

ORIGINAL ARTICLE

Circulating Osteoblast-Lineage Cells in Humans

Guiti Z. Eghbali-Fatourechi, M.D., Jesse Lamsam, M.S., Daniel Fraser, Ph.D.,
David Nagel, A.B., B. Lawrence Riggs, M.D., and Sundeep Khosla, M.D.

ABSTRACT

BACKGROUND

Although current evidence suggests that only a minuscule number of osteoblast-lineage cells are present in peripheral blood, we hypothesized that such cells circulate but that their concentration has been vastly underestimated owing to the use of assays that required adherence to plastic. We further reasoned that the concentration of these cells is elevated during times of increased bone formation, such as during pubertal growth.

METHODS

We used flow cytometry with antibodies to bone-specific proteins to identify circulating osteoblast-lineage cells in 11 adolescent males and 11 adult males (mean [\pm SD] age, 14.5 \pm 0.7 vs. 37.7 \pm 7.6 years). Gene expression and *in vitro* and *in vivo* bone-forming assays were used to establish the osteoblastic lineage of sorted cells.

RESULTS

Cells positive for osteocalcin and cells positive for bone-specific alkaline phosphatase were detected in the peripheral blood of adult subjects (1 to 2 percent of mononuclear cells). There were more than five times as many cells positive for osteocalcin in the circulation of adolescent boys (whose markers of bone formation were clearly increased as a result of pubertal growth) as compared with adult subjects ($P < 0.001$). The percentage of cells positive for osteocalcin correlated with markers of bone formation. Sorted osteocalcin-positive cells expressed osteoblastic genes, formed mineralized nodules *in vitro*, and formed bone in an *in vivo* transplantation assay. Increased values were also found in three adults with recent fractures.

CONCLUSIONS

Osteoblast-lineage cells circulate in physiologically significant numbers, correlate with markers of bone formation, and are markedly higher during pubertal growth; therefore, they may represent a previously unrecognized circulatory component to the process of bone formation.

From the Endocrine Research Unit, Mayo Clinic College of Medicine, Rochester, Minn. Address reprint requests to Dr. Khosla at the Endocrine Research Unit, Mayo Clinic College of Medicine, 200 First St., SW, 5-194 Joseph, Rochester, MN 55905, or at khosla.sundeep@mayo.edu.

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BONE MARROW CONTAINS BOTH OSTEOBLAST and osteoclast precursors that can differentiate into the mature osteoblasts and osteoclasts, respectively,¹ that are believed to be needed for normal bone remodeling on trabecular surfaces contiguous to bone marrow. In addition to residing in the bone marrow, substantial numbers of osteoclast precursors are detectable in the peripheral circulation,² and these cells may be able to travel to sites of active bone remodeling distant from red marrow. However, it is unclear whether a parallel process involving circulating osteoblast-lineage cells exists and, if so, what role these osteoblastic cells might play in normal or pathologic bone remodeling.

Osteoblastic cells in the bone marrow have traditionally been identified by their adherence to standard tissue-culture plastic and by the subsequent formation of mineralized nodules.^{3,4} Using these criteria, previous investigators have provided evidence that osteoblastic cells that adhere to plastic are, indeed, present in the circulation in some species,^{5,6} but in exceedingly low numbers (less than 1 in 10⁸ peripheral-blood mononuclear cells in adult humans⁶).

In addition to these adherent osteoblastic cells, Long et al.^{7,8} have identified a population of cells in the bone marrow that have osteogenic potential and are nonadherent and positive for osteocalcin, bone-specific alkaline phosphatase, or both. Moreover, a recent study showed that bone marrow cells that do not adhere to plastic have bone-repopulating activity in lethally irradiated mice that is more than 10 times as great as that of adherent cells from the marrow.⁹ If nonadherent osteogenic bone marrow cells are present in the peripheral circulation, assays that rely on adherence to plastic would detect only the rare adherent circulating osteoblastic cell,^{5,6} resulting in a substantial underestimation of the actual number of osteoblastic cells in the circulation.

