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Hepatocyte growth factor is secreted by osteoblasts and cooperatively permits the survival of haematopoietic progenitors

Russell S. Taichman,[1] Marcelle J. Reilly,[1] Rama S. Verma,[2] Karen Ehrenman[2] and Stephen G. Emerson[2]
[1]*Department of Periodontics, Prevention and Geriatrics, School of Dentistry, University of Michigan, Ann Arbor, MI, and*
[2]*Departments of Internal Medicine and Paediatrics, University of Pennsylvania, Philadelphia, PA, USA*

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Summary. Human osteoblasts (HOBs) support the growth of human haematopoietic progenitor cells, and support the survival and limited expansion of long-term culture-initiating cells. Using human CD34$^+$ cells and the murine myelomonocytic cell line NFS-60 as targets, we previously found that one component of HOB-derived haematopoietic activity is cell-associated granulocyte colony-stimulating factor (G-CSF). However, antibody failed to neutralize all the activity, suggesting that more than one factor supports haematopoietic cells. In the present investigations, we asked whether the HOB-derived, non-G-CSF secreted activity was as a result of other known growth factors. We found that, among the cytokines expressed by HOBs, only hepatocyte growth factor (HGF) and G-CSF stimulated NFS-60 cell proliferation. HOB cells and osteosarcoma cells secreted biologically active HGF, although the levels varied considerably. Moreover, addition of neutralizing HGF antibody to CD34$^+$ cell/HOB co-cultures resulted in a significant reduction (≈50%) in the ability of the HOBs to support haematopoietic progenitor cells. These results suggest that a major component of osteoblast-derived haematopoietic activity is HGF. Secretion of HGF, in concert with cell-associated cytokines such as G-CSF, may account for the stem cell-stimulating activities of osteogenic cells and, thereby, the unique stem cell-supporting role of the osteoblasts within the bone marrow microenvironment.

Keywords: osteoblasts, progenitor cells, bone marrow, hepatocyte growth factor, stem cells.

Haematopoiesis occurs in unique microenvironments *in vivo* that facilitate the maintenance of haematopoietic stem cells as pluripotent, while supporting lineage commitment of the expanding blood cell populations. Each of these activities may require different growth factors and microenvironments, the identities of which have yet to be determined. Within the bone marrow microenvironment, haematopoietic growth factors are produced by resident stromal cells (Dexter *et al*, 1977; Eaves *et al*, 1991; Guba *et al*, 1992; Kittler *et al*, 1992). Marrow stromal cells are comprised of several different populations including fibroblasts, macrophages, endothelial cells and adipocytes. Moreover, each of these adherent cells probably represents local bone marrow specializations of a widely expressed lineage.

One stromal cell type with a highly restricted expression is the osteoblast. It has long been appreciated that endosteal osteoblasts and haematopoietic stem cells are immediately juxtaposed *in vivo* (Lord *et al*, 1975; Gong, 1978; Gartner & Kaplan, 1980; Deldar *et al*, 1985; Hermans *et al*, 1989; Lord, 1990), but the functional implications of this apposition remains unknown. Recently, we have begun to address the haematopoietic role of osteoblasts using normal primary osteoblasts derived from human bone explants (Taichman & Emerson, 1994; Taichman *et al*, 1996, 1997a, b) and osteosarcoma cell lines (Taichman & Emerson, 1994; Taichman *et al*, 1996). We have determined that primary human osteogenic cells (HOBs) produce several haematopoietic growth factors that stimulate haematopoietic progenitor cell maintenance and proliferation, and that human osteogenic cells support the limited expansion of long-term culture-initiating cells (LTC-ICs) derived from human CD34$^+$ bone marrow cells *in vitro* (Taichman & Emerson, 1994; Taichman *et al*, 1996). To biochemically characterize the haematopoietic activities of HOBs, we have utilized transformed cell lines to model both early haematopoietic

Correspondence: Russell S. Taichman, Department of Periodontics, Prevention and Geriatrics, University of Michigan School of Dentistry, 1011 North University Avenue, Ann Arbor, MI 48109–1078, USA. E-mail: rtaich@umich.edu

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cells and osteogenic cells. Using the murine myelomyocytic cell line NFS-60, we have determined that the part of the activity produced by HOBs that is active on human $CD34^+$ bone marrow cells is granulocyte colony-stimulating factor (G-CSF). G-CSF is currently the only human growth factor/cytokine known to stimulate proliferation of NFS-60 cells (Shirafuji *et al*, 1989). However, not all of the activity that stimulates NFS-60 cell growth produced by primary osteogenic cells or human osteosarcomas is owing to G-CSF (Taichman & Emerson, 1994).

In this report, we present data indicating that HOBs and several osteosarcoma cell lines express and secrete hepatocyte growth factor (HGF). Moreover, the secreted HGF, in collaboration with other haematopoietic activities secreted by osteogenic cells including G-CSF, is required to support the survival of early haematopoietic progenitor cells and the proliferation of the NFS-60 cell line. HGF derived from normal osteogenic cells also appears to contribute to the support of normal progenitor cell proliferation. These results suggest that a major portion of osteoblast-derived haematopoietic cell stimulatory activities may be owing to HGF. Secretion of HGF may account for the stem cell stimulation of osteogenic cells and thereby the unique stem cell supporting role of the osteoblast within the bone marrow microenvironment.

MATERIALS AND METHODS

Cell lines. Several human osteosarcoma cell lines were purchased from the American Tissue Type Collection (ATCC; Rockville, MD, USA) including G292 (CRL1423), HOS TE85 (CRL1743), MG-63 (ATCC CRL1424), SaOS-2 (ATCC 85-HTB) and U-2 OS (ATCC HTB96). The cell lines were maintained in Dulbecco's minimal essential medium (DMEM) with Earle's salts or McCoys' 5A medium, 10% heat-inactivated fetal bovine serum (FBS) and antibiotics. Murine NFS-60 cells, a myeloid progenitor line that proliferates in response to multiple murine cytokines but is only known to proliferate in response to human G-CSF (a kind gift from Dr James Ihle, St Jude Children's Research Hospital, Memphis, TN, USA), were maintained in Roswell Park Memorial Institute (RPMI)-1640 medium with 10% FBS and 10% WEHI-3 (ATCC TIB68) conditioned medium. Unless otherwise stated, all tissue culture reagents were purchased from Life Technologies, Grand Island, NY, USA.

