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Human osteoblast-like cells and osteosarcoma cell lines synthesize macrophage inhibitory protein 1α in response to interleukin 1β and tumour necrosis factor α stimulation *in vitro*

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Summary. Recent investigations have demonstrated that macrophage inhibitory protein 1α (MIP-1α) plays a critical role in haematopoiesis. In part, MIP-1α limits the differentiation of early haematopoietic cells, thereby ensuring that sufficient quantities of blood precursors are available to meet haematopoietic demands. MIP-1α is produced by cells of the marrow microenvironment (marrow stromal cells) in response to a variety of stimuli, including interleukin 1β (IL-1β) and tumour necrosis factor α (TNF-α). Our recent investigations demonstrated that normal human osteoblast-like cells (HOBs) maintain the early phenotype of haematopoietic precursors, like other members of the bone marrow stroma. Although the precise molecular mechanisms for these observations have not been determined, the production of MIP-1α remains one such possibility. In the present study, we investigated whether cells of the osteoblast lineage under basal, IL-1β and/or TNF-α stimulation produce MIP-1α. We observed that IL-1β and TNF-α stimulated HOBs and human osteosarcoma cells to rapidly express MIP-1α mRNA and to secrete large quantities of the protein. MIP-1α mRNA and protein was not, however, detected under basal conditions. Perhaps more importantly, enriched human $CD34^+$ bone marrow cells in co-culture may be capable of stimulating the expression of MIP-1α mRNA by HOBs *in vitro*. These findings suggest that human osteoblast-like cells may produce MIP-1α *in vivo* to support haematopoiesis at sites where osteoblasts and haematopoietic cells are closely associated.

Keywords: osteoblasts, haematopoiesis, microenvironment, macrophage inhibitory protein 1α, bone marrow stromal cells.

Normally, haematopoietic cell development depends upon intimate interactions with the adherent cell populations of the bone marrow. These cells, known collectively as bone marrow stromal cells (BMSCs), include fibroblasts, adipocytes, macrophages and endothelial cells. Although morphologically distinct, all BMSCs probably display overlapping haematopoietic activities. For example, most clonally derived or mixed BMSC populations secrete low levels of granulocyte/macrophage colony-stimulating factor (GM-CSF), acid and basic fibroblast growth factors (FGF), tumour necrosis factor α (TNF-α), transforming growth factor β (TGF-β), stem cell factor (SCF) and interleukins (IL-1β, -4, -5, -6, -7, -11, -17) to support basal haematopoiesis (Metcalf, 1988; Roberts *et al*, 1988; Dexter *et al*, 1990; Hirano, 1990; Ruscetti *et al*, 1991; Toksoz *et al*, 1992; Kittler & Quesenberry, 1993). In the presence of inflammatory monokines IL-1β and TNF-α, BMSCs secrete enhanced quantities of G-CSF and GM-CSF (Guba *et al*, 1992; Fibbe *et al*, 1988; Kittler *et al*, 1992). Differences are known *in vitro*, particularly with regard to the ability to support early haematopoietic cells. The distinctions may be due to the differential expression of cell adhesion molecules, extracellular matrix proteins and/or the production of novel cytokines and inhibitors.

Osteoblasts are also members of the bone marrow microenvironment. However, their role in haematopoiesis has not yet been completely defined. Although it is widely recognized that osteoblasts may regulate the activity and/or development of osteoclasts, which are derived from

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haematopoietic precursors, there remains limited direct evidence for a more global role of osteoblasts in haematopoiesis (Rodan & Martin, 1982; Fuller *et al*, 1991; Kassem *et al*, 1991; Roodman, 1991). Nevertheless, a substantial body of evidence now shows that: (i) endosteal osteoblasts may share a common precursor with other bone marrow stromal cells (Dorheim *et al*, 1993); (ii) osteoblast-like cells produce cytokines *in vitro,* which if produced *in vivo* would have activities on haematopoietic cells (Gowen *et al*, 1990; Littlewood *et al*, 1990; Felix *et al*, 1991; Birch *et al*, 1993; Lacey *et al*, 1994; Taichman & Emerson, 1994); and (iii) early haematopoietic precursors reside in close proximity to endosteal surfaces (Lord & Hendry, 1972; Lord *et al*, 1975; Gong, 1978; Deldar *et al*, 1985; Hermans *et al*, 1989; Lord, 1990; Cui *et al*, 1996).

We have begun to address the role of osteoblasts in haematopoiesis by examining primary human osteoblast-like cells (HOBs) for their ability to support the proliferation and/or maintenance of human $CD34^+$ bone marrow cells. We have observed that HOBs support the limited expansion of long-term culture initiating cells (LTC-ICs) and haematopoietic progenitor cells *in vitro* (Taichman *et al*, 1996). In part, these activities are probably mediated through the secretion of soluble cytokines and through cell–cell adhesions (unpublished observations). Moreover, the production of IL-6 by HOBs is augmented by the presence of early haematopoietic precursors bearing the $CD34^+$ marker *in vitro* (Taichman *et al*, 1997). Additionally, like other members of the bone marrow stroma, osteoblasts respond to IL-1β and TNF-α by secreting enhanced cytokine levels (Lacey *et al*, 1993; Avioli & Chaudhary, 1994). Together, these findings imply the existence of a functional dialogue between osteoblasts and early haematopoietic precursors.