We reasoned that the use of flow cytometry with antibodies to bone-specific proteins (osteocalcin and alkaline phosphatase) would better identify circulating osteoblast-lineage cells. Furthermore, if these circulating osteoblastic cells play a physiologic role in bone formation, their concentration in peripheral blood would increase under conditions of increased bone formation. Therefore, we determined the concentration of these cells in the circulation in adult men as compared with adolescent boys, who had markedly increased bone-formation indexes, since they were going through the pubertal growth spurt.

METHODS

STUDY SUBJECTS

Study subjects included 11 consecutively recruited healthy adult males 28 to 49 years of age and 11 consecutively recruited adolescent boys 13 to 15 years of age. For some studies, buffy coats were obtained from male subjects at the Mayo Transfusion Center. All studies were approved by the Mayo Clinic's institutional review board, and all subjects, or their parents, provided written informed consent; subjects less than 18 years of age provided assent. The study was conducted between May 2003 and January 2005.

SERUM BIOCHEMICAL AND HORMONAL MEASUREMENTS

Serum osteocalcin was measured with a two-site immunoradiometric assay (ELSA-OSTEO, CisBio) (interassay coefficient of variation, less than 12 percent), and serum bone alkaline phosphatase was measured by immunoassay (Quidel) (interassay coefficient of variation, less than 8 percent). Serum insulin-like growth factor I (IGF-I) and IGF-binding protein 3 were measured with immunoradiometric assays (Diagnostic Systems Laboratories) (interassay coefficient of variation, less than 15 percent for both). Serum testosterone was measured by a competitive immunoassay (Access Immunoassay System, Beckman Coulter) (coefficient of variation, less than 10 percent), and serum estradiol was measured with a double antibody radioimmunoassay (Diagnostic Products) (coefficient of variation, less than 8 percent).

EXTRACTION AND IMMUNOSTAINING OF MONONUCLEAR CELLS

Samples of whole blood or buffy coats were layered over Ficoll-Paque density gradients, and mononuclear cells were isolated and subsequently processed for immunostaining as previously described.¹⁰ Primary antibodies were a monoclonal anti-bone alkaline phosphatase (B4-78 hybridoma, Developmental Studies Hybridoma Bank, University of Iowa) or a goat polyclonal antihuman osteocalcin (Santa Cruz Biotechnology) antibody. Control isotype antibodies were used at the same concentrations as the primary antibodies. An anti-CD15 antibody (Becton Dickinson), a granulocyte-specific marker, excluded contamination of isolated cells with granulocyte lineages. After incubation with primary antibodies, cells were stained with conjugated secondary antibodies including phycoerythrin-conjugated Affini-

tyPure f(ab')₂ fragment donkey antimouse IgG and fluorescein isothiocyanate-conjugated AffinityPure IgG f(ab')₂ fragment donkey antigoat (Jackson ImmunoResearch) antibodies.

FLOW CYTOMETRY AND FLUORESCENCE-ACTIVATED CELL-SORTER ANALYSIS

We used the procedure for flow cytometry that was previously described by our laboratory.¹⁰ For some studies, mononuclear cells stained with the anti-osteocalcin or anti-bone alkaline phosphatase antibody and the corresponding secondary antibody were also sorted by flow cytometry (FACSVantage SE, Becton Dickinson) directly into a growth medium consisting of MesenCult Basal Medium supplemented with 15 percent osteogenic fetal-calf serum (StemCell Technologies).

PRIMARY CULTURE OF OSTEOBLASTIC CELLS

Sorted cells were suspended in growth medium and plated at a density of 1×10^5 cells per well in fibronectin-coated plates (Becton Dickinson). On days 8 and 14 after plating, supernatants that contained nonadherent cells were centrifuged, the media were changed, and nonadherent cell pellets were returned to the original wells. On day 21 and then weekly thereafter, media were changed to the differentiation medium (MesenCult Basal Medium with 15 percent osteogenic stimulatory supplements, further supplemented with a 3.5 mM final concentration of β -glycerophosphate, 10^{-8} M dexamethasone, and 50 μ g per milliliter of ascorbic acid [StemCell Technologies]), each time preserving nonadherent cells. After three weeks in the differentiation medium, cells were fixed and stained with von Kossa's stain to detect any calcified nodules.