Primary human osteoblast-like cells (HOBs). Enriched human osteoblast cultures were established using a modifications of methods described by Robey and Termine (1985). Normal human trabecular bone was obtained from patients undergoing orthopedic surgery in accordance with the University of Michigan's Investigational Review Board. Bone cleaned of loosely adherent tissue was ground to produce a uniform particle size (size ≤ 1 mm^2) (BioComp Minimill, W. Lorenz, Jacksonville, FL, USA) and incubated in 1 mg/ml bacterial collagenase (Type P, Boehringer Mannheim Biologicals, Indianapolis, IN, USA). The explants were placed into culture until confluent monolayers were produced in a 1:1 (v/v) mixture of Ham's F12/DMEM (Biofluids, Rockville, MD, USA) with low Ca^{2+} and 10% heat-inactivated FBS. Thereafter, the cultures were maintained in calcium-replete Ham's F12/DMEM (1:1 v/v) medium containing 10% heat-inactivated antibiotics, 10 mmol/l β-glycerol phosphate and 10 μg/ml L-ascorbate. To verify that the cells expressed an osteoblast phenotype, the cultures were screened for the expression of the osteoblast-specific protein osteocalcin (osteocalcin$^+$) using reverse transcriptase-polymerase chain reaction (RT-PCR), as previously detailed (Taichman & Emerson, 1994, 1996). In addition, the HOB cells express several additional features of the osteoblast phenotype. These include expression of mRNA, matrix Gla protein, osteonectin and type I collagen (Gerstenfeld *et al*, 1987; Robey *et al*, 1987), high levels of alkaline phosphatase activity are expressed by the HOB cells, which, in primary culture, mineralize their extracellular matrix, albeit weakly and only after extended culture periods (Taichman & Hauschka, 1992). The cells do not, however, express mRNA for bone sialoprotein (BSP), nor do they appear to express parathyroid hormone (PTH) receptors, as they fail to generate cAMP in response to PTH or express mRNA for the receptor (unpublished observations).

To evaluate the purity of the osteoblastic cells, we utilitized stem cell factor as a marker of bone marrow fibroblasts to determine the level of contamination we can detect in fibroblast populations in our osteoblast preparations. We found that by mixing $SCF^{(-)}$ HOB populations with $SCF^{(+)}$ stromal populations, we were able to detect a 1·0% contamination of bone marrow stromal cells in a given 'osteoblast' preparation (Taichman *et al*, 2000a). Therefore, as commonly used, the HOB populations are at least 99% pure of contaminating stromal fibroblasts, with a possible maximal contamination of 1% stromal cells. However, until a 'universal' human stromal cell marker is established that clearly distinguishes immature osteoblasts from stromal cells (i.e. not dependent upon late osteoblasts maturation markers), it is not possible to rule out some level of stromal fibroblastic cell contamination.

NFS-60 cytokine proliferation assays. NFS-60 cells were washed three times in phosphate-buffered saline (PBS) and 1×10^4 cells were plated into triplicate 96-well flat-bottomed tissue culture plates (Corning) in 0·1 ml of Ham's F12/DMEM (1:1 v/v), containing 10% heat-inactivated FBS, 100 U/ml penicillin G, 100 μg/ml streptomycin sulphate, 10 mmol/l β-glycerol phosphate and 10 μg/ml L-ascorbate. Log serial dilutions of recombinant human cytokines were added alone or in combination with G-CSF (0·001–10 ng/ml) over the following dose ranges; bone morphogenic proteins (BMPs)-4, 5, 6, 7 (0·10–10 ng/ml, Genetics Institute, Cambridge, MA, USA), endothelial growth factor (EGF; 0·03–30 ng/ml), erythropoietin (0·02–20 U/ml, Stem Cell Technologies, Vancouver, BC, Canada), basic fibroblast growth factor (bFGF; 0·1–100 ng/ml), FLT2/3-ligand (FL; 0·1–100 ng/ml, 200 ng/ml, Genzyme, Cambridge, MA, USA), granulocyte macrophage colony-stimulating factor (GM-CSF; 0·02–200 ng/ml), human growth factor (HGF; 0·1–100 ng/ml, R & D Systems), interleukin-1 alpha (IL-1α; 0·025–25 U/ml), IL-1β (0·03–30 pg/ml), IL-3 (0·01–10 ng/ml), IL-4 (0·03–30 ng/ml),

IL-5 (0·03–30 ng/ml), IL-6 (0·001–10 ng/ml), IL-7 (0·001–10 ng/ml), IL-10 (0·02–20 ng/ml, Peprotech, Rockyhill, MN, USA), IL-11 (0·01–10 ng/ml), stem cell factor (SCF; 0·05–50 ng/ml), leukaemia inhibitory factor (LIF; 0·01–100 U/ml), lymphotoxin (LT; 0·01–10 ng/ml), macrophage CSF (M-CSF; 0·001–100 U/ml), macrophage inflammatory protein-1α (MIP-1α; 0·1–10 ng/ml), oncostatin M (0·02–20 ng/ml), parathyroid hormone (PTH; 1×10^{-11}–1×10^{-8} mol/l), PTH-related protein (PTHrP; 1×10^{-11}–1×10^{-8} mol/l), transforming growht factor-β_1 (TGF-β_1; 0·1–100 ng/ml), thrombopoietin (TPO; 0·05–5 ng/ml, 20 ng/ml), tumour necrosis factor-α (TNF-α; 1–100 U/ml, 500 U/ml). All cytokine reagents were purchased from R & D Systems except where indicated. Thereafter, the cultures were incubated in an atmosphere of 5% CO_2 and 5% O_2 at 37°C for 4 d. Proliferation was quantified using a colorimetric assay with 3-(4,5-dimethyl-azol-2yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, St. Louis, MS, USA) and read on a multiwell scanning spectrophotometer (Molecular Devices, Sunnyvale, CA, USA).

In some cases, proliferative responses to growth factors were measured by [^{3}H]-thymidine incorporation. For these assays, at 90 h of culture, before the cultures reached confluence, 18·5 kBq of [^{3}H]-thymidine (SA = 248·9 GBq/mmol/l, New England Nuclear, Beverly, MA, USA) was added to each well and the cells were harvested 6 h later onto glass filters for scintillation counting. All data are presented as either OD_{560} or CPM (mean ± standard deviation) for triplicate determinations from three independent experiments.

NFS-60 cell proliferation in dual-chambered co-culture with MG-63 osteosarcomas. MG-63 human osteosarcoma or HOB cells were seeded at an initial density of $2{\cdot}0 \times 10^4$ cells/cm^2 in Ham's F12/DMEM (1:1 v/v) (Life Technologies) containing 10% heat-inactivated FBS, antibiotics, 10 mmol/l β-glycerol phosphate and 10 μg/ml L-ascorbate (Sigma, St. Louis, MO, USA). After 5 days, the osteosarcoma cells were washed twice in warm PBS and 1×10^4 NFS-60 cells were seeded into the top chamber of the TransWell 24-well plates (0·4 μmol/l pore size, Corning Costar, Boston, MA, USA) with the confluent human osteosarcoma cells or HOBs in the bottom chamber. Where indicated, neutralizing rabbit polyclonal anti-human cytokine serum (or control) was added daily to a final concentration of 0·05 μg/ml IL-1α, IL-1β, G-CSF and TNF-α, 1 μg/ml HGF, 5 μg/ml aFGF and LIF, and 30 μg/ml LT, IL-3, IL-4, KL and MIP-1α (R & D Systems, Minneapolis, MN, USA or Genzyme, Cambridge, MA, USA). Murine monoclonal antibodies to human GM-CSF, IL-6, TGF-β or isotype-matched control (MOPC21, Sigma) were added daily to 30 μg/ml final concentrations. In some cases, 1×10^4 NFS-60 cells were seeded into the top chamber of 24-well plates (non-contact) or in direct contact with confluent HOBs or MG-63 osteosarcoma cells in the bottom chamber, with 10 μg/ml of an affinity-purified IgG fraction of neutralizing goat anti-human G-CSF serum or 10 μg/ml of normal goat IgG serum or murine monoclonal HGF (10 μg/ml) or control (MOPC21 IgG) added daily. At 4 d, the NFS-60 cells were recovered and absolute NFS-60 cell numbers were determined by manual haemocytometer counting in PBS containing 0·4% trypan blue (Sigma) and reported as mean ± standard deviation ($n = 3$).