Recently, it was demonstrated that IL-1β and TNF-α stimulate several BMSC lineages, including macrophages, fibroblasts, endothelial and T cells, to produce macrophage inflammatory protein 1α (MIP-1α) (Wright *et al*, 1980; Fahey *et al*, 1990; Eaves *et al*, 1993; Orolsky *et al*, 1995). MIP-1α is a member of a group of cytokines best known for their ability to attract host cells to sites of inflammation. Consequently, MIP-1α is essential for the mounting of an effective inflammatory response to viral infections (Cocchi *et al*, 1995; Cook *et al*, 1995), although receptors for MIP-1α may act as co-receptors for viral absorption (Atchison *et al*, 1996). In haematopoiesis, Lord *et al* (1976) first demonstrated that soluble factor(s) produced by BMSCs were capable of inhibiting the proliferation of early haematopoietic cells by demonstrating that rapidly proliferating haematopoietic spleen colony-forming cells (CFUs) were protected from the lethal effects of large doses of tritiated thymidine. Similar inhibitory effects were observed in long-term bone marrow cultures (Toksoz *et al*, 1980). Later, Graham *et al* (1990) identified the active molecule as MIP-1α using sequence analysis and specific antibody blockade. As an inhibitor of stem cell proliferation, recent investigation has focused upon the ability of MIP-1α to provide protection from agents that target the DNA synthesis phase of the cell cycle (Dunlop *et al*, 1992; Lord *et al*, 1992). Also important is the recognition that most primitive haematopoietic cells are normally quiescent *in vivo*, whereas their leukaemic counterparts maintain a rapid turnover. For this reason, the use of MIP-1α is currently being examined for its ability to protect stem cells against the effects of cytotoxic agents used for therapy of rapidly proliferating leukaemic cells (Dunlop *et al*, 1992; Lord *et al*, 1992).

Although the precise molecular mechanisms with respect to how osteoblasts support haematopoiesis have yet to be determined, by virtue of the fact that osteoblasts maintain LTC-ICs *in vitro* the production of MIP-1α remains one such possibility. In bone, IL-1β or TNF-α are known to stimulate bone resorption by osteoclasts in conjunction with soluble activities produced by osteoblasts (Roodman, 1991). Further, osteoclastic mobility is activated by MIP-1α (Fuller *et al*, 1995). Whether osteoblasts synthesize MIP-1α in response to IL-1β or TNF-α remains unknown. Kukita *et al* (1992) reported that MIP-1α mRNA is localized to osteoblasts in bone-remodelling sites in the marrow and that osteoclasts were frequently observed within the vicinity of these cells. If osteoblasts synthesize MIP-1α then this protein may be part of the normal mechanisms used by osteoblasts to regulate bone resorption.

Based upon these observations, we hypothesized that osteoblasts synthesize MIP-1α. We investigated whether primary human osteoblast-like cells and several human osteosarcoma cell lines expressed MIP-α mRNA or protein under basal, IL-1β and/or TNF-α stimulation. We observed that IL-1β and TNF-α stimulated cells of the osteoblastic lineage to rapidly express MIP-1α mRNA and to secrete large quantities of the protein. MIP-1α mRNA and protein was not, however, detected under basal conditions. Perhaps more importantly, enriched human $CD34^+$ bone marrow cells in two out of three experiments stimulated the expression of MIP-1α mRNA in mixed HOB/$CD34^+$ cell co-culture. Together, these findings suggest that human osteoblast-like cells may produce MIP-1α *in vivo* to support haematopoiesis at sites where osteoblasts and haematopoietic cells are closely associated.

MATERIALS AND METHODS

Human osteoblast-like cells (HOBs) and osteosarcoma cell lines. Enriched human osteoblast cultures were established using modifications of the methods described by Robey & Termine (1985). Normal human trabecular bone was obtained from patients undergoing orthopaedic 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 $\leq 1\,mm^2$; 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/Dulbecco's minimal essential medium (DMEM, Biofluids, Rockville, MD, USA) with low Ca^{2+} and 10% fetal bovine serum (FBS). Thereafter, the cultures were maintained in calcium-replete Ham's F12/DMEM (1:1 v/v) medium containing 10% heat-inactivated FBS (Gibco BRL, Life

Technologies Laboratories, Grand Island, NY, USA), antibiotics, 10 mM β-glycerol phosphate and 10 μg/ml L-ascorbate, hereafter referred to as experimental medium. To verify that the cells expressed an osteoblast phenotype, the cultures were screened for the expression of the osteoblast-specific protein osteocalcin (osteocalcin$^+$) by reverse transcriptase polymerase chain reaction (RT-PCR) as previously described (Taichman & Emerson, 1994).