RNA EXTRACTION AND REAL-TIME REVERSE-TRANSCRIPTASE-POLYMERASE-CHAIN-REACTION (RT-PCR) ANALYSIS

Total RNA was extracted from cells collected by fluorescence-activated cell sorting with Absolutely RNA Microprep kit (Stratagene). A complementary-DNA template was constructed with the use of AMV Reverse Transcriptase, Random Primer p(dN)₆, RNase inhibitor, deoxynucleoside triphosphates, and oligodT (Roche). PCR analyses were performed on the iCycler iQ/Real-Time PCR Detection System apparatus with a 2X iQ SYBR Green Supermix (Bio-Rad). Gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase.

IN VIVO BONE-FORMING ASSAY

Osteocalcin-positive or osteocalcin-negative peripheral-blood cells that had been sorted by fluorescence-activated cell sorting, as well as unsorted peripheral-blood mononuclear cells (10^6 cells each), were suspended in 1 ml of alpha-Dulbecco's modified Eagle's medium (Invitrogen) and mixed with 40 mg of hydroxyapatite and tricalcium phosphate powder (in a 20:80 ratio; particle size, 0.1 to 0.5 mm; Berkeley Advanced Biomaterials). A secondary matrix was formed by mixing 15 μ l of mouse fibrinogen (3.2 mg per milliliter in phosphate-buffered saline) and 15 μ l of mouse thrombin (25 U per milliliter in 2 percent calcium chloride; Sigma-Aldrich) to form a solid fibrin gel. The transplants were placed in subcutaneous pockets in an eight-week-old immunocompromised mouse (Beige Nude XIX, Harlan). After eight weeks, we looked at radiographs of the pellets, and then the pellets were recovered, placed in 70 percent ethanol, and analyzed with the use of quantitative computed tomography¹¹ (Stratec XCT Research SA+, Orthometrix).

HISTOLOGY

The pellets were dehydrated in 95 percent ethanol for one day and then in 100 percent ethanol for five days before they were immersed for four days in glycol methylmethacrylate and embedded by controlled polymerization. The samples were then cut into 5- μ m sections and stained with Goldner's stain.¹²

STATISTICAL ANALYSIS

All data are presented as means \pm SD. Comparisons between the adult and adolescent subjects were performed with the use of unpaired t-tests. Spearman rank correlations were used to relate serum biochemical and hormonal measurements to the percentage of cells positive for osteocalcin or bone alkaline phosphatase. A P value of less than 0.05 was considered significant, and all reported P values are two-sided.

RESULTS

Table 1 shows the biochemical and hormonal data of the study subjects. Owing to the dramatic increase in bone formation during pubertal growth, the adolescent boys had significantly increased indexes of bone formation as well as increased serum levels of IGF-I and IGF-binding protein 3, reflect-

ing increased secretion of growth hormone.¹³ Serum testosterone levels were similar in the two groups, whereas serum estradiol levels were lower in the adolescent boys than in the adult men.

As shown in Figure 1, cells positive for osteocalcin, but not cells positive for bone alkaline phosphatase, were more than five times as great in the circulation of the adolescent boys as in the adult men (mean percentage, 5.13 vs. 0.93). Moreover, in the two groups of subjects combined, the percentage of osteocalcin-positive cells significantly correlated with serum levels of osteocalcin and bone alkaline phosphatase ($r=0.72$ and $r=0.77$, respectively; $P<0.001$ for both). The percentage of osteocalcin-positive cells was also positively correlated with serum levels of IGF-I and IGF-binding protein 3 ($r=0.74$, $P<0.001$, and $r=0.61$, $P<0.01$, respectively). Although serum testosterone levels were not correlated with the percentage of osteocalcin-positive cells ($r=-0.12$, $P=0.62$), there was a suggestion of an inverse association between these cells and serum estradiol levels ($r=-0.40$, $P=0.09$). No significant associations were noted between markers of bone turnover and the percentage of cells positive for bone alkaline phosphatase, or between the hormonal variables and the percentage of cells positive for bone alkaline phosphatase (data not shown).