Reverse transcription polymerase chain reaction (RT-PCR). HOBs and the osteosarcoma cells were plated at an initial density of $2{\cdot}0 \times 10^5$ cells/cm^2 with complete medium changes on d 5, d 7, d 10 and d 14 in medium containing freshly prepared β-glycerol phosphate (10 mmol/l) and L-ascorbate (50 μg/ml). Total cellular RNA was recovered on d 14 by lysing the cells directly in Stat-60 according to the directions of the manufacturer (Tel-Test, Friendswood, TX, USA). RNA integrity and purity was checked using electrophoresis with ethidium bromide and absorbency at A_{260}/A_{280}. RNA (1·0 μg), 10× RT buffer (1× RT buffer: 50 mmol/l Tris HCl, pH 8·3, 50 mmol/l KCl, 8·0 mmol/l $MgCl_2$ and 10 mmol/l dithiothreitol), 25 mmol/l dXTP mix (25 mmol/l of each dXTP; ACGT), 3·0 μg of oligo d(T) and 2·5 U of reverse transcriptase (M-MLV Reverse Transcriptase, Life Technologies) were incubated together at 38°C for 1 h. One-fifth of the double-stranded product was mixed with 10× Taq buffer (1× Taq/RT buffer: 10 mmol/l Tris, pH 8·3, 50 mmol/l KCl, 1·5 mmol/l $MgCl_2$, 0·01% gelatin and 2·0 mmol/l dithiothreitol), 1 mmol/l dXTP mix, 500 ng of each sense and antisense oligonucleotides, and 2·5 U of Taq polymerase (Taq polymerase, Life Technologies). Sense and antisense primers were prepared by the oligonucleotide synthesis core at the University of Michigan and were designed to cross intron/exon boundaries including BMP-2 (5′-ACC-CGG-GAG-AAG-GAG-GAG-G and 3′-CGG-TGA-TGG-AAA-CTA-CTA-TTG, expected size: 657 bp; Oida *et al*, 1995), BMP-4 (5′-GCC-ATT-CCG-TAG-TGC-CAT-CC and 3′-AAA-TAC-TCC-AAT-ACT-TCG-GG, expected size 688 bp; Oida *et al*, 1995), bFGF (5′-GCC-TTC-CCG-CCC-GGC-CAC-TTC-AAG-G and 3′-GCA-CAC-ACT-CCT-TTG-ATA-GAC-ACA-A, expected size 179 bp; Zaheer *et al*, 1995), FLT2/3 ligand (5′-AAT-CCG-TGA-GCT-GTC-TGA-CTA-C and 3′-GGA-GAA-GTT-CTG-GCG-AGT-GAT expected sizes 272 bp and 312 bp; Zaheer *et al*, 1995), HGF primer set I (5′-CTC-CCC-ATC-GCC-ATC-CCC and 3′-CAC-CAT-GGC-CTC-GCC-TGG, expected size 749 bp; Borset *et al*, 1996), HGF primer set II (5′-TTC-AAC-TTT-GAA-CAC-TGA-CC and 3′-ACAATG CCTCTGGTTCCCCTT, expected size 384 bp; Sensebe *et al*, 1997), IL-1 (5′-ATG-GCC-AAA-GTT-CGA-GAC-ATG and 3′-CTA-CGC-CTG-GTT-TTC-CAG-TAT-CTG-AAA-GTC-AGT, expected size 815 bp; Brenner *et al*, 1989), IL-5 (5′-ATG-AGG-ATG-CTT-CTG-CAT-TTG and 3′-TCA-ACT-TTC-TAT-TAT-CCA-CTC-GGT-GTT-CAT-TAC, expected size 664 bp; Tanabe *et al*, 1987), IL-7 (5′-GGA-CTT-CCT-CCC-CTG-ATC-CTT-G and 3′-CTT-TGT-TGG-TTG-GGC-TTC-ACC-CAG, expected size 377 bp; Thalmeier *et al*, 1996), IL-10 (5′-ATG-CCC-CAA-GCT-GAG-AAC-CCA-AGA and 3′-GTC-GGG-TCA-GCT-ATC-CCA-GAG-C, expected size 340 bp; Huettner *et al*, 1995), IL-11 (5′-ATC-AAC-TGT-GTT-TGC-CGC-CTG-GTC and 3′-CGT-CAG-CTG-GGA-ATT-TGT-CCT-TC, expected size 201 bp; Thalmeier *et al*, 1996), LIF (5′-CCT-GTG-CCA-TAC-GCC-ACC-CAT-G and 3′-GCC-TGG-GCC-AAC-ACG-GCG-ATG, expected size 503 bp; Thalmeier *et al*, 1996), M-CSF (5′-CCG-AGG-AGG-TGT-CGG-AGT-ACT-G and 3′-CTG-GCA-GTT-CCA-

CCT-GTC-TGT-C, expected size 671 bp; Thalmeier *et al*, 1996), MIP-1α (5′-CTC-AGA-ATC-ATG-CAG-GTC-TCC-A and 3′-GGC-AAC-AAC-CAG-TCC-ATA-GAA-G, expected size 405 bp; Davatelis *et al*, 1989), TPO (5′-TCC-TCC-ACCA-GCA-GAC-TGA-GCC-AGT and 3′-GAG-TCC-ACG-AGT-TCC-ATT-CAA-GAG, expected size 656 bp; Bartley *et al*, 1994) or as previously detailed (Taichman & Emerson, 1994, 1996; Taichman *et al*, 1997a).

The samples underwent thermal cycling at 94°C for 1 min, 60°C for 1 min and 72°C for 1 min for 35 cycles, followed by a 10-min extension at 72°C (Perkin Elmer Cetus DNA thermal cycler, Foster City, CA, USA). The products were electrophoresed in 3% agarose and visualized using ethidium bromide. To control for false positives or DNA contamination, reverse transcriptase was omitted from the reaction (data not presented). Positive controls included RNA from IL-1β- and TNF-α-stimulated stromal cells or peripheral blood lymphocytes stimulated with phytohaemagglutinin (PHA) and phorbol 12-myristate 13-acetate (PMA). Whole blood cells were separated by density centrifugation over Ficoll-Hypaque and separated by adherence to plastic. The non-adherent blood cells were cultured overnight in Iscove's modified Dulbecco's medium (IMDM) with 10% FBS, 10% horse serum, 1 μmol/l hydrocortisone with the non-adherent layers removed after 48 h and stimulated for a further 48 h with IL-1β (250 pmol/l) and TNF-α (1·0 nmol/l) (R & D Systems). Human bone marrow stromal cells were obtained from healthy adult volunteers by iliac crest puncture and aspiration into preservative-free heparin under a protocol approved by the University of Michigan's Investigational Review Board. Mononuclear cells were isolated by density separation on Ficoll-Hypaque (specific gravity 1·077). Plastic adherence at 37°C was performed in modified Dexter's medium (IMDM, 10% FBS, 10% equine serum, 1 μmol/l hydrocortisone, penicillin/streptomycin; Life Technologies). Following overnight adherence, the non-adherent cells were removed and the cultures were passaged at confluence three times. For positive controls, at the final passage, the bone marrow stromal cells were replated at an initial density of 1×10^4 cells/cm^2 and stimulated at confluence with IL-1β (250 pmol/l) and TNF-α (1·0 nmol/l) for 48 h.

