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# Phenotypic Expression of Marrow Cells When Grown on Various Substrata

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**Abstract** Our aim was to study the role of various extracellular matrices (ECM) on growth and differentiation of marrow stromal cells in vitro. Morphology changes, gene expression, and enzymatic activities were monitored in stromal osteoblastic MBA-15 and adipocytic 14F1.1 cells. These stromal cells were plated on dishes precoated with different substrata, such as matrigel (basement membrane), collagen type I, and endothelial ECM, and compared with cells plated on protein-free dishes. Striking morphological differences were observed when the cells grew on these different substrata. Changes in cell shape and growth also led to differential mRNA expression and enzymatic activities. When MBA-15 cells were plated on collagen, there was a decrease in mRNA for alkaline phosphatase (ALK-P), osteopontin (OP), and osteonectin (ON), and an increase in mRNA for procollagen (I). A differential effect was noted on 14F1.1 cells, the mRNA for ALK-P increased, the expressions of OP and ON lowered, and no expression for procollagen (I) was monitored. MBA-15 cells cultured on matrigel had decreased mRNA for ALK-P and OP, while they had increased ON mRNA expression and remained unchanged for procollagen I. No change in mRNA expression by 14F1.1 cells was monitored when cultured on matrigel. Functional enzymatic activities of ALK-P markedly decreased in MBA-15 cells cultured on various substrata, and increased or were unchanged in 14F1.1 cells. An additional enzyme, neutral endopeptidase (CD10/NEP), altered differentially in both cell types; this enzymatic activity increased or was unchanged when cells were cultured on these matrices. The results indicate a specific role for different ECM on various stromal cell types and their function. 

**Key words:** marrow stromal cells, Cell morphogenesis, attachment, ECM, mRNA expression

Specific extracellular matrix (ECM) is the natural substratum that acts in coordination with chemotaxis and migration followed by attachment of cells. The ECM acts as a specialized coordinator in each microenvironment. Previously reported investigations on several bone matrix macromolecules monitored the cellular adhesion process in vitro [Benayahu et al., 1995a; Clezardin et al., 1989; Clover et al., 1992; Grzesik et al., 1993, 1994; Oldberg et al., 1988; Somerman et al., 1987, 1989]. The cell interaction with ECM promotes the cellular expression and differentiation, and may activate or, alternately, inhibit the cells' maturation. The relationships between cellular architecture and cell functions such as morphology, growth, and phenotypic expression affected by ECM substrata comprise the prime objective of this study.

Osteoprogenitor cells exist in the marrow stromal system and are intimately involved with bone formation at the endosteal surface of bone. Osteoblasts are in close proximity to bone matrix, that is known to serve as a major regulator of the cells that are in contact with it. However, there is little information concerning the regulation of osteogenesis within the medullary cavity of bones. Other cell types belonging to the stromal compartment are fibroblast, adipocyte, and endothelial cells. The local interactions between the cells and the matrix construct the three-dimensional architecture that enables their growth and differentiation. Specific ECM affects the cellular responses through transmembrane molecules that act as stimulators and/or inhibitors in the stromal compartment. The transmembrane molecules assemble at cell–cell and cell–ECM contact sites [Ben-Ze'ev, 1992].

