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ARTICLES

Differential Effects of Retinoic Acid and Growth Factors on Osteoblastic Markers and CD10/NEP Activity in Stromal-Derived Osteoblasts

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Abstract The effects of retinoic acid (RA) on the expression of osteoblastic-related cell markers was examined. A marrow stromal osteogenic cell line, MBA-15, was analyzed by Northern blotting for the expression of bone matrix proteins. These cells constitutively express mRNA encoding for procollagen α_2 (I), osteonectin, osteopontin, biglycan, and alkaline phosphatase (ALK-P). Gene expression was unchanged in response to RA triggering for 24 hr. Furthermore, cell growth and enzymatic activities of ALK-P and neutral endopeptidase (CD10/NEP) were studied. These parameters were examined in MBA-15 and clonal populations representing different stages of differentiation. The cell's growth rate was unchanged, while ALK-P activity was greatly increased during the culture period under RA treatment in MBA-15 and in the clonal cell lines examined while CD10/NEP activity displayed a different pattern. MBA-15.4, a preosteoblast cell line, exhibited an inhibition in CD10/NEP activity at the beginning of the culture period, reaching basal level with time. This activity was greatly increased over control level in MBA-15.6, a mature stage of osteoblasts. Furthermore, the response of cell lines to various growth factors was tested subsequent to priming the cultures with RA. A synergistic effect was monitored for ALK-P activity in MBA-15.4 and MBA-15.6 cells under rh-bone morphogenic protein (BMP-2) and purified osteogenin (BMP-3), and an antagonist effect was measured when cells were exposed to transforming growth factor β (TGFβ). Contrarily, BMP-2 and BMP-3 inhibited the CD10/NEP activity that had remained unchanged following priming of the cell with RA. Insulin-like growth factor I (IGF-I) and basic fibroblast growth factors (bFGF) did not affect either ALK-P nor CD10/NEP activities in both cloned cells. Cellular response to bone-seeking hormone, parathyroid hormone (PTH), and prostaglandin E_2 (PGE_2) was monitored by activation of intracellular cAMP. Treatment with RA caused a dramatic decrease in MBA-15.6 cell responses to PTH and PGE_2, but no significant effects could be observed in other clonal lines. © 1994 Wiley-Liss, Inc.

Key words: vitamin A, growth factors, marrow stromal osteoblasts, bone matrix proteins, CD10/NEP, neutral endopeptidase

Retinoic acid (RA), a physiological metabolite of vitamin A, has been described as playing a fundamental role in limb development and osteogenesis in vivo [Eichele, 1989]. It modulates differentiation and proliferation in a variety of cell types. The RA effects vary according to the differentiation stage of the responding cell [Heath et al., 1989, 1992; Lee et al., 1988; Ng et al., 1988; Nakayama et al., 1990]. Its regulatory role in bone development was studied in vitro in organ culture [Dickson et al., 1989] as well as on cells of osteogenic origin including tumorogenic cell lines [Heath et al., 1989, 1992; Imai et al., 1988; Lee et al., 1988; Livesey et al., 1985; Nakayama et al., 1990; Ng et al., 1988; Suzuki et al., 1993; Zhou et al., 1991]. RA affects gene expression in some cell types regulating transcription control elements of specific genes [Imai et al., 1988; Livesey et al., 1985; Suzuki et al., 1993; Zhou et al., 1991]. The diversity of responses among bone cells may reflect various

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specialized cell subpopulations that belong to the osteoblastic family. The differences may also be due to the fact that the cells are derived from various anatomical sites. Arresting cells at different stages on the differentiation pathway may accentuate the variance in their responses.

A central question in developmental biology is how differentiated cells arise from progenitor cells. Growth factors and lymphokines act as local regulators for specific microenvironments, as was demonstrated for the hemopoietic compartment [Metcalf 1991; Zipori, 1990]. Although the stromal compartment serves as a source for osteogenic cells [Benayahu et al., 1989; Owen and Friedenstein, 1988], little is known regarding their growth and differentiation. Various factors that have a role in the mesenchymal cell differentiation were extracted from bone matrix and have been proposed to affect growth and differentiation of bone cell in vitro and in vivo [Canalis et al., 1988; Oyajobi and Russell, 1992; Rodan, 1992]. It is believed that systemic hormones serve as mediators that alter cell responses to local growth factors by modifying their differentiation functions.

The marrow stromal compartment contains osteogenic cells that are closely associated with bone formation at the endosteal surface of bone. An advantage of working with osteogenic cell populations of the marrow stroma is the probability of encountering less differentiated cells, considering their origin. The marrow-derived osteogenic cell line MBA-15 expresses osteoblastic-associated features in vitro and forms bone in vivo [Benayahu et al., 1989, in press, a]. Clonal lines were derived from a parental MBA-15 line, presumably "arrested" at a particular stage along the osteogenic lineage [Fried et al., 1993]. The MBA-15 clonal lines differ in their morphology, proliferation rate, synthesis of extracellular matrix proteins, and levels of ALK-P and CD10/NEP activities [Fried et al., 1993; Indig et al., 1990]. 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, limited proliferation ability, synthesizes twice as much noncollagenous proteins as collagen, is high in ALK-P activity, and responds strongly to PTH. The properties of other clones ranged between these two categories. These differential properties directly reflected 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 [Fried et al., 1993]. These cells serve as a useful model to study the processes involved in the differentiation of bone-forming cells.

Our attention was also directed to the expression of an additional marker, neutral endopeptidase (meprilysin; NEP), by the stromal osteoblasts. We have previously shown that CD10/NEP is present on marrow stromal cell subpopulations and that it is highly expressed on osteoblastic cells [Indig et al., 1990]. NEP is an ectoenzyme present on the surface of many cell types and is widely distributed in mammalian tissue. The amino acid sequence of (human) NEP is identical with that of the CALLA antigen (CD10) and is highly conserved in different species [Letarte et al., 1988; Shipp et al., 1989]. This enzyme has been associated with the metabolism and regulation of a variety of peptides [Erdos et al., 1989].

Here, we focused on the effect of RA during growth and modulation of differentiation in MBA-15 and clonal lines. mRNA expression of bone matrix proteins and the changes in responsiveness to PTH and PGE_2, following the RA treatment, were studied in MBA-15 cells. We further examined the RA effects on cell growth, ALK-P, and CD10/NEP activities of these osteoblastic cells. Furthermore, the ALK-P and CD10/NEP activities of the clonal MBA-15.4 and MBA-15.6 cell lines in response to various growth factors were tested subsequent to priming the cultures with RA.