Cytokine ELISAs. ELISAs were performed using the double-antibody sandwich method with commercially available kits (R & D Systems). Based upon parallel assays of known standards, the sensitivities of the assays of unconcentrated conditioned medium were: G-CSF 39–2500 pg/ml; GM-CSF 8–500 pg/ml. Aliquots of conditioned medium were concentrated twofold by centrifugation at 1000 ***g*** in a 25°C fixed angle JA-17 rotor (Beckman, Palo Alto, CA, USA) in Centricon-10 concentrators (Amicon Division of W. R. Grace, Danvers, MA, USA) until the desired volume was reached. Used in conjunction with concentrated medium, the G-CSF and GM-CSF ELISAs were able to detect concentrations as low as 19 pg/ml and 4 pg/ml respectively. Matched antibody capture and detection antibodies were utilized to construct an ELISA for HGF from commercially available components (R & D Systems).

Isolation of human CD34$^+$ bone marrow cells. Human bone marrow cells were obtained from healthy adult volunteers by iliac crest puncture and aspiration into preservative-free heparin under a protocol approved by the University of Michigan's Investigational Review Board. Mononuclear cells were isolated by density separation on Ficoll-Hypaque (specific gravity 1·077). Plastic adherence at 37°C was performed in modified Dexter's medium (IMDM, 10% FBS, 10% equine serum, 1 μmol/l hydrocortisone, penicillin/streptomycin; Life Technologies). Following overnight adherence, the non-adherent cells were recovered and CD34$^+$ bone marrow cells isolated by positive immunomagnetic selection using the QUIND/10 antibody (Miltenyi Biotec, Sunnyvale, CA, USA). In some experiments, the non-adherent bone marrow mononuclear CD34$^+$ cells that were isolated by positive immunomagnetic selection (Miltenyi) were stained with a phycoerythrin conjugate of the anti-CD34 antibody, HPCA-2 (Becton Dickinson, San Jose, CA, USA), and sorted by fluorescence-activated cell sorting (EPICS C, Coulter, Hialeah, FL, USA) to evaluate purity. Under these conditions, $92{\cdot}5 \pm 5\%$ ($n = 2$) of the recovered cells expressed the CD34 antigen.

Liquid co-culture of CD34$^+$ bone marrow cells and primary human osteogenic cells. Primary human osteogenic cells (HOBs) at confluence were harvested by trypsinization (20 min, 37°C, 0·05% trypsin/0·5 mmol/l EDTA, Gibco-BRL) and seeded into a 96-well plate at a final density of 2×10^4/cm^2 in Ca^{+2}-replete Ham's F12/DMEM (1:1 v/v) with 10% heat-inactivated FBS, antibiotics, 10 mmol/l β-glycerol phosphate and 10 μg/ml L-ascorbate. After 7 d, the osteoblast monolayers were washed twice and CD34$^+$ bone marrow cells were seeded onto the osteoblast monolayers. After 2 weeks, all of the cultures were harvested by trypsinization (20 min, 37°C, 0·05% trypsin/0·5 mmol/l EDTA) and either prepared for light microscopy (cell number and morphology was determined) or the cells were replated in colony-forming progenitor cell assays in methylcellulose containing IL-3, GM-CSF and erythropoietin. The effect of osteoblast-derived HGF or G-CSF on the progenitor cell survival was compared by daily addition (to a final concentration of 1 μg/ml) of neutralizing anti-HGF monoclonal antibodies (R & D Systems), IgG1 control (MOPC 21, Sigma) or vehicle during the 2 week co-culture period. After 2 weeks, haematopoietic colonies were scored as granulocyte macrophage colony-forming units (CFU-GM), erythroid burst-forming units (BFU-E) or mixed lineage CFUs (CFU-GEMM) using light microscopy. In some cases, the effect of increasing does of G-CSF or HGF were directly incorporated into the progenitor assays.

Statistical analyses. Analysis of variance (ANOVA) was utilized to determine statistical significance to a level of $P < 0{\cdot}05$.

RESULTS

We recently reported that normal human osteogenic cells, as well as human osteosarcoma cell lines, constitutively produce G-CSF capable of stimulating the proliferation of normal human CD34$^+$. Based on experiments using neutralizing antibodies to human G-CSF and porous

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micromembrane assays, we determined that not all the osteoblast-derived stimulatory activity was attributable to G-CSF (Hara *et al*, 1988; Metcalf, 1989; Shirafuji *et al*, 1989;Taichman & Emerson, 1994). Therefore, to determine if other known cytokines were responsible for the additional non-G-CSF haematopoietic activities produced by osteogenic cells, we addressed the following questions: (i) what known cytokines do osteogenic cells produce?, (ii) do haematopoietic cells proliferate in response to other known cytokines alone or in combination with G-CSF?, and (iii) can we inhibit haematopoietic cell proliferative activity produced by osteoblast cell lines with specific anti-cytokine neutralizing antibodies? To address these questions we used the murine myelocytic leukaemia cell line NFS-60 to model osteoblast–haematopoietic cell interactions and subsequently confirmed these findings with $CD34^+$ cells.

Expression of cytokines by human osteosarcomas and osteoblasts
MG-63 human osteosarcoma cell lines were cultured in the presence of L-ascorbate and β-glycerol phosphate to induce osteoblast phenotype as previously detailed (Taichman & Emerson, 1996). After 14 d, RNA was isolated and examined for mRNA expression of several cytokines. Using the RT-PCR, we determined that MG-63 cells constitutively expressed mRNA for BMP-4, bFGF, FLT-ligand (FL), IL-7, M-CSF and LIF. No mRNA was consistently detected for BMP-2, IL-5, IL-10, oncostatin M and thrombopoietin (TPO) in MG-63 cells under the culture conditions tested (Fig 1). These data supplement our previous report that MG-63 cells express mRNA for G-CSF, IL-1β, IL-6, GM-CSF, TGF-β_1 and TNF-α, but failed to express mRNA for KL, IL-3 or LT (Taichman & Emerson, 1996).

Using RT-PCR we also determined that mRNA for HGF was expressed by both MG-63 cells and primary human explant-derived osteogenic cells (HOBs) (Fig 2A). This was of considerable interest as HGF has recently been demonstrated to stimulate NFS-60 proliferation when in direct contact with a preadipocyte cell line (Satoh *et al*, 1997). To further verify that the expression of HGF mRNA is a general feature of osteosarcomas, we evaluated whether G292, HOS TE85, SaOS-2 and U2–0S cells also expressed HGF. Here, we noted that HOS TE85 and U2–0S cells expressed HGF mRNA, whereas G292 and U2–0S did not (Fig 2B), yet NFS-60 do proliferate on these cells, suggesting an as yet unidentified activity (data not presented). Using a sandwich ELISA, we found that MG-63 cells secrete easily detectable quantities of HGF ($15\,542 \pm 878$ pg/ml/10^4cell/4 d, $n = 3$). Primary HOBs also secreted detectable HGF, although the levels produced by primary HOB cells varied from marrow donor to donor (3547 ± 604 vs133 ± 45 pg/ml/10^4cell/4 d) (Fig 2C).

NFS-60 cell proliferation in G-CSF alone and in combination with test cytokines
We next measured the ability of NFS-60 cells to proliferate in response to recombinant cytokines whose mRNA had been detected by RT-PCR in osteosarcoma cells. Over 4 d, NFS-60 cells proliferated in response to G-CSF, but failed to proliferate in response to BMP-4, aFGF, IL-1α, IL-1β, IL-4, IL-5, IL-6, IL10, IL-ll, FL, GM-CSF, oncostatin M, PTHrP, LIF, LT, TNF-α or TPO. Similarly, NFS-60 cells did not proliferate

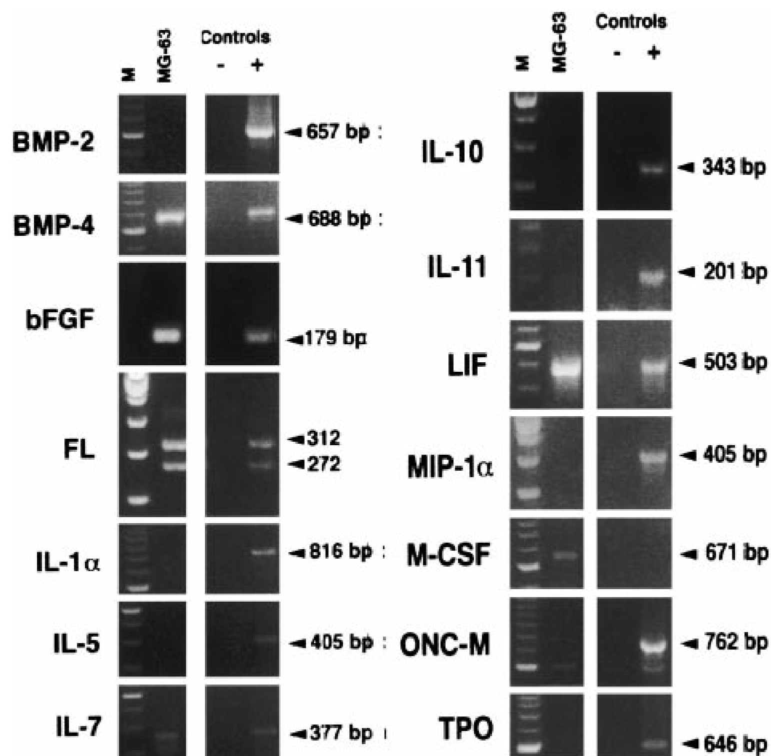

Fig 1. Reverse transcriptase PCR (RT-PCR) detection of cytokine mRNAs by MG-63 cells. RT-PCR was performed using primers designed to cross intron–exon boundires. Negative controls omitted reverse transcriptase from the reverse transcription reaction. Where indicated, positive controls included RNA from IL-1β- and TNF-α-stimulated stromal cells or peripheral blood lymphocytes stimulated with PHA and PMA.

in response to BMP-5, BMP-6, BMP-7, EGF, EPO, IL-2, IL-3, IL-12, SCF or MIP-1α. Furthermore, none of these cytokines increased the ability of NFS-60 cells to proliferate in response to G-CSF (Fig 3A).

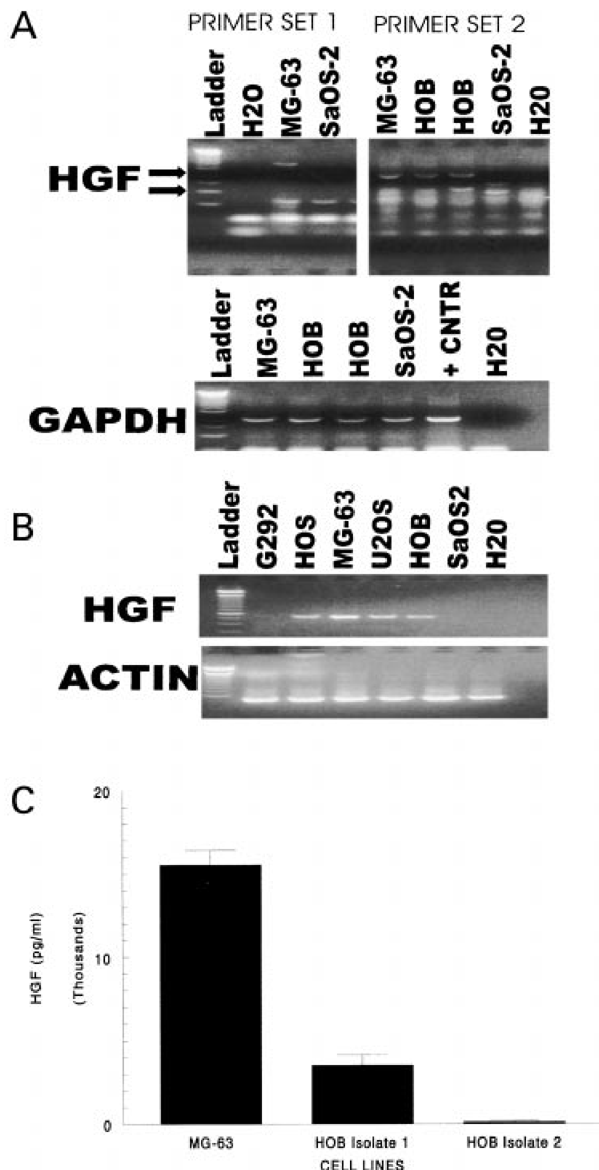

Fig 2. Synthesis of HGF by HOB cells and human osteosarcomas. A. RT-PCR was performed (35 cycles) to detect mRNA for GADPH and two sets of HGF primers from mRNA recovered from primary human osteogenic cells and MG-63. B. RT-PCR was performed on mRNA recovered from G292, HOB, HOS TE85, MG-63, SaOS-2 and U2-OS for HGF and actin. Primers were designed to cross intron/exon boundaries. Negative controls included omitting reverse transcriptase (not shown) from the reverse transcription reaction or H_20 (neg control). C. HOBs and MG-63 cells were seeded ($1{\cdot}0 \times 10^4$) in 24-well tissue culture plates with 10% FBS. 96 h after confluence, the cells were washed and re-fed. Conditioned medium was collected and assayed for HGF by ELISA. Representative data from three independent experiments are presented as a mean ± standard pg/ml HGF deviation for triplicate determinations.

In contrast, the concentrations of HGF secreted by both MG-63 cells and primary HOBs were sufficient to stimulate the proliferation of NFS-60 cells. HGF alone, at concentrations of 1·0 ng/ml or higher, stimulated NFS-60 cell proliferation, although the magnitude of the response was not robust. However, when combined with low (0·01 ng/ml) concentrations of G-CSF, HGF synergized to stimulate the NFS-60 cell proliferation (Fig 3B).

NFS-60 cell proliferation in dual-chambered co-culture with human osteosarcomas

Previously, we observed that osteogenic cells stimulate the proliferation of NFS-60 cells in co-culture. However, osteoblast-conditioned medium failed to induce NFS-60 proliferation (Taichman & Emerson, 1994). Verfaillie (1992) demonstrated that stromal cell-dependent haematopoiesis depends in part on short-lived soluble factors that may require high local concentrations for activity. Based upon these observations, NFS-60 cells were placed in TransWell co-culture with MG-63 monolayers for 4 d. Thereafter, the NFS-60 cells were recovered by washing the membranes with trypsin to ensure that all the plated cells were recovered. On average, the cell number increased fivefold during the culture period, demonstrating that some of the osteoblast-derived activity did not require cell-to-cell contact.

Using this dual-chambered co-culture system, neutralizing antibodies to each of the cytokines whose messages were detected by RT-PCR were added to individual cultures. Of these, the only anti-human cytokine antibody that reduced NFS-60 proliferation was G-CSF and only when the cells were in direct contact with one another (Taichman & Emerson, 1994; Taichman *et al*, 1996). When the cells were separated from one another by porous micromembranes, however, antibody to G-CSF did not alter proliferation of the NFS-60 cells (Fig 4A). These data are consistent with our previous findings that primary osteoblasts and osteosarcoma cell lines express G-CSF preferentially in a cell-associated, rather than soluble, form in the absence of inflammatory mediators (Taichman & Emerson, 1994; Taichman *et al*, 1996). Incubation with anti-TGF-β antibody increased NFS-60 cell proliferation (Fig 4A).

Next, we confirmed that the immunoreactive HGF protein secreted by osteoblasts and osteosarcoma cells was biologically active. When NFS-60 cells were cultured while separated from the adherent cells by porous membranes (Fig 3B) or in direct contact with the adherent cells (Fig 4B), NFS-60 proliferation was observed. Neutralizing antibody to human HGF caused a significant decrease in proliferation of the NFS-60 cells under both contact and non-contact conditions on MG-63 cells. Furthermore, under culture conditions in which HOB and NFS-60 cells were in direct contact, the inhibitory effect of anti-HGF antibody was enhanced by the addition of an anti-G-CSF antibody (Fig 4B).

Does HGF produced by osteogenic cells support haematopoietic progenitor cells?

To directly determine if HGF produced by HOBs contributes

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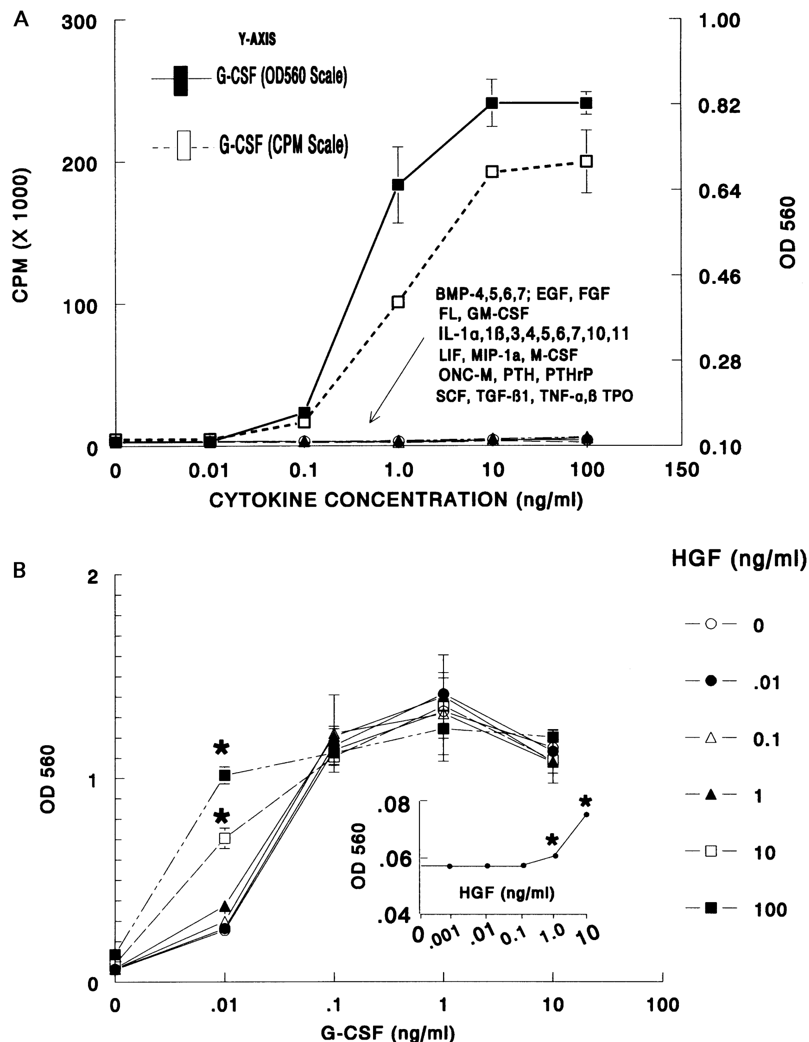

Fig 3. NFS-60 proliferation in recombinant human cytokines. A. Log serial dilutions of recombinant human cytokines were added to 1×10^4 NFS-60 cells as reported in the *Patients and Methods*. Proliferation was quantified at the conclusion of 4 d of culture by colorimetric assay using 3-(4,5 dimethylazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) at A_{560} (OD_{560} vertical scale) on an ELISA plate reader (Molecular Devices) or by [^{3}H]-thymidine incorporation over a 6-h period (CPM vertical scale). B. Log dilutions of recombinant human HGF alone (insert) or in combination with G-CSF were added to 1×10^4 NFS-60 cells. Data are presented as mean ± standard deviation for triplicate determinations from a minimum of three independent experiments. *Significant difference from 0 HGF (insert) or HGF alone, $P < 0{\cdot}05$.

to the survival of haematopoietic progenitor cells *in vitro*, $CD34^+$ cells were cultured for 2 weeks in the presence of human osteogenic cells and/or neutralizing anti-HGF antibody. At the conclusion of the assay, the cells were recovered, cell numbers normalized and assays performed for progenitor cell activity. In the absence of HOB monolayers, no colony formation was observed (Fig 5). In the presence of HOB monolayers, maintenance of progenitor cell activity was observed (92·3 ± 7% input). The addition of the anti-HGF antibody resulted in a significant reversal in the ability of osteogenic cells to support haematopoietic progenitor cells, reducing the ability of the HOBs to support progenitor cell proliferation by approximately 50% (Fig 5).

Finally, to directly assay the role of HGF in the ability of HOBs to support normal human haematopoietic progenitor cells *in vitro*, we determined whether the combination of G-CSF and HGF alone was sufficient to support the formation of haematopoietic colonies *in vitro*. For these investigations, $CD34^+$ bone marrow cells were cultured for 2 weeks in methylcellulose and the presence or absence of increasing doses of recombinant G-CSF and HGF. At the conclusion of the culture period, progenitor cell activity was as shown in Fig 6. Increasing concentrations of both G-CSF and HGF produced significantly greater colonies together, than either cytokine alone.