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Throughout the past decade, we have been studying marrow stromal cell subpopulations, especially two established cell lines classified according to their morphology, attachment, growth, biochemical properties, and mRNA expression [Benayahu et al., 1989, 1994a,b,c, 1995a,b]. While the stromal MBA-15 is an osteoblastic subpopulation, 14F1.1 cells are adipocytic cells. These cell lines are variable in their expression of ECM proteins, biochemical activities, and hormonal responses [Benayahu et al., 1991]. CALLA antigen (CD10) has also been associated with metabolism and regulation of a variety of peptides by its endopeptidase (NEP) activity [Erdos et al., 1989]. We have previously shown that CD10/NEP is present on marrow stromal cell subpopulations and is highly expressed on osteoblastic cells [Indig et al., 1990]. Furthermore, the osteogenic potential of MBA-15 cells to form bone tissue was demonstrated within diffusion chambers [Benayahu et al., 1989] as well as in ectopic sites [Benayahu et al., 1994a]. The relationships of the osteoblastic MBA-15 cells to hemopoiesis regulation were studied by us, both in vitro [Benayahu et al., 1992] and in vivo [Benayahu et al., 1994a]. The adipocyte 14F1.1 cells were shown to have a broad range of function in the support of the lymphopoieses–myelopoieses in vitro. These cells were also able to support stem cell maintenance and renewal [Zipori, 1992]. Furthermore, we could demonstrate the induction of myeloblastic cells to osteoclasts when they were cocultured with 14F1.1 cells [Benayahu et al., 1994d].

We have recently demonstrated the potential role of matrix components in elucidating the variable attachment capabilities of stromal cells to bone ECM proteins [Benayahu et al., 1995a]. In the present work we studied the interaction of the stromal cells with various matrices, such as collagen type I, which is a major ECM component in bone. In addition the interaction of stromal cells with basement membrane produced by the endothelial cells that vascularize bone. This is a specifically important relationship as it takes place at the bone–marrow interface. The endothelial cells' basement membrane components were examined using matrigel and ECM extracted from bovine eye corneas with stromal cells. We used these ECM components in order to expand upon the characterization of stromal osteoblastic and adipocyte cells' function in a specific microenvironment. Our primary interest involved the examination of stromal osteogenic and nonosteogenic expression of various properties based on the cellular–matrix contact that we considered especially important. We focused upon the identification of the responses of these stromal cells and the determination of their phenotypic expression in response to the varying matrices. This experimental system may serve as a model to define cellular–matrix interactions. It may shed light on the nature of the cell–matrix relationships within the marrow stroma and at the endosteal bone surfaces, as well as in the medullary cavity as is expressed in situ.

## MATERIAL AND METHODS

### Cells

Bone marrow–derived stromal osteoblastic cell line MBA-15 and 14F1.1 adipocyte cells [Benayahu et al., 1989, 1991] were grown on culture plates (Costar, Cambridge, MA) in high-glucose Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS) (Bet-Haemek, Israel). Cultures were incubated at 37°C in a humidified atmosphere of 10% $CO_2$ in air. Cells for experiments were trypsinized, counted, and seeded at a concentration of $0.5 \times 10^4$ cells/cm$^2$ on dishes with or without protein coating as indicated for each experiment.

### Extracellular Matrix and Protein-Coated Dishes

We used ECM proteins, collagen (3 mg/ml) [Pepsin-solubilized collagen (Coll I) from bovine dermal collagen] (Vitrogen 100, Collagen Corp., Palo Alto, CA) and basement membrane matrigel (Collaborative Research, Massachusetts). These matrices were prepared as a thin layer on non-tissue culture dishes used for coating, as detailed earlier [Benayahu et al., 1995a] or according to the manufacturer's instructions. Non-tissue cultures dishes coated with bovine corneal ECMs from steer eyes were a kind gift from Prof. I. Vlodavsky (Hadassa Medical School, Jerusalem) and used according to instruction [Fridmann et al., 1985; Rogelj et al., 1989; Bar-Shavit et al., 1991].

### Enzymatic Determination

Confluent 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 activ-

ity and protein content. Alkaline phosphatase (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 at 37°C by a two-stage technique using the synthetic substratum Suc-Ala-Ala-Leu-NH-Np supplemented with streptomyces griseus aminopeptidase I (SGAPI) [Indig et al., 1990]. Protein was measured by Lowry et al.'s method [Lowry et al., 1951] with human serum albumin as a standard. Enzymatic activity and protein determinations were performed in microwell plates and read by a kinetic microplate reader (Molecular Devices Corp., Palo Alto, CA).

