factors from the response obtained when cells were challenged with the growth

factors alone (Figs. 1, 3B). Priming of MBA-15.6 cells with $1,25(OH)_2D_3$ had a synergetic effect with BMP-2 and BMP-3 (Fig. 3D). No changes in response to TGFß and IGF-I were elicited by MBA-15.6 cells when primed with $1,25(OH)_2D_3$ (Fig. 3D).

CD10/NEP activity was measured under the same culture conditions (Fig. 4). The basic inhibition effect of the growth factors BMP-2, BMP-3 and TGFß was noted when MBA-15.4 cells were also primed with PTH (Fig. 4A) or with $1,25\text{-}(OH)_2D_3$ (Fig. 4C). MBA-15.4 cells exposed to IGF-I following priming with both hormones did not alter the IGF-I's effect (Fig. 4A,C). Pretreatment of MBA-15.6 cells with PTH followed by growth factors exposure did not alter the basic growth factors effects (Fig. 4B). Although $1,25(OH)_2D_3$ stimulated CD10/NEP activity in MBA-15.6 (Fig. 4D), this activity was reduced when growth factors of the TGF superfamily were added, but was not altered in the presence of IGF-I. The basic CD10/NEP activity in the presence of growth factors alone was not altered by both hormones being introduced to cultures as primers to the growth factors challenged.

DISCUSSION

Most of the local bone growth factors have been characterized by studying differentiation of bone-related activities. The role assignment of the various factors at a particular point in the bone formation scheme is not a strict one, and certain factors may influence different cells. It is important to note that, in addition to these physiological dynamics, the systemic hormones are playing a concomitant role and may alter the cellular response to the growth factors. The effects of growth factors on marrow stromal osteoblasts was examined on MBA-15 cells in previous studies (9,13,21): ALK-P activity was shown to be increased following exposure to BMP-2 and BMP-3, while a decrease in enzyme activity was observed following TGFß treatment (9,21). Neither IGF-I, PDGF (9) nor bFGF (21) produced any changes in ALK-P activity and cell growth. In order to clarify the biological activities of local and systemic factors on the osteoblastic cell at different stages of differentiation, we used the clonal cell lines, MBA-15.4 and MBA-15.6. Priming of MBA-15.6 and MBA-15.4 cells with retinoic acid (RA) (13) and then exposing them to growth factors resulted in a synergistic effect on ALK-P activity with BMP-2 and BMP-3, but no changes were observed when IGF-I or bFGF were added. TGFß antagonized the RA increase of ALK-P activity. In contrast, CD10/NEP activity was changed under the same conditions, with an inhibitory effect being seen when exposed to BMP-2, BMP-3 and TGFß, but with no changes being observed under IGF-I and bFGF. In this study, we demonstrated synergistic effects of PTH or $1,25(OH)_2D_3$ with BMP-2 and BMP-3 and an antagonistic effect with TGFß in both the stromal preosteoblast, MBA-15.4 and the more mature cells, MBA-15.6. Pretreatment with both hormones did not alter the inhibitory effects of TGFß as was observed when cells were primed with RA. Cell response to the IGF-I effect was unchanged following either $1,25\text{-}(OH)_2D_3$ or PTH

priming. These defined clonal osteoblastic cells strengthen the evidence that growth factors differentially affect cellular activities. As depicted by our studies, combined exposure to systemic hormones (PTH, 1,25-$(OH)_2D_3$ and RA) and BMPs on osteoblast cells enhanced their ALK-P activity and potential in bone formation. Using combined exposure of the osteoblastic clonal cells to hormones and growth factors may widen the understanding of the series of events leading to net bone formation.

The effects of TGFß depend on cell type, cell density, and culture conditions (22-24). Thies et al (25) have shown that BMP-2 increased ALK-P activity and osteocalcin production in bone marrow stromal cells, raising the possibility that BMP-2 may be involved in the differentiation of osteoblasts from progenitor cells. Osteogenin, which has been shown to be identical to BMP-3, increased ALK-P activity and collagen synthesis in rat bone cells in vitro (5,17). The BMPs and TGFß were also studied on endochondral bone formation in vivo. Furthermore, PTH was shown to have an anabolic effect on bone formation which appears to be mediated, at least in part, by an increased synthesis of IGF-I. The stimulation of IGF-I synthesis is due to an increase in cAMP, while other inducers of cAMP mimic the effect of PTH on IGF-I production (2,26). It is believed that the synthesis and binding of IGF-I to its bone cell is regulated by various hormones that affect the bone cell function. $1,25(OH)_2D_3$ was reported as being necessary for normal growth and bone mineralization. It was also shown to increase the binding of IGF-I to its receptor in cells of the osteoblastic lineage, and to stimulate the synthesis of selected IGF-binding proteins that may modify IGF actions and concentrations (2,27).

The CD10 was identified by MoAbs as a 100-Kd cell surface antigen. In the marrow stromal cellular subpopulations CD10 also had NEP activity that was highly correlated to the osteoblastic cells (12,13,28-30). MBA-15 and clonal subpopulations were demonstrated to have CD10/NEP activity, and a diverse pattern upon modulation by RA was observed as well (13). This enzyme activity in clones MBA-15.33, MBA-15.31 and MBA-15.4 which was either inhibited or unchanged, was elevated in MBA-15.6 cells. A difference in ALK-P and CD10/NEP activity in response to 1,25-$(OH)_2D_3$ treatment had been previously observed in clonal MBA-15.30 and MBA-15.33 cells: their CD10/NEP activity increased in a dose-dependent manner with no parallel change in ALK-P activity (12). In the present study, we were able to demonstrate that PTH did not affect CD10/NEP activity, while 1,25-$(OH)_2D_3$ had a stimulatory effect only in the more mature MBA-15.6 osteoblastic cell, and that the inhibitory effect of BMPs-2,3 and TGFß is observed when PTH or $1.25(OH)_2D_3$ is used for pretreatment of cultures. IGF-I is the only growth factor that was stimulatory for CD10/NEP activity. Both enzymes (ALK-P and CD10/NEP) existed on the surface of stromal cells. The intense activity of these enzymes suggests their role as local regulators for various functions of bone cells in the bone local microenvironment.

Our findings support the possibility that marrow osteoblasts vary among different stages of differentiation, as demonstrated by the clonal subpopulations

described in this study. This may open new approaches in the study of hormones, growth factors and the combination of their action as therapeutic agents in clinical use.

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