Discordant Expression of Osteoblast Markers in MC3T3-E1 Cells that Synthesize a High Turnover Matrix*

Richard J. Wenstrup‡§, John L. Fowlkes, David P. Witte, and Jane B. Florer‡

From the Divisions of Human Genetics and Pathology, Cincinnati Children’s Hospital, Cincinnati, Ohio 45229 and the Department of Pediatrics, Duke University Medical Center, Durham, North Carolina 27710

To examine the autocrine effects that an organizing extracellular matrix has on osteoblast precursors, we created MC3T3-E1 cell lines that stably expressed pro-\(\alpha_1(1)\) collagen chains with a truncated triple helical domain. Cells that had incorporated the pro-\(\alpha_1(1)\) expression plasmid (pMG155) expressed shortened pro-\(\alpha_1(1)\) transcripts at high levels and efficiently secreted the expression gene products into culture media. Those cells lost over 30% of newly deposited collagenous matrix compared with virtually no loss in control cultures, and media from the abnormal cells had qualitative differences in matrix metalloproteinase production. Electron micrographs strongly suggested that type I collagen molecules containing the truncated pro-\(\alpha_1(1)\) chains dramatically interfered with collagen fibrillogenesis in newly forming osteoblast matrix. Abnormal collagen fibrillogenesis was also associated with altered characteristics of cellular differentiation in that abnormal cells displayed a delayed and attenuated increase in alkaline phosphatase activity. Surprisingly, synthesis of osteocalcin was more than 5-fold higher than control cells. These findings demonstrate that osteoblasts require a normally structured collagenous matrix for regulation of alkaline phosphatase activity. However, in the presence of rapid turnover of osteoblast matrix, osteocalcin gene expression may be up-regulated in response to local signals by an unknown mechanism.

Since techniques have become available for isolation and analysis of osteogenic precursor cells in culture (1–6), there has been considerable interest in using these cell culture systems to understand the controlling events that regulate cessation of cell division and the initiation of cellular events that are the hallmarks of differentiation. In both primary osteoblasts (2, 3, 5, 7) and in the cloned mouse calvarial cell line MC3T3-E1 (1, 4, 6), a period of rapid cell division is followed by a transitional period that is characterized by deposition of an insoluble, type I collagen-rich extracellular matrix and an initial rise in alkaline phosphatase activity. Later, a third stage occurs, beginning approximately 2 weeks after plating, which is characterized by further increases in ALP activity, expression of osteocalcin, and mineral deposition.

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‡To whom correspondence should be addressed: Division of Human Genetics, TCHR Room 1033, 3333 Burnet Ave., Cincinnati, OH 45229. Tel.: 513-559-4471; Fax: 513-559-4373; E-mail: wenstrjr@ucbeh.san.uc.edu.

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EXPERIMENTAL PROCEDURES

Cell Culture—Stock cultures of MC3T3-E1 cells were grown in \(\alpha\)-MEM\(^1\) (minimum essential medium containing 10% fetal bovine se-

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\(^{*}\) The abbreviation used is: MEM, minimal essential medium.
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rum, penicillin (100 units/ml), and streptomycin (100 μg/ml) with 5 mm β-glycerophosphate and 25 μg/ml of ascorbic acid. Cells were grown in a humidified atmosphere of 9% CO₂ and 90% air at 37 °C. Until the time of study, cells were subcultured every 3–5 days using 0.05% trypsin to achieve cell detachment. For studies characterizing the temporal sequence of osteoblast differentiation, 50,000 or 400,000 cells were plated into 35-mm diameter multiwell dishes or 100-mm plates and grown for periods ranging from 3 to 28 days. Cultures were fed with fresh media twice a week.

Assay of Cell Replication—At the completion of the incubation period, cells were harvested by removing the media, washing twice with phosphate-buffered saline, and treated for 5 min with 0.05% trypsin to achieve cell detachment. Cell number was determined at the various time points by direct counting with a hemocytometer.