### Isolation of RNA and Northern Blotting Analysis

Cells were seeded at $5 \times 10^4$ on 35 mm matrix-free dishes (control) and on precoated matrix constituent dishes (experiments). Cultures were scraped off with a rubber policeman 7 days later. Total RNA was extracted according to the method of Chomoczynski 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 which encode for various bone matrix proteins and, 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]. The 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 samples 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 used. This cDNA was a kind gift from Prof. G. Rodan (Merck Sharp and Dohme, Pennsylvania). A cDNA probe encoding rat procollagen, which was obtained from plasmid Pucl and released with PstI and a 0.9 kb $P\alpha_2R_2$ fragment [Genovese et al., 1984], was kindly provided by Dr. D. Rowe (University of Connecticut, Farmington, CT). All probes were previously tested and found to be crossreactive with mouse mRNA [Benayahu et al., 1994b,c].

## RESULTS

### Growth and Morphology Properties

MBA-15 and 14F1.1 cells were applied at a concentration of $5 \times 10^4$/dish on 35 mm dishes that were coated with matrigel, collagen I, or endothelial ECM. The attachment capability of these cells on different matrix components had been studied by us and found to be variable [Benayahu et al., 1995a]. Furthermore, the differences in 14F1.1 and MBA-15 cells' attachment capability was shown to affect the cells' spread, growth, and morphology. The 14F1.1 cells were examined at the proliferation stage, 3 days (Fig. 1A–D), and then cultures reached 7 days (Fig. 1E–H). Variability in cellular morphology was clearly demonstrated by the cells' appearance. In sparse cultures, the 14F1.1 cells had a polygonal shape (Fig. 1A), and their growth appearance profiles changed when they were seeded on various matrices when plated at the same density (Fig. 1B–D). The 14F1.1 cells' morphology acquired a fibroblastoid appearance on matrigel (Fig. 1B), while those on endothelial ECM became slender in shape and had few cytoplasmic extensions (Fig. 1D). Cells plated on collagen retained their large cuboidal morphology (Fig. 1C) and had the lowest density since they were restricted in their growth capacity. The 14F1.1 cells (control) grown on protein-free dishes acquired the morphology of large cells that accumulated adipose droplets (Fig. 1E). Such morphology was not observed when these cells were plated on matrigel (Fig. 1F), collagen type I (Fig. 1G), or endothelial ECM (Fig. 1H), upon which they acquired a heterogenous shape with a fibroblast-to-cuboidal appearance.

The MBA-15 cells which were plated on matrix-free dishes are large polygonal cells on day 7, when they reached confluence (Fig. 2A), and acquired a different profile appearance for each matrix. They had a flattened fibroblastoid appearance on matrigel (Fig. 2B), a polygonal shape when plated on collagen type I (Fig. 2C), and grew more confluent in comparison to the uncoated culture dishes. Those cells plated on the endothelial ECM (Fig. 2D) assumed a fibroblastoid appearance of slender shape with few cytoplasmic extensions upon reaching confluence.

### mRNA Expression of Matrix Proteins

We examined the function of these cells when cultured on uncoated culture dishes compared to cells grown on bovine serum albumin (BSA),

