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Effects of Interferon Alpha on Human Osteoprogenitor Cell Growth and Differentiation In Vitro

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Abstract The specific effects of interferon alpha (IFNα), on the differentiation pathways of human osteogenic cells are not known. The aim of this study was to investigate possible effects of IFNα on osteogenic development by investigating cell differentiation, colony formation (colony forming unit-fibroblastic, CFU-F), cell proliferation, and gene expression, in particular bone morphogenetic protein (BMP) expression, of human bone marrow osteoprogenitor cells. Human bone marrow fibroblasts were cultured with or without the addition of IFNα (5–1,000 IU/ml) in the presence and absence of dexamethasone (10 nM) and ascorbate (100 μM), which are agents known to affect osteogenic differentiation. IFNα produced a significant dose-dependent inhibition of cell proliferation and alkaline phosphatase specific activity at concentrations as low as 50 IU/ml. IFNα (50–1,000 IU/ml) inhibited the stimulation of alkaline phosphatase specific activity induced by ascorbate and dexamethasone. Examination of CFU-F showed dose- and time-dependent inhibitions of colony formation and reductions in both colony size and alkaline phosphatase-positive CFU-F colonies particularly at earlier times. Reactivity with an antibody specific for osteoprogenitors (HOP-26), was reduced in IFNα-treated cultures. Northern blot analysis showed a significant dose-dependent up-regulation of BMP-2 mRNA, estrogen receptor alpha mRNA and osteocalcin mRNA expression in ascorbate/dexamethasone cultures. In contrast, IFNα significantly inhibited BMP-2 mRNA expression in the absence of ascorbate and dexamethasone. In conclusion, IFNα inhibits human osteoprogenitor cell proliferation, CFU- F formation, HOP-26 expression, and alkaline phosphatase specific activity and modulates BMP-2 gene expression. These results suggest a role for IFNα in local bone turnover through the specific and direct modulation of osteoprogenitor proliferation and differentiation. J. Cell. Biochem. 74:372–385, 1999.

Key words: interferon alpha; osteoprogenitor; differentiation; osteoblast; bone marrow

The regulation of human bone formation is subject to the interplay between an array of regulatory growth factors, hormones, and cytokines [Triffitt and Oreffo, 1999]. The role of interferon alpha (IFNα), an immunomodulatory cytokine, in bone turnover has not been studied extensively. To date, the effects of IFNα on osteogenic cell development in vitro in respect to cell-colony formation, proliferation, and differentiation of human bone marrow fibroblasts have not been studied in detail. Interferons (IFNs) comprise a multigene family and three major classes have been identified: IFNα, IFNβ, and IFNγ [Pestka et al., 1987; Aguet, 1991; Gutterman, 1994; Skalla, 1996]. IFNs have been further classified by their receptor binding; IFNα and β share the same receptor structure (Type I receptor) while IFNγ utilises a separate receptor system (Type II receptor) [Colamonici and Pfeffer, 1991; Uze et al., 1994].

Early work focussed on the antiviral role of IFNs, by which they were first identified. However, a variety of studies have implicated additional roles for IFNα in malignant, immunologic, angiogenic, inflammatory, and fibrotic diseases [Dorr, 1993; Agarwala and Kirkwood, 1995; Haria and Benfield, 1995; Gutterman, 1994]. The inhibition of tumour cell growth by IFNs has led to the suggestion that these cytokines may function as tumour suppressor genes and that IFNs act to inhibit cell proliferation and induce differentiation [Thomas and Balkwill, 1991; Jaramillo et al., 1995; Lengyel, 1993]. Studies have shown IFNα will induce G_0/G_1

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arrest, down regulate G_1 cyclins, cyclin A, and inhibit c-myc expression and inhibit the mitogenic activity of tissue and haematopoietic growth factors [Creasy et al., 1980; Aman et al., 1994, 1996].