Transfections—To create MC3T3-E1 cells that stably express a mutated type I collagen, subconfluent 100-mm dishes of cells were cotransfected with the expression plasmid pCDNA3 (Invitrogen Corp., San Diego, CA), which contains a neomycin resistance cassette, and pMG155 (18) (generously provided by Darwin Prockop, Thomas Jefferson University, Philadelphia, PA). pMG155 is a human pro-1(I) minigene cloned into NotI/BamHI sites of Bluescript (Strategene Corp., La Jolla, CA); the insert contains 2.5 kilobases of promoter and genomic sequences that include exons 1–5 ligated to exons 47–52. Transfection was carried out overnight in serum-free α-MEM by lipofection using LipofectamineTM (Life Technologies, Inc.). Cells were then re-fed, and selection was carried out by addition of 400 μg/ml G418 (Life Technologies, Inc.) 2 h after transfection. The polymerase chain reaction product was confirmed to be osteocalcin by sequence analysis and comparison with a short segment of the mouse sequence (27).

Measurement of Collagenous Matrix—To quantify the accumulation of collagenous matrix deposited by MC3T3-E1 cells, colorimetric analysis of the hydroxyproline content of the cell layer was performed after hydrolysis of the cell layer with 6 N HCl (22).

Analysis of Turnover—Twenty-day-old cultures were pulse-labeled with 200 μCi of [³H]proline in serum-free medium supplemented with 50 μg/ml ascorbate and 5 mm β-glycerophosphate. After 48 h, labeling media were removed, and cultures were grown under normal conditions for 14 days longer (to day 28 after passage). Cell layers from duplicate dishes were collected on days 4, 7, and 14 of chase and suspended in 1 ml of 6 N HCl and hydrolyzed for 24 h at 100 °C. Hydrolysates were dried under vacuum at 50°C and reconstituted in 1 ml of 0.005 N HCl. [³H]hydroxyproline was separated from [³H]proline by paper chromatography; using a solvent mixture of butanol:acetic acid:H₂O (61:15:1), 15–20 μl of sample, and 20 μg of each amino acid as standard, hydroxyproline and proline were detected as discrete spots by developing first with 0.2% isatin to identify proline and p-dimethylaminobenzaldehyde to identify hydroxyproline. Hydroxyproline spots were cut out of the chromatogram, and ³H-generated counts per minute were determined by scintillation counting.

Analysis of Conditioned Media by Gelatin-Substrate Zymography—Gelatin-substrate zymography was performed as described elsewhere, with modifications (1). Aliquots of day 14 serum-free conditioned media from mutant and control clones of MC3T3-E1 cells were separated on 9.5% SDS-polyacrylamide gels containing 1 mg/ml gelatin (Sigma). Gels were electrophoresed for 1 h at 30 mA and then washed for 1 h at 4 °C in 2.5% Triton X-100. After extensive washing in distilled H₂O, the gels were incubated in 50 mM Tris, 5 mM CaCl₂, pH 8.0, for 18 h at 37 °C on a shaking platform to activate gelatinases present in the samples. To visualize gelatinase activity present in the samples, gels were stained with Coomassie Blue, and gelatinase activity appeared as cleared areas within a dark blue background. The M₀ of the gelatinases was calculated based on their relative migration in relation to protein standards.

Assessment of Cell Maturation—Alkaline phosphatase enzyme activity was measured in cell layers by colorimetric assay as previously reported (23).

Analysis of Proteins Secreted by MC3T3-E1 Cells—Media and cells were harvested separately as described previously into protease inhibitors (24). Samples used for collagen analysis were dialyzed either against phosphate-buffered saline and directly analyzed or were dialyzed against 0.5% acetic acid and lyophilized. Before proteins were harvested from media or cell layer at different time points after passages, serum was removed from media and the cells were re-fed for 16 h prior to harvest. Cell layers were harvested into guanidinium HCl.

