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Expression of osteoblastic markers in cultured human bone and fracture callus cells

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Abstract We compared the expression of osteoblastic markers in cultured human cells isolated from fracture calluses of various histological states of development with that in cells from adult and fetal bone. Adult osteoblasts and all callus cells produced almost exclusively type I collagen, whereas fetal osteoblasts produced also considerable amounts of type III collagen in vitro. 1,25-Dihydroxyvitamin D_3 induced the synthesis of osteocalcin in all bone and callus cells but to varying extents. Fetal bone cells and early-stage callus cells synthesized less than 10% the amount of osteocalcin produced by adult bone cells. Late-stage callus cells produced intermediate levels of osteocalcin. Fetal bone cells and early-stage callus cells responded to parathyroid hormone with a less pronounced increase in intracellular cAMP than did adult bone cells. Late-stage callus cells showed the best response to parathyroid hormone. The activity of alkaline phosphatase was highest in fetal bone cells. These observations show that cells isolated from fetal bone and from fracture callus tissues express a pattern of markers clearly relating them to the osteoblastic lineage. On the basis of the different patterns of osteoblastic markers expressed in vitro we conclude that functionally distinct subtypes of osteoblasts do exist in different mineralized tissues and at different developmental stages.

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Abbreviations *DMEM* Dulbecco's modified Eagle's medium · *FCS* Fetal calf serum · *PTH* Parathyroid hormone · *1,25D₃* 1,25-Dihydroxyvitamin D_3 · *AP* Alkaline phosphatase

Introduction

Formation of bone is not a uniform process, and ossification occurs on different occasions. During early embryonic development the formation of bone tissues is initiated by desmal and endochondral ossification [1]. During fetal, postnatal, and pubertal development the volume, length, and shape of almost all bones are changed by appositional growth, ephiphyseal endochondral bone formation, and dynamic modeling, respectively. In adult bone the functional adaptation on mechanical strain and the role of bone as a reservoir for calcium require locally defined remodeling processes, i.e., the replacement of resorbed bone by the formation of new bone tissue. The repair of fractured bone by the development of a fracture callus comprises another situation in which formation and development of bone tissue take place [2, 3].

Bone is the product of osteoblasts differentiating in multiple steps from pluripotent mesenchymal stem cells. Osteoblasts must fulfill many different functions: rapid cartilage-replacing or de novo formation of bone matrix of low structural complexity in woven bone [4], production of an organic matrix which involves the spatial and chronological synchronization of numerous bone forming cells leading to alternating layers of perfectly paralleled collagen fibrils in lamellar sheets, and the subsequent incrustation of the organic matrix with highly ordered chemically heterogenous mineral [5]. Osteoblastic cells also represent the targets for many hormones involved in calcium homoeostasis [6, 7] and transform systemic signals into locally acting factors, a process which probably directs resorption to places of low mechanical stress [8].

In addition to their involvement in reception and translating of systemic signals for bone resorption, osteoblasts prepare the bone surface for osteoclastic resorption by enzymatically degrading the osteoid [9]. Although this remains far from clear, postresorptional bone formation must also follow precise functional demands [10].

To find out whether all these functions are conferred by a single uniform cell type, or whether different functions are performed by specialized osteoblastic cell types we studied the expression of functionally important osteoblastic markers in cells from fracture callus during different developmental stages in comparison to those from fetal and adult bone.

Materials and methods

Source of cells

Samples of normal femoral compact bone were obtained during surgery from three adults aged 20–30 years and from the femurs of three fetuses (18–21 weeks of gestation) after legal abortion. Late-stage callus tissue was obtained from two male patients aged 30 (patient A) and 15 (patient B) years, respectively. The callus at the mid-diaphysis of the right femur of patient A had to be removed during insertion of an intramedullar rod. The callus from the distal right radius of patient B was removed during orthopedic refracturing and corrective splinting of the radius. The biopsy of early-stage callus tissue was obtained from the proximal humerus of a 25-year-old male patient (patient C) during corrective plate osteosynthesis. None of the patients/fetuses had underlying systemic bone disease. The studies were approved by the local ethics committee.