MATERIALS AND METHODS

Cell Culture

MBA-15 cells [Benayahu et al., 1989] and clonal subpopulations [Fried et al., 1993] were previously described in detail. 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 d. All cultures were incubated at 37°C in a humidified atmosphere of 10% CO_2 in air. The clonal cell lines, MBA-15.4, 15.6, 15.30, 15.31, and 15.33, were passaged more than 20 times, and were frozen from subcultures soon after cloning. All experiments described herein were performed with the 20th to 30th passage of cells.

For final experiments, cells were trypsinized and seeded at 1×10^4 cells/ml, 1 ml in 24-well plates. After 24 hr, the medium was removed and the cells were supplemented with DMEM containing 2% charcoaled FCS (chFCS) with or without 10^{-6} M RA. Cell growth and enzymatic activity were performed on various days during the cultivation period, as indicated in each experiment. In priming experiments, cells were first exposed to RA for 24 hr and then the medium was changed and supplemented with or left without the growth factors for 48 hr, as described in Table I.

Retinoic Acid (RA)

RA (a generous gift from Hoffmann-La Roche Ltd.) was dissolved in ethanol and added to culture medium containing 2% charcoaled FCS; ethanol (0.01% v/v) was used in control cultures.

Growth Factors (GF)

rh-Bone morphogenic protein 2 (BMP-2) was a generous gift from the Genetic Institute (Cambridge, MA). Purified osteogenin (BMP-3) [Luyten et al., 1989], transforming growth factor β (TGFβ), insulin-like growth factor (IGF-I), and basic fibroblast growth factors (bFGF) were purchased from Collaborative Research (Bedford, MA). 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.

Colorimetric Assay for Cell Growth

Cell growth was measured in a 24-well plate using a colorimetric method [Mosmann, 1983]. Stock solution (5 mg/ml) of 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide (MTT) (Sigma Chemical Co., St. Louis, MO) was prepared and 100 μl was added to the assayed well. Following 3 hr incubation at 37°C, acid isopropanol was added and mixed to dissolve the dark blue crystals. A 250 μl sample was transferred to a 96-well plate and read in a kinetic microplate reader (Molecular Devices Corp., Palo Alto, CA) using a test wavelength of 550 nm and a reference wavelength of 630 nm.

Enzymatic Determination

Cultured cells were rinsed twice with PBS, scraped with a Teflon spatula, transferred to 0.25 M sucrose, sonicated on ice for 30 sec at high speed, and assayed for enzyme activity and protein content. ALK-P activity was determined with 2 mM 4-nitrophenyl phosphate at 37°C in 0.1 M 2-amino-2-methyl-1-propanol and 2 mM $MgCl_2$ [Majeska and Rodan, 1982]. 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) [Indig et al., 1989]. The CD10/NEP activity of MBA-15 and clonal cells was abolished in the presence of phosphoramidon (1.8 μM) [Indig et al., 1990]. In this study, the enzymatic activity in several experiments was selectively inhibited up to 98% by 1.0 μM thiorphan. Protein was measured by the Lowry and collegues method [Lowry et al., 1951] 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).

Hormonal Stimulation

Cells were seeded at 2.5×10^4 cells/ml, 2 ml in 35 mm plates and grown to confluence. The medium was then removed and the cultures were preincubated with fresh medium containing 0.5% bovine serum albumin (BSA) and 5×10^{-4} M isobutylmethylxanthine (IBMX) (Sigma Chemical Co., St. Louis, MO) at 37°C for 15 min. Cells were then challenged with fresh medium containing either 100 ng/ml of 1–34 human parathyroid hormone (hPTH) (Sigma Chemical Co., St. Louis, MO) dissolved in 1% Na-acetate (pH 4.0) containing 0.1% BSA for 5 min, or 500

TABLE I. Design of Priming Experiments

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

ng/ml PGE_2 (Sigma Chemical Co., St. Louis, MO) dissolved in 0.4% ethanol for 10 min. All hormonal stock solutions were frozen in aliquots, and dilutions were freshly prepared in serum-free medium immediately before use. Culture cAMP was determined after incubation.

cAMP Determination

Intracellular cAMP was determined using two assays, and the results were highly correlated. One assay was based on a protein binding technique with a [^{3}H]-cAMP kit (KAPH2, Diagnostic Products Corporation, France). For this assay, the cells were rinsed with PBS, scraped off, suspended in 40 mM Na-acetate containing 4 mM EDTA (pH 4.2), sonicated on ice for 30 sec, and then heated for 2 min in boiling water. After centrifugation, the supernatant was removed, lyophilized, and reconstituted in a kit buffer, then underwent further quantitation according to the manufacturer's instructions. The second assay was based on the radioimmunoassay technique according to Harper and Brooker [1975]. In this assay, the culture exposure to hormones was terminated by the addition of perchloric acid neutralized with potassium bicarbonate, and this was followed by acetylation of the samples. cAMP was quantitated using polyclonal αcAMP antibodies (Bio-Makor, Israel) and radiolabeled [^{125}I]-cAMP (NEN, England). Protein A (Bio-Makor, Israel) was used to separate the bound from the free ligand.

Isolation of RNA and Northern Blot Analysis

Cells were seeded at two cell densities, 1×10^4/ml and 3×10^4/ml, 10 ml in 90 mm plates. After 48 hr, the medium was removed and the cultures were supplemented with DMEM containing 2% chFCS with or without 10^{-6} M RA for 24 hr. Total RNA was extracted by the acid guanidinium thiocyanate-phenol-chloroform method [Chomodzynski and Sacchi, 1987] and fractionated on formaldehyde agarose gels [Kroczek and Siebert, 1990]. The RNA was transferred onto Gene Screen Plus nylon membranes according to the manufacturer's specifications and hybridized with random primed and labeled cDNA fragments to enable encoding for various bone matrix proteins and β-actin. After hybridization for 12 hr at 65°C, the membranes were washed and exposed to X-ray film.

The cDNA encoding h-osteonectin mRNA was isolated by digesting plasmid HON-2 [Young et al., 1990a] with EcoRI, which liberated two contiguous fragments of 0.6 kb (5′ end) and 1.9 kb (3′ end); the 1.9 kb fragment was used in this study. cDNA encoding h-biglycan (PgI) was isolated by restricting plasmid P16 [Fisher et al., 1989] with EcoRI, resulting in the release of a 1.7 kb cDNA. cDNA for h-osteopontin was obtained by digesting plasmid OP-10 [Young et al., 1990b] with XbaI and XhoI to release the insert DNA of 1.5 kb. These cDNA were generous gifts from Dr. M. Young (National Institute of Dental Research, Bethesda, MD). The cDNA encoding for h-ALK-P was obtained by treating plasmid pS3-1 with EcoRI [Weiss et al., 1986]. A 2.5 kb fragment of cDNA containing the entire coding region was a generous gift from Prof. G. Rodan (Merck Sharp and Dohme, PA). A cDNA probe encoding the α_2(I) chain of rat procollagen was obtained from plasmid Pucl and released with PstI [Genovese et al., 1984]. A 0.9 kb $p\alpha_2R_2$ fragment used in this study was kindly provided by Dr. D. Rowe (University of Connecticut, Framington).

