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**Brief Definitive Report**

**Human Osteoblasts Support Hematopoiesis through the Production of Granulocyte Colony-stimulating Factor**

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**Summary**

Previous attempts at identifying the constitutive source(s) of granulocyte colony-stimulating factor (G-CSF) in human bone marrow have been unsuccessful despite the fact that normal bone marrow supports abundant myelopoiesis in vivo. We hypothesized that the intimate physical association between bone and hematopoietic cells facilitates interactions between osteoblasts and hematopoietic stem cells. Here we provide the first direct evidence that human osteoblasts participate in hematopoiesis by constitutively producing G-CSF and present the protein in a membrane-associated fashion to human hematopoietic progenitors. These results suggest a direct and central role for osteoblasts in normal myelopoiesis.

Hematopoietic stem cell differentiation after birth is largely restricted to the bone marrow cavity. Whether osteoblasts and bone matrix provide essential adhesive, stimulatory, or regulatory signals to stem cells is not known. Overall, the unique physiological conditions that bone tissue provides for hematopoietic cells is essentially a mystery. Mesenchymal cells of fibroblast origin, dispersed throughout the marrow cavity, secrete cytokines such as GM-CSF, IL-6, and c-kit ligand to support basal hematopoiesis, and respond to inflammatory monokines by secreting large quantities of GM-CSF and G-CSF (1-7). This cytokine secretion profile is not, however, specific to bone marrow and is shared by fibroblasts from many tissues. In addition, in the absence of inflammation human fibroblasts do not produce G-CSF, and the source of basal G-CSF production that supports normal granulopoiesis is unknown (6, 7).

The development of the bone marrow cavity is a coordinated process in which blood precursors migrate and colonize spaces carved out of embryonic bone and cartilage (8). Thus, an intimate physical association between bone cells and blood cells is established early in life. Since (a) osteoblasts are found on endosteal marrow surfaces; (b) primitive hematopoietic stem cells are closely approximated with the endosteal surfaces rather than randomly distributed throughout the marrow cavity; and (c) many stromal cell lines share several phenotypic characteristics with osteoblasts (9-14), we hypothesized that osteoblasts might directly support stem cell survival and/or differentiation. In particular we asked whether osteoblasts might be a heretofore undetermined source of constitutively produced G-CSF in the bone marrow microenvironment.

**Materials and Methods**

**Human Osteoblasts.** Human osteoblasts were obtained using a modification of methods described by Robey and Termine (15). Normal human trabecular bone was obtained from patients undergoing orthopedic surgery in accordance with the University of Michigan’s Policies for Human Subjects. Bone cleaned of loosely adherent tissue was ground to produce a uniform particle size (size <1 mm²) (BioComp MinimiU; W. Lorenz, Jacksonville, FL) and incubated in 1 mg/ml bacterial collagenase (Type P; Boehringer Mannheim Biologicals, Indianapolis, IN). The explants were placed into culture until confluent monolayers were produced in a 1:1 (vol/vol) mixture of F12/DMEM medium (Biofluids Inc., Rockville, MD) with low Ca²⁺ and 10% FCS. Thereafter, cultures were maintained in calcium-replete DMEM/F12 (1:1 vol/vol) medium containing 10% heat inactivated FCS, antibiotics, 10 mM β-glycerophosphate, and 10 mg/ml l-ascorbate. To verify that the cells were osteoblasts, several histochemical assays were performed including in vitro mineralization, and expression of high levels of alkaline phosphatase (16).

**RNA Preparation.** Total cellular RNA was recovered from osteoblasts as reported in (7). RNA quantity and integrity were checked by gel electrophoresis with ethidium bromide and absorbance at A₂₆₀/A₂₈₀.

**Oligonucleotide Primers.** Sense and antisense primers were prepared by the oligonucleotide synthesis core at the University of Michigan. The primers used in these investigations were reported previously (7) with the exceptions of those for osteocalcin (nucleotides 1046-1066 sense) GGCAGCGAGGTAGTGAAGAG and (nucleotides 1364-1384, antisense) GATGTGGTCAGCACA-TG.

**Reverse Transcriptase Polymerase Chain Reaction (RT-PCR).** RT-PCR was performed for 35 cycles as previously reported (7). PCR products were electrophoresed in 3% agarose and visualized with...
ethidium bromide. As positive cytokine controls, RNA was isolated from either peripheral blood lymphocytes stimulated with PHA (3.0 μg/ml) and PMA (3 ng/ml) or IL-1α (25 U/ml) stimulated human bone marrow stromal cells. To control for DNA contamination, reverse transcriptase was omitted from the reverse transcriptase reaction.