Harvested proteins were analyzed by electrophoresis on SDS-polyacrylamide gels and then electroblotted to a polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA). The membranes were reacted with polyclonal antiserum to a peptide sequence from the carboxyl propeptide of human type I procollagen (LF41), a peptide sequence from murine decorin (LF113) (25), murine bone osteocalcin (LF23), or anti- osteonectin (both from C. Pham-Nguyen Biotech, Inc.). Quantitation of osteocalcin in culture media was performed by radioimmunosay using reagents from Biomedical Technologies, Inc. (Stoughton, MA).

Measurement of Steady-state mRNAs from MC3T3-E1 Cells—In some experiments, total cellular RNA was isolated by guanidinium isothiocyanate extraction (26) using TRIzol reagent (Life Technologies Inc.), and 10 μg was loaded on 1% agarose formaldehyde gels and separated by electrophoresis. RNA was transferred overnight onto GeneScreen Plus (DuPont) in 10 × SSPE buffer and baked at 80 °C for 2 h. Uniformity of RNA loading was determined by methylene blue staining of blots. The filters were hybridized to a ³²P-labeled 600-base pair insert from plasmid H677, a human pro-1(I) cDNA (21), or to an approximately 500-base pair murine osteocalcin cDNA segment that was generated by reverse transcriptase-polymerase chain reaction from total cellular RNA of 21-day MC3T3-E1 cells grown under differentiating conditions, using primers designed from the human sequence (27). The polymerase chain reaction product was confirmed to be osteocalcin by sequence analysis and comparison with a short segment of the mouse sequence (27).

Electron Microscopy—MC3T3-E1 cells grown for 14 days under differentiating conditions were fixed by immersion in Karnovsky’s solution containing 2.5% gluteraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer. Samples were postfixed in 1% osmic acid, dehydrated through graded series of alcohols, and embedded in epon, which was polymerized at 60 °C for 36–48 h. Following polymerization the blocks were trimmed, and ultrathin sections were cut with a diamond knife using a Reichart Ultra-Cut E ultramicrotome. Sections were stabilized on a mesh copper grid post stained with lead citrate solution and viewed in the Zeiss 912 electron microscope.

Materials—[3,3,4,5-³H]proline and [³²P]-CTP were from DuPont NEN. All culture materials, including α-MEM and fetal bovine serum, were obtained from Life Technologies, Inc.

RESULTS

We tested the ability of MC3T3-E1 cells to stably express a human pro-α(I) minigene construct (pMG155) that contained genomic sequences including 2.5 kilobases of the pro-α(I) promoter and exons 1–5 and 47–52 (18). Cells were transfected with pMG155 and the neo-containing expression plasmid pcDNA3. G418-resistant clones were analyzed by Northern blot analysis for expression of shortened pro-α(I) transcripts (Fig. 1A), which were found in approximately one-half of G418-resistant clones. Immuno blot analysis of procollagens harvested from media and cell layer indicated that contrary to expectation, type I collagen molecules incorporating the minigene product are efficiently secreted from MC3T3-E1 cells rather than being degraded intracellularly (Fig. 1B). When several minigene expressing clones were analyzed, there was surprisingly little heterogeneity in the expression of the minigene product at the level of steady-state RNA or secreted protein; expression either approached that of the endogenous pro-α(I) gene product or was undetectable even on prolonged exposures of Northern blots.