DISCUSSION

These data demonstrate that osteoblast-derived HGF plays an important role in the ability of osteogenic cells to support haematopoiesis. Using RT-PCR and ELISA methods, we determined that primary human osteogenic cells and MG-63 cell lines constitutively secrete HGF. NFS-60 cells were also shown to proliferate in response to HGF and the proliferative activity was synergistic with suboptimal doses of G-CSF. When HGF activity was neutralized using monoclonal antibodies, a significant reduction in the ability of osteogenic cells to support NFS-60 cell proliferation was observed. Most importantly, neutralization of HGF resulted in a reduction in the ability of osteogenic cells to support the survival of primary haematopoietic progenitor cells. Furthermore, the proliferative activity produced by osteogenic cells

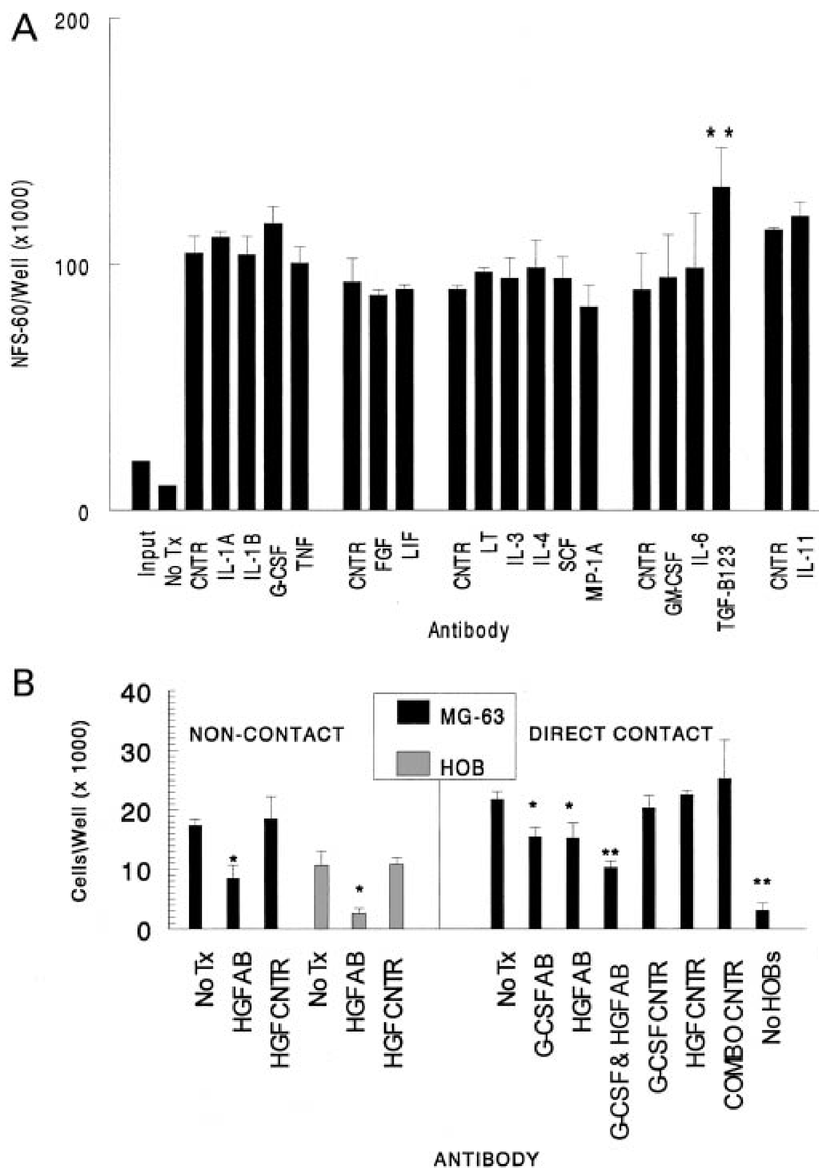

Fig 4. Antibody inhibition of NFS-60 cell proliferation in dual chambered co-culture with MG-63 osteosarcoma cells. A. NFS-60 cells (2×10^4) were seeded into the top chamber of TransWell 24-well plates (0·4 μmol/l pore size) with confluent MG-63 osteosarcoma cells in the bottom chamber. Where indicated, neutralizing rabbit polyclonal anti-human cytokine serum (or control serum) was added daily to a final concentration of 0·5 μg/ml (IL-1α, IL-1β, G-CSF, TNF-α), 5 μg/ml (aFGF, LIF) or 30 μg/ml (LT, IL-3, IL4, KL, MIP-1α). Murine monoclonal antibodies to GM-CSF, IL-6, TGF-beta or isotype-matched control were added daily to 30 μg/ml final concentrations. Input was defined as the number of NFS-60 cells placed in culture at culture initiation. B. NFS-60 cells (2×10^4) were seeded into the top chamber of TransWell 24-well plates (0·4 μmol/l pore size) with confluent HOBs or MG-63 osteosarcoma cells in the bottom chamber (non-contact), or in direct contact with MG-63 cells at a final density of 1×10^4 cells/well in 96-well tissue culture plates for 4 d (direct contact). Where indicated, (i) vehicle (No Tx), (ii) 10 μg/ml of an affinity purified IgG fraction of neutralizing goat anti-human G-CSF serum, or (iii) 10 μg/ml of normal goat IgG serum or murine monoclonal HGF (10 μg/ml) or control (MOPC21) were added daily (R & D Systems). Absolute NFS-60 cell numbers were determined by manual haemocytometer counting and reported as mean ± standard deviation ($n = 4$) at 4 d. *Significant difference from antibody or vehicle control, $P < 0{\cdot}05$ or $^{**}P < 0{\cdot}01$.

was not as a result of the BMPs (2, 4, 5, 6, 7), Epo, bFGF, FL, GM-CSF, IL-1α IL-1β, IL-3, IL-4, IL-5, IL-6, IL-7, IL-11, SCF, LIF, LT, M-CSF, MIP-1α, oncostatin M, PTH, TNF-α or TGF-β.

These findings contribute to our fundamental understanding of the role of osteogenic cells within the haematopoietic microenvironment and the mechanisms by which osteogenic cells and haematopoietic stem cells interact. Previous studies have demonstrated that osteogenic cells support primitive haematopoietic stem cell survival and proliferation, and support stem cell engraftment in bone marrow transplantation (El-Badri *et al*, 1998; Nelissen *et al*, 2000). However, the biochemical basis of the haematopoietic activity of osteogenic cells has been relatively obscure. Previous biochemical studies have failed to detect secreted G-CSF from osteogenic cells, while only cell-associated G-CSF has been demonstrated by Western blot analysis and immunoprecipitation (Taichman *et al*, 1996). Of note, other haematopoietic co-mitogens such as SCF have not been found to be produced by, or responsible for, the haematopoietic supportive activity of the osteogenic cells in our systems, further underscoring the concept that haematopoiesis occurs on a network of cells of which differentiated osteoblasts are a component. Within this scope, our data suggest that HGF, in collaboration with other haematopoietic growth factors such as G-CSF, may be a central molecular player in stem cell survival and proliferation supported by osteoblastic cells within the bone marrow microenvironment. However, other haematopoietic activities produced by human osteoblasts remain to be identified.