ELISAs. G-CSF ELISAs were performed using the double-antibody sandwich method (R&D Systems Inc., Minneapolis, MN). Based upon parallel assays of known diluted standards, the sensitivities of the assays for G-CSF in unconcentrated medium is 5–2,000 pg/ml. Aliquots of conditioned medium were concentrated 4–10-fold by centrifugation at 1,000 g in a 25°C fixed angle JA-17 rotor (Beckman Instruments Inc., Fullerton, CA) in Centricon-10 concentrators (Amicon Division of W. R. Grace and Co., Beverly, MA) until the desired volume was reached. Used in conjunction with concentrated medium, the G-CSF ELISA was able to detect concentrations as low as 4 pg/ml.

Immunohistochemistry. Indirect immunohistochemistry phase-contrast microscopy was performed for G-CSF on 2% paraformaldehyde-fixed primary human osteoblasts grown in 96-well tissue culture plates. Paraformaldehyde-fixed primary human osteoblasts were incubated with 10 μg/ml of a murine monoclonal anti-human G-CSF in PBS (M7; kind gift of Dr. Michael Widmer, Immunex Corporation, Seattle, WA) or an isotype control (POPC-21, Sigma Chemical Co., St. Louis, MO) at 4°C for two hours followed by a rabbit antimurine FITC-conjugated serum at a 1/64 dilution (Sigma Chemical Co.). Nonspecific binding was blocked with 10% normal rabbit serum.

Isolation of CD34⁺ Cells. Bone marrow cells obtained from healthy adult volunteers were diluted 1:4 (vol/vol) in IMDM and separated by density separation on Ficoll-Hypaque (specific gravity 1.077) to recover mononuclear cells. After two rounds of plastic adherence at 37°C for 1 h each in IMDM medium with 20% FCS (to remove monocytes, platelets, and megakaryocytes), the nonadherent cells were recovered. CD34⁺ hematopoietic progenitor populations were isolated using an avidin-biotin immunofluorescence process (CellPro Inc., Bothell, WA).

Coculture of NFS-60, CD34⁺, and Osteoblasts. CD34⁺ or NFS-60 cells were seeded directly onto osteocalcin⁺, c-kit ligand-confuent osteoblast monolayers in 96-well tissue culture plates at a final density of 10⁴ cells/well and incubated for 3–14 d. Either vehicle, 10 μg/well of an affinity purified IgG fraction of neutralizing goat anti-human G-CSF serum (R&D Systems), or 10 μg/well of normal goat IgG serum were added to cultures daily. Absolute cell numbers were determined by manual hemocytometer counting in PBS containing 0.4% trypan blue (Sigma Chemical Co.).

Results and Discussion

We first determined whether unstimulated primary human osteoblasts are a normal source of G-CSF. Primary osteoblasts were obtained using the methods of Robey and Termine (15), where osteoblasts emerge from collagenase-digested human bone during the second to third week of culture. We found that these cells express several functional characteristics of osteoblasts including the expression of mRNA for matrix Gla protein (not shown) and the osteoblast-specific protein, osteocalcin (bone Gla protein) (16–18) (Fig. 1), mineralization of their extracellular matrix, and the expression of alkaline phosphatase examined by histochemical methods (data not shown) (16).

Primary human osteoblast cultures were examined for their expression of mRNA for several cytokines using RT-PCR. As shown in Fig. 1, osteoblasts constitutively express mRNA for several cytokines including TNF-α, IL-6, GM-CSF, and G-CSF, but failed to express mRNA for IL-1α, IL-3, and c-kit ligand, and osteocalcin. Negative controls omitted reverse transcriptase from the reverse transcription reaction. RNA from IL-1β and TNF-α stimulated stromal cells served as positive controls for G-CSF and c-kit ligand.

Figure 1. RT-PCR detection of cytokine mRNA by (A) primary human osteoblasts and (B) stromal cells. RT-PCR was performed using primers for IL-1α, TNF, lymphotoxin (LT), IL-6, G-CSF, GM-CSF, IL-3, c-kit ligand, and osteocalcin. Negative controls omitted reverse transcriptase from the reverse transcription reaction. RNA from IL-1β and TNF-α stimulated stromal cells served as positive controls for G-CSF and c-kit ligand.
tern of cytokine mRNAs expressed by the human osteosarcoma cell lines, MG-63 and SaOS-2 (data not shown). The RT-PCR results suggest that osteoblasts constitutively express G-CSF mRNA. We next asked whether the G-CSF message is translated and how the protein is presented to hematopoietic progenitors. To determine whether the G-CSF message is translated, we performed immunohistochemical staining for G-CSF on paraformaldehyde-fixed primary human osteoblasts using a monoclonal anti-human G-CSF antibody. As shown in Fig. 2, G-CSF is localized on the cell membrane of the osteoblasts and not in the interstitial extracellular matrix. To quantify G-CSF production from unstimulated osteoblasts expressing G-CSF mRNA, salt extracts of extracellular matrix (20, 21) and concentrated, conditioned medium were analyzed for G-CSF by ELISA (7). As positive controls for G-CSF production, osteoblasts or stromal cells were exposed to lipopolysaccharide or IL-1α, respectively (22–24). LPS stimulation of osteoblasts resulted in the production of 21 pg/ml/24 h/10⁴ cells of soluble G-CSF. No G-CSF could be detected in the medium or extracellular matrix of unstimulated osteoblasts, suggesting that under basal conditions G-CSF may not be released as a soluble protein.