In addition to the increases in bone formation during the adolescent growth spurt, there are also

significant increases after a fracture.¹⁴ One adult subject in this study had a severe fracture of the distal forearm six weeks after his initial data were collected. Thus, we had the serendipitous opportunity to examine sequential changes in his osteocalcin-positive and bone alkaline phosphatase-positive cells before and after the fracture. A blood sample obtained 20 days after the fracture showed a more than ninefold increase in circulating osteocalcin-positive cells (from 0.6 to 5.9 percent) and a twofold increase in circulating cells positive for bone alkaline phosphatase (from 1.9 to 4.1 percent) as compared with the percentages at baseline.

We subsequently studied two additional men, 40 and 34 years of age, 10 days after fractures of the distal forearm and tibial shaft, respectively. The proportions of circulating osteocalcin-positive cells in these subjects were 3.7 and 2.2 percent, respectively, and those of bone alkaline phosphatase-positive cells were 3.0 and 8.0 percent, respectively. The combined mean values for cells positive for osteocalcin and bone alkaline phosphatase in the three patients studied after fractures were 3.9 percent and 5.0 percent, respectively. Relative to the normal means for these cells in age-matched subjects (Fig. 1), these percentages represent approximately fourfold and twofold increases, respectively.

We performed a number of studies to characterize more completely the cells that were positive for osteocalcin and bone alkaline phosphatase. Since platelets can express low levels of osteocalcin,¹⁵ the possibility that platelets confounded the results was addressed by testing a sample of platelet-rich plasma and applying the appropriate gates to the flow cytometry data for mononuclear cells, as was done in all analyses.¹⁰ None of the cells in this gate setting expressed osteocalcin (data not shown). Similarly, granulocyte-lineage cells in peripheral blood express alkaline phosphatase.¹⁶ However, the gating around mononuclear cells effectively excluded virtually all CD15+ cells (a granulocyte marker), and double staining with an anti-CD15 antibody and the anti-bone alkaline phosphatase antibody indicated that less than 0.1 percent of the mononuclear cells coexpressed both markers.

Since the percentages of circulating osteocalcin-positive cells were markedly increased during the adolescent growth spurt, we performed further studies of these cells. The ability of sorted osteocalcin-positive cells to form mineralized nodules *in vitro* was assessed. Culture of these cells required

Table 1. Age and Serum Biochemical and Hormonal Data in the 11 Adult and 11 Adolescent Males.*

Characteristic	Adult Males	Adolescent Males	Difference (95% CI)	P Value
Age — yr	37.7±7.6	14.5±0.7	—	—
Osteocalcin — ng/ml	25±4	145±58	120 (84 to 156)	<0.001
Bone alkaline phosphatase — U/liter	29±9	148±64	119 (78 to 159)	<0.001
IGF-I — ng/ml	249±93	629±232	380 (223 to 537)	<0.001
IGF-binding protein 3 — ng/ml	4325±900	5604±1006	1279 (433 to 2124)	0.005
Testosterone — ng/dl	340±126	318±163	22 (-107 to 151)	0.73
Estradiol — pg/ml	26±7	16±10	10 (3 to 18)	0.01

* Plus-minus values are means ±SD. CI denotes confidence interval, dashes not applicable, and IGF insulin-like growth factor. To convert values for osteocalcin to nanomoles per liter, multiply by 0.172. To convert values for IGF-I to nanomoles per liter, multiply by 0.13. To convert values for IGF-binding protein 3 to nanomoles per liter, multiply by 0.035. To convert values for testosterone to nanomoles per liter, multiply by 0.3467. To convert values for estradiol to picomoles per liter, multiply by 3.671.

the preservation of nonadherent cells. However, the majority of the cells gradually became adherent, and when osteocalcin-positive cells were placed in osteoblast-differentiation medium, they could form mineralized nodules (Fig. 2). In addition, freshly sorted osteocalcin-positive cells, as compared with unsorted cells and as compared with osteocalcin-negative cells, were markedly enriched for expression of the bone-related genes — osteocalcin, bone alkaline phosphatase, and collagen type I ($\alpha 2$ chain) (Fig. 3).