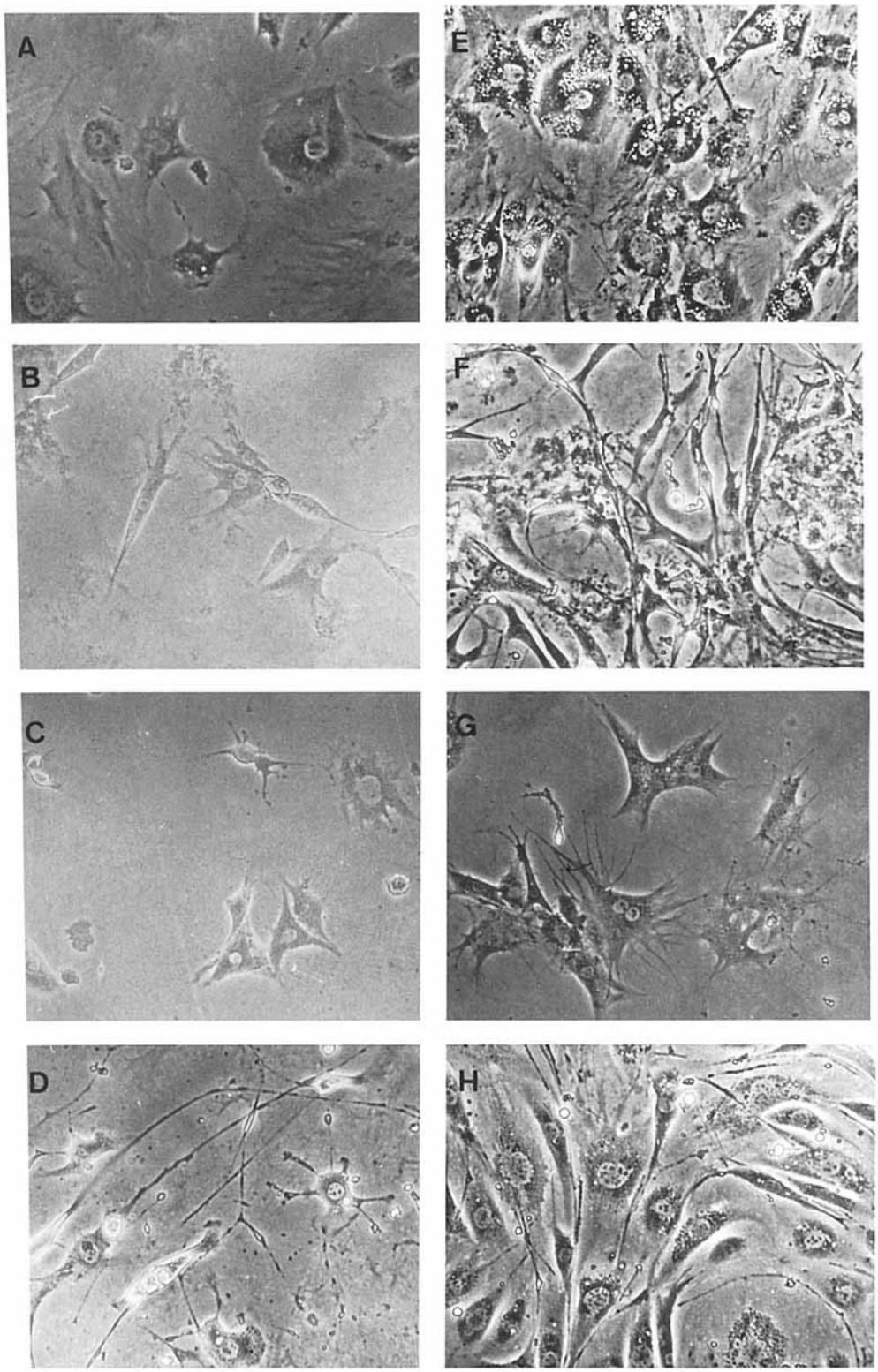


**Fig. 1.** Morphological appearance of 14F1.1 cells grown on protein-free dishes (**A,E** ), and on plates precoated with matrigel (**B,F**), collagen type I (**C,G**), or endothelial ECM (**D,H**). Cells were grown for 3 (A–D) or 7 (G–H) days. The large polygonal adipocytic cells acquired a fibroblastoid morphology when grown on matrigel (F), less on endothelial ECM (H).