When culture medium is supplemented with 50 μg/ml ascorbate and 5 mm β-glycerophosphate, MC3T3-E1 cells deposit a type I collagen-rich extracellular matrix that increases in a linear manner and is first measurable at the end of the first week of culture. Compared with cells from a representative control done F6 (that had been simultaneously co-transfected with pcDNA3 and pMG155, selected with G418, and subse-
quently found not to express the minigene), cells from the minigene expressing clone C3 deposited approximately the same amount of collagen in culture dishes as measured by total hydroxyproline content (Fig. 2). To determine whether secretion of abnormal type I collagen molecules reduced the stability of collagen fibrils formed in the extracellular matrix, we performed pulse-chase studies in which cells were labeled for 48 h with \(^{3}H\)proline beginning at 12 days after plating. Rates of disappearance of \(^{3}H\)hydroxyproline from the insoluble cell layer were measured for a 2-week chase period. Cultures expressing the pro-\(\alpha_1(II)\) minigene retained only 66.3 ± 4.7% of newly deposited \(^{3}H\)hydroxyproline in osteoblast matrix during the 2-week chase compared with 97 ± 7.5% in control cultures (Fig. 3). Electron microscopy was performed to determine whether the relative instability of collagenous matrix produced by pro-\(\alpha_1(II)\) minigene-expressing cells could be related to abnormal collagen fibrillar structure. Images of collagen fibrils synthesized by control cells clearly show collagen fibrils recognizable by 67-nm periodicity that results from the nonintegral quarter stagger array of type I collagen monomers in fibrils; cells expressing the pro-\(\alpha_1(II)\) minigene synthesize collagen fibrils that were thinner and more disorganized and completely lacked the 67-nm pattern seen in controls (Fig. 4). In an attempt to determine whether the loss of \(^{3}H\)hydroxypro-
Expression of a dominant negative type I collagen mutation in the murine calvarial osteoblast cell line MC3T3-E1 resulted in deposition of a type I collagen matrix that has relatively high rates of turnover compared with control cultures. Deposition of the abnormal matrix resulted in suppression of one marker of osteoblast differentiation (alkaline phosphatase activity) and a significant increase in another (osteocalcin secretion). There was no compensatory up-regulation of the expression of other bone matrix proteins including type I collagen, decorin, bone sialoprotein, and osteonectin.

The human pro-\(\alpha\)1(I) minigene product expressed by the pMG155 expression plasmid contains an intact carboxyl propeptide required for molecular assembly and 23 uninterupted Gly-X-Y triplets of the adjacent triple helical domain; it would therefore be expected to co-assemble with wild-type mouse pro-\(\alpha\) chains, and SDS-polyacrylamide gel electrophoresis under nonreducing conditions showed that the shortened pro-\(\alpha\)1(I) chains were not secreted as monomers (data not shown). The turnover of \(^{3}H\)hydroxyproline from the collagen matrix is due primarily to loss of type I collagen molecules.

**DISCUSSION**

We then examined alkaline phosphatase activity in control and minigene-expressing cells to determine whether abnormal collagen fibrillogenesis had an effect on osteoblast differentiation. In the presence of ascorbate and 5 mM collagen fibrillogenesis had an effect on osteoblast differentiation of osteoblast clones (C3, lanes 1 and 2, and C2, lanes 3 and 4) or a control clone (F6, lanes 5 and 6) were analyzed by gelatin-substrate zymography. The 38-kDa gelatinase produced at high levels by mutant osteoblasts is denoted by the arrow.

![Image](http://www.jbc.org/Downloaded from)
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why the collagenous matrix deposition by 21 days is not much lower in cells secreting the truncated proα1(I) chains is not entirely clear but may be due to the fact that there were higher cell numbers in the minigene-expressing clones that were measured for hydroxyproline deposition compared with the control clones.

These experiments, which use a dominant negative type I collagen mutation to effect a targeted disruption of collagen fibrillogenesis, augment previous in vitro studies of collagen-osteoblast interaction in two ways. First, they lend support to the theory that suppression of osteoblast characteristics by ascorbate deprivation (2, 4, 6, 9) or by the addition of proline analogs (4, 9) is due at least in part to the inhibition of collagen deposition. Second, these data indicate that the increase in alkaline phosphatase activity associated with osteoblast differentiation depends not only on the overall quantity of collagen deposited by also on normal collagen structure.

An unanticipated result of these studies was that the relative instability and abnormal structure of the extracellular matrix in cells expressing the proα1(I) minigene was associated with a dramatic increase in osteocalcin gene expression, particularly in earlier stages of cellular differentiation. This elevation of osteocalcin expression was in contrast to what was observed in studies in which collagen deposition was merely reduced in quantity by ascorbate deprivation or by collagen synthesis inhibitors; in the latter cases, both alkaline phosphatase activity and osteocalcin gene expression failed to increase during prolonged osteoblast cultures (4, 9).

The stimulation of osteocalcin gene expression in the presence of a rapid matrix turnover suggests a more complex mechanism for its regulation. Basal expression of osteocalcin associated with cellular differentiation (33, 34) is detectable after about 14 days in MC3T3-E1 cells cultured under differentiating conditions (Fig. 8B); in cells that expressed truncated proα1(I) chains, osteocalcin gene expression began at day 7, shortly after collagen deposition is detectable but before the expected onset of basal expression that is associated with cellular differentiation. It is also evident that the up-regulation of osteocalcin expression occurred through a mechanism distinct from that which is initiated by pharmacological doses of 1,25-dihydroxycholecalciferol (vitamin D); however, our experiments do not exclude the possibility that trace amounts of vitamin D present in bovine serum had a permissive effect on osteocalcin expression in cells making an abnormal matrix.

It appeared that the osteocalcin gene responded differently to high rates of turnover than the genes coding for other components of the extracellular matrix (reviewed in Ref. 35). For example, type I collagen, decorin, and osteonectin were not increased, even in longer cultures. Osteocalcin differs from these matrix components in that it is thought to play a role in bone turnover (36–38). The data contained in this report suggest that osteocalcin may be regulated by factors released from osteoblast matrix undergoing high rates of turnover. The identity of that factor or factors, the signal pathway that might be used, and target sequences on the osteocalcin promoter are the objects of further study. It is interesting to note that the abnormal matrix did not induce an increase in the secretion of bone sialoprotein, an approximately 80-kDa protein found in skeletal tissues. Its function is not known, but it has been hypothesized to play a role in attachment of osteoclasts to bone because it contains an RGD sequence and supports the attachment of osteoclasts in vitro, perhaps through ligation of the α3β1 integrin (39).

The mechanism by which deposition of a collagen-rich osteoblast matrix facilitates the transition from immature, rapidly dividing preosteoblasts to differentiated cells is unknown. Type
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I collagen may act directly on cellular receptors, or it might be merely required as a latticework for other matrix proteins which themselves interact directly with cells. It is unclear whether signals initiated by insoluble matrix molecules act directly to promote osteoblast-specific gene expression or whether cell-matrix interactions have indirect effects by which they might change in the repertoire of cellular receptors for hormones or cytokines present in the extracellular matrix (40, 41). These suggest a need for better understanding of extracellular matrix receptors and downstream signaling pathways by which matrix components modulate gene expression in differentiating osteoblasts.

Although stable incorporation of informative genes in MC3T3-E1 cells is a powerful means to alter the extracellular environment of osteoblastic precursor cells, the clonal selection required for this strategy to work allows for the possibility that observed differences in osteoblast characteristics may be due to clonal variation rather than a response to the altered extracellular matrix.

In these studies, several steps were taken to minimize the effect of clonal drift. All control clones were chosen from cells that had been transfected with both pCDNA3 and pMG155 and had undergone clonal selection with G418 but did not express the pro-α1(I) minigene. Second, all clones undergoing selection were “passed” by brief trypsinization in the dishes twice a week to maintain characteristics of differentiated osteoblasts. Finally, several minigene-expressing and nonexpressing clones were examined to confirm that discordant expression of osteocalcin and alkaline phosphatase was a characteristic of clones that expressed the pro-α1(I) minigene.

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