An *in vivo* transplantation assay in which osteocalcin-positive, osteocalcin-negative, or unsorted peripheral-blood mononuclear cells were implanted subcutaneously into immunocompromised mice was used to obtain further evidence of the osteogenic potential of the osteocalcin-positive cells. As shown in Figure 4A, both radiography and quantitative computed tomography showed higher radiodensity and volumetric bone mineral density in the area of the osteocalcin-positive cell suspensions than in the osteocalcin-negative cells. Histologic examination of the implanted cell suspensions showed clear evidence of mineralized bone formation by the osteocalcin-positive cells, which was lacking in the osteocalcin-negative cells (Fig. 4B). Figure 4C is a higher-power view of the mineralized bone formed in the osteocalcin-positive implant. In the same sample, polarized light shows the presence of lamellar bone (Fig. 4D).

Finally, additional studies were performed to characterize circulating cells positive for bone alkaline phosphatase. Gene expression studies using real-time RT-PCR indicated that cells positive for bone alkaline phosphatase were also enriched for bone-related genes (2-fold for osteocalcin, >200-fold for bone alkaline phosphatase, and 5-fold for collagen type I, relative to unsorted cells). In addition, in preliminary studies, these cells seem to be able to be induced to form mineralized nodules *in vitro*, similar to the osteocalcin-positive cells (data not shown).

DISCUSSION

The present study provides clear evidence that osteoblast-lineage cells are present in the human circulation in significant numbers and that the percentage of circulating osteocalcin-positive cells is higher during the adolescent growth spurt, which is associated with a marked stimulation of bone

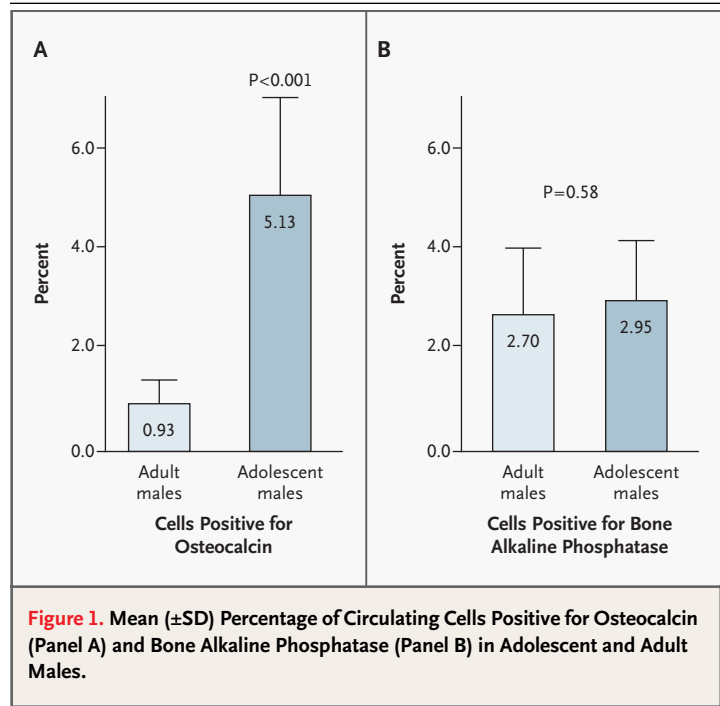


Figure 1. Mean (\pm SD) Percentage of Circulating Cells Positive for Osteocalcin (Panel A) and Bone Alkaline Phosphatase (Panel B) in Adolescent and Adult Males.