Isolation and culture of bone and callus cells

To avoid contaminations of nonosseous tissues the greatest care was taken to select and excise regions from which cells were to be isolated. Bone and callus cells were isolated and cultured as described previously [11]. In brief, specimens of cortical bone or apparently well mineralized, cartilage-free callus tissue were cleaned from adherent tissue, minced into small pieces, and incubated for 4 h at 37°C with 400 U/ml collagenase type IV (Sigma). Cells set free by collagenase digestion were discarded. The remaining collagenase-treated particles were placed into Ca^{2+}-free Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum (FCS), ascorbate (50 µg/ml), penicillin (400 U/ml), nethilmycin (50 µg/ml), and glutamine (2 mM). Cells growing out of the particles were cultured until they had reached confluency and trypsinized and used for experiments in the second and third passage.

In vitro labeling and analysis of collagen

Cells seeded at a density of 10^4 cells/cm^2 were cultivated for 3 days under conditions described above before incubation in fresh medium without antibiotics for 24 h. The cells were labeled with 10 µCi L-[5-^{3}H]proline (Amersham, specific activity 35 Ci/mM) in DMEM without serum and glutamine supplemented with fresh ascorbate and β-aminoproprionitrile (100 µg/ml). Cells were scrapped into the medium, lysed by repeated freeze-thaw cycles, and dialysed against 0.5% acetic acid. For analysis of different types of collagen produced in vitro aliquots of the protein-bound radioactivity were incubated for 5 h with 0.1 mg/ml pepsin (Boehringer) at 14°C to degrade noncollagenous material. Thereafter samples were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis using the protocol of the delayed reduction method described by Sykes et al. [12]. After electrophoresis gels were impregnated with 2,5-diphenyloxazole, dried, and exposed to preflashed films (Hyperfilm, Amersham). Fluorograms were scanned with a laser densitometer (Ultroscan XL, Pharmacia) and evaluated using a calibration curve to determine the relative intensities of single bands.

Determination of osteocalcin production

Cells (4×10^4) were seeded into 4 cm^2 wells and cultured under conditions described above for 10 days before the medium was changed to DMEM, 10% FCS with or without 10^{-8} M 1,25-dihydroxyvitamin D_3 ($1,25D_3$; kindly provided by Dr. U. Fischer, Hoffmann-LaRoche AG, Basel, Switzerland), 10^{-8} M vitamin K (Sigma), and ascorbate (50 µg/ml). After 24 h the medium was changed to DMEM, 0.5% FCS with or without $1,25D_3$, vitamin K, and ascorbate as described above. Cells were cultured for 48 h before the conditioned cell culture medium was harvested, and stored at –70°C after the addition of proteinase inhibitors (phenylmethylsulfonylfuoride 50 µg/ml, *N*-ethylmaleimide 50 µg/ml, EDTA 5 mM). Aliquots of the cell culture supernatant were used for the determination of osteocalcin by a commercially available ^{125}I-labeled RIA kit (OSCAtest, Henning, Berlin). The values were corrected by subtraction of osteocalcin concentrations found in unconditioned medium supplemented with 0.5% FCS. Cell number was determined in parallel. All measurements were performed in triplicate.

Response to parathyroid hormone

Cells (4×10^4) were seeded in 4 cm^2 wells and cultured for 10 days with DMEM, 10% FCS. Two hours before the administration of parathyroid hormone (PTH) the medium was changed to DMEM, 0.5% FCS; 15 min before the administration of PTH 0.5 mM isobutylmethylxanthine (Sigma) was added to the culture medium. Cells were incubated for 5 min with or without 10^{-7} M human parathyroid hormone 1–34 (Sigma), 0.5 mM isobutylmethylxanthine in DMEM, 0.5% FCS. The incubation was stopped by placing the cells on ice. Cells were lysed by two sequential extractions in 0.75 ml acidified 65% ethanol at 4°C for 1 h. The content of intracellular cAMP in aliquots of the cell extract was determined by a commercially available ^{125}I-labeled RIA kit (Amersham, single range). All measurements were performed in triplicate.