The human osteosarcoma cell lines HOS T85 (CRL1743), MG-63 (ATCC CRL1424), SaOS-2 (ATCC 85-HTB) and U2-OS (ATCC HTB96) were maintained in either MEM with Earle's salts or McCoy's 5A medium with 10% heat-inactivated FBS. For experiments, cells were plated to an initial density of $2{\cdot}0\times10^5$ cells/cm^2 with experimental medium changes on days 5, 7, 10 and 14.

Human CD34+ bone marrow cells and bone marrow stromal cells. Human bone marrow cells were obtained from healthy adult volunteers by iliac crest puncture and aspiration into preservative-free heparin or from endosteal marrow scrapings during orthopaedic surgery 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 Iscoves modified Dulbecco's medium (IMDM), 10% FBS, 10% equine serum, 1 mM hydrocortisone, penicillin/streptomycin (Dexter *et al*, 1973; Gibco BRL). Plastic adherent cells (BMSCs) were washed and cultured until confluent. Mixed human bone marrow stromal cells (BMSCs) were obtained as described and were utilized after the third passage (Guba *et al*, 1992). For experiments, cells were plated to an initial density of $2{\cdot}0\times10^4$ cells/cm^2 with experimental medium changes on days 5, 7, 10 and 14. After overnight adherence, the non-adherent cells were recovered and the CD34$^+$ bone marrow cells were isolated by positive immunomagnetic selection using the QUIND/10 antibody with the Mini-MACs System (Miltenyi Biotec, Sunnyvale, CA, USA). Where possible, the positive selected cells were stained with a phycoerythrin conjugate of the anti-CD34 antibody HPCA-2 (Becton Dickinson, San Jose, CA, USA) to evaluate purity by fluorescence activated cell sorting (EPICS C, Coulter Corp. Hialeah, FL, USA). Under these conditions, 92·5 ± 3·5% ($n=2$) of the recovered cells expressed the CD34 antigen.

Reverse transcriptase polymerase chain reaction (RT-PCR). Total cellular RNA was recovered 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 by electrophoresis with ethidium bromide and absorbency at A_{260}/A_{280}. RNA (1·0 μg), 10× RT buffer (1× RT buffer: 50 mM Tris, pH 8·3, 50 mM KCl, 8·0 mM $MgCl_2$ and 10 mM dithiothreitol), 25 mM dXTP mix [25 mM of each dXTP (ACGT)], 3·0 μg oligo-(dT), and 2·5 U reverse transcriptase (M-MLV Reverse Transcriptase, Gibco BRL, Gaithersburg, MD, USA) were incubated at 38°C for 1 h. One-fifth of the double-stranded product was then mixed with 10×*Taq*/RT buffer (1×*Taq*/RT buffer: 10 mM Tris, pH 8·3, 50 mM KCl, 1·5 mM $MgCl_2$, 0·01% gelatine and 2·0 mM dithiothreitol), 1 mM dXTP mix, 500 ng of each sense and antisense oligonucleotide and 2·5 U *Taq* polymerase (AmpliTaq DNA Polymerase; Perkin Elmer Cetus, Norwalk, CT, USA). The samples underwent thermal cycling at 94°C for 1 min, 60°C for 1 min and 72°C for 2 min for 35 cycles, followed by a 10-min extension at 72°C (Perkin Elmer Cetus DNA thermal cycler). The products were electrophoresed in 3% agarose and visualized using ethidium bromide. Where indicated, positive RNA specimens were included in the analyses. These included IL-1β- and TNF-α-stimulated human bone marrow stroma (MIP-1α), or in some cases *E. coli* lipopolysaccharide (LPS) was utilized to stimulate MIP-1α expression in HOBs (25 μg/ml, Sigma Chemical Co., St. Louis MO, USA). The oligonucleotide synthesis centre at the University of Michigan prepared the sense and antisense primers. To control for false positives due to 'overamplification' or DNA contamination, reverse transcriptase was omitted from selected reactions and primers were designed to cross intron/exon boundaries. The primers used in these investigations were: MIP-1α, sense CTCAGAATCATGCAGGTCTCCA, antisense GGCAACAACCAGTCCATAGAAG, expected size 405 bp (Davatelis *et al*, 1989); β-actin, sense GTGGGGCGCCCCCA GGCACCA, antisense CTCCTTAATGTCACGCACGATTTC, expected size 548 bp, where the β-actin antisense primers incorporated additional sequences for the T7 promotor; and TGF-β_1, sense ACCACTGCCGCACAACTCCGGTGAC, antisense ATCTATGACAAGTTCAAGCAGAGTA, expected size 268 bp (Brenner *et al*, 1989).

Production of cell-conditioned medium. Confluent osteoblast-like cells or osteosarcoma cell lines were washed twice and fed with experimental medium. For cytokine stimulations, IL-1β (0–250 pM), TNF-α (0–1·0 nM) or vehicle were added directly to the cultures (R & D Systems, Minneapolis, MN, USA). At 24, 48, 72 or 96 h, the conditioned medium was harvested, spun at 12 000 r.p.m. at 4°C for 15 min and frozen at −80°C until further assay.

Co-culture of CD34+ bone marrow cells and primary human osteoblasts. Primary HOBs at confluence were harvested by trypsinization (20 min, 37°C, 0·05% trypsin/0·5 mM EDTA; Gibco BRL) and seeded into 96-well or 24-well flat-bottomed tissue culture plates to a final density of 2×10^4/cm^2 in experimental medium. After 7 d, the HOBs monolayers were washed twice and 1×10^4 CD34$^+$ bone marrow cells were seeded onto the osteoblast monolayers. Thereafter, conditioned medium was collected over the first 120 h of the osteoblast/blood cell co-culture. In some cases, CD34$^+$ bone marrow cells ($1{\cdot}0\times10^4$) were seeded into the top chamber of TransWell® (Corning Costar Corporation, Cambridge, MA, USA) dual-chambered 24-well plates (0·4 mM pore size) with confluent primary human osteoblasts in the bottom chamber.

Cytokine enzyme-linked immunosorbent assays (ELISAs). Conditioned medium was collected at the times indicated and stored at −80°C until assayed for cytokine levels using the double-antibody sandwich method assembled with commercially available components according to the directions of the manufacturer (sensitivity 15·0 pg/ml, range 31·25–1000 pg/ml; R & D Systems). Cytokine levels are presented as means ± standard errors (μg/ml) for triplicate determinations.

Statistical analyses. Arithmetic means and standard

deviations were calculated using INSTAT 1·14 (GraphPad, San Diego, CA, USA) software, and presented as mean MIP-1α (pg/ml) produced by HOBs (or osteosarcomas) in the absence of IL-1β and TNF-α. Analysis of variance (ANOVA) was utilized to determine statistical significance to a level of $P<0{\cdot}05$. Turkey's HSD *post hoc* test was utilized for pairwise comparisons.

RESULTS

Expression of MIP-1α and TGF-β1 mRNA by human osteoblast-like cells (HOBs)

To determine whether primary HOBs or human osteosarcoma cell lines express message for MIP-1α, cultures were established over a 14-d period in the presence of L-ascorbate and β-glycerol phosphate to facilitate the expression of a mature osteoblast phenotype (Taichman & Emerson, 1996). At the conclusion of the culture period, total cellular RNA was isolated and examined for MIP-1α mRNA using RT-PCR. As a positive control for MIP-1α expression, mixed bone marrow stromal cells were cultured under similar conditions and stimulated with IL-1β (250 pM) and TNF-α (1 nM) for 48 h. To control for our ability to detect similar sized transcripts, matched samples were examined for TGF-β1 mRNA. Under these conditions, only the positive controls expressed MIP-1α mRNA, however all of the cells examined, including HOBs, HOS T85, MG-63, SaOS-2 and U2-OS, expressed TGF-β_1 transcripts (Fig 1).

As IL-1 and TNF-β have been implicated in the regulation of bone resorption mediated through osteoblast-like cells (Littlewood *et al*, 1991), the inflammatory monokines were evaluated for their ability to induce the expression of MIP-1α mRNA by cells of the osteoblastic lineage. For these experiments, IL-1β and TNF-α were added in combination to the cultures. At 48 h, total cellular RNA was isolated and examined for MIP-1α expression. As shown in Fig 2, normal human osteoblast-like cells expressed MIP-1α transcripts in

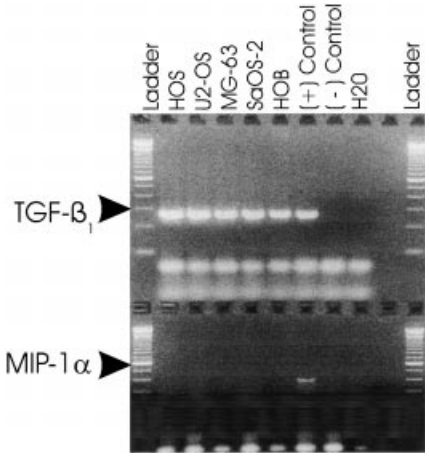

Fig. 1. Human osteoblast-like cells (HOBs) and human osteosarcoma cell lines do not constitutively express MIP-1α mRNA. Total cellular RNA was isolated and examined for MIP-1α and TGF-β_1 mRNA transcripts by RT-PCR (35 cycles). As positive controls for MIP-1α expression, mixed bone marrow stromal cells were cultured under similar conditions and stimulated with IL-1β (250 pM) and TNF-α (1 nM) for 48 h. To control for the ability to detect a similar sized transcript, matched samples were examined for TGF-β1 mRNA. Negative controls omitted reverse transcriptase from the positive controls.

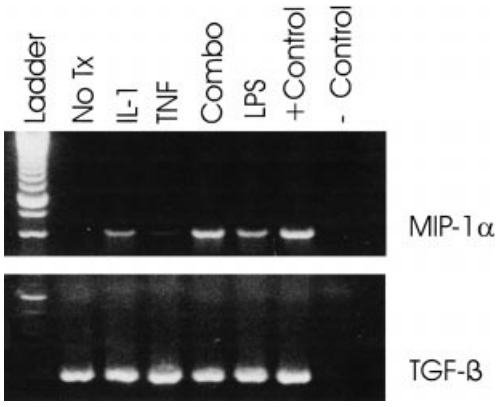

Fig. 2. IL-1β and TNF-α stimulate the expression of MIP-1α mRNA by human osteoblast-like cells (HOBs). Total cellular RNA was isolated from primary HOBs and examined for the expression of β-actin or MIP-1α mRNA transcripts by RT-PCR (35 cycles). Where indicated, HOBs cultures were stimulated with IL-1β (250 pM), TNF-α (1 nM) or both for 48 h. To control for our ability to detect similar sized transcripts, matched samples were examined for TGF-β1 mRNA As positive controls for MIP-1α expression, mixed bone marrow stromal cells were cultured under similar conditions and stimulated with IL-1β (250 pM) and TNF-α (1 nM) for 48 h or HOBs were stimulated with *Escherichia coli* LPS (25 μg/ml) (Sigma Chemical Co., St. Louis, MO, USA). For negative controls, reverse transcriptase was omitted from the positive control.

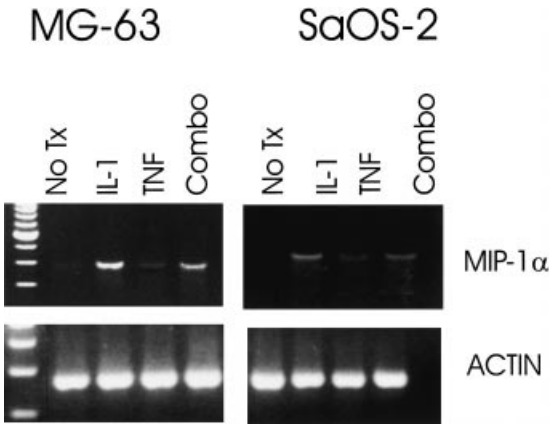

Fig. 3. IL-1β and TNF-α stimulate the expression MIP-1α mRNA by human MG-63 and SaOS-2 osteosarcomas. Total cellular RNA was isolated from MG-63 and SaOS-2 osteosarcomas and examined for MIP-1α transcripts by RT-PCR (35 cycles). Where indicated, the osteosarcoma cultures were stimulated with IL-1β (250 pM), TNF-α (1 nM) or both for 48 h. As positive controls for MIP-1α expression, mixed bone marrow stromal cells were cultured under similar conditions and stimulated with IL-1β (250 pM) and TNF-α (1 nM) for 48 h (data not presented). Negative controls omitted reverse transcriptase from the positive control.

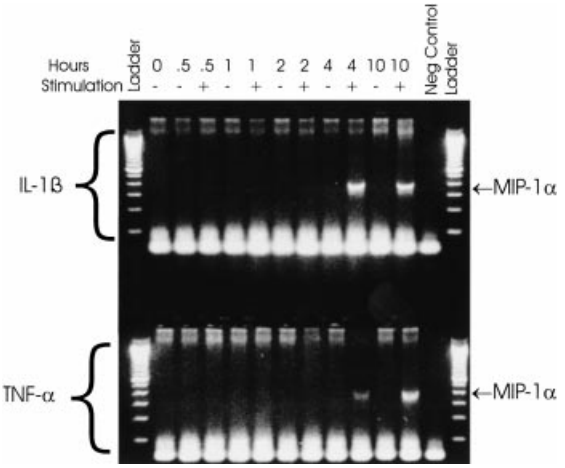

Fig. 4. Time course for MIP-1α mRNA expression stimulated by IL-1β and TNF-α MG-63 cells were stimulated with IL-1β (250 pM) or TNF-α (1 nM) over the course of 72 h where a plus sign signifies IL-1α or TNF-α stimulation and a minus sign indicates vehicle controls. At times 0, 0·5, 1, 2, 4, 10, 24, 48 and 72 h, total cellular RNA was collected and examined for MIP-1α mRNA by RT-PCR. Data for the 24- to 72-h period were not presented, but were positive. Negative controls omitted reverse transcriptase from the positive controls.

response to IL-1β and TNF-α. IL-1β and TNF-α also induced MIP-1α expression by MG-63 and SaOS-2 cells (Fig 3). Similar data were obtained for HOBs, T85 and U2-OS (data not presented).

To determine the time frame in which MIP-1α mRNA expression occurs in response IL-1β and TNF-α, MG-63 and SaOS0-2 cells were stimulated with IL-1β or TNF-α over a 72-h period. At various time points, RNA was collected and reverse transcribed for PCR analysis. By 4 h, MIP-1α transcripts were observed in response to both IL-1β and TNF-α (Fig 4; data for the 24- to 72-h period are not presented because all time points beyond 4 h were positive). IL-1β or TNF-α also induced MIP-1α transcripts in Sa0S-2 cells (data not presented).

Secretion of MIP-1α protein by human osteoblast-like cells in response to IL-1β or TNF-α stimulation

The preceding results suggested that osteoblasts do not constitutively express MIP-1α mRNA, however MIP-1α mRNA expression was rapidly induced by IL-1β or TNF-α. Therefore, we next evaluated whether the MIP-1α mRNA is translated. Here, primary HOBs were stimulated with IL-1β and TNF-α and the levels of MIP-1α present in the conditioned medium assayed by sandwich ELISA. As shown in Fig 5, IL-1β stimulated the production of MIP-1α ($n = 3$) protein for the three HOB isolates tested, although considerable variation in the magnitude of the response was observed. Similarly, TNF-α stimulation of the HOBs also resulted in the production of MIP-1α. Together, IL-1β and TNF-α stimulated MIP-1α production to a considerably greater degree than either treatment alone (relative expression of the combination; increase over IL-1β or TNF-α alone was 61·8 ± 9·8% and 91·1 ± 8·7% respectively). No

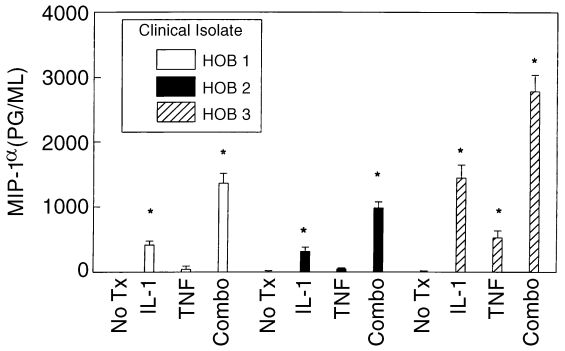

Fig. 5. IL-1β and TNF-α stimulates the secretion of MIP-1α by human osteoblast-like (HOBs) cells. Primary HOBs from three individuals were stimulated with IL-1β (250 pM), TNF-α (1 nM) or both (combo) for 4 d. The levels of MIP-1α present in the conditioned medium were assayed by sandwich ELISA. Results are reported as means ± standard deviations ($n = 3$). An asterisk indicates a significant difference from untreated controls (no treatment) ($P < 0{\cdot}05$) as determined by ANOVA.

MIP-1α protein was detected in the medium of unstimulated HOBs.

To determine whether IL-1β and TNF-α also stimulated MIP-1α production by MG-63 and SaOS-2 cells, these cell lines cells were stimulated with log-fold serial dilutions of IL-1β (0–250 pM) or TNF-α (0–1·0 nM) and the conditioned medium was assayed for MIP-1α protein levels after 4 d. This time point was selected as one in which protein accumulation was most easily observed (data not presented). Both IL-1β and TNF-α significantly stimulated the accumulation of MIP-1α protein in MG-63 and SaOS-2 culture supernatants, however IL-1β was more potent in stimulating MIP-1α production (Figs 6 and 7). Synergistic stimulation (defined as more than additive) of MIP-1α production by IL-1β and TNF-α was observed in some experiments, however this was not a consistent finding.

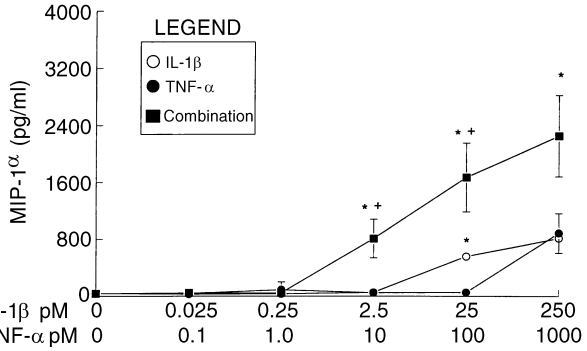

Fig. 6. IL-1β and TNF-α stimulate the secretion of MIP-1α by MG-63 cells. At confluence, MG-63 human osteosarcomas were stimulated with log-serial dilutions of IL-1β (0–250 pM), TNF-α (0–1 nM) or both for 4 d, and MIP-1α levels were assayed by sandwich ELISA. Results are reported as means ± standard deviations ($n = 3$). An asterisk indicates significant difference from untreated controls (no Tx) ($P < 0{\cdot}05$) as determined by ANOVA. A plus sign indicates synergistic interactions between IL-1 or TNF treatments ($P < 0{\cdot}05$) as determined by ANOVA.

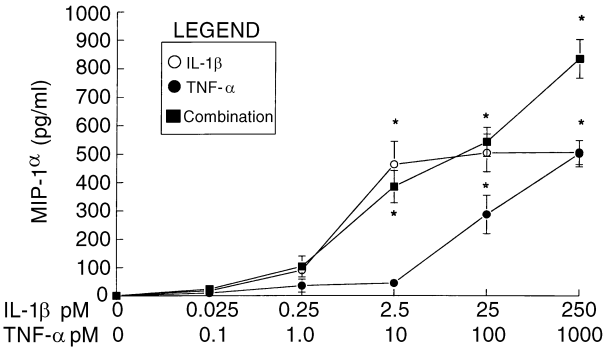

Fig. 7. IL-1β and TNF-α stimulate the secretion of MIP-1α by SaOS-2 cells. At confluence, SaOS-2 human osteosarcomas were stimulated with log-serial dilutions of IL-1β (0–250 pM), TNF-α (0–1 nM) or both for 4 d, and MIP-1α levels were assayed by sandwich ELISA. Results are reported as means ± standard deviations ($n = 3$). An asterisk indicates significant difference from untreated controls (no Tx) ($P < 0{\cdot}05$) as determined by ANOVA.

Normal human osteoblast-like cells may express MIP-1α mRNA in response to human haematopoietic CD34$^+$ bone marrow progenitors

Previously, we have noted that human bone marrow haematopoietic CD34$^+$ cells were able to enhance the levels of IL-6 produced by human osteoblast-like cells. Other groups have reported that CD34$^+$ cells express mRNA for IL-1β (Watari *et al*, 1996). As IL-1β and TNF-α induce MIP-1α production, we hypothesized that human osteoblasts would respond to the presence of early haematopoietic cells by producing MIP-1α. For these experiments, explant-derived HOBs monolayers were seeded with enriched human CD34$^+$ bone marrow cells either in direct contact or separated from the HOBs by a porous micromembrane (TransWell®). Over the 4-d period, immunoreactive MIP-1α protein was not detected by ELISA, however MIP-1α mRNA was expressed by the HOBs in direct contact with enriched CD34$^+$ cell populations in two out of three experiments (Fig 8). Under conditions where osteoblasts and haematopoietic cells were separated, in one of the two positive cases, MIP-1α mRNA was expressed by the HOBs (Fig 8). Together these data raise the possibility that osteoblasts may respond to the presence of early haematopoietic cells by expressing MIP-1α mRNA. No clear correlations could be drawn between the expression of MIP-1α mRNA in the co-cultures and IL-1β and TNF-α transcripts expressed by the haematopoietic cells (data not shown).

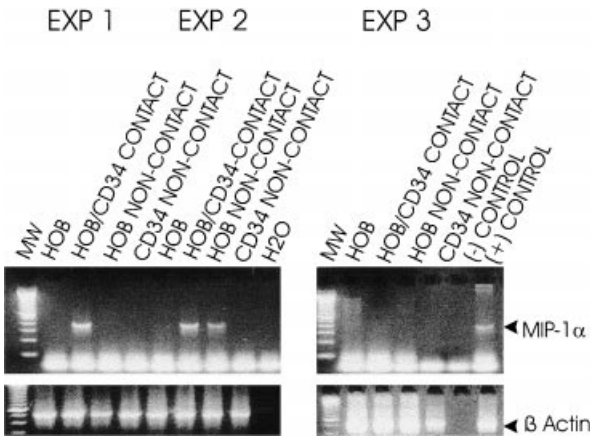

Fig. 8. CD34$^+$ bone marrow cells stimulate (RT-PCR) detection of MIP-1α mRNA. CD34$^+$ bone marrow cells and primary HOBs were cultured either alone or together in direct contact or separated by porous micromembranes for 96 h in three independent experiments. At the conclusion of the culture period, mRNA from osteoblasts only (HOBs), mRNA from osteoblasts and CD34$^+$ cells in co-culture (HOBs/CD34 contact), mRNA from osteoblasts recovered from dual-chambered co-culture (HOBs/non-contact), mRNA from CD34$^+$ cells recovered from dual-chambered co-culture (CD34/non-contact). Each sample was examined by RT-PCR for the expression of MIP-1α or β-actin. Negative controls were by omission of reverse transcriptase (not shown) from the reverse transcription reaction or by use of H_20. MW, molecular weight ladder.

DISCUSSION

Hematopoiesis is a tightly regulated process in which specialized cells of the bone marrow microenvironment produce both physiological inhibitors and stimulators of early haematopoietic cells. As events localized to endosteal surfaces are probably critical for the maintenance of haematopoietic stem and progenitor cells, we have begun to explore this tissue compartment with several models of endosteal haematopoiesis. In the present study, we examined osteoblast-like cells for their ability to produce MIP-1α. MIP-1α is a member of a family of at least 10 cytokines of 8–10 kDa exhibiting homology ranging from 20% to 45%. Members of this family have both proinflammatory as well as reparative activities. In addition, MIP-1α may inhibit the progression of haematopoietic stem cells to the cell cycle (Broxmeyer *et al*, 1991; Mancini *et al*, 1992; Eaves *et al*, 1993; Maltman *et al*, 1993; Verfaillie *et al*, 1994; Bonnet *et al*, 1995; Mayani *et al*, 1995). The effects of MIP-1α, however, appeared to be highly variable, depending upon the maturational status of the target cells. For example, early haematopoietic progenitors were maintained better in cultures supplemented with MIP-1α and IL-3 (Verfaillie & Miller, 1994; Verfaillie *et al*, 1994). MIP-1α did not inhibit the ability of haematopoietic progenitors to form colonies, but decreased the number of progenitors activated in myelodepressed animals without a significant effect on the long-term reconstituting populations (Eaves *et al*, 1993; Keller *et al*, 1994). On more mature populations, MIP-1α may have few inhibitory effects (Mancini *et al*, 1992; Mayani *et al*, 1995). In part, differences may be due to alterations of receptor density and/or activation. Here, both high- and low-affinity receptors, with apparent molecular masses of 92 kDa and 52 kDa, are expressed by haematopoietic cells (Avalos *et al*, 1994). The K_d for the high-affinity receptors correlates with MIP-1α concentrations required to induce biological effects on stem cells (Avalos *et al*, 1994). Receptors for MIP-1α are present on cells enriched for CD34 expression from patients with chronic myeloid leukaemia (CML) such that it has been suggested that *in vitro* CML progenitor cells may be refractory to the growth inhibitory effects of MIP-1α (Chasty *et al*, 1995).

Previously, we observed that primary human osteoblast-like cells support early blood cell lineages as assayed by the maintenance of long-term culture initiating activity *in vitro* (Taichman *et al,* 1996). As MIP-1α mRNA has been detected in many members of the bone marrow microenvironment cells including macrophages, fibroblasts and endothelial cells, we hypothesized that osteoblast-like cells would also express MIP-1α (Fahey *et al*, 1990; Eaves *et al*, 1993; Orolsky *et al*, 1995). As such, we hypothesized that part of the molecular basis for the ability of osteoblasts to support the maintenance of early blood progenitors as immature cells may be owing to MIP-1α.

In our *in vitro* experiments, we did not find evidence for the expression of MIP-1α mRNA in cells of the osteoblast lineage in the absence of inflammatory stimuli. We therefore asked whether the inflammatory cytokines IL-1β and TNF-α would stimulate MIP-1α production. These inflammatory mediators were chosen because of their role in regulating mineralized tissue metabolism (Dewhirst *et al*, 1986; Fibbe *et al*, 1988; Linkhart *et al*, 1991; Chaudhary *et al*, 1992;Taichman & Hauschka, 1992; Avioli & Chaudhary, 1994) and mRNA for IL-1β has been characterized in early human haematopoietic cells (Watari *et al*, 1996). We observed that IL-1β and TNF-α stimulated the expression MIP-1α mRNA in mixed populations of bone marrow stromal cells, but more importantly we observed that primary human osteoblast-like cells and several human osteosarcoma cell lines expressed MIP-1α mRNA after the cytokine stimulation. mRNA for MIP-1α was expressed as early as 4 h in the cells examined (MG-63 and SaOS-2), but significant protein levels were not detected for 2–3 d (unpublished observations).

Previously, it has been reported that $CD34^+$ cells express mRNA for IL-1β (Watari *et al*, 1996). Therefore, we wished to determined whether human osteoblasts respond to the IL-1β produced by these early cells by producing MIP-1α. Although MIP-1α protein was not detected in any of our co-cultures, in some cases mRNA for MIP-1α was detected in samples collected from mixed osteoblast/haematopoietic cells. At present, it is not clear which of the cells in the mixed culture ($CD34^+$ or HOBs) expressed the MIP-1α mRNA. In this regard, $CD34^+$ bone marrow cells may express MIP-1α mRNA, depending on the methods utilized to isolate them (Watari *et al*, 1996). Just as likely, however, remains the possibility that osteoblasts may themselves express MIP-1α message in the co-culture because in one case MIP-1α transcripts were expressed by HOBs that were separated from the haematopoietic cells by porous micromembranes. Unfortunately, because of the small sample size and the possibility of interpatient variability, we were not able to determine whether the haematopoietic cells activated the expression of MIP-1α mRNA through IL-1β or TNF-α pathways (data not presented). Alternatively, it may be possible that the $CD34^+$ cells stimulate MIP-1α production through IL-1β- and TNF-α-independent pathways as was observed when $CD34^+$ cells induced HOBs to secrete elevated IL-6 (Taichman *et al*, 1997).

If MIP-1α protein is secreted by osteoblasts in co-cultures, this activity may in part be responsible for the maintenance of primitive haematopoietic cells in long-term cultures. As defects in the responsiveness of CML neoplastic progenitors to MIP-1α are believed to have an important role in their uncontrolled proliferation, it would be as interesting to determine whether differences exist among the ability of haematopoietic progenitor cells to stimulate MIP-1α mRNA (Chasty *et al*, 1995; Watari *et al*, 1996). Clearly, these considerations will require further experimentation.

In summary, we demonstrated that primary human osteoblast-like cells and osteosarcoma cell lines expressed MIP-1α mRNA and secrete MIP-1α protein in response to IL-1β and TNF-α exposure. Furthermore, human osteoblast-like cells may produce MIP-1α in response to the presence of early haematopoietic cell populations, although from our data we could not correlate the expression of MIP-1α mRNA with the expression of IL-1β and TNF-α transcripts by the haematopoietic cells (data not presented). These findings suggest that *in vivo* human osteoblast-like cells may produce MIP-1α to nevertheless support normal haematopoiesis processes.

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