Proliferative Response of Different Human Osteoblast-like Cell Models to Proinflammatory Cytokines

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ABSTRACT

Children with inflammatory bowel disease are known to be at risk of osteopenia. The cause of this osteopenia is likely to be multifactorial, but the inflammatory process with its characteristic overproduction of cytokines has been implicated. To investigate this possible contribution of the disease activity to the development of osteopenia, we performed *in vitro* assays of the proliferation of osteoblast-like cells of differing origins in response to the inflammatory cytokines tumor necrosis factor-α and IL-1β. Osteoblast-like cells derived from pediatric bone explants, adherent stromal cells derived from bone marrow (osteoprogenitors), MG-63 osteosarcoma cells, and SV-40 virally transformed osteoprogenitor cells (HCC1) were studied. Tumor necrosis factor-α stimulated the proliferation of cells in primary cultures (*i.e.* from explants and marrow samples) in a linear, dose-dependent manner. In contrast, inhibition of proliferation was observed with the established cell lines (MG-63 and HCC1). IL-1β stimulated proliferation of all cells apart from the immortalized human bone marrow cell line, HCC1, in which case potent inhibition was observed. We conclude that proinflammatory cytokines are potent regulators of osteoblast-like cell proliferation, and that the responses are specific to cell type. The opposite results obtained with established cell lines compared with the primary cultures suggest that careful consideration should be given to choosing the most suitable cell line for *in vitro* studies relating to *in vivo* mechanisms predisposing to osteopenia. (*Pediatr Res* **48: 163–168, 2000**)

Abbreviations
IBD, inflammatory bowel disease
TNF, tumor necrosis factor
BMC, bone mineral content
MEM, minimum essential medium
DMEM, Dulbecco's modified Eagle medium

IBD predisposes patients to the development of osteopenia and thus increases the risk of fracture (1–4). In extreme cases, such as a 16-year-old boy reported by our group (5) who presented with Crohn's disease of the small intestine, fractures of the vertebrae may produce severe adverse effects including pain and loss of mobility. This case prompted us to undertake the first population-based study of BMC in children with IBD, and the results (6) reveal that reduced BMC is indeed a common finding in these patients.

Osteopenia may be important in childhood because the rate of bone accretion increases markedly during puberty and peak bone mass, achieved in early adult life, determines lifetime risk of fracture (7). Bone turnover is greater in children, and therefore factors that influence bone metabolism at this time are likely to have potent effects on the future bone health and morbidity of this population.

The inappropriate and aggressive inflammatory response present in the gut during relapse of IBD produces proinflammatory cytokines (8, 9). Activated macrophages release TNF-α and IL-1β, and these cytokines initiate a cascade of further cytokines, such as IL-6, in a variety of tissue and cell types (10). Circulating concentrations of TNF-α, IL-1β, IL-6, and others have been found to be elevated in individuals with IBD (11–15), although this finding is still contentious (16). Inflammation in the gut damages the mucosal barrier and renders it more permeable to bacterial antigens (17). These circulating antigens may initiate inflammatory events throughout the body, and local production of TNF-α and IL-1β may ensue. We hypothesize that proinflammatory cytokines modulate bone cell function by inhibiting normal mineralization, thus contributing to osteopenia.

During the past few decades, research into bone biology has used as model systems, human osteoblast-like cells derived from a variety of sources. Human bone and bone marrow samples are often difficult to obtain, although the cells obtained from such samples are deemed to be representative of mature (bone-derived) and immature (marrow-derived) osteoblasts. To

assess the contribution of the inflammatory process in the development of osteopenia, we have tested the influence of TNF-α and IL-1β on proliferation of different types of cells derived from bone and bone marrow. In addition, we have studied the effects of these cytokines on two established human osteoblast-like cell lines, tumor-derived MG-63 osteosarcoma cells and SV40 virally transformed osteoprogenitor HCC1 cells.