Statistical Analysis

The significance of differences between experimental and control groups was analyzed by the Duncans's multiple range test. Statistical analysis of data presented as "percentage of control" was based on a variance of discrete random variables.

RESULTS

mRNA Expression of Matrix Proteins

We examined the expression of several genes of collagen, noncollagenous proteins, and ALK-P, which have been shown to be involved in osteoblastic differentiation, and their response to RA. Cultures were grown in subconfluence and confluence densities with or without RA. Total RNA from cultures was extracted, hybridized with specific matrix protein, probed, and analyzed by Northern blotting. The MBA-15 cells constitutively expressed mRNA for procollagen α2(I), osteonectin, ALK-P, osteopontin, and the proteoglycan-biglycan. Addition of RA to growth medium did not affect matrix gene expression or ALK-P mRNA levels (Fig. 1). This does not necessarily reflect the level of proteins actually synthesized by the cell. The same pattern of cell response was detected at both culture densities.

Cell Proliferation and Enzyme Activities

MBA-15 cells and various clonal subpopulations were treated with RA for up to 96 hr. Cell

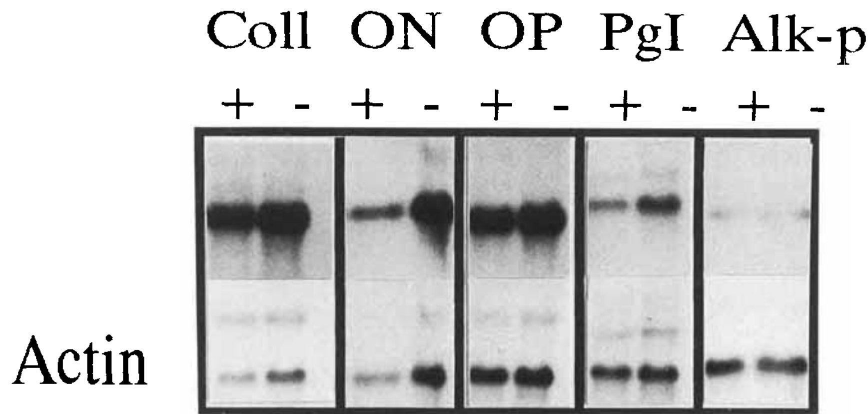

Fig. 1. Northern blot illustrating the constitutive expression of osteoblastic-related matrix proteins and alkaline phosphatase mRNA level in confluent cultures of marrow stromal of the MBA-15 cell line. Total RNA was extracted from untreated cultures (−) and retinoic acid (10^{-6} M) exposure for 24 hr cultures (+). This panel represents an autoradiogram of random primed ^{32}P-labeled cDNA hybridized to mRNA. Migration to 4.7 kb for procollagen α_2(I) (COLL), 2.2 kb for osteonectin (ON), 1.4 kb for osteopontin (OP), 2.6 kb for biglycan (Pgl), 2.5 kb for alkaline phosphatase (ALK-P), and 2.1 kb for β actin mRNA are shown. Quantitation and normalization of mRNA levels using actin showed differences in a range of 10% between RA-treated cells and control.

growth was examined at various time points along the exposure period, and no changes were detected in any cells examined (Figs. 2, 4A,B, Table II). Under the same conditions, however, the cells did express changes in enzymatic activities. Enzymatic response to RA demonstrated an increase in ALK-P levels and a different pattern for CD10/NEP activity. ALK-P is an established osteoblastic marker whereas CD10/NEP was shown by us to be expressed by marrow stromal cell members, with the highest levels of activity being exhibited by osteoblastic cells [Indig et al., 1990]. In the parental cell line MBA-15, the marked increase seen in ALK-P activity (12–18 times that of control levels) was in accordance with the exposure time in culture (Fig. 3A). A similar pattern was noted in clonal subpopulations, regardless of the differentiation stage of tested cells. This enzyme activity was increased during the proliferation period and reached its maximal activity expressed by a plateau at confluent stages (Fig. 4C,D, Table II). It is noteworthy that the increase in ALK-P was coincident with a cessation of cell growth in

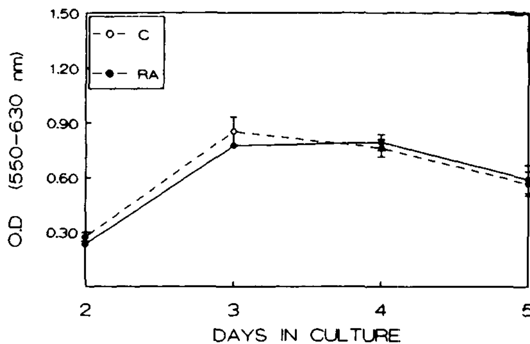

Fig. 2. Growth curve of MBA-15 cell exposed for 4 d to 10^{-6} M RA. Cell growth was examined along the culture period using the MTT assay. Results are mean ± SEM of four separated wells.

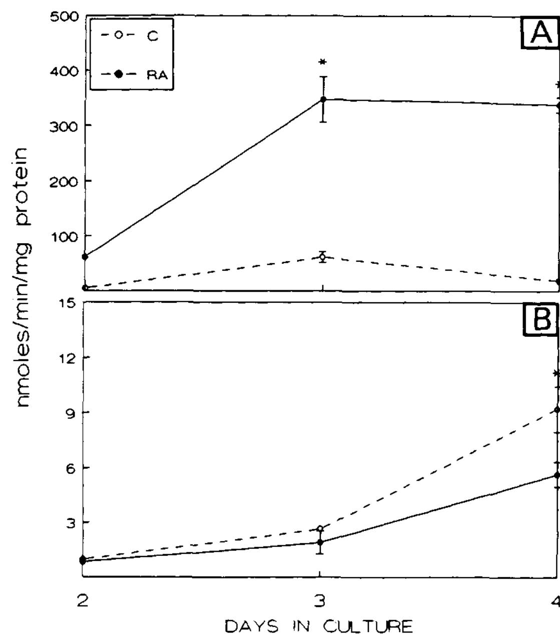

Fig. 3. Enzymatic activities of ALK-P (**A**) and CD10/NEP (**B**) *determined in MBA-15 cell exposed for 4 d to 10^{-6} M RA.* Results are mean ± SEM of triplicate determination of five separated wells ($P < 0.05$; Duncan's multiple range test).

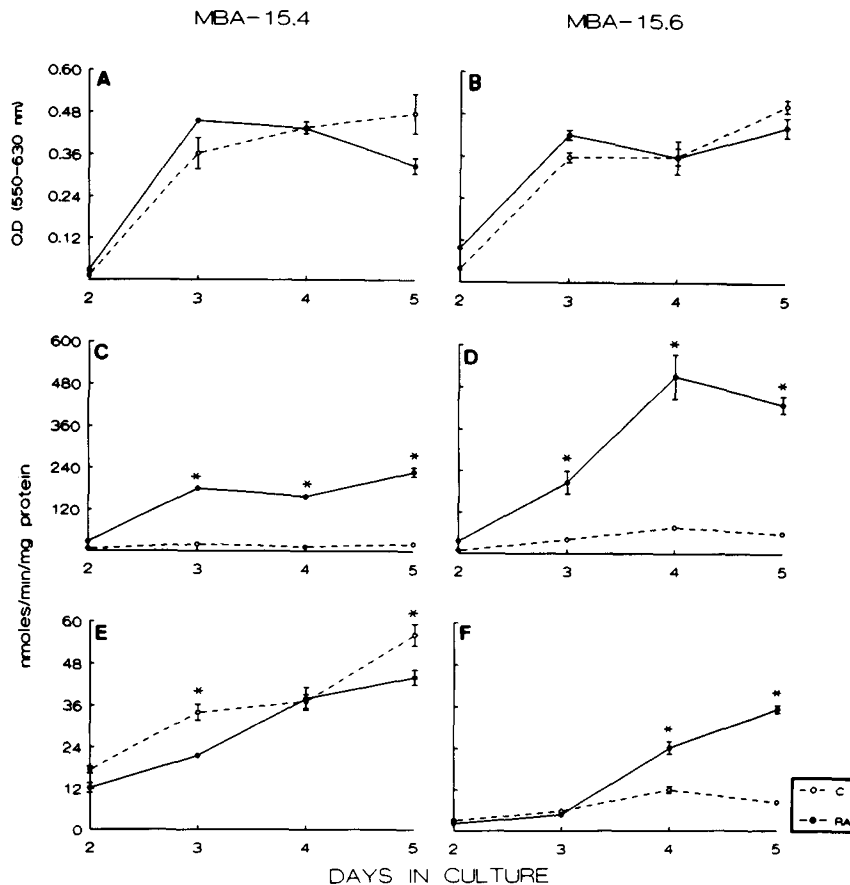

Fig. 4. Clonal subpopulations, MBA-15.4 and MBA-15.6, exposed for 4 d to 10^{-6} M RA. Cell growth (**A, B**), ALK-P (**C, D**), and CD10/NEP activities (**E, F**). Assays were performed in triplicate on four to six separate cultures. The results are mean ± SEM ($P < 0.05$; Duncan's multiple range test).

controls and that the inhibitory effect of RA was independent of cell growth. CD10/NEP activity exhibited a different pattern; the various cell responses shown to RA were not uniform (Figs. 3B, 4E,F, Table II). In the parental cell line MBA-15, the increase in enzyme levels with time in culture never reached control levels (Fig. 3B). In MBA-15.4 cells, a decrease in CD10/NEP activity at early culture stages was noted, after which the enzyme activity reached the basal level (Fig. 4E). MBA-15.6 cells expressed CD10/NEP activity that was increased with time in culture from day 3 to day 5 (Fig. 4F). Thus, RA modulated the enzymatic response pattern of ALK-P differently from that of CD10/NEP in the stromal osteoblastic clonal lines.

Effects of Growth Factors on Cells Primed by RA

MBA-15 cells have been shown to respond to different growth factors [Benayahu, 1991; Benayahu et al., 1993]. We further examined the effects of BMP-2, BMP-3, TGFβ, bFGF, and IGF-I on the clonal cells MBA-15.4 and MBA-15.6. No changes were detected in cell growth (data not shown), but an alteration in cell enzymatic activities was observed. In both MBA-15.4. and MBA-15.6, ALK-P was elevated by BMP-2 up to fivefold (Fig. 5A) and 2.5-fold (Fig. 5E), respectively, and to a lesser extent following BMP-3 (Fig. 5A,E). TGFβ antagonized ALK-P enzyme activity, while IGF-I and bFGF had no effect (Fig. 5A,E). When cells were exposed to RA for 24 hr, the hormonal triggering caused a five- to eightfold increase in ALK-P activity (Fig. 5A,E). Following priming by RA for 24 hr, the cultures were further challenged for 48 hr with one of the indicated growth factors. The results showed synergistic effects between the RA and BMP-2 on ALK-P elevated activity of up to 15-fold in MBA-15.4 (Fig. 5B) and 10-fold in MBA-15.6 (Fig. 5F), respectively, and to a lesser extent when treated with BMP-3.

TABLE II. Cell Growth and Enzymatic Activity of Clonal Subpopulations†

		Cell viability MTT (OD)	Enzyme activity nmoles/min/mg protein ALK-P	CD10/NEP
MBA-15.33	C	1.03 ± 0.02	6.45 ± 0.73*	3.75 ± 0.37
	RA	1.22 ± 0.03	128.24 ± 20.15*	3.81 ± 0.53
MBA-15.31	C	1.18 ± 0.02	15.54 ± 1.25	14.53 ± 1.11
	RA	1.22 ± 0.01	137.96 ± 10.29*	10.84 ± 1.72
MBA-15.4	C	0.47 ± 0.056	16.79 ± 3.36	9.49 ± 0.43
	RA	0.32 ± 0.022	234.32 ± 4.44*	10.12 ± 0.33
MBA-15.30	C	ND	87.97 ± 7.14	14.46 ± 1.06
	RA	ND	265.42 ± 24.39*	89.61 ± 1.68*
MBA-15.6	C	0.97 ± 0.04	43.13 ± 3.03	13.35 ± 0.24
	RA	0.82 ± 0.01	448.25 ± 16.63*	19.10 ± 0.01*

†The clonal populations were exposed to RA for 3 d. Cell growth was determined using the MTT method. The enzymatic activity for ALK-P and CD10/NEP was quantitated as described in *Materials and Methods*. Results are summarized as mean ± SEM of *triplicate* determination of four separate wells.
*Difference between control and RA-treated culture. $P < 0.05$; Duncan's multiple range test.

In both clones, TGFβ inhibited the increase of ALK-P caused by RA alone. No effect was observed following the exposure to IGF-I or bFGF (Fig. 5B,F). Under the same experimental protocol, CD10/NEP activity was decreased following BMP-2, BMP-3, and TGFβ exposure, while bFGF and IGF-I did not affect these cell activities (Fig. 5C,G). Pretreatment with RA following exposure to growth factors did not alter the response compared to the effects of growth factors alone (Fig. 5D,H).

Hormonal Responses

The clonal populations expressed different patterns in their basal response to PTH or PGE_2. MBA-15.6 cells exhibited the highest PTH response, while other clonal cell lines were more responsive to PGE_2 [Fried et al., 1993]. Intracellular cAMP changes were measured in RA-pretreated cultures in response to short exposures of PTH or PGE_2. The retinoid-modulated adenylate cyclase activity in MBA-15.6 cells, and

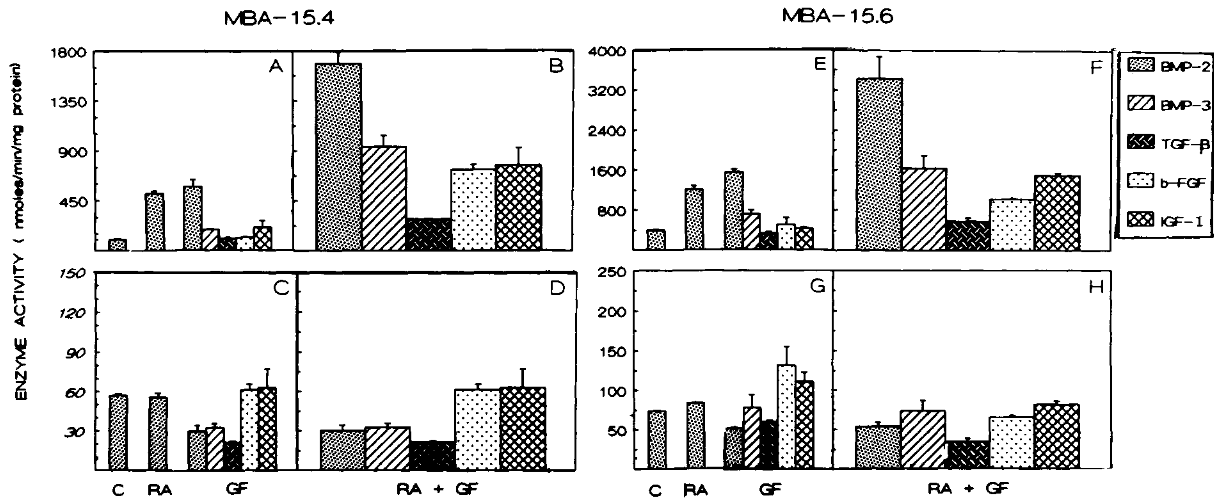

Fig. 5. Enzymatic activity in clonal subpopulations, MBA-15.4 and MBA-15.6. Cells were exposed for 24 hr to 10^{-6} M RA and then challenged with or without growth factors. Basal levels of ALK-P (**A, E**) or CD10/NEP activities (**C, G**) under RA or growth factor alone. Growth factors added were BMP-2 (100 ng/ml), BMP-3 (10 ng/ml), TGFβ (1 ng/ml), IGF-I (10 ng/ml), and bFGF (1 ng/ml). Priming of the cell with RA and then challenge with the growth factors were analyzed for ALK-P (**B, F**) or CD10/NEP (**D, H**) activities. Assays were performed in triplicate on four to six separate cultures. The results are mean ± SEM ($P < 0.05$; Duncan's multiple range test).

their response to PTH and PGE_2, was significantly lowered following RA treatment. RA did not alter the ability of any of the other clones to respond to PGE_2 or PTH exposure (Fig. 6).

DISCUSSION

RA has been shown to be a natural morphogen responsible for pattern formation during embryogenesis [Thaller and Eischele, 1987, 1990; Wagner et al., 1990]. Retinoids regulate growth and differentiation in a variety of cell lines including osteogenic cells, for example, RCT, an immortalized cell line [Heath et al., 1989, 1992], and MC3T3-E1 [Nakayama et al., 1990], ROS 17/2.8 [Imai et al., 1988], and UMR,

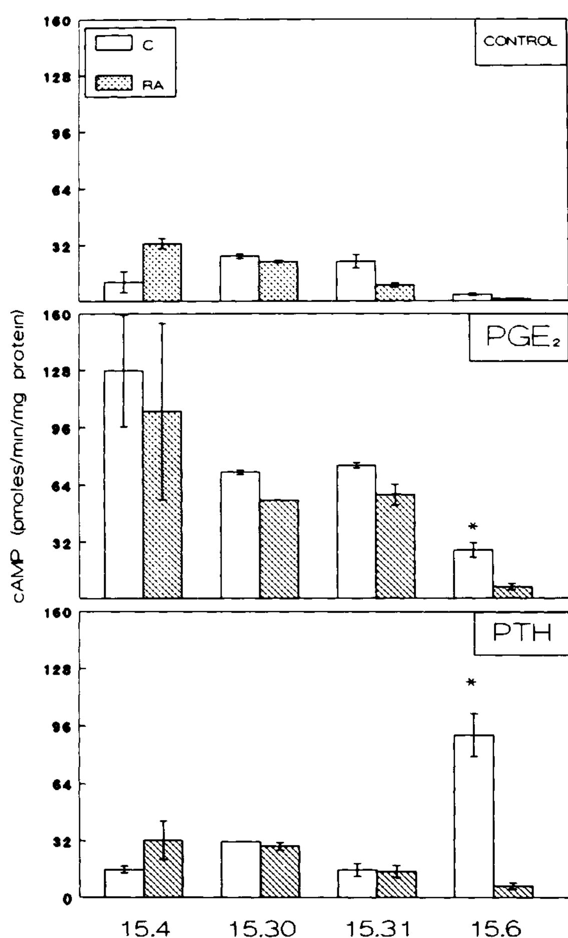

Fig. 6. Hormonal responsiveness of clonal cell lines was measured by changes in intracellular cAMP with and without 10^{-6} M RA pretreatment. Clonal cells were exposed for 3 d to RA, then triggered for 10 min with PTH (100 ng/ml) or PGE_2 (500 ng/ml), and cAMP was determined. Results are summarized as mean ± SEM of triplicate determination of three to five separated wells. Asterisk marks the difference between control and RA-treated culture. $P < 0.05$; Duncan's multiple range test.

which are osteosarcoma cell lines [Livesey et al., 1985; Zhou et al., 1991]. It is now recognized that the retinoic acid receptors (RARs) are members of the steroid receptor superfamily that acts through direct modulation of gene transcription. The modulation of these cells is probably mediated through specific nuclear RARs [Petkovich et al., 1987]. Recent studies have demonstrated the existence of three distinct RAR subtypes, designated αRAR, βRAR, and γRAR [de The et al., 1987; Giguere et al., 1987; Krust et al., 1989; Zelent et al., 1989], that were identified in MC3T3-E1 cells [Nakayama et al., 1990] and in other osteoblastic lines examined [Zhou et al., 1991].