Recently, Satoh *et al* (1997) observed that the preadipose cell line MC3T3-G2/PA6 (PA6) stimulated the proliferation of NFS-60 cells when cultured in direct contact with each

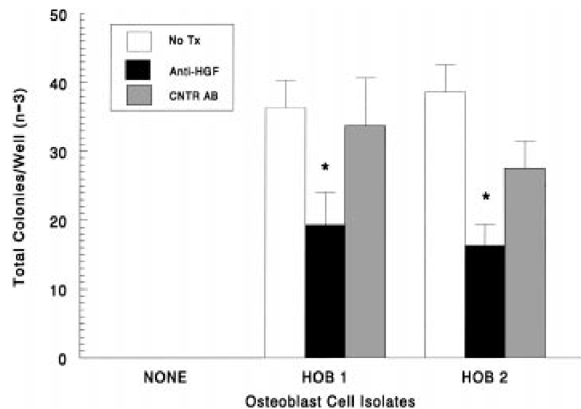

Fig 5. Survival of haematopoietic progenitor cells on HOBs. Human CD34$^+$ bone marrow cells were seeded onto confluent human osteoblast monolayers for 2 weeks in the presence or absence of neutralizing monoclonal antibody to HGF. After 2 weeks, the cells were recovered, counted and replated in methylcellulose in the presence of cytokines (IL-3, GM-CSF and erythropoietin). After two more weeks, total haematopoietic colonies were scored using light microscopy. *Indicates significant difference from GF control ($P < 0{\cdot}05$).

other, in part owing to the production of HGF. In contrast to these observations, we found that the proliferation of NFS-60 cells in our osteosarcoma system was cell-contact independent and could be attributed to secreted HGF. However, it should also be noted that additional non-HGF activity may be produced by osteosarcoma cells that may support haematopoiesis, as G-292 and U2-OS cells support NFS-60 proliferation without apparently producing HGF (Fig 2B) in our system. Takai *et al* (1997) recently found that HGF was produced within the bone marrow stromal microenvironment. The present studies confirmed these results and show that the secretion of HGF by stromal osteogenic cells makes a substantial contribution to haematopoietic cell proliferation (Takai *et al*, 1997). These results may explain, at least in part, the recent findings that osteogenic cells directly support the proliferation of stem cells and their engraftment following allogeneic bone marrow transplantation (El-Badri *et al*, 1998).

These observations suggest that HGF may be a critical cytokine linking the physiology of osteogenic cells and the haematopoietic marrow. Recombinant HGF is known to stimulate the formation of colonies from haematopoietic CD34$^+$ progenitor cells from human bone marrow, peripheral blood or umbilical cord blood (Nishino *et al*, 1995; Zarnegar & Michalopoulos, 1995; Goff *et al*, 1996). This effect synergizes with the activity of G-CSF, GM-CSF, IL-3 and SCF (Galimi *et al*, 1994; Nishino *et al*, 1995; Goff *et al*, 1996, 1997) by either direct or indirect mechanisms (Ratajczak *et al*, 1997). Conversely, HGF has been directly linked to the metabolism of bone. HGF induces the formation of osteoclasts in the presence of osteogenic cells and it may even activate bone resorption (Fuller *et al*, 1995; Ohnishi *et al*, 1997). HGF has been reported to both enhance or decrease osteoblast proliferation (Inaba *et al*, 1993; Grano *et al*, 1996) and to increase expression of alkaline phosphatase (Inaba *et al*, 1993). Two classes of HGF receptors have been identified. Expression of the high affinity HGF receptor c-Met is observed predominantly in epithelial cells, but may be expressed by a variety of cells, including bone marrow stromal cells, osteogenic cells and osteosarcomas (Kmiecik *et al*, 1992; Gohda *et al*, 1994; Takai *et al*, 1997).

HGF production by fibroblasts is enhanced by the addition of heparin or phorbol ester, whereas dexamethasone and TGF-β inhibit HGF production. Fibroblast HGF production is upregulated by IL-1β and TNFα by increasing their basal production of HGF (Matsumoto *et al*, 1992; Inaba *et al*, 1993; Gohda *et al*, 1994; Takai *et al*, 1997). Other agents, including EGF, platelet-derived growth factor (PDGF), aFGF, bFGF (Gohda *et al*, 1994) and cAMP-elevating agents such as forskolin, cholera toxin and PGE_2 (Tamura *et al*, 1993; Matsunaga *et al*, 1994; Sugiyama *et al*, 1996) markedly simulate the production of HGF. In contrast, little is known concerning what regulates the production of HGF produced by osteogenic cells. In preliminary investigations, it does not appear that IL-1β and TNFα alter HGF production by

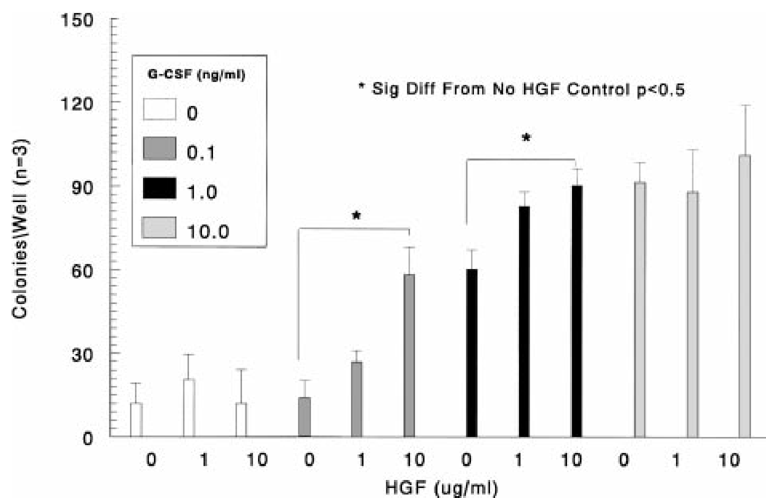

Fig 6. G-CSF and HGF stimulate haematopoietic progenitor cells colony formation. To directly assay the role of HGF in the ability of HOBs to support normal human haematopoietic progenitor cells *in vitro*, CD34$^+$ bone marrow cells were cultured for 2 weeks in methylcellulose in the presence or absence of increasing does of recombinant G-CSF and HGF. At the conclusion of the culture period, progenitor cell activity was determined. After two more weeks, total haematopoietic colonies were scored using light microscopy. *Indicates significant difference from GF control ($P < 0{\cdot}05$).

MG-63 cells (unpublished observations). We do not yet know whether the haematopoietic cells themselves cooperate with osteogenic cells in the production of the HGF, as is the case for osteoblast-derived IL-6 and possibly MIP-1α (Taichman *et al*, 1997b, 2000b).

In summary, the present data demonstrate that HGF contributes to the support of haematopoiesis by osteogenic cells. The data support a model in which osteogenic cells, by virtue of their direct proximity to primitive repopulating stem cells and their production of HGF and G-CSF, play a unique role in supporting stem cell survival and proliferation within the bone marrow microenvironment. Further investigation will be required in order to determine the biological basis of osteoblast-supported haematopoiesis, its regulation and its precise role in stem cell survival, maintenance and proliferation.

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