METHODS

Cell culture. Human osteoblast-like cells were cultured from bone explants derived from four children not suffering from diseases likely to have adverse effects on osteoblast function. The Bro Taf Local Research Ethics Committee granted ethical approval for this work. Informed consent (from patient or parent as appropriate) was obtained in each case. Samples were obtained from *1)* the digits of a foot from a 9-month-old girl after amputation of the leg because of agenesis of the fibula, *2)* a 13-year-old boy undergoing epiphysiodesis of the distal femur and proximal tibia and fibula of the normal leg, *3)* a 13-year-old girl undergoing open reduction of a fractured radial head, and *4)* a 6-year-old girl after femoral osteotomy. Bone explants were washed extensively with PBS, placed in 24-well plates, and cultured in α-MEM containing HEPES (25 mM), penicillin (100 U/mL), streptomycin (100 μg/mL), and 10% FCS in a humidified 5% CO_2 atmosphere at 37°C. At confluence, the osteoblast-like cells were transferred to 25-cm^2 flasks for routine culturing with the same growth conditions as those described above for primary cultures but in an aerobic incubator at 37°C. Confluent monolayers of cells were subcultured using trypsin (0.025%, wt/vol) and EDTA (0.025%, wt/vol) in PBS. All assays were performed with cells at passage 8 or lower.

Adherent marrow stromal cells were isolated from bone marrow samples, obtained by iliac aspiration as waste from two patients (a 15-year-old boy and a 9-year-old girl) in remission from hematologic malignancy, as previously described by Cheng *et al.* (18). Briefly, the sample (1–2 mL) was centrifuged at 500 $\times$ g for 5 min, and the pelleted cells were resuspended in 10 mL of α-MEM (10% FCS). Seven milliliters of lymphocyte separation medium (Life Technologies Ltd, Paisley, U.K.) was underlayered, and the sample was centrifuged at 500 $\times$ g for 30 min to separate cells. Cells at the layer interface were harvested and centrifuged at 500 $\times$ g for 5 min. The cell pellet was then resuspended in α-MEM (as described above) and cultured in 25-cm^2 flasks. After cells adhered to the plastic substratum (5 d), nonadherent cells were washed away during the first medium change. All assays were performed with cells at passage 6 or lower.

MG-63 osteoblast-like cells derived from an osteosarcoma (19) were maintained in 25-cm^2 flasks in DMEM with penicillin (100 U/mL), streptomycin (100 μg/mL), and 5% FCS at 37°C in a humidified atmosphere (95% air, 5% CO_2).

HCC1, an immortalized human bone marrow cell line, was kindly donated by Dr. Brian Ashton, Department of Rheumatology, Robert Jones and Agnes Hunt Orthopaedic Hospital, Oswestry, Shropshire, U.K. They were maintained in MEM containing penicillin (100 U/mL), streptomycin (100 μg/mL), and 5% FCS. The bipotential nature of this cell line has previously been reported (20). With manipulation of the culture conditions, the clone has been shown (using enzyme assay, reverse transcription-PCR, and histologic analysis) to differentiate along both adipocytic and osteoblastic lineages.

Cytokines. Recombinant human TNF-α and IL-1β were purchased from R&D Systems Europe, LTD (Abingdon, U.K.). The relevant concentrations were prepared in PBS before use in cell cultures.

Proliferation assay. Cells were seeded into 96-well plates at a density of approximately 1500 cells per well, and allowed to settle overnight. The medium used for all cell types was α-MEM containing 5% FCS, and cells were incubated in a humidified incubator at 37°C in 95% air and 5% CO_2. Treatments were added on d 1, and plates were assayed at intervals during the next 11 d. Media and treatments were replenished every 3 d. The CellTitre 96 Aqueous Non-Radioactive Cell Proliferation Assay (Promega UK, Southampton, U.K.) kit was used to assess the numbers of cells in each well. This is a spectrophotometric method based on conversion of a tetrazolium compound to a colored formazan by the mitochondrial activity of living cells. The outside wells of the 96-well plate were not used, leaving the inner 6 $\times$ 10 array. Twelve wells were assigned to each treatment used. After washing in PBS, cells were incubated for 2 h at 37°C with 120 μL of a solution of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and phenazine methosulfate (PMS) in phenol red–free DMEM (Life Technologies Ltd, Paisley, U.K.) and according to the manufacturer's instructions. Absorbances were read on a Packard Spectra-Count Microplate Photometer at 490 nm. We have demonstrated similar results with proliferation bioassays comparing this assay with a method estimating total protein content of cells (21) (data not shown).

Characterization of the osteoblast phenotype. The osteoblastic phenotype of cells derived from bone explants was confirmed by staining for the presence of alkaline phosphatase with nitroblue tetrazolium salt and 5 bromo-4-chloro-3-indolylphosphate (22, 23). Furthermore, all cell lines derived from bone explants were shown to produce collagen type 1 C-terminal propeptide (a marker of collagen synthesis) using the Prolagen-C EIA kit [Metra Biosystems (UK) Ltd, Oxford, U.K.]. The cells were not monitored for alkaline phosphatase or collagen production during the assay time courses.

Statistics. The statistical analysis presented was performed on a minimum of 12 replicates using the unpaired *t* test. Differences were considered to be significant when *p* values were $\leq$0.05. Results are presented as mean $\pm$2 SEM.

RESULTS

Human osteoblast-like cells. Both TNF-α and IL-1β, at low concentrations of 0.01–1 ng/mL (Fig. 1) and at higher concentrations of 1–100 ng/mL (data not shown), stimulated proliferation of primary human osteoblast-like cells in a linear, dose-dependent fashion. This effect was highly reproducible, both between different assays with the same cell line and
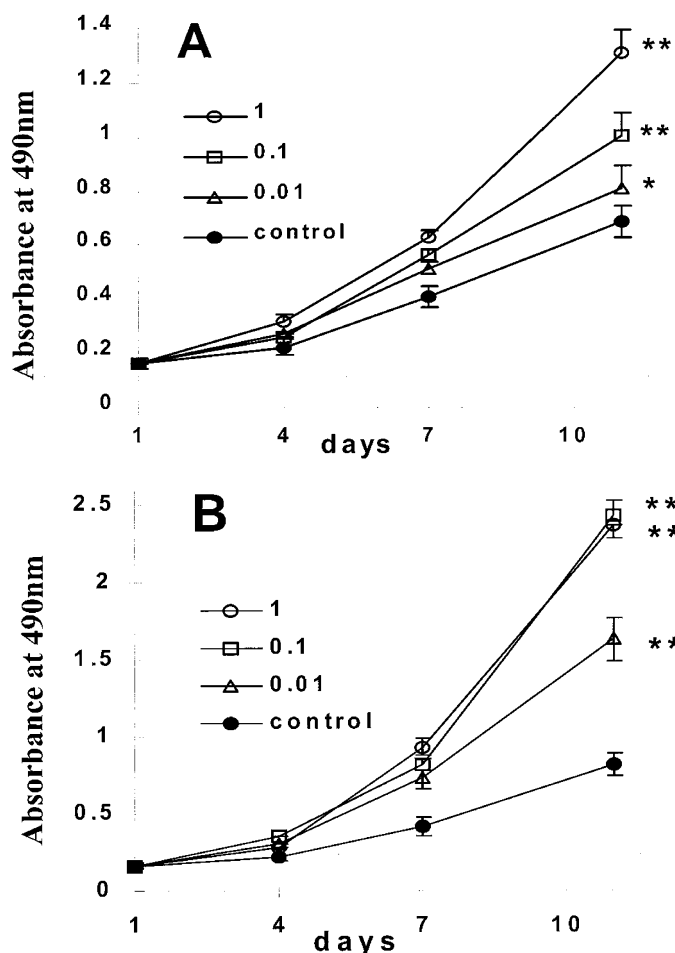