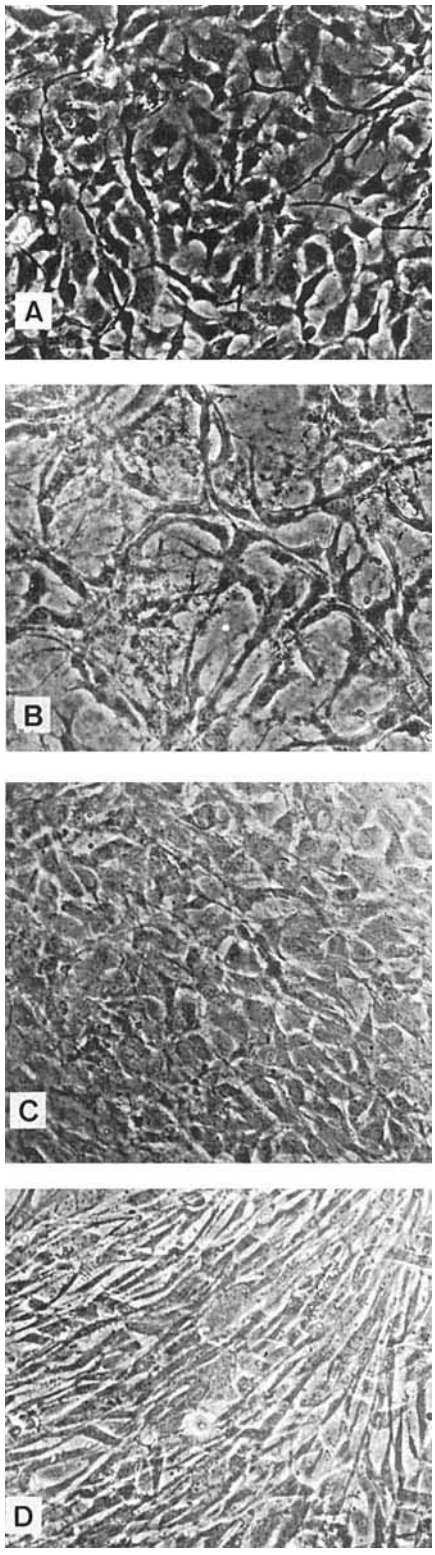


**Fig. 2.** Morphological appearance of MBA-15 cells grown on protein-free dishes (**A**) compared to plates precoated with matrigel (**B**), collagen (I) (**C**), or endothelial ECM (**D**). Cultures were grown for 7 days, when MBA-15 cells were large cuboidal cells in control cultures. They became fibroblastoid in shape when grown on matrigel and endothelial ECM. The polygonal-shaped cells were maintained on collagen-precoated dishes.

matrigel, or collagen substrata. mRNA expressions of collagen (I) and noncollagenous proteins, such as osteopontin (OP), osteonectin (ON), and ALK-P, were studied. We used total RNA extracted from MBA-15 and 14F1.1 cells and hybridized with specific cDNA probes, and used Northern blotting for analysis. The mRNA for procollagen (I) was constitutively expressed by MBA-15 (the osteoblastic cells) but not by 14F1.1 cells (adipocytic cells) (Fig. 3). The mRNA expressions of ALK-P and OP were reduced in MBA-15 cells when grown on BSA, collagen, or matrigel, in comparison to those plated on protein-free dishes (Fig. 3A,B). The 14F1.1 cells' mRNA for ALK-P grown on collagen showed increased expression, but was unchanged on the other matrices. The mRNA for OP exhibited a different pattern, and increased by 40% when the 14F1.1 cells were grown on either BSA or matrigel, while it was unaffected when grown on collagen substratum. A decrease in ON mRNA expression was observed only when both cells were cultured on collagen, remaining unchanged on other matrices. MBA-15 cells expressed an elevation in mRNA for procollagen (I) on all three matrices examined. The 14F1.1 cells did not express mRNA for procollagen (I), and no induction in this gene expression by the cells was detected under the three culture conditions (Fig. 3A,B).

### Enzymatic Activities

The 14F1.1 and MBA-15 cells were examined for their enzymatic activities of ALK-P and CD10/NEP when cultures reached confluence. The cells expressed changes in enzymatic activities when they were plated on each of the matrices. Inhibition was demonstrated in the ALK-P levels of the MBA-15 cells that were grown on matrigel- (40%), collagen- (30%), or endothelial ECM–coated dishes (80%) in comparison to control levels (Fig. 4). The 14F1.1 cells expressed a different pattern: an elevation in ALK-P activity appeared for the cells when cultured on matrigel, but there were no observable changes for those cultured on endothelial ECM (Fig. 4). Due to low cell growth on collagen we were unable to detect enzymatic activity under this culture condition. Expression of CD10/NEP activity was elevated and there was a differential pattern between cell type and the various matrices. The enzymatic activity of MBA-15 cells on matrigel was increased up to ninefold, and up to fourfold for cells on endothelial ECM, but was unchanged in cells on collagen-precoated dishes.

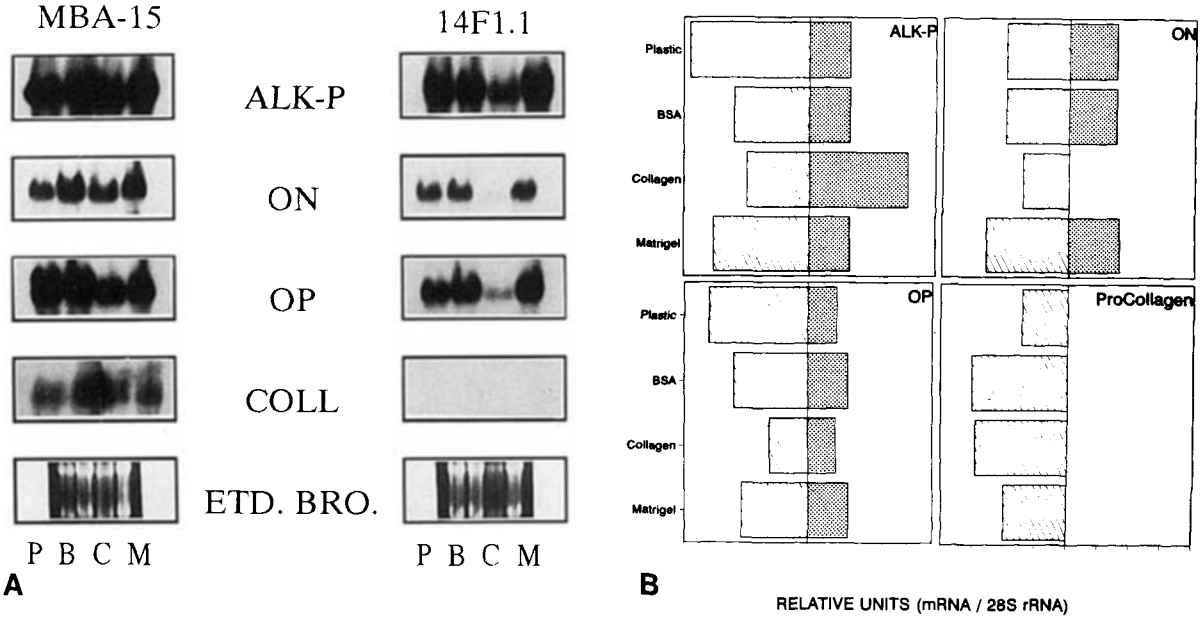


**Fig. 3.** The growth of osteoblastic MBA-15 and adipocyte 14F1.1 cells on different matrices were studied for determining the modulation of mRNA. The Northern blots illustrate the constitutive expression of osteoblastic-related matrix proteins and the alkaline phosphatase mRNA level in cultured cells. Total RNA was extracted from cultures grown on uncoated plastic (P) and on precoated tissue culture dishes. Cultures were grown on BSA (B), collagen (C), or matrigel (M). **A** represents an autoradiogram of reprobing of a Northern blot with random-primed $^{32}$P-labeled cDNA hybridized to mRNA migration, 4.7 kb for procollagen (I) (COLL), 2.2 kb for osteonectin (ON), 1.4 kb for osteopontin (OP), and 2.5 kb for alkaline phosphatase (ALK-P). Quantitation and normalization of mRNA levels using 28SrRNA, represented by the ethidium bromide (ETD. BRO.) staining, showed differences of culture conditions of cells, calculated as relative units in **B.** The stripped bars represent MBA-15 cells and the dotted bars 14F1.1 cells.

The 14F1.1 cells on matrigel expressed an elevation up to eightfold of CD10/NEP activity, but when cells were cultured on endothelial ECM enzyme activity was unchanged (Fig. 4).

## DISCUSSION

It is well recognized that the ECM proteins enhance proliferation and differentiation in vivo and do so in many culture systems. It is believed that ECM is a specific coordinator in the microenvironment that further modulates the cells' differentiation or changes their response to hormones and growth factors. The cells' interactions with a specific ECM may also specifically control the development of various tissues. Bone matrix contains mainly collagen and a smaller amount of noncollagenous proteins that are ultimately mineralized. Osteoprogenitor cells of the bone marrow stroma express their osteogenic potential only on endosteal bone surfaces. At this stage, they interact with bone matrix, and this may induce subsequent maturation of cell expression. There are other stromal cells in the medullary cavity, including fibroblasts, endothelial, reticular cells, and adipocytes [Benayahu et al., 1991; Owen and Friedenstein, 1988]. An attempt was made earlier to characterize the interaction of the marrow stromal subtypes with ECM proteins from bone [Benayahu et al., 1995a]. The ECM, which plays a role in the anchoring of cells to the substrata, was studied in the attachment potential of the various stromal cells. We were able to demonstrate the variable attachment properties according to the stromal cell stage of differentiation and to various substrata such as fibronectin, types of collagen, and proteins from the noncollagenous constituents of the bone matrix [Benayahu et al., 1995a]. Based on these data, the pattern of cell morphology and phenotypic expression was now further studied. In the current study, we focused on collagen type I, a major bone matrix protein, ECM components from endothelial cells, and matrigel (basement membrane), and explored their roles in the pathways of stromal cell–matrix interactions.

Following the stromal cell–ECM interaction, there were multiple observable effects of the cells' changes in morphology and phenotypic expression, as was measured by mRNA expression and changes of enzymatic activities. The stromal osteoblasts, the MBA-15 cells, were com-

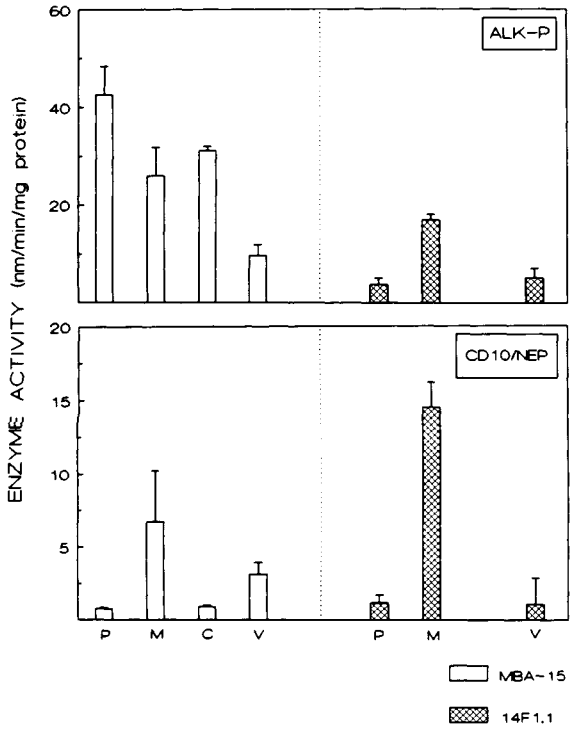


**Fig. 4.** Enzymatic activities of ALK-P and CD10/NEP determined in MBA-15 and 14F1.1 cells grown for 4 days on various matrices: plastic (P), matrigel (M), collagen (C), and endothelial ECM (V). Results are mean ± SEM of triplicate determination of five separate wells ($P < 0.05$; Duncan's multiple range test).

pared to the adipocyte, 14F1.1 cells when cultured on three different substrata. The adhesion of these cells and their spreading on the substrata further differentially modulated their morphologies. The changes in cell shape (cytoarchitecture) also reflected their expression of differentiated cell function, measured by mRNA and biochemical enzymatic activities. Functional osteoblastic-related gene expression for various matrix proteins, collagenous and noncollagenous, was examined. The constitutive mRNA expression of procollagen (I) modulated its expression in MBA-15 cells, while it was neither expressed nor induced in 14F1.1 cells. The mRNAs for ON, OP, and ALK-P were differentially changed in both cell types. We had earlier shown that procollagen (I) and ON appear as early markers before mineralization in MBA-15 cells, and increase under mineralization conditions [Benayahu et al., 1994b]. These mRNA were unaffected under retinoic acid (RA) treatment. Although the ALK-P mRNA was unaffected, this enzyme activity was markedly increased in MBA-15 cells and in their clonal cell lines under RA treatment [Benayahu et al., 1994c]. The functional mode of ALK-P activity in other osteoblastic cells had variable regulatory responses according to the cell stage of differentiation. An increase in UMR-201 cells when exposed to RA was observed [Ng et al., 1988]. In contrast, other investigators reported that 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], and ROS 17/2.8 [Imai et al., 1988]. These cells' mRNA further displayed variable changes when cultured on various matrices and in the presence of RA [Traianedes et al., 1993].

We also studied the regulation of CD10/NEP enzymatic activity. This enzyme was identified as a CALLA antigen and had been reported to be highly correlated to the osteoblastic cells in the marrow stroma [Howell et al., 1993; Ibbotson et al., 1992; Indig et al., 1990; Kee et al., 1992]. CD10/NEP is an endopeptidase that is believed to act as a regulator by its proteolytic activity on local growth factors, while ALK-P is active mainly in the mineralization process and is widely used as an osteoblastic marker. A specific alteration of these enzymes' activities was monitored; the CD10/NEP that was differentially modulated from the ALK-P activity. The latter was dramatically inhibited on precoated culture dishes with ECM, while these cultures demonstrated an increase in CD10/NEP activity. A difference in modulation of these enzymes was monitored in an earlier study in response to 1,25-dihydroxyvitamin $D_3$ treatment: this hormone affected CD10/NEP activity by causing an increase in a dose-dependent manner with no concomitant change in ALK-P activity [Indig et al., 1990]. CD10/NEP activity in human osteoblast-like cells was upregulated 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]. Further studies with RA [Benayahu et al., 1994c] and growth factor modulation on MBA-15 cells [Benayahu et al., 1994c, 1995b] emphasized the difference in the modulation of these enzymes' expression by osteoblastic cells. In the present study, the adipocyte 14F1.1 cells were shown to express a modulation pattern different from that of the osteoblastic MBA-15 cells.

The study of the stromal cells' expression and their regulation upon growth on specific matrices is a prerequisite for characterizing their function. Our demonstration of stromal cells' physiological assemblies at cell–ECM sites may shed light on the specific responses of cells within

the marrow stroma compartment. The phenotypic expression of osteoblastic and nonosteoblastic cells was shown to be differentially regulated. The results of this study clearly depict the role of ECM in terms of being a functional native regulator for the expression of the marrow stromal cells. These interactions are important in maintaining the physiology of bone and hematopoiesis, and an understanding of them may shed light on the structure and function of these tissues in situ.

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