The present study is the first report of RA effects on osteoblastic stromal-derived cell lines. MBA-15 nontransformed cells were examined for gene expression of bone matrix proteins, cell growth, and biochemical modulation. These cells were shown by us to produce collagen type I [Benayahu et al., 1989]. We have also shown that collagen and osteonectin appear as early markers before mineralization, and that their mRNA expression increases under mineralization conditions [Benayahu et al., in press, b]. Procollagen α_2(I) and other matrix proteins were unaffected under RA treatment in MBA-15 cells. It is clear that there were no significant changes in mRNA for some of the bone matrix proteins. However, this does not necessarily reflect the level of proteins actually synthesized by the cells. A seemingly paradoxical pattern was observed in the study of mRNA for procollagen α(I) in UMR cells under RA treatment. A decrease in gene expression was noted in UMR-106-06, while there was an increase in UMR-201 cells [Zhou et al., 1991]. This may be due to differences in stages of differentiation and maturation. ALK-P activity is involved mainly with phosphate degradation for the mineralization process. Its activity is remarkably dependent on culture stage [Stein et al., 1992]. This enzyme activity markedly increased in MBA-15 cells and in its clonal cell lines under RA treatment, although no change was detected in the ALK-P mRNA expression level. When osteoblastic cell cultures were under active mineralization and in the presence of dexamethasone, ALK-P mRNA expression was either unchanged [Benayahu et al., in press, b] or decreased [Arnow et al., 1990; Gerstenfeld et al., 1987]. The functional mode of ALK-P activity in calvaria-derived RCT-1 undif-

ferentiated cells was upregulated upon treatment with RA. The same mode of RA function has been reported in relatively undifferentiated UMR-201 cells [Ng et al., 1988]. In contrast, RA inhibited ALK-P expression in differentiated osteoblasts, such as in the late passage of rat calvarial cells RCT-3 [Heath et al., 1989], UMR-106-06 [Livesey et al., 1985], or ROS 17/2.8 [Imai et al., 1988]. The changes in ALK-P–specific activity with no accompanying effect on mRNA levels may indicate post-translational modulation.

The response of osteoblast cells to PTH and PGE_2 exhibits diverse patterns which are correlative with their differentiation stage, as was demonstrated by MBA-15 clonal lines [Fried et al., 1993]. The RA effect on PTH-responsive adenylate cyclase shows an upregulation in RCT cells [Heath et al., 1989] and in UMR-106-06 [Livesey et al., 1985]. A decrease in cell response associated with a more differentiated stage was demonstrated in ROS 17/2.8 [Imai et al., 1988] and in MBA-15.6 cells in this study. A significant decrease in PGE_2 response of MBA-15.6 was also noted. No changes were observed in PTH or PGE_2 responsiveness in other MBA-15 clonal cells, thereby reflecting the variation in their differentiation stages.

The regulation of bone remodeling involves many local factors as well as systemic hormones. In the bone local microenvironment, the activities of these components may be regulated by proteolytic enzymes such as CD10/NEP. In the marrow stromal cellular subpopulations, CD10/NEP activity was highly correlated to the osteoblastic cells [Howell et al., 1993; Ibbotson et al., 1992; Indig et al., 1990; Kee et al., 1992]. MBA-15 and clonal subpopulations demonstrated a diverse pattern in CD10/NEP activity upon modulation by RA. CD10/NEP activity increased with time during the culture period. Under exposure to RA, this enzyme activity in clones MBA-15.33, MBA-15.31, and MBA-15.4 was either inhibited or unchanged. MBA-15.6 cells expressed an elevation throughout the entire culture experimental period. A difference in ALK-P and CD10/NEP activity in response to 1,25-dihydroxyvitamin D_3 treatment had been previously observed in clonal MBA-15.30 and MBA-15.33. Their CD10/NEP activity increased in a dose-dependent manner with no change in ALK-P activity [Indig et al., 1990]. CD10/NEP activity in human osteoblast-like cells was also up-regulated by 1,25-dihydroxyvitamin D_3 and calcitonin and decreased by phorbol 12-myristate-13-acetate in a dose-dependent manner [Howell et al., 1993]. This enzyme has been suggested as a possible inactivator/modulator peptidase of peptide hormones, such as calcitonin, or of neuropeptides, such as enkephalines and Substance P [Erdos and Skidgel, 1989; Howell et al., 1993]. Such neuropeptides may play a role in the growth control of different cell types [Zachary et al., 1987]. Indeed, extensive proenkephalin gene expression was observed in bone-derived cells [Rosen et al., 1991]. The high activity of this peptidase on the surface of stromal cells may suggest their possible role as local regulators for various functions of bone cells, both in formation and in resorption.

Most of the local bone growth factors have been described by studying differentiation of bone-related activities. The growth factors are considered to have therapeutic potential for induced bone formation properties. Increase bone formation properties are needed in cases of replaced bone graft or in metabolic disorders associated with reduction of bone formation. 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. In this study, the effects were exerted by various growth factors from the TGFβ superfamily, TGFβ, BMP-2, and BMP-3, and other growth factors, IGF-I, and bFGF. These growth factors are found in bone matrix extracts and have been proposed to contribute to growth and differentiation of bone cells in vitro and in vivo. The effects of growth factors on marrow stromal osteoblasts, the source for the endosteal osteoblastic cells, have not been fully characterized. We examined the effects of growth factors on MBA-15 cells in previous studies [Benayahu, 1991; Benayahu et al., 1993]. ALK-P activity was shown to be increased following exposure to BMP-2 and BMP-3, while a decrease in enzymatic activity was observed following TGFβ treatment [Benayahu et al., 1993]. IGF-I, PDGF [Benayahu et al., 1993], and bFGF [Benayahu, 1991] had no effect. Cell growth was not affected by these growth factors. In order to elucidate 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 MBA-15.6 and MBA-15.4 cells with RA and then exposing them to growth factors resulted in a synergistic effect on ALK-P activity under RA followed by BMP-2 and BMP-3, but no changes were observed when IGF-I or bFGF was added. TGFβ antagonized the RA increase of ALK-P activity. On the contrary, CD10/NEP activity was changed under the same conditions, an inhibition was monitored when exposed to BMP-2, BMP-3, and TGFβ, but no changes were observed under IGF-I and bFGF. These effects were not altered by RA priming in either clonal cell line. These defined clonal supopulations of osteoblastic cells strenghten the evidence that growth factors affect differentially cellular activities. As we can picture from this study, the intent of combined exposure to RA and BMPs on osteoblast cells enhanced their ALK-P activity and potential in bone formation. The combined exposure of osteoblastic cells when exposed to RA and growth factors may enrich the understanding of the intricate series of events leading to net bone formation. The benefit from this information may advance and open new approaches to study the growth factors as therapeutic agents in clinical use.