Determination of the alkaline phosphatase activity

Cells (5×10^4) were lysed in 100 µl Triton buffer (20 mM Tris, pH 10; 0.5 mM $MgCl_2$, 0.1 mM ZnCl, 0.1% Triton X-100). Intracellular alkaline phosphatase (AP) activity was assayed in triplicate using a commercially available kit (Sigma, 104-LL). One unit of AP activity is defined as 1 nM *p*-nitrophenol produced by 10^6 cells/min. All measurements were carried out in triplicate.

Evaluation of data

Mean values were calculated from the respective means of the individual, triplicate samples. Because of low case numbers statistical analysis was not performed.

Results

Histological examination of callus tissue

The histological features of the callus samples obtained from patients A and B showed virtually the same pattern. This callus tissue consisted mainly of moderately hyper-

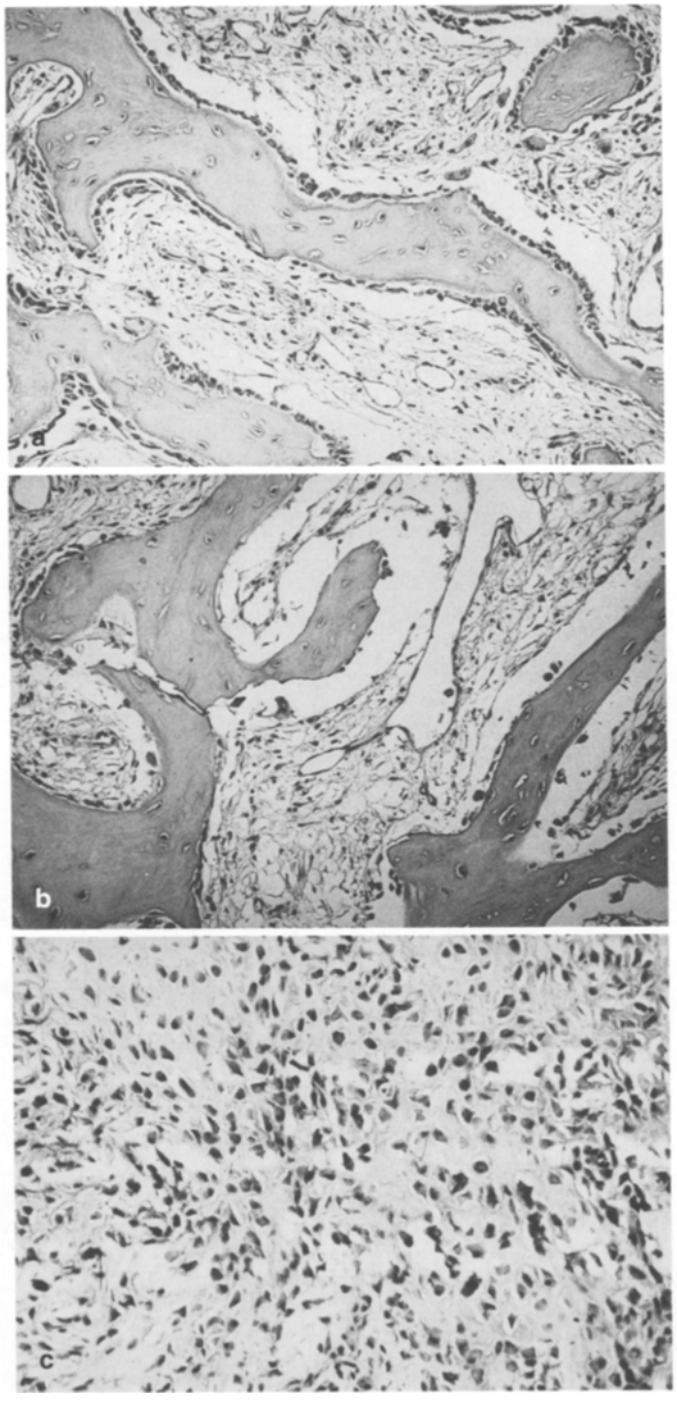

Fig. 1 a–c Histological features of maturating (late-stage) callus tissue (patients A, B) showing trabecular woven bone with broad osteoblastic seams and loosely fibrous tissue in the intertrabecular spaces. H&E, original magnification ×160 **c** Histomorphological aspect of the immature (early-stage) callus tissue (patient C) composed of multiple spindle-shaped cells with some nuclear pleomorphism. Between these cells a scarce latticelike osteoid production is seen. H&E, original magnification ×400

