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GENE EXPRESSION AT SINGLE CELL RESOLUTION ASSOCIATED WITH DEVELOPMENT OF THE BONE CELL PHENOTYPE: ULTRASTRUCTURAL AND IN SITU HYBRIDIZATION ANALYSIS.

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Introduction

The ability to culture normal diploid calvarial cells under conditions that support progressive development of the osteoblast phenotype and a bone tissue-like organization has provided a viable in vitro model system for examining the selective expression of genes and physiological signalling mechanisms that mediate osteoblast growth and differentiation. The tissue-like organization in vitro is reflected by the progressive development of nodules of multilayered cells in a mineralized extracellular matrix with orthogonally organized type I collagen fibrils. The ordered deposition of mineral within the collagen fibers initiates and is primarily associated with the nodule areas (Owen et al., 1990; Pockwinse et al., 1992; Bhargava et al., 1988).

A sequential expression of genes has defined a developmental sequence in primary cultures of normal diploid osteoblasts that contains three principal periods and two transition points where signalling mechanisms in the regulation of osteoblast phenotype expression are operative (Owen et al., 1990; Stein et al., 1990). During the first period, actively proliferating osteoblasts express a series of genes supporting cell growth and extracellular matrix biosynthesis, including histone, TGFß, fibronectin and collagen. At the first transition point with the down-regulation of proliferation, expression of genes involved with the maturation and organization of an extracellular matrix competent for mineralization is initiated, e.g. alkaline phosphatase mRNA and enzyme activity peaks. Then, at the second principal transition point, extracellular matrix mineralization is initiated, and defines the onset of the third period, at which time expression of genes which include osteopontin and osteocalcin are up-regulated.

Two key questions that necessitate resolution are concerns of: 1.) potential heterogeneity of the isolated cells that would generate discrete bone-like nodules; and 2.) the extent to which the temporal expression of genes and peak periods of gene expression coincide with discrete areas of cellular organization or reflect a uniform level of expression in all cells throughout the culture. We therefore utilized in situ hybridization to examine histone, osteocalcin and osteopontin gene expression at the single cell level during key periods of the osteoblast developmental sequence in relationship to modifications in cell ultrastructure and organization as revealed by transmission electron microscopy and scanning electron microscopy. Hormone responsiveness of the cells in relation to developing tissue organization was also examined.

Methods

Fetal rat calvarial derived osteoblasts were isolated and cultured as described in Owen et al., 1990. To utilize in situ hybridization for assessing the sequential expression of genes during development of the osteoblast phenotype at the single cell level, cells were cultured on plastic (Thermanox) coverslips. On the Thermanox coverslips, cell growth lagged behind that of the tissue culture plastic; however, the same temporal pattern of alkaline phosphatase activity and osteocalcin synthesis was observed and the same morphologic organization of mineralized nodules developed (Pockwinse et al., 1992). Preparation of samples for transmission and scanning electron microscopy and conditions for in situ hybridization were as described in Pockwinse et al., 1992.

Results

Morphologic Analyses of Normal Diploid Osteoblast Cultures

The surface morphology of the osteoblast cultures is clearly illustrated by the scanning electron micrographs in Figure 1. Early stage proliferating osteoblasts have the characteristic cuboidal morphology and the absence of polarity or orientation (Figure 1a). Post-proliferatively, as the organization of the extracellular matrix is ongoing and the cell density has significantly increased, the orientation of cells with respect to the nodule is visible (Figure 1b). In the mature multilayered cellular nodules of mineralized extracellular matrix, the orientation of internodular cells towards the apex of the nodule is pronounced (Figure 1c).

FIG.1

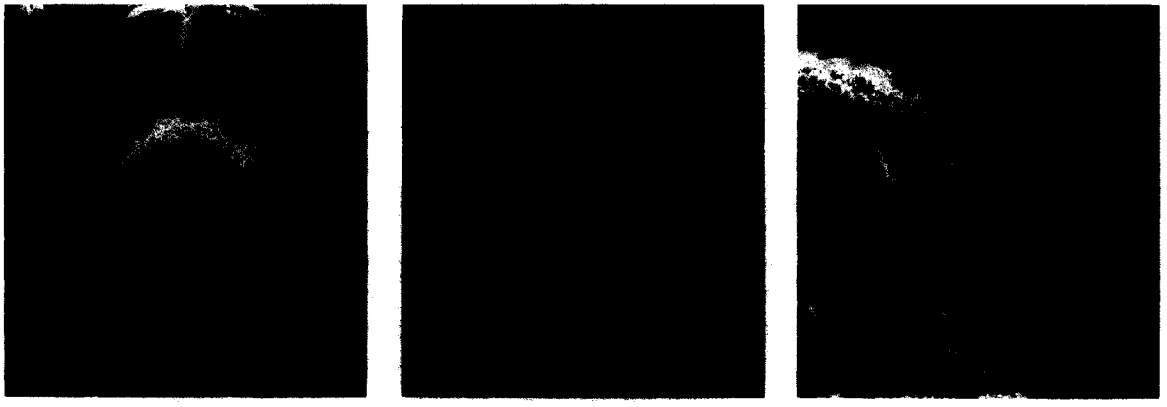

Figure 1. SCANNING ELECTRON MICROSCOPY OF RAT OSTEOBLAST CULTURES. A. During the proliferation period, cells exhibit a uniform distribution with flattened morphology. A prophase mitotic cell exhibiting a rounded morphology is located in the center of the field, day 7, 500X. B. Numerous mineralized nodules throughout the culture, day 18, 8X. C. Nodule at higher magnification illustrating rough surface morphology resulting from the deposition of mineral. Note the orientation of cells towards the apex of the nodule 103X.

Transmission electron microscopy of normal diploid osteoblasts in primary culture indicates that the cells exhibit a morphology characterized by distinct changes in cell shape and orientation in relation to the developing nodule during the progressive differentiation of the osteoblast phenotype. The majority of the cells isolated from the third enzymatic digest of 21 day fetal rat calvaria exhibit the cuboidal morphology characteristic of osteoblasts in bone (Holtrop, 1975). Formation of the extracellular matrix is reflected by the presence of fine collagen fibrils being actively secreted by these osteoblasts (Figure 2a). Such cells were still observed after 38 days of culture, but were confined to the internodule region and exhibited a marked increase in the representation of glycogen-containing vesicles. Cells in the mineralized nodule have the appearance of a young osteocytic cell. They contain abundant and well organized granular endoplasmic reticulum, a large golgi, lysosomal bodies and some lipid deposits (Figure 2b). Cytoplasmic processes are being initiated and appear lacking the extensive cytoplasmic organelles present in the main cell body. The overall organization of the mature culture is reflected by an alignment and orientation of the cells, with respect to the nodules, at the periphery of the mineralized nodule. Note that these cells are flattened and elongated with large euchromatic nuclei, i.e. reflecting active transcription of genes (Figure 2c).

FIG. 2

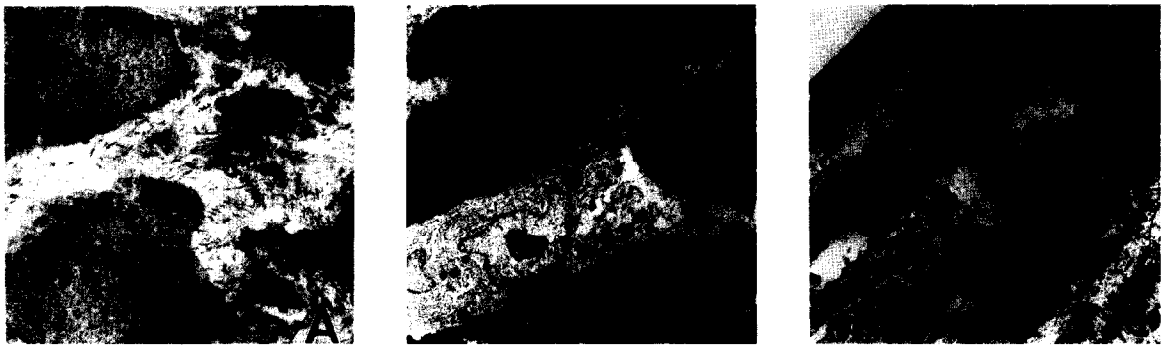

Figure 2. ULTRASTRUCTURAL DETAIL OF CELLULAR DIFFERENTIATION IN CULTURED OSTEOBLASTS.
A. Typical large cuboidal cells which are actively secreting collagen fibrils 2,250X. B. Osteocytic cells in mineralized nodule 3,000 X. C. The cells immediately adjacent to and surrounding a mineralized nodule are flattened, elongated and parallel with respect to each other 2,450X.

In Situ Hybridization Analysis of Cell Growth and Differentiation-Associated Genes in Normal Diploid Osteoblast Cultures

In primary cultures, an apparently homogeneous population of proliferating calvarial-derived cells progressively differentiates into cells with a characteristic osteocytic morphology within multilayered nodules exhibiting a bone tissue-like organization. It is, therefore, necessary to determine at the single cell level, which cells express specific genes characteristic of both the developing and mature osteoblast phenotype during the differentiation process. Thus we pursued in

situ hybridization analysis of the cultures using an H4 histone gene probe to monitor proliferation, based upon the tight coupling of histone gene expression to DNA synthesis (Figure 3a). It is evident that during the early proliferative period intense cytoplasmic labelling is apparent in 50% of the cells. Since histone mRNA is present only during the S-phase of the cell cycle, which is 9 hrs of the 20 hr doubling time for these cells, the 50% labelling indicates that 98% of the cells are actively proliferating. In contrast, hybridization with the histone gene probe was not detected in either the nodules or internodular region of the culture in the post-proliferative period.

Similarly, hybridization analysis was carried out using osteopontin and osteocalcin gene probes based on expression of these two genes in association with extracellular matrix maturation and mineralization. Labelling of cells with the osteopontin gene probe was observed during proliferation prior to nodule formation in a few cells. However, as seen in Figure 3b, all cells associated with the nodule were intensely labelled following hybridization with the osteopontin gene probe.

Osteocalcin gene expression is not observed prior to the initiation of nodule formation. However, beginning at the early stage of nodule formation, all nodule-associated cells hybridize with the radiolabelled osteocalcin gene probe. The expression of the osteocalcin gene is restricted to cells within or immediately associated with the mineralizing nodule. Cells at the peripheral region of the nodule are also intensely labelled by the osteocalcin probe. Interestingly, 1.25 (OH) $2D_3$ which increases osteocalcin gene transcription 3-10 fold, results in dramatic changes of cell morphology within the mature nodules: from cuboidal to elongated shaped cells which exhibit a parallel alignment with orientation towards the nodule apex (Figure 3c). In these elongated cells, increased levels of osteocalcin expression is observed. Taken together, these results indicate that both osteopontin and osteocalcin are expressed in a developmental manner. Both genes are co-expressed during the later period of the osteoblast developmental sequence, in association with extracellular matrix mineralization and the development of bone tissue-like organization.

FIG. 3

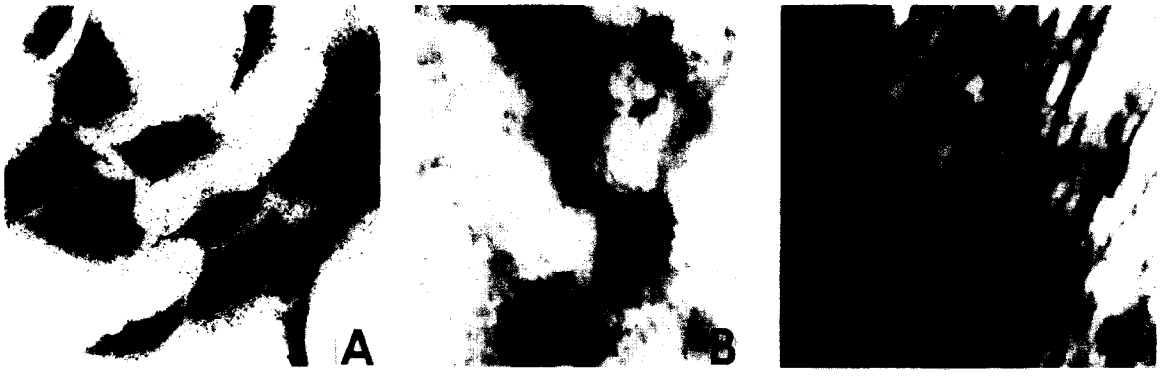

Figure 3. IN SITU HYBRIDIZATION FOR GROWTH AND DIFFERENTIATION RELATED GENES IN RAT OSTEOBLAST CULTURES.
A. Histone H4 gene, reflecting proliferation 320X. B. Osteopontin gene localization in developing nodules 45X. C. Osteocalcin gene expression in 10^{-8} M 1,25(0H) $2D_3$ - treated cultures (48 hrs) was detected in nodule associated cells 320X.

Discussion

These studies demonstrate that growth and the ordered developmental sequence of osteoblast differentiation reflected by a temporal expression of genes encoding cell growth and bone cell proteins are related to the developing tissue organization. Clearly, osteopontin and osteocalcin expression are localized to cells of the post-proliferative developing nodule with maximal expression in cells of the actively mineralizing nodule. We now show a selectivity of osteopontin and osteocalcin expression in mature osteoblasts associated with mineralized nodules. Thus, while the protein binds to hydroxyapatite it is also produced by cells in mineralized areas. These in situ hybridization observations are consistent with increased levels of osteocalcin and osteopontin mRNA in mineralizing cultures. Since both genes were expressed concomitantly, a key question is whether the same cells expressed both genes. Indeed, the present studies demonstrate concomitant expression restricted to cells within the mineralizing nodules. The mechanisms involved in the dramatic vitamin D mediated modification of cell morphology selectively observed in the mature nodules, remains to be established. However, the in situ hybridization analysis clearly demonstrates that vitamin D enhances osteocalcin gene expression only in the nodule associated cells and does not induce detectable levels of osteocalcin expression in the internodular cells. It therefore appears that cells within the mature nodules exhibiting gene expression associated with the phenotypic properties of mature osteoblasts, are competent to respond. Thus, the internodular cells do not appear to respond to the differentiation promoting properties of vitamin D documented for osteoblasts residing within nodules, as well as several cellular phenotypes including myelocytic cells, keratinocytes, chondrocytes (Suda et al., 1990).

The observations from scanning electron microscopy and transmission electron microscopy indicate an orientation of these cells toward the nodule. Such organization of cells with respect to the maturing nodule may be related to either: 1) interchange of cellular signals for osteoblast growth and differentiation; or 2) the organization of intracellular matrices for expression of genes related to initiation and/or propagation of mineralization. With respect to the latter possibility, it is evident that both the nuclear matrix (Dworetzky et al., 1990) and the cytoskeleton (Egan et al., 1991) undergo modifications in composition or organization during the osteoblast developmental sequence which correlate with and may be functionally related to changes in gene expression associated with progressive development of the bone cell phenotype. Another possibility that should be considered is that the orientation and polarity of the cells may reflect cellular migration which may contribute to the multilayering of cells in formation of the nodules.

In summary, the biological relevance of the diploid culture system is predicated upon the extent to which osteoblast growth and differentiation are expressed in relation to the bone tissue-like organization of the mineralized nodule. The localization of osteocalcin and osteopontin expression in the maturing nodule is consistent with both the developmental expression of these genes in fetal rat (Yoon et al., 1987) and the in vivo in situ expression in developing long bone in osteoblasts at the zone of mineralization (Weinreb et al., 1990). While a broad spectrum of genes expressed in an ordered and functionally related manner have been established in primary cultures of normal diploid osteoblasts, the rate limiting regulatory mechanisms remain to be determined. To begin to understand the regulatory mechanisms involving bone cell differentiation, the expression of cell growth and phenotype marker genes must be further defined at the single cell level and in relationship to the development of the nodule and the extracellular matrix mineralization process.

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