Our findings support the possibility that marrow osteoblastic phenotypic expression varies among different clonal subpopulations. The characteristic responses of mature ostoeblastic cells, MBA-15.6, were reflected by RA in their ALK-P and CD10/NEP activities and cAMP response to PTH and PGE_2. These subline responses were different from those observed with other clonal lines, especially with the less mature MBA-15.4 cells. It points out that precise differentiated functions are differentially controlled, and this regulation augments the cell's ability to perform specific activities.

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REFERENCES

Aronow MA, Gerstenfeld LC, Owen TA, Tassinari MS, Stein GS, Lian JB (1990): Factors that promote progressive development of the osteoblast phenotype in cultured fetal rat calvaria cells. J Cell Physiol 143:213–221.

Benayahu D (1991): Mechanisms of interactions between osteogenic and nonosteogenic cells in the marrow. PhD Thesis, Tel-Aviv University, Israel, pp 1–135.

Benayahu D, Kletter Y, Zipori D, Wientroub S (1989): Bone marrow–derived stromal cell line expressing osteoblastic phenotype in vitro and osteogenic capacity in vivo. J Cell Physiol 140:1–7.

Benayahu D, Zipori D, Wientroub S (1993): Marrow adipocytes regulate growth and differentiation of osteoblasts. Biochem Biophys Res Commun, 197:1245–1252.

Benayahu D, Gurevitch O, Z Wientroub S (in press, a): Bone formation by marrow osteogenic cells (MBA-15) is not accompanied by osteoclastogenesis and generation of hemopoietic supportive microenvironment. J Bone Miner Res.

Benayahu D, Kompier R, Shamay A, Kadouri A, Zipori Z, Wientroub S (in press, b): Mineralization of marrow-stromal osteoblasts MBA-15 on three-dimensional carriers. Calcif Tissue Int.

Canalis E, McCarthy T, Centralla M (1988): Growth factors and the regulation of bone remodeling. J Clin Invest 81:277–281.

Chomoczynski P, Sacchi N (1987): Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159.

Dickson IR, Walls J, Webb S (1989): Vitamin A and bone formation. Different responses to retinol and retinoic acid of chick bone cells in organ culture. Biochim Biophys Acta 1013:254–258.

Eichele G (1989): Retinoids and vertebrate limb pattern formation. Trends Genet 5:246–251.

Erdos EG, Skidgel RA (1989): Neutral endopeptidase 24.11 (enkephalinase) and related regulators of peptide hormones. FASEB J 3:145–151.

Fisher LW, Termine JD, Young MF (1989): Deduced protein sequence of bone small proteoglycan I (biglycan) shows homology with proteoglycan II (decorin) and several non-connective tissue proteins in a variety of species. J Biol Chem 264:4571–4576.

Fried A, Benayahu D, Wientroub S (1993): Marrow stroma–derived osteogenic clonal cell lines: Putative stages in osteoblastic differentiation. J Cell Physiol 155:472–482.

Genovese C, Rowe D, Kream B (1984): Construction of DNA sequences complementary to rat α1 and α2 collagen mRNA and their use in studying the regulation of type I collagen synthesis by 1,25-Dihydroxyvitamin D. Biochemistry 23: 6210–6216.

Gerstenfeld LC, Chipman SD, Glowacki J, Lian JB (1987): Expression of differentiated function by mineralizing cultures of chicken osteoblasts. Dev Biol 122:49–60.

Giguere V, Ong ES, Segui P, Evans RM (1987): Identification of a receptor for the morphogen retinoic acid. Nature 330:624–629.

Harper GF, Brooker G (1975): Fentomole sensitive radioimmunoassay for cyclic AMP and cyclic GMP after 2′-O-acetylation by acetic anhydride in aqueous solutions. J Cyclic Nucleotide Res 1:207–218.

Heath JK, Rodan SB, Yoon K, Rodan GA (1989): Rat calvaria cell lines immortalized with SV-40 large T antigen: Constitutive and retinoic acid inducible expression of osteoblastic features. Endocrinology 124:3060–3068.

Heath JK, Suva LJ, Yoon K, Kiledjian M, Martin TJ, Rodan GA (1992): Retinoic acid stimulates transcriptional activity from the alkaline phosphatase promotor in immortalized rat calvarial cell line, RCT-1. Mol Endocrinol 6:636–646.

Howell S, Caswell AM, Kenny AJ, Turner AJ (1993): Membrane peptidases on human osteoblastic-like cells in culture: Hydrolysis of calcitonin and hormonal regulation of endopeptidase-24.11. Biochem J 290:159–164.

Ibbotson KJ, D'souza SM, Deschodt-Lanckman M, Appelboom TE (1992): Inhibition of bone resorption in vitro by human enkephalinase (EC 3.4.24.11), a neutral metalloendopeptidase. J Bone Miner Res 7:273–279.

Imai Y, Rodan SB, Rodan GA (1988): Effects of retinoic acid on alkaline phosphatase messenger ribonucleic acid, catecholamine receptors, and G proteins in ROS 17.2.8 cells. Endocrinology 122:456–463.

Indig FE, Ben-Meir D, Spungin A, Blumberg S (1989): Investigation of neutral endopeptidases (EC 3.4.24.11) and of neutral proteinases (EC 3.4.24.4) using a new sensitive two stage enzymatic reaction. FEBS Lett 255: 237–240.

Indig FE, Benayahu D, Fried A, Wientroub S, Blumberg S (1990): Neutral endopeptidase (EC 3.4.24.11) is highly expressed on osteoblastic cells and other marrow stromal cell types. Biochem Biophys Res Commun 172:620–626.

Kee BL, Paige CJ, Letarte M (1992): Characterization of murine CD10, an endopeptidase expressed on bone marrow adherent cells. Int Immunol 4:1041–1047.

Kroczek RA, Siebert E (1990): Optimization of Northern analysis by vacuum blotting RNA transfer visualization and ultraviolet fixation. Anal Biochem 184:90–95.

Krust A, Kastner P, Petkovich M, Zelent A, Chambon P (1989): A third human retinoic acid receptor, hRARτ. Proc Natl Acad Sci USA 86:5310–5314.

Lee KL, Petkovitch PM, Heerche JNM (1988): The effects of sodium butyrate on the retinoic acid–induced changes in 1,25-Dihydroxyvitamin D_3 receptors in tumorigenic and nontumorigenic bone derived cell lines. Endocrinology 122:2399–2406.

Letarte M, Vera S, Tran R, Addis JBL, Onizuka RJ, Quackenbush EJ, Jongeneel CV, McInnes RR (1988): Common acute lymphocytic leukemia antigen is identical to neutral endopeptidase. J Exp Med 168:1247–1253.

Livesey SA, Ng KW, Collier GR, Kubota M, Steiner AL, Martin TJ (1985): Effect of retinoic acid on cellular content and human parathyroid hormone activation of cyclic adenosine 3′:5′-Monophosphate–dependent protein kinase isoenzyme in clonal rat osteogenic sarcoma cells. Cancer Res 45:5734–5740.

Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ (1951): Protein measurement with folin-phenol reagent. J Biol Chem 193:265–275.

Luyten FP, Cunningham NS, Ma S, Muthukumara N, Hammonds RG, Nevis WB, Wood WI, Reddi AH (1989): Purification and partial amino acid sequence of osteogenin, a protein initiating bone differentiation. J Biol Chem 264: 13377–13380.

Majeska RL, Rodan GA (1982): Alkaline phosphatase inhibition by parathyroid hormone and isoproterenol in a clonal rat osteosarcoma cell line. Possible mediation by cyclic AMP. Calcif Tissue Int 34:59–66.

Metcalf D (1991): Lineage commitment of hemopoietic cells in developing blast cell colonies: Influence of colony-stimulating factors. Proc Natl Acad Sci USA 88:11310–11314.

Mosmann T (1983): Rapid colorimetric assay for cellular growth and survival application to proliferation cytotoxity assays. J Immunol Methods 65:55–63.

Nakayama Y, Takahashi K, Noji S, Muto K, Nishijima K, Taniguchi S (1990): Functional modes of retinoic acid in mouse osteoblastic clone MC-3T3-E1, proved as a target cell for retinoic acid. FEBS Lett 261:93–96.

Ng KW, Gummer PR, Michelangeli VP, Bateman JF, Mascara T, Cole WG, Martin J (1988): Regulation of alkaline phosphatase expression in neonatal rat calvarial cell strain by retinoic acid. J Bone Miner Res 3:53–61.

Owen M, Friedenstein AJ (1988): Stromal stem cells: Marrow derived osteogenic precursors. In Everd D, Harnett S (eds): "Cell and Molecular Biology of Vertebrate Hard Tissue," Vol 136. Ciba Fund Symposium. New York: J. Wiley & Sons, pp 42–60.

Oyajobi BO, Russell RGG (1992): Bone remodeling cytokines and joint disease. In Kuettner KE, Schleyerbach R, Peyron JG, Hascall VC (eds): "Articular Cartilage and Osteoarthritis." New York: Raven Press Ltd, pp 333–348.

Petkovich M, Brand MJ, Krust A, Chambon P (1987): A human retinoic acid receptor which belongs to the family of nuclear receptors. Nature 312:724–729.

Rodan GA (1992): Introduction to bone biology. Bone 13:S3–S6.

Rosen H, Polakiewicz RD, Benzakine S, Bar-Shavit Z (1991): Proenkephalin A in bone-derived cells. Proc Natl Acad Sci USA 88:3705–3709.

Shipp MA, Vijayaraghavan J, Schmidt EV, Masteller EL, D'Adamio L, Hersh LB, Reinherz EL (1989): Common acute lymphoblastic leukemia antigen (CALLA) is active neutral endopeptidase 24.11 ("enkephalinase"): Direct evidence by cDNA transfection analysis. Proc Natl Acad Sci USA 86:297–301.

Stein GS, Lian JB, Owen TA, Bidwell JP, Shalhoub V, Breen E, Van-Wijnen AJ, Stein JL (1992): Contribution of cell structure to integrated expression of cell growth and tissue-specific genes during osteoblast differentiation. In Slavkin H, Price P (eds): "Chemistry and Biology of Mineralized Tissues." Amsterdam: Elsevier Science Pub., pp 277–286.

Suzuki S, Koga M, Takaoka K, Ono K, Sato B (1993): Effects of retinoic acid on steroid and vitamin D_3 receptors in cultured mouse osteosarcoma cells. Bone 14:7–12.

Thaller C, Eischele G (1987): Identification and spatial distribution of retinoids in the developing chick limb bud. Nature 327:625–628.

Thaller C, Eischele G (1990): Isolation of 3,4-didehydroretinoic acid, a novel morphogenic signal in the chick wing bud. Nature 345:815–819.

de The H, Marchi A, Tiollais P, Dejean A (1987): A novel steroid thyroid hormone receptor-related gene inappropriately expressed in human hepatocellular carcinoma. Nature 330:667–670.

Wagner M, Thaller C, Jessell T, Eichele G (1990): Polarizing activity and retinoid synthesis in the floor plate of the neural tube. Nature 345:819–822.

Weiss MJ, Henthorn PS, Lafferty MA, Slaughter C, Rauducha M, Harris H (1986): Isolation and characterization of a cDNA encoding a human liver/bone/kidney type alkaline phosphatase. Proc Natl Acad Sci USA 83:7182–7186.

Young MF, Day AA, Dominguez P, McQuillan CI, Fisher LW, Termine JD (1990a): Structure and expression of osteonectin in human tissue. Connect Tissue Res 24:17–28.

Young MF, Kerr JM, Termine JD, Wewer UM, Wang M-G, McBride OW, Fischer LW (1990b): cDNA cloning, mRNA distribution and heterogeneity, chromosomal location, and RELP analysis of human osteopontin (OPN). Genomics 7:491–502.

Zachary I, Woll PJ, Rozengurt E (1987): A role of neuropeptides in the control of cell proliferation. Dev Biol 124:298–308.

Zelent A, Krust A, Petkovich M, Kastner P, Chambon P (1989): Cloning of murine retinoic acid receptor α and β cDNA and of a novel third receptor τ predominantly expressed in skin. Nature 339:714–717.

Zhou H, Hammonds RG, Findlay DM, Fuller PJ, Martin TJ, Ng KW (1991): Retinoic acid modulation of mRNA levels in malignant, nontransformed and immortalized osteoblasts. J Bone Miner Res 6:767–777.

Zipori D (1990): Regulation of hemopoiesis by cytokines that restrict options for growth and differentiation. Cancer Cells 2:205–211.