To determine whether the G-CSF detected by immunohistochemical methods has functional activity, we evaluated the proliferation of the G-CSF-dependent cell line NFS-60 in coculture with osteocalcin⁺, c-kit ligand⁻ human explant osteoblasts (25). A neutralizing antibody to human G-CSF was used to directly test whether osteoblast-derived G-CSF has biological activity. As shown in Fig. 3, NFS-60 cells proliferate in the presence of osteoblast monolayers but not in medium alone. Inclusion of a neutralizing anti-G-CSF antibody in the cultures caused a 41% reduction in the proliferation of the NFS-60 cells. The proliferation of NFS-60 cells was not affected by a control antiserum nor were significant differences in proliferation of the NFS-60 cells observed when the cells were grown in osteoblast-conditioned medium (50% vol/vol) alone, with or without the neutralizing anti-human G-CSF antibody.

These results obtained with the NFS-60 cell line suggest that osteoblasts might stimulate the proliferation of human

Figure 2. Immunohistochemical detection of G-CSF on osteoblast cell surfaces. 2% paraformaldehyde-fixed primary human osteoblasts (A) were incubated with (C) 10 μg/ml of murine monoclonal anti-human G-CSF in PBS or (B) an isotype control at 4°C for 2 h followed by a rabbit antimurine FITC-conjugated serum at a 1:64 dilution (Sigma Chemical Co.). Nonspecific binding was blocked with 10% normal rabbit serum.

Figure 3. NFS-60 cell proliferation on osteoblast monolayers. NFS-60 cells were seeded onto confluent human osteoblasts at a final density of 10⁴ cells/well in 96-well tissue culture plates for 3 d. Where indicated vehicle (no AB), 10 μg/well of an affinity purified IgG fraction of neutralizing goat anti-human G-CSF serum (Anti-G-CSF) or 10 μg/well of normal goat IgG serum (control AB) were added daily. Absolute NFS-60 cell numbers were determined by manual hemocytometer counting and reported as mean ± SD (n = 4). (Asterisk) Significant difference from antibody or vehicle control, p <0.01.
hematopoietic progenitors by producing G-CSF. To examine this possibility, CD34+ hematopoietic progenitor cells were isolated and seeded into confluent osteocalcin+, c-kit ligand-, human osteoblast monolayers. At 14 d the hematopoietic cells were recovered and counted. The significance of G-CSF in this system was tested by the addition of an antiserum to human osteoblast monolayers at a final density of 10⁴ cells/well and incubated for 14 d. The Results of one of two experiments are presented. Where indicated vehicle (no AB), 10 µg/well of either neutralizing goat anti-human G-CSF serum or goat IgG serum (control AB) were added daily (R&D Systems). Cell counts were determined by manual hemocytometer counting and are reported as mean ± SD (n = 3). The hematopoietic cells recovered after 2 wk of coculture maintained an immature (23 ± 6% myeloblasts/promyelocytes; 51.7 ± 5.5% myelocytes/metamyelocytes; 21 ± 5% bands/polymorphonuclear neutrophils; and 4.3 ± 0.6% monocytes, treatment with anti-G-CSF reduced the recovery of band/polymorphonuclear neutrophils to 8.7 ± 10%.

(Asterisk) Significant difference from antibody or vehicle control, p < 0.01.

**Figure 4.** Human CD34+ cells obtained by affinity purification of nonadherent low density mononuclear bone marrow cells (CellPro Inc.) were seeded directly into 96-well tissue culture plates or onto confluent osteocalcin+, c-kit ligand-, human osteoblast monolayers at a final density of 10⁴ cells/well and incubated for 14 d. The Results of one of two experiments are presented. Where indicated vehicle (no AB), 10 µg/well of either neutralizing goat anti-human G-CSF serum or goat IgG serum (control AB) were added daily (R&D Systems). Cell counts were determined by manual hemocytometer counting and are reported as mean ± SD (n = 3). The hematopoietic cells recovered after 2 wk of coculture maintained an immature (23 ± 6% myeloblasts/promyelocytes; 51.7 ± 5.5% myelocytes/metamyelocytes; 21 ± 5% bands/polymorphonuclear neutrophils; and 4.3 ± 0.6% monocytes, treatment with anti-G-CSF reduced the recovery of band/polymorphonuclear neutrophils to 8.7 ± 10%.

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