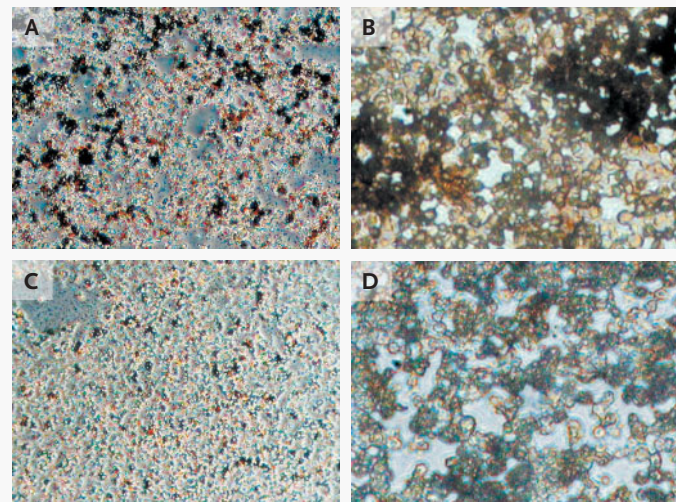


Figure 2. Formation of Mineralized Nodules *In Vitro* by Osteocalcin-Positive Cells.

Sorted osteocalcin-positive cells (Panels A and B) and unsorted cells (Panels C and D) were cultured for three weeks to expand cell numbers. Nonadherent cells were preserved with each change of medium. The osteocalcin-positive and unsorted cells were then placed in osteoblast-differentiation medium, where the osteocalcin-positive cells formed mineralized nodules, shown as the dark areas in Panels A and B (von Kossa's stain).

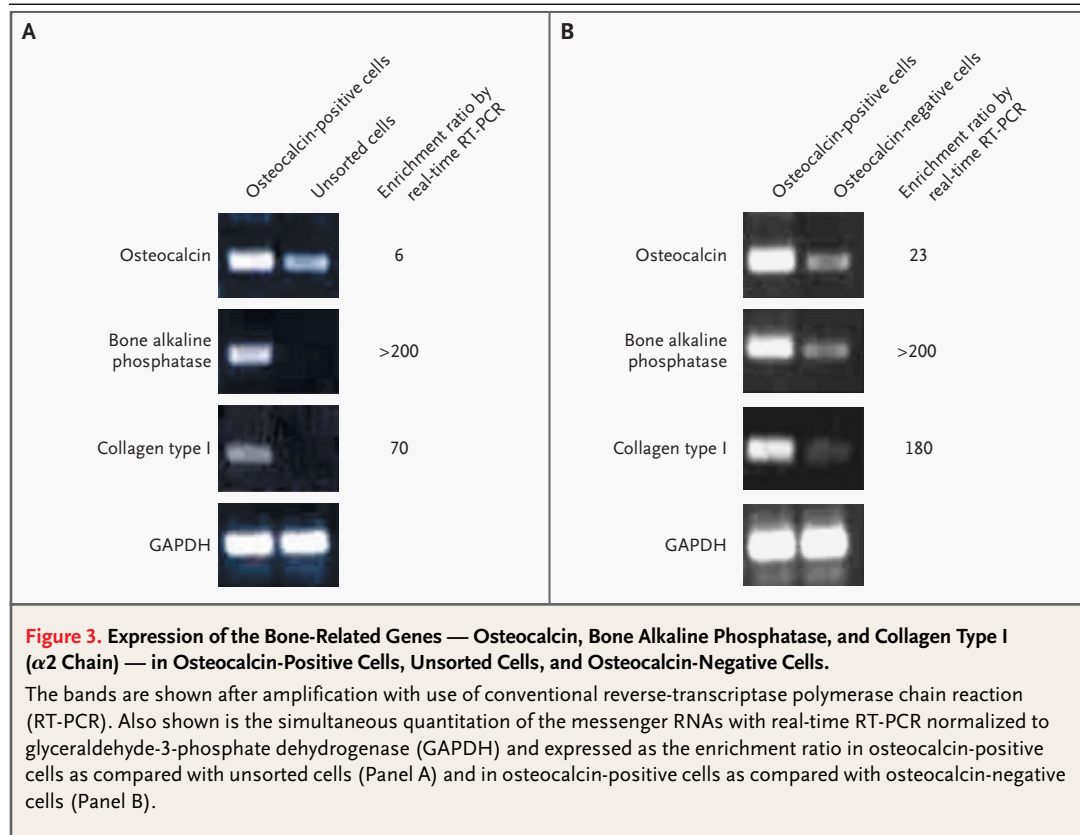
formation, than in adulthood.¹⁷ Previous studies showed the presence of these cells in peripheral blood in humans,^{5,6} but at exceedingly low concentrations. Indeed, in this study, we detected concentrations of these cells in the human circulation approximately six orders of magnitude greater than previously measured.⁶ The principal reason for this difference is that earlier studies required adherence to plastic as a criterion for selecting these cells.^{3,4} Since the majority of circulating osteoblast-lineage cells may lack the capacity to adhere to plastic, we used a different approach, involving the detection of bone-related proteins on the cell surface.