cellular bone trabeculas which were composed of woven bone with increased osteoblastic surface activity. The marrow space was filled with a loosely textured collagenous matrix. Occasionally areas of fibrocartilage were interspersed. No significant inflammatory reaction was present. In the third sample (patient C) representing an earlier stage of callus development – in addition to loosely and in part densely packed collagen fibers – areas of irregulary arranged osteoblastic cells with surrounding scarce osteoid matrix were noted. In these areas high numbers of cells were found. These regions were ill defined, and focally a transition to fibrocartilage was seen. There was no major inflammatory infiltrate.

In summary, all tissue samples showed typical features of normal callus formation, the first two representing advanced stages of a mainly endesmal ossification process (Fig. 1A/B), while the third tissue sample could be attributed to early endesmal (membraneous) ossification (Fig. 1C).

Cell culture and collagen metabolism

The morphology and growth of all bone and callus cells in culture was similar (not shown). The pattern of collagen types produced by cells from early- and late-stage callus and from adult bone consisted predominantly of type I collagen (Table 1). The low levels of type III collagen indicates the absence of significant contaminations by fibroblastic cells in these cell populations. In contrast, cells from fetal bone produced increased amounts of types III and V collagen. The ratio of collagen type I $\alpha 1$ and $\alpha 2$ chains was similar in all bone and callus cells, indicating the absence of major contamination by chondrocytes.

Osteocalcin

In the absence of $1,25D_3$ neither bone nor callus cells produced detectable amounts of osteocalcin (not shown). After incubation with 10^{-8} M $1,25D_3$ all bone and callus cells secreted osteocalcin although in different amounts (Table 2). This is in contrast to skin fibroblasts which were analyzed in parallel and did not produce detectable amounts of osteocalcin (≤ 0.01 ng/10^6 cells) even in the presence of $1,25D_3$ (not shown). Cells from the immature callus produced low but clearly detectable amount of osteocalcin. Levels of osteocalcin secreted by adult bone cells were about ten times higher than in fetal bone cells. Cells from late-stage calluses produced levels of osteocalcin ranging between fetal and adult osteoblasts.

Response to parathyroid hormone

Incubation with 10^{-7} M human PTH increased the intracellular level of cAMP in all cells (Table 2). Cells from early-stage callus and from fetal bone responded less

Table 1 Qualitative analysis of collagen produced by bone and callus cells in vitro. Cultures of cells established from the indicated tissues were labeled for 24 h with [^{3}H]proline. The pattern of collagen types was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and densitometric evaluation of pepsin-digested samples

	Bone cells		Callus cells	
	fetal n=3[a]	adult n=3[a]	early stage n=1	late stage n=2
Relative amounts (%) of				
collagen type I[b]	89.6 ± 1.2	96.3 ± 0.9	96	98/98
collagen type III[b]	9.2 ± 0.8	3.3 ± 0.4	4	2/2
collagen type V[b]	1.2 ± 0.2	0.4 ± 0.2	0	0/0
α1(I)/α2(I)	2.3 ± 0.2	2.4 ± 0.3	2.3	2.2/2.6

[a] Mean ± SEM of different individuals
[b] Percentage of total collagen

Table 2 1,25-dihydroxyvitamin D_3 induced amounts of osteocalcin, PTH response and alkaline phosphatase activity of human bone and callus cells in vitro. Cell cultures established from the indicated tissues were incubated with 1,25-dihydroxyvitamin D_3 and osteocalcin was determined by RIA in the medium supernatant. Intracellular level of cAMP was determined by RIA after incubation of the cells with/without 10^{-7} M hPTH. Activity of alkaline phosphatase was determined in cell lysates cleaving paranitrophenolphosphate.

	Bone cells		Callus cells	
	fetal n=3[a]	adult n=3[a]	early stage n = 1	late stage n=2
Osteocalcin[b]	7.4±2.8	107 ±37.0	1.1	45.6/42.6
Response to PTH[c]	4.2±1.7	11.5±2.3	4.8	17.3/32.6
AP activity[d]	137 ±42	76 ±38	40	31.3/58

[a] Mean ± SEM of different individuals
[b] ng 10^{-6} cells
[c] relative increase of intracellular cAMP induced by 10^{-7} M PTH in relation to basal intracellular cAMP
[d] nmol Pi 10^{-6} cells min^{-1}

than cells from adult bone. Cells from the late-stage calluses showed the most pronounced response to PTH.

Alkaline phosphatase acitivty

High activities of alkaline phosphatase were found in cultures of all bone and callus cells examined. AP activity was higher in fetal bone cells than in adult bone cells (Table 2). Cells from early- and late-stage calluses expressed AP activities similar to adult bone cells.

Discussion

The present study is the first report on the expression of markers indicating the osteoblastic phenotype not only in cultured cells from fetal and adult human bone [13], but also in cells from human fracture calluses of different developmental stages. The pattern of markers expressed by bone and callus cells in vitro clearly designates all cells under examination as belonging to the osteoblastic lineage [14] although single parameters showed a broad range of expression in the different cell types. Diversity of the osteoblastic phenotype has been described previously by authors studying cloned bone cells or cell populations isolated from the same tissue sample by sequential enzymatic release [15, 16]. The isolation of cells expressing different patterns of osteoblastic markers from one tissue was interpreted to reflect the process of maturation of osteoblastic cells starting with the undifferentiated fibroblastlike cell type and ending with the mature active osteoblast. On the other hand, the presence of active osteoblasts, osteocytes, and bone lining cells indicates that distinct types of stably differentiated cells of the osteoblastic lineage coexist within one tissue [6, 17]. Our observations suggest that distinct subtypes of osteoblastic cells predominate in different mineralized tissues corresponding to their developmental and functional stage.

Obviously there are some discrepancies between in vitro and in vivo observations. First of all, in vivo mature osteoblasts or osteocytes do not proliferate, but propagation of these cells in vitro selects for proliferation. The expression of specific osteoblastic markers in proliferating cell poulations indicates that cell division does not affect the phenotypic expression of differentiated functions. Furthermore, the elevated expression of types III and V collagen in fetal bone cells is in agreement with previous results on native bone tissue [20]. The actual quantitative level of collagen production, AP activity, and other features of cells in vitro, however, may rather reflect the metabolic capacities of these cells than the actual in vivo activities of the cells, which depend largely

on the presence or absence of growth factors and other parameters of the local environment.

Despite these restrictions our observations might be interpreted in the following way taking into account the functional demands of the different tissues: High activity of AP in cells derived from fetal bone indicates progessive mineralization during appositional and enchondral ossification processes. The increased response of adult bone cells and particularly of late-stage fracture callus cells to PTH and vitamin D_3 indicates a high sensitivity of both cell types to signals regulating bone resorption in relation to calcium homeostasis. This may be due to the well-known function of adult bone to serve not only as mechanical support but also as an ion reservoir. The poor response of fetal bone cells and early-stage callus cells to PTH and vitamin D_3 might be interpreted in the sense that in developing bone the ion reservoir function is of minor importance. Another important parameter distinguishing cells from mature and developing bony tissues is the capacity to produce osteocalcin. Recent publications associate the appearance of osteocalcin with a late phase in bone matrix formation [22], possibly signaling the availability for osteoclastic bone resorption [23, 24]. Our observations suggest a correlation of the capacity to synthesize large amounts of osteocalcin in vitro and the origin of these cells from bone tissue in which remodeling processes predominate modeling and growth.

In conclusion, the results of our studies on bone and callus cells in vitro are consistent with the hypothesis about the predominance of distinct subtypes of osteoblastic cells in mineralized tissues of different developmental states reflecting different functional demands of their tissues of origin.

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