Figure 1. Effects of (A) TNF-α (0, 0.01, 0.1, and 1 ng/mL) and (B) IL-1β (0, 0.01, 0.1, and 1 ng/mL) on proliferation of osteoblast-like cells derived from a 9-month-old girl. The y-axis shows absorbance at 490 nm after incubation of cells with MTS-PMS for 2 h. Absorbances are linearly proportional to cell numbers (data not shown). Each point is the mean of 12 replicates, and error bars represent ±2 SEM. TNF-α and IL-1β stimulate cell proliferation in a dose-dependent manner. * $p < 0.05$; ** $p < 0.01$ (treatment vs control).
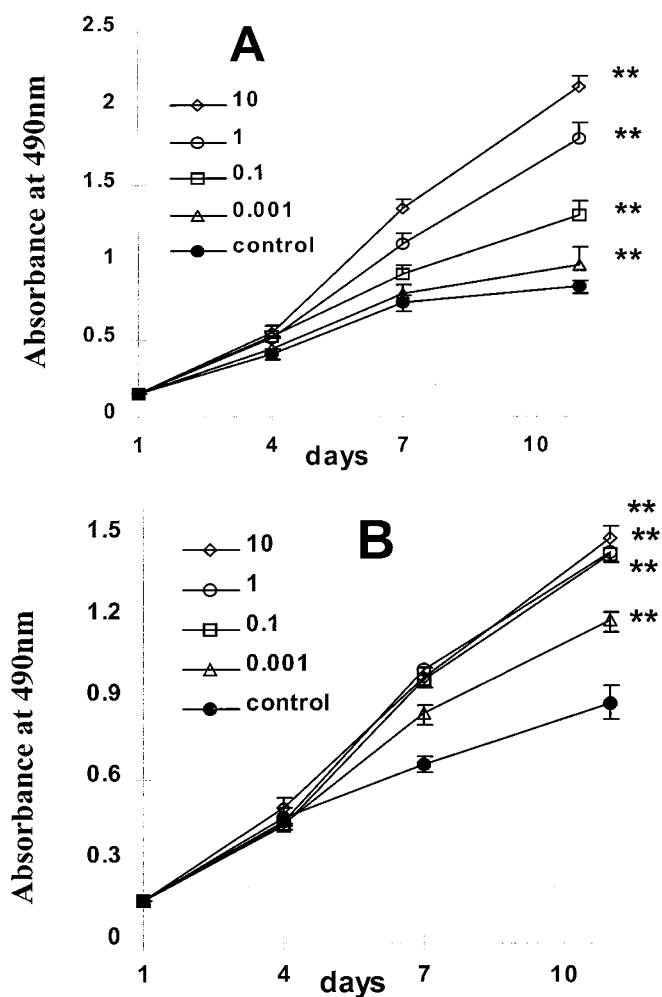
Figure 2. Effects of (A) TNF-α (0, 0.001, 0.1, 1, and 10 ng/mL) and (B) IL-1β (0, 0.001, 0.1, 1, and 10 ng/mL) on proliferation of bone marrow stromal cells derived from a 15-year-old boy. The y-axis shows absorbance at 490 nm after incubation of cells with MTS-PMS for 2 h. Absorbances are linearly proportional to cell numbers (data not shown). Each point is the mean of 12 replicates, and error bars represent ±2 SEM. TNF-α and IL-1β stimulate proliferation in a dose-dependent manner. ** $p < 0.01$ (treatment vs control).

between four different cell lines. By the 11th d of incubation, there was a significant ($p < 0.05$) difference between the cell numbers in the control and cytokine-treated (0.01–1 ng/mL and 1–100 ng/mL) wells. Cells treated with 0.001 ng/mL TNF-α, however, did not proliferate faster than untreated controls (data not shown). In later experiments, cells derived from adult bone were found to respond similarly after coincubation with TNF-α or IL-1β (0.01–1 ng/mL and 1–100 ng/mL; data not shown).

Marrow stromal cells. Bone marrow adherent cells are known to contain the early osteoblast precursors (osteoprogenitors). TNF-α and IL-1β, at low concentrations of 0.01–1 ng/mL ($p < 0.01$) and at higher concentrations of 1–100 ng/mL ($p < 0.01$), stimulated the proliferation of these cells in a linear, dose-dependent fashion. Representative results are shown in Figure 2. Similar results were obtained with two other cell lines.

MG-63 osteosarcoma cells. When grown under standard conditions *in vitro*, MG-63 osteosarcoma cells proliferate much more rapidly than osteoblast-like cells obtained from normal bone explants (results not shown). TNF-α, when

present at high concentrations (10 ng/mL), significantly ($p < 0.01$) inhibited (80%, 88%, and 94% of control at d 4, 7, and 10, respectively) the proliferation of these MG63 cells (Fig. 3A). In contrast, IL-1β (10 ng/mL) significantly stimulated ($p < 0.01$) cell proliferation (120% of control; Fig. 3B). In general, however, the effect of cytokines on MG-63 cells was of lower magnitude than that seen in the primary osteoblast-like cells.

HCC1 osteoprogenitor cells. TNF-α (0.01–10 ng/mL) and IL-1β (0.1–10 ng/mL) strongly inhibited the proliferation of these cells (Fig. 4). This inhibition of proliferation was much greater than that previously observed with TNF-α and MG-63 cells.

DISCUSSION

We have postulated that childhood IBD activity, mediated through the production and action of inflammatory cytokines, can affect the metabolism of bone directly, with adverse con-
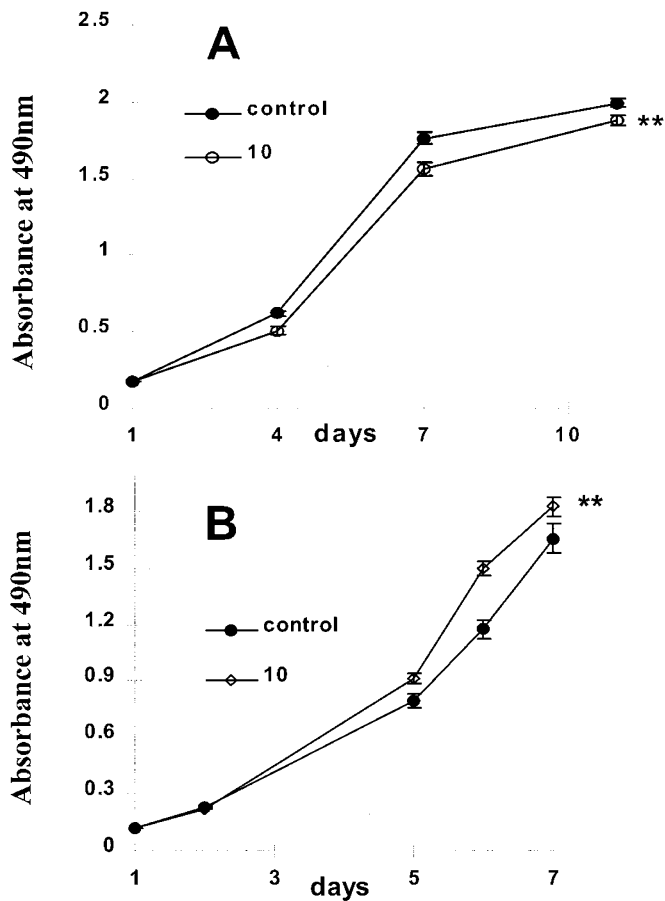
Figure 3. Effects of (*A*) TNF-α (0 and 10 ng/mL) and (*B*) IL-1β (0 and 10 ng/mL) on proliferation of MG-63 osteosarcoma cells. The *y*-axis shows absorbance at 490 nm after incubation of cells with MTS-PMS for 2 h. Absorbances are linearly proportional to cell numbers (data not shown). Each point is the mean of 12 replicates, and error bars represent ±2 SEM. TNF-α inhibits, but IL-1β stimulates cell proliferation. ** $p < 0.01$ (treatment *vs* control).

Figure 4. Effects of (*A*) TNF-α (0, 0.01, 0.1, 1, and 10 ng/mL) and (*B*) IL-1β (0, 0.01, 0.1, 1, and 10 ng/mL) on proliferation of HCC1 osteoprogenitor cells. The *y*-axis shows absorbance at 490 nm after incubation of cells with MTS-PMS for 2 h. Each point is the mean of 12 replicates, and error bars represent ±2 SEM. TNF-α (at all concentrations tested) and IL-1β (at 0.1, 1, and 10 ng/mL) inhibited cell proliferation. * $p < 0.05$; ** $p < 0.01$ (treatment *vs* control).

sequences. We have selected various cell types possessing osteoblast-like phenotypes and evaluated the effect of these proinflammatory cytokines on their proliferation. At the outset, we had hypothesized that these cytokines would reduce osteoblast cell numbers, and that osteopenia *in vivo* might thus be a consequence of a decreased abundance of cells. However, TNF-α and IL-1β caused an increase in proliferation rate in all primary cell cultures tested. It is possible that this stimulation is mechanistically brought about by the induction of the transcription factors NF-κB (24, 25) and AP-1 (26), whose target genes include cyclin D1 (27) and the proto-oncogene *c-myc* (28–31). Increased proliferative activity is characteristic of the early stages of the differentiation pathway of osteoblasts (32). One possibility for reconciling osteopenia *in vivo* with stimulated proliferation *in vitro* is that cytokines may lengthen the immature period of osteoblast development and disrupt or delay the differentiation sequence.

An inhibition of proliferation was seen with the tumor-derived MG-63 cells (TNF-α only) and the virally transformed cell line HCC1 (TNF-α and IL-1β). The specific assay we used does not address the mechanism of growth inhibition seen with
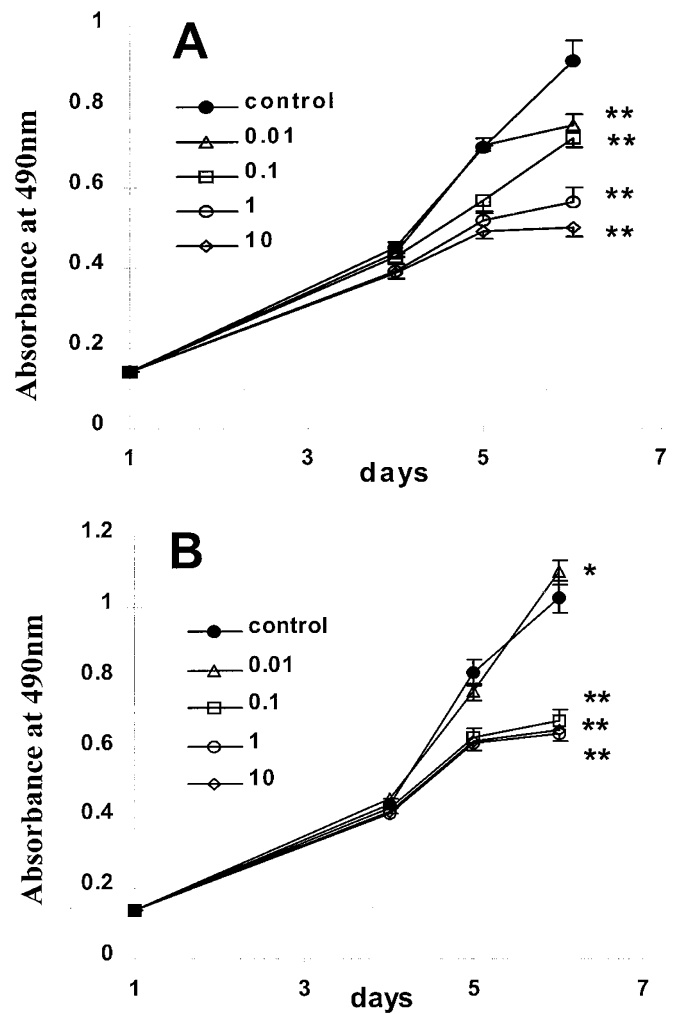
TNF-α in both cases and IL-1β in the case of HCC1 cells. Conceivably, both apoptotic events and growth arrest could be occurring.

Other workers have reported the effect of TNF-α and IL-1β on the proliferation of human osteoblast-like cells obtained from a variety of sources (33–38). The methodologies used (*e.g.* tissue culture medium, cell densities, and proliferation assays) and results obtained are extremely varied. Only one of these studies (33) has investigated the effects of cytokines on cells derived from bone explants and osteosarcoma cells at the same time and therefore using the same conditions. This one study differs from ours in that it demonstrates an inhibition of growth of primary osteoblasts as well as MG63 cells after incubation of cells with TNF-α or IL-1β. Frost *et al.* (38), however, demonstrate a stimulation of proliferation of primary osteoblast-like cells, although their results differ from ours in that they report that the response to IL-1β is delayed at least 4 d compared with TNF-α. They conclude that TNF-α and IL-1β

must have different intracellular mechanisms causing cell growth. Taichman and Hauschka (37) report that both cytokines stimulated proliferation of MG63 cells. We observed a stimulation of proliferation of MG63 cells with IL-1β, but report an inhibition with these cells and TNF-α.

A view is emerging that the change to an immortal phenotype via multistep mutation or viral infection may render a cell sensitive to a latent killing action possessed by TNF-α (but not IL-1β) (39, 40). Caspase molecules are activated by the ligand-bound TNF-α receptor (41). These molecules are a subset of the apoptotic machinery that is responsible for cleavage of various vital cellular components. In primary cells, caspase activity is curtailed by NF-κB, but also activated by TNF-α (42–44). However, the expression of certain oncogenes appears to repress NF-κB activity, and, thus, the pathway conferring survival from caspase activity is not available to these cells (40).

It is possible that the magnitude of growth inhibition of MG-63 cells compared with the HCC1 cells may be explained by their relative abilities to induce the tumor suppressor gene p53 (45). p53, induced by cytokines, is able to arrest growth and provoke apoptosis in oncogene-expressing cells. Thus, in HCC1 cells, the observed growth inhibition may be a combined effect of p53 and caspase activity. However, MG-63 cells lack functional p53 activity owing to rearrangements in the gene for p53 (46), and thus only caspases may initiate death. Growth inhibition of HCC1 with IL-1β may be caused solely by p53 inasmuch as the IL-1β pathway does not invoke caspase activity.

Bone mass depends on the balance between bone formation by osteoblasts and bone resorption by osteoclasts. Bone loss is thus the result of an imbalance of the bone remodeling process, in which bone resorption exceeds bone formation. Other workers have investigated the effects of TNF-α on osteoclasts, and it is thought that TNF-α targets osteoclast precursors and promotes differentiation of these preosteoclasts into mature osteoclasts, resulting in increased bone resorption (47). Others have also thought, however, that TNF-α depresses bone formation by osteoblasts in addition to enhancing bone resorption by osteoclasts, a situation that would exacerbate bone loss (48). Our work supports this view. Furthermore, it has recently been shown that cells of the osteoblast lineage play an essential role in the activation of osteoclast function. It seems that ultimately, the process of osteoclast formation is dependent on hemopoietic precursors being presented to the appropriate osteoblasts or stromal cells in an environment that provides appropriate stimulatory factors (49).

The differential proliferative responses observed in this study highlight the problem of selecting appropriate and representative bone cell models for studying the effects of disease. Transformed cell lines, although of uniform phenotype, have unrepressed replication activity and fail to display the normal coupling of differentiation and growth arrest. We propose that the primary cultures are more representative of the *in vivo* osteoblast and hence provide more relevant experimental models than MG63 cells for the further study of inflammation-induced osteopenia.

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