The number of circulating osteocalcin-positive cells correlated positively with serum levels of osteocalcin and bone alkaline phosphatase. Moreover, they correlated with serum levels of IGF-I and IGF-binding protein 3. Since IGF-I and IGF-binding protein 3 reflect growth hormone secretion,¹³ it is possible that increases in circulating osteocalcin-positive cells during puberty are driven, at least in part, by IGF-I, growth hormone, or both. Although these associations collectively provide evidence in support of the biologic relevance of increases in

circulating osteocalcin-positive cells during adolescence, further studies are needed to define more precisely the factors regulating the concentration of these cells in peripheral blood. In addition, whether the increase in circulating osteocalcin-positive cells during growth is due to an increase in the pool of precursors or to less programmed cell death is, at present, an open question.

The data from the three patients with fractures, which included sequential changes in the percentage of circulating osteoblastic cells before and after fracture in one patient, indicate that the concentration of both circulating cells positive for osteocalcin and cells positive for bone alkaline phosphatase may also increase after fractures. Why both populations of cells may be elevated after fracture but only populations of osteocalcin-positive cells increase in adolescent boys is unclear. Our findings suggest that the cells positive for osteocalcin and bone alkaline phosphatase probably represent somewhat different subpopulations of cells, indicating a need for further characterization.

Of the three patients with fractures, the one with the most clinically severe fracture had the highest



percentage of osteocalcin-positive cells. Although studies are lacking that correlate the severity of fracture with the increase in circulating osteoblastic cells, as well as the time course of changes in these cells after fracture, the present findings raise the possibility that circulating cells positive for bone alkaline phosphatase, osteocalcin, or both play a functional role in healing fractures in portions of the peripheral skeleton not adjacent to red marrow. Indeed, studies in mice have shown that systemically infused bone marrow stromal cells can localize to the fracture callus and potentially participate in fracture repair.¹⁸

The present findings, although new, might be anticipated, given the context of extensive work by Long and colleagues,^{7,8} who showed that a population of nonadherent bone marrow cells expressing osteocalcin, bone alkaline phosphatase, or both on their surface also have the capacity to differentiate into mature osteoblasts. In addition, there is recent evidence of highly osteogenic cells in the nonadher-

ent cell population from bone marrow.⁹ It is plausible that the cells we have detected in peripheral blood are identical to the nonadherent osteogenic cells from the bone marrow that have previously been identified,⁷⁻⁹ but further detailed characterization of both cell populations would be required to test this possibility. In addition, how the nonadherent osteocalcin-positive and bone alkaline phosphatase-positive cells differ from the classic, plastic-adherent osteoblastic cells from the bone marrow^{3,4} remains to be better defined.

Our observations may resolve a fundamental inconsistency in the traditional view of bone remodeling that has arisen from the work of Hauge and colleagues.¹⁹ It has long been known that remodeling in cancellous bone occurs on the surfaces of trabeculae, at the interface of the bone and bone marrow. Remodeling involves both osteoclasts and osteoblasts, which form the basic multicellular unit.²⁰ Since osteoblasts are known to differentiate from mesenchymal precursors in the bone marrow,³ the

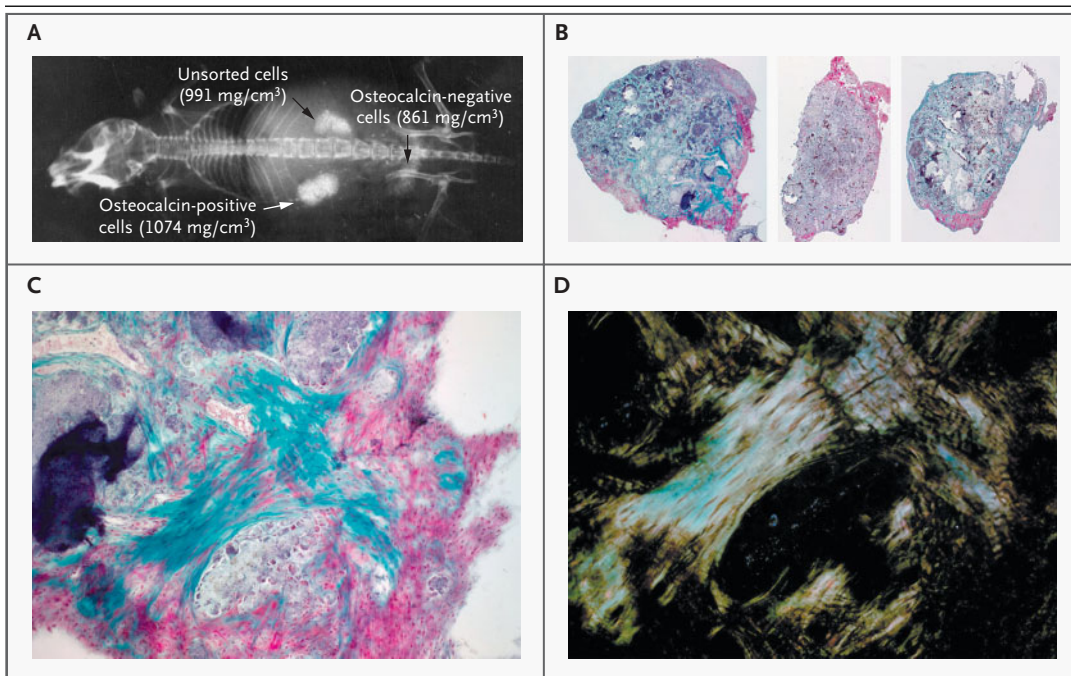


Figure 4. Evidence of the Osteogenic Potential of Osteocalcin-Positive Cells Implanted into Immunocompromised Mice.

Panel A shows a radiograph of an immunocompromised mouse that was implanted with cell suspensions containing osteocalcin-positive cells, osteocalcin-negative cells, and unsorted cells. The numbers indicate the volumetric bone mineral density of the cell suspensions, measured by quantitative computed tomography. In Panel B, Goldner's staining, in which turquoise indicates mineralized bone, highlights histologic sections of the cell suspensions containing osteocalcin-positive cells (left image), osteocalcin-negative cells (center image), and unsorted cells (right image). Panel C shows a higher-power view of the histologic sections of the cell suspensions containing the osteocalcin-positive cells, showing clear areas of bone formation, which, under polarized light (Panel D), demonstrate the presence of lamellar bone.

traditional view held that osteoblasts traveled directly from the marrow to bone surfaces as the need arose in each basic multicellular unit. However, Hauge et al.¹⁹ provided convincing histologic evidence that each basic multicellular unit has a roof of flattened cells that form a protective compartment over most sites of remodeling in cancellous bone, which raises the question of how osteoblastic cells in the marrow are able to gain access to the bone surface. Since the protective canopy of cells is often penetrated by capillary sinusoids of the marrow,¹⁹ our data suggest that the previously identified nonadherent osteogenic cells from the marrow⁷⁻⁹ may traverse these sinusoids and thus gain access to the basic multicellular unit. As part of this process, these cells probably also access the peripheral circulation.

In conclusion, the present study indicates that

osteoblast-lineage cells are present in the human circulation in physiologically relevant numbers. Collectively, our data suggest a circulatory component to the process of bone formation and raise the possibility that these cells may also be involved in the healing of fractures. Moreover, circulating osteoblast-lineage cells might also have therapeutic potential. Developing a method to harvest these cells from peripheral blood, expand them in culture, and subsequently implant them into sites of impaired bone healing might lead to new approaches to bone regeneration.

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