The effects of IFNs on bone cells in vitro have been studied in a variety of different models. IFNα will inhibit the proliferation of normal human bone-derived cells and a human osteogenic osteosarcoma cell line TE 85 (HOS) [Beresford et al., 1990]. Significant differences were observed between normal trabecular bone-derived cells and osteosarcoma cells in their response to this cytokine. Differential effects of IFNα and γ have been reported on SaOS-2 and U2-OS cells [Harju et al., 1990]. Proliferation of SaOS-2 cells was inhibited by low concentrations of IFNα or γ whereas even high concentrations of IFNα or γ (10,000 U/ml) did not affect U2-OS growth. Other workers have shown that IFNγ will i) inhibit cell proliferation, collagen synthesis and $1,25(OH)_2D_3$-stimulated osteocalcin production in trabecular bone cell cultures [Gowen et al., 1988], ii) inhibit DNA synthesis in rat calvarial osteoblast cultures and inhibit bone resorption [Smith et al., 1986, Gowen et al., 1986], iii) if over expressed in transgenic mice, give rise to a complex chondro-osseous lesion diagnosed as osteochondrodysplasia in which granulomatous lesions and residual degenerating cartilaginous masses are present in the bones [Nii et al., 1997], and iv) depending on the cell line used, significantly modulate alkaline phosphatase activity [Harju et al., 1990; Yoshihara et al., 1990].

In vivo studies have shown that the growth of human osteosarcoma xenografts in nude mice can be inhibited by human IFNα [Brosjo et al., 1985, 1989; Forster et al., 1988]. Growth inhibition was associated with the mineralisation and partial replacement of the tumour by normal bone and this was initially thought to be due to the induction of differentiation of the tumour cells by IFNα. However, the use of species-specific antibodies to both murine and human type I collagens, showed that IFNα induced the production of a bone-inductive agent by the human osteosarcoma cells and that this resulted in the formation of new heterotopic mouse bone tissue [Forster et al., 1988]. Although the mechanism of bone induction by IFNα in these osteosarcoma cells remains unclear, it is likely that bone morphogenetic proteins (BMPs), which play a central role in osteogenesis, may be involved. The expression and role of the BMPs in the control of mesenchymal cell differentiation has been reported extensively and it is clear that BMPs are key factors in the augmentation of bone formation [reviewed in Hogan, 1996]. However, the regulation of expression of BMPs in osteoblastic cultures by IFNα has not been studied.

A number of reports have described the direct anti-proliferative effects of IFNs on haematopoietic progenitors. IFNα will inhibit colony formation by haematopoietic progenitor cells, including colony-forming unit-granulocyte, -erythroid, -macrophage, -megakaryocyte (CFU-GEMM), day 7 colony-forming unit granulocyte-macrophage (CFU-GM), pluripotent haematopoietic stem cells (CFU-S), and burst-forming unit-erythroid (BFU-E) [Broxmeyer et al., 1983; Mazur et al., 1986]. Aman and coworkers [1994, 1996] demonstrated that IFNα acted on human bone marrow stromal cells to inhibit production of a number of haematopoietic growth factors including granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte-colony-stimulating factor (G-CSF), interleukin-1 (IL-1), and interleukin-11 (IL-11). In a preliminary study using rabbit and human marrow fibroblasts Wang et al. [1992] showed that IFNα inhibited colony formation of bone marrow fibroblast osteoprogenitors (CFU-F). However there is a lack of information on the action of IFNα on stem cells present within the bone marrow, which give rise to colony forming-units fibroblasts (CFU-F), and on osteoprogenitors. Furthermore, the effects of IFNα on the expression of osteoblastic markers in human osteoprogenitors, the modulation of colony formation and identification of the target cells in differentiating marrow fibroblastic cultures affected remain undefined. The recent development of a monoclonal antibody, HOP-26, a specific marker for early cells of the osteogenic lineage [Joyner et al., 1997], provides a novel tool to investigate the action of IFNα on early osteoprogenitors. The aim of the current study was to learn more about the effects of this agent on the development of bone-forming cells and to investigate the effect of IFNα on the osteogenic differentiation, colony formation (CFU-F), cell proliferation, and expression of bone marker proteins in human bone marrow fibroblast cultures.

MATERIALS AND METHODS

Tissue culture reagents were obtained from Gibco/BRL (Paisley, Scotland). Reagents for RNA extraction and hybridisation were of molecular biology grade from Amersham International plc (Aylesbury, Bucks., England) and Biogenesis Ltd. (Poole, England). $1,25(OH)_2D_3$ was a generous gift from Roche Products Ltd. (Welwyn Garden City, Herts., England). Methyl [^{3}H] thymidine was obtained from Amersham International plc (Aylesbury, Bucks., England). Human recombinant interferon-alpha 2c (IFNα specific activity 2×10^8 IU/mg protein) was kindly provided by Dr. H. Bauer (Department of Orthopaedics, Karolinska Institute, Karolinska Hospital, Stockholm, Sweden). Dexamethasone, alkaline phosphatase kits and all other biochemical reagents were from Sigma Chemical Company (Poole, Dorset).

Cell Culture

Bone marrow samples were obtained from haematologically normal patients undergoing routine total hip replacement surgery. Only tissue which would have been discarded was used, with the approval of the local hospital management committee. Primary cultures of bone marrow fibroblasts were established as previously described [Oreffo et al., 1997]. In brief, marrow cells were harvested using Minimal Essential Medium-alpha modification (αMEM) from trabecular bone marrow samples and pelleted by centrifugation at 500*g* for 5 min at 4°C. The cell pellet was resuspended in 10 ml αMEM and passaged through nylon mesh (90 micron pore size; Lockertex, Warrington, England). Samples of cell suspension were diluted with 0.5% ($^w/_v$) trypan blue in 0.16 M ammonium chloride and the number and viability of nucleated cells determined. Cells were plated at 1×10^4 to 2×10^4/ well in 24-well plates, 2×10^6 cells in 25 cm^2 flasks or at 10^7 cells in 175 cm^2 flasks for Northern analysis. Cells were cultured in αMEM supplemented with 10% ($^v/_v$) foetal calf serum (FCS) alone (basal medium) or dexamethasone (10 nM) and ascorbate-2-phosphate (100 μM) in the presence and absence of IFNα (5, 50, 100, 250, and 1,000 IU/ml). Fibroblastic cultures were refed every 3 days and at the completion of cell culture, the media was removed, the cell layer washed in phosphate buffered saline (PBS), and the cell layer used for alkaline phosphatase, protein, and DNA determinations. For osteocalcin immunocytochemistry studies, all cell cultures were treated with $1,25(OH)_2D_3$ (10 nM) in αMEM supplemented with 10% ($^v/_v$) foetal calf serum for the final 48 h of the culture period. For Northern analysis, cells were washed twice in PBS and stored at −135°C until total RNA was isolated.

Assays for Alkaline Phosphatase Activity and DNA Content

Cell layers were washed with phosphate buffered saline (PBS) and stored at −70°C until assayed for alkaline phosphatase activity. For assay, the cell layer from each well was scraped into 0.5 ml 0.1% ($^v/_v$) triton-X-100. Alkaline phosphatase activity was measured using p-nitrophenyl phosphate as substrate in 2-amino-2-methyl-1-propanol alkaline buffer solution (1.5 M, pH 10.3 at 25°C). DNA content was measured according to the method of West et al. [1985]. Alkaline phosphatase specific activity was expressed as nanomoles of p-nitrophenol/h/μg DNA.

Cell Proliferation Assay

DNA synthesis during cell proliferation was determined by radiolabelled thymidine incorporation. Primary cultures were passaged and plated at a density of 1×10^4 cells/well in 24-well plates in αMEM supplemented with 10% FCS. After 48 h, cells were rinsed twice in PBS, and serum starved for 24 h by incubation in αMEM alone before incubation with and without test agents in αMEM supplemented with 10% ($^v/_v$) FCS for a further 72 h. Cells were pulsed with 3H thymidine (1 μCi/well) for the last 4 h of the incubation period and the amount of 3H thymidine incorporated was measured after trichloroacetic acid precipitation using liquid scintillation counting [Inui et al., 1997].

Immunohistochemistry

Colonies were assessed for HOP-26 immunostaining after 9 days culture as previously described [Joyner et al., 1997]. In brief, cultures were fixed in 4% ($^v/_v$) phosphate-buffered formalin (pH 7.4) for 30 min, blocked with human AB serum diluted 1:10 in TBS (15 min), and incubated with undiluted hybridoma conditioned medium (30 min at 15°C). HOP-26 antibody binding was detected using alkaline phosphatase- or fluorescein isothiocyanate (FITC)-conjugated secondary antibodies (30 min at 15°C)

preabsorbed against human Ig (Dakopatts, Glostrup, Denmark). Flasks were counterstained using Mayer's haematoxylin and non-immunoreactive antibody controls were included in all assays. Light microscopy examination of the cultures was performed using a Zeiss Axiophot photomicroscope (Carl Zeiss Ltd., Welwyn Garden City, Herts, UK). HOP-26 staining was scored (mean ± SEM) from three pooled experiments (22 colonies scored /flask, three flasks per experiments). Reactivity to the osteocalcin specific monoclonal antibody OS35 was assessed after fixation of the cells in 4% ($^{w}/_{v}$) paraformaldehyde in PBS (pH 7.4). Fixed cultures (day 28) were permeabilised using 0.05% Nonidet P40 in TBS (15 min), preblocked with goat serum 1:10 in TBS (15 min), and incubated with OS35 (1:100; 30 min at 15°C). Goat-anti mouse-alkaline phosphatase (1:200) was used as secondary antibody and localisation of enzymic reaction produced with fast red determined microscopically. Flasks were counterstained with Mayer's haematoxylin.

Colony Formation Assay

Primary cultures of bone marrow fibroblastic cells were established as described above except cells were plated out in 25 cm^2 plastic tissue culture flasks at 2×10^6 nucleated cells/flask in αMEM supplemented with 10% ($^{v}/_{v}$) foetal calf serum (FCS), or αMEM supplemented with 10%($^{v}/_{v}$) FCS containing dexamethasone (10 nM)/ascorbate-2-phosphate (100 μM), penicillin (5,000 units/100 ml), and streptomycin sulphate (5 mg/100ml) in the presence or absence of IFNα (0–1,000 IU/ml). Cultures were maintained at 37°C in a gassed incubator, 5% CO_2 in air. The medium was changed after 6 days and then at day 9 and cultures stopped at day 12. Cells grown in the presence of dexamethasone/ascorbate-2-phosphate were stopped on day 9 to prevent merger of the colonies. In time course studies to determine the onset of action of IFNα, cultures were either incubated with IFNα (i) throughout the culture period (ii) for the first 6 days or (iii) for the final 3–6 days of the culture period. At the completion of cell culture, the media was removed, the cell layer washed in phosphate-buffered saline (PBS) and cultures fixed in 95% ($^{v}/_{v}$) ethanol. Microscopy examination of the cultures was performed using a Zeiss Axiophot photomicroscope (Carl Zeiss Ltd, Welwyn Garden City, Herts, UK), and recorded on Kodak Ektachrome (64T) colour reversal film (Kodak, Hemel Hempstead, UK).

Colony Counting and Colony Size Measurement

As shown in previous studies, individual distinct colonies were present at day 12, and these samples provided reproducible stable colony numbers [Oreffo et al., 1998]. Total and alkaline phosphatase-positive colonies were counted by eye using an Anderman colony counter (Anderman and Co. Ltd, Kingston-on-Thames, UK). All counts were performed by an independent observer without prior knowledge of the sample characteristics and the counts were repeated to confirm reproducibility of counts obtained. Mean values for each group were derived from three to six flasks. For quantitative measurement of colony sizes, colony images were captured with a video camera, digitised, and analysed using OPTIMAS image analysis software (DataCell Ltd, Maidenhead, Berks, England). Mean colony sizes (± SEM; cm^2) for each group, were determined from 10 colonies by random selection procedures (three flasks per group).

Histochemical Staining—Alkaline Phosphatase Activity

Cultures were rinsed three times in PBS and fixed in 95% ($^{v}/_{v}$) ethanol and stained using an alkaline phosphatase kit (Sigma kit no.85) according to the manufacturer's instructions. Colonies were considered to be alkaline phosphatase-positive if any cells showed significant staining by light microscopy.

Isolation of Total RNA and Northern Blot Analysis

Total RNA was isolated from T-175cm^2 flasks using RNAzol B according to the manufacturer's instructions (Biogenesis, Poole, England). Poly $(A)^+$ RNA was purified from 22 μg of total RNA using oligo (dT) coated magnetic microspheres (Dynabeads; Dynal UK Ltd., Wirral, UK) according to the manufacturer's instructions and electrophoresced on 1% ($^{w}/_{v}$) agarose gel before blotting on Biodyne nylon membrane (Pall Ultrafine Filtration Corporation) under alkaline conditions. The membrane was baked for 45 min at 80°C to immobilise the transferred RNA. The blot was hybridised with [^{32}P]-labelled riboprobes (Amersham International, England) for BMP-2, estrogen receptor alpha, β-actin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcribed from linearised

plasmids and random primed cDNA probes (Megaprime kit, Amersham International, England) for osteocalcin and alkaline phosphatase. Prehybridisation and hybridisation solution consisted of 50% ($^{v}/_{v}$) formamide, 1% ($^{v}/_{v}$) SDS, 4 × SSPE (0.18 M NaCl, 0.01 M NaH_2PO_4, 0.001 M Na_2EDTA, pH7.7), 0.05% ($^{w}/_{v}$) non-fat dried milk, and 300 µg/ml denatured salmon sperm DNA. Probes were used at ~1 × 10^6 cpm/ml for hybridisation at 68°C for BMP-2, 60°C for estrogen receptor alpha, 42°C for alkaline phosphatase and osteocalcin, and, 65°C for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Post-hybridisation washes were in 2 × SSC with 0.1% ($^{v}/_{v}$) SDS followed by 0.1 × SSC with 0.1% ($^{v}/_{v}$) SDS at the respective temperatures. Oligo(dT)15–18 were labelled using a 3'-end labelling kit (Promega Ltd., Southampton, England). Prehybridisation (2 to 3 h) and hybridisation (overnight) were carried out in a solution consisting of SSC (5×), SDS (0.5% [$^{w}/_{v}$]), Tris-HCL (10 mM, pH 7), and Denhardt's solution (1×; ficoll (0.02% [$^{w}/_{v}$]), polyvinylpyrroloidone (0.02% [$^{w}/_{v}$]), BSA (0.02% [$^{w}/_{v}$]). Hybridisation was performed at 37°C overnight after which the membranes were washed three times in SSC (2×) with SDS (0.1% [$^{w}/_{v}$]) at room temperature. The signal was obtained on autoradiographs by exposing the blot to pre-flashed Kodak X-OMAT film (Kodak Ltd., Hemel Hempstead, England) at −70°C. For quantitative measurement of the relative levels of the mRNAs, autoradiographs were digitised with a video camera and analysed using OPTIMAS image analysis software (DataCell Ltd, Maidenhead, Berks, England). mRNA expression was normalised to Oligo $(dT)_{15–18}$ levels and the results expressed as mRNA levels relative to control/untreated samples (mean ± SD from triplicate samples).

Statistics

Values are expressed as mean ± SEM. Statistical analysis was performed by Kruskal-Wallis (non-parametric) one-way analysis of variance (ANOVA) with the Dunn multiple comparison post-test and by Student's *t*-test.

RESULTS

Effects of IFNα on Primary Human Bone Marrow Cells

Alkaline phosphatase activity. IFNα produced a dose-dependent inhibition of alkaline phosphatase specific activity with a significant reduction in activity compared to cultures in αMEM supplemented with 10% ($^{v}/_{v}$) FCS alone (basal control) or with ascorbate-2-phosphate/dexamethasone at doses as low as 50 IU/ml (Fig. 1a). In ascorbate-2-phosphate/dexamethasone supplemented cultures, the basal level of enzyme activity was increased approximately seven-fold compared with that in αMEM supplemented with 10% FCS alone.

IFNα inhibited alkaline phosphatase specific activity in primary cultures when added either throughout the culture period or for only the first 6 days of the culture period, indicating an inhibitory action on early progenitors of human bone marrow fibroblasts (not shown).

Cell proliferation. Cell numbers, as assessed by DNA content, were reduced following addition of IFNα. IFNα produced a dose-dependent inhibition of cell growth in primary cultures of human bone marrow fibroblasts cul-

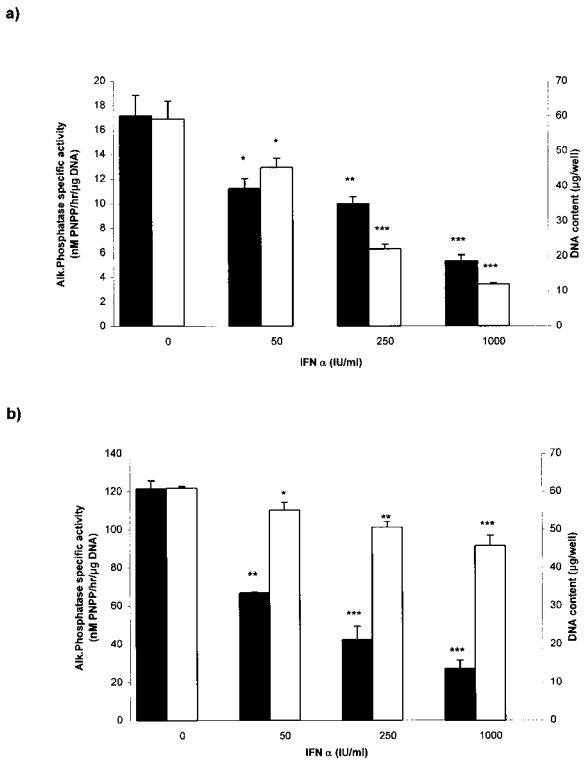

Fig. 1. Effect of IFNα on alkaline phosphatase specific activity (■) and DNA content (□) in primary human bone marrow cells cultured in αMEM supplemented with 10% FCS **(a)** and ascorbate-2-phosphate and dexamethasone **(b)**. Cells cultured for 10 days with the indicated groups as described in Materials and Methods (mean ± SEM, n = 3–9). *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$ vs. respective control (a) αMEM/FCS (b) ascorbate-2-phosphate/dexamethasone.

tured in basal medium (Fig. 1b) or in the presence of ascorbate-2-phosphate/dexamethasone (Fig. 1b). ^{3}H thymidine incorporation indicated a reduction in cell proliferation following addition of IFNα and a significant effect was seen at concentrations as low as 50 IU/ml (Table 1).

Effects of IFNα on HOP-26 Expression

To determine whether IFNα affected cell differentiation in CFU-F cultures, expression of HOP-26, a marker of early CFU-F colonies was examined. Colonies derived from individual CFU-Fs were observed to be heterogenous in size, morphology, endogenous alkaline phosphatase activity, and expression of HOP-26. However, the majority of CFU-F cultured in 10% FCS αMEM remained HOP-26-positive until day 9 (Fig. 2). IFNα alone and dexamethasone/ascorbate-2-phosphate alone reduced the levels of HOP-26 expression in human bone marrow cultures compared with those grown in FCS alone with a higher proportion of the colonies, as assessed by perinuclear staining in three separate experiments showing weak staining under these conditions. Addition of IFNα and dexamethasone/ascorbate-2-phosphate together also reduced the level of HOP-26 expression compared to cells grown in the absence of IFNα and dexamethasone (Fig. 2).

Effects of IFNα on Osteocalcin Expression

1,25$(OH)_2D_3$-induced osteocalcin expression was detected immunocytochemically in primary human bone marrow cells cultured for 28 days in either αMEM supplemented with 10% FCS or with ascorbate-2-phosphate/dexamethasone in the presence and absence of IFNα (1,000 IU/ml) throughout the period of culture (Fig. 2). Addition of IFNα (1,000 IU/ml) did not grossly affect osteocalcin expression as assessed by immunocytochemistry whether added from day 7, from day 14, or throughout the culture period (not shown).

TABLE I. Effect of IFNα on [^{3}H] Thymidine Incorporation Into Primary Human Bone Marrow Cells[a]

Treatment group IFNα (IU/ml)	[^{3}H] Thymidine incorporation (DPM ± SEM/well)
0	6314 ± 282
5	6844 ± 52
50	4721 ± 199**
100	2909 ± 122***
250	2311 ± 221***
1000	831 ± 75***

[a]Cells plated at 1×10^4/well for 48 h in αMEM supplemented with 10% FCS, rinsed with serum-free medium, and incubated for a further 72 h in the presence and absence of IFNα (5–1,000 IU/ml). [^{3}H] Thymidine data (dpm) presented as the mean ± SEM, n = 3.
**$P < 0.01$, ***$P < 0.001$ vs. FCS control.

Effects of IFNα on Colony Formation in HBM Cells

Examination of colony formation in αMEM supplemented with 10% FCS on day 12, showed a dose-dependent inhibition of colony formation and alkaline phosphatase-positive CFU-F colonies (Fig. 3a). In the absence of IFNα and the presence of dexamethasone and ascorbate-2-phosphate (Fig. 3b), colony number and alkaline phosphatase-positive CFU-F colonies were significantly stimulated ($P \leq 0.001$ and $P \leq 0.05$, respectively) above levels observed in those cultures maintained in FCS alone (Fig. 3a). Addition of IFNα under these conditions inhibited CFU-F number and alkaline phosphatase-positive CFU-F colony number similarly to that seen with FCS alone (Fig. 3b).

The effects of IFNα on modulation of colony formation in αMEM supplemented with 10% FCS were dependent on time of addition (Fig. 4). Addition of IFNα throughout the culture period reduced colony formation and alkaline phosphatase-positive colonies compared to addition of IFNα from days 7–12 (Fig. 4).

Effects of IFNα on Colony Size in Human Osteoprogenitors

To determine if the addition of IFNα was required throughout the culture period for the modulation of colony growth, alkaline phosphatase expression, and colony number, IFNα was added for only the last 6 days (days 7–12) of the culture period or for the duration of the study (0–12 days). Analysis of colony size showed a significant reduction in colony size in cultures treated with IFNα (250 IU/ml) from day 0 for the whole of the study in cultures maintained either in FCS or in dexamethasone and ascorbate-2-phosphate (Fig. 5a,b). In cultures supplemented with ascorbate-2-phosphate/dexamethasone, this decrease was significant even with 50 IU/ml IFNα. With addition of IFNα at day 7 for the remainder of the culture period there was no effect in the absence of ascorbate-

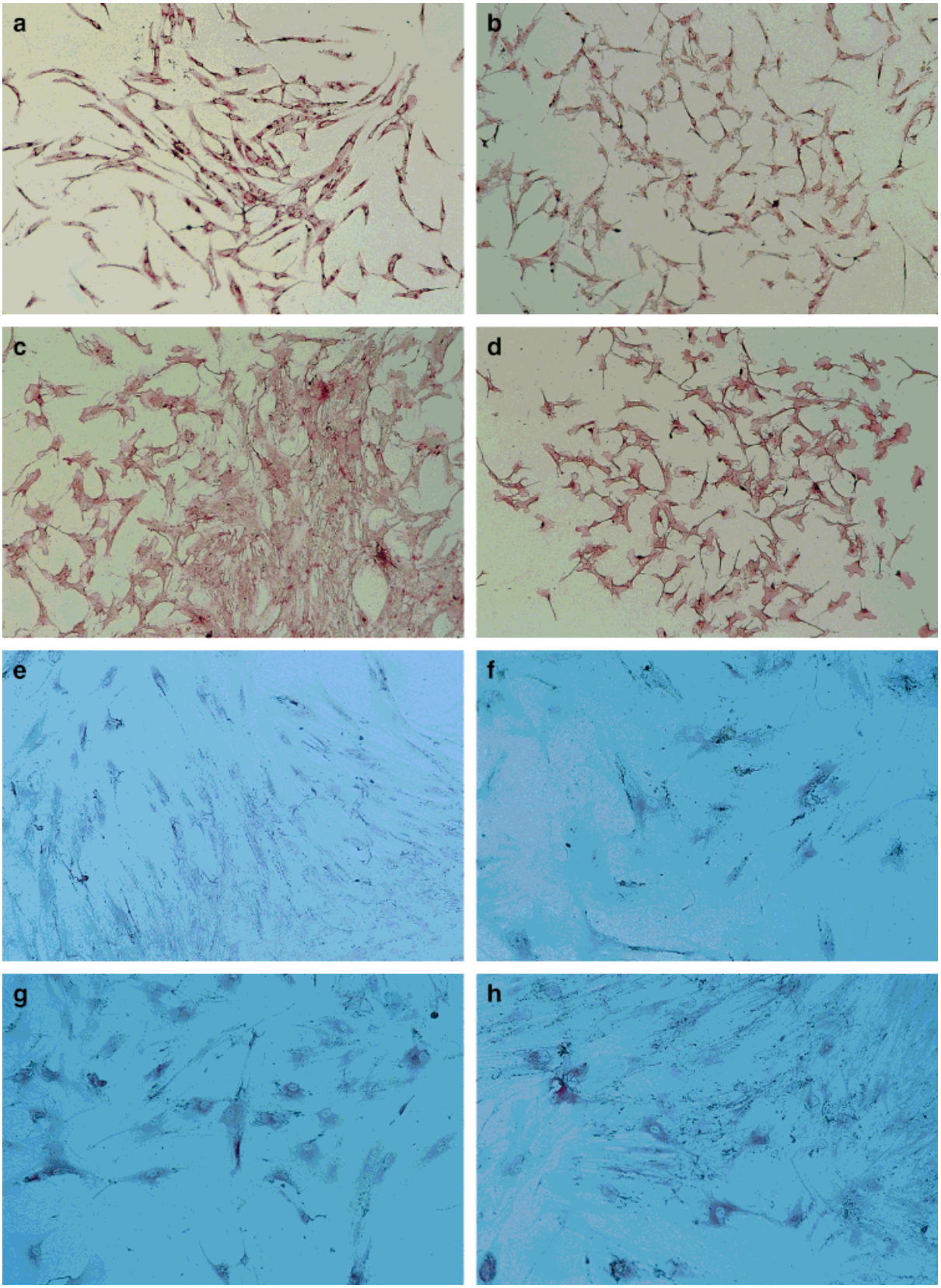

Fig. 2. Immunolocalisation of HOP-26 expression in human bone marrow cells cultured for 7 days in **(a)** basal media, **(b)** basal media containing IFNα (1,000 IU/ml), **(c)** ascorbate-2-phosphate and dexamethasone, **(d)** ascorbate-2-phosphate and dexamethasone containing IFNα (1,000 IU/ml). Osteocalcin expression in human bone marrow cells as assessed by immunocytochemistry under the following culture regimes **(e)** basal media, **(f)** basal media containing IFNα (1,000 IU/ml), **(g)** ascorbate-2-phosphate and dexamethasone, **(h)** ascorbate-2-phosphate and dexamethasone containing IFNα (1,000 IU/ml). Magnification ×100.

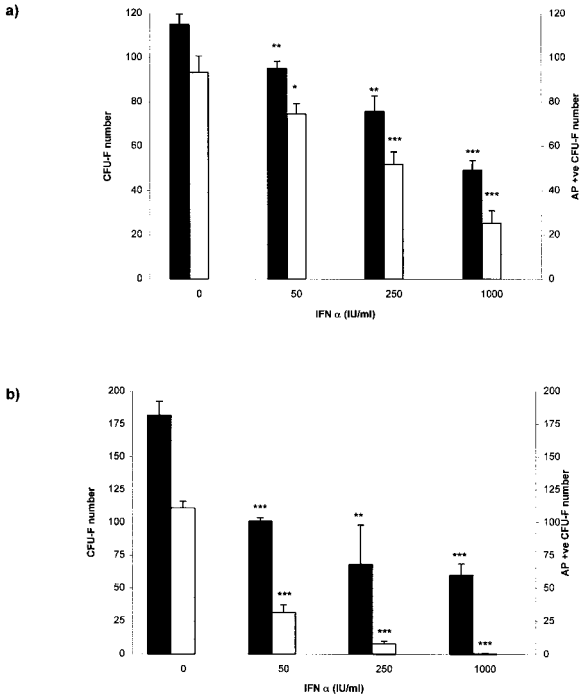

Fig. 3. Variation in CFU-F number (■) and alkaline phosphatase-positive CFU-F number (□)/25 cm^2 culture flask in human bone marrow cells with exposure to IFNα. Cells cultured (**a**) in the presence and absence of IFNα in basal media for 12 days or (**b**) in the presence and absence of IFNα in ascorbate-2-phosphate and dexamethasone for 9 days as described in Materials and Methods. Each point represents mean ± SEM, n = 4–6. $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$ vs. respective control (a) αMEM/FCS control (b) ascorbate-2-phosphate/dexamethasone.

2-phosphate/dexamethasone (Fig. 5a) but significant decreases still in the presence of these agents (Fig. 5b).

Examination of cell density within each colony by image analysis showed looser cell organisation within colonies from IFNα treated cultures compared to control cultures (not shown).

Effects of IFNα on Passaged Human Bone Marrow Fibroblastic Cells

IFNα (50–1,000 IU/ml) produced a dose-dependent and consistent inhibition of alkaline phosphatase specific activity after 7 days in passaged human bone marrow cells cultured in αMEM supplemented with 10% FCS (Fig. 6). This decrease was approximately 55% at 1,000 IU/ml although the magnitude of the response to IFNα was slightly reduced compared to primary marrow cultures where the decrease was up to 70% (Fig. 1). In contrast to the consistent inhibition of cell proliferation observed in primary marrow cultures (Fig. 1), IFNα did not produce a significant reduction in cell numbers, as assessed by DNA content (Fig. 6).

Modulation of Gene Expression by IFNα

Alkaline phosphatase, estrogen receptor alpha (ERα), and osteocalcin mRNA expression was unchanged following treatment with IFNα (50–1,000 IU/ml) as assessed by Northern blot analysis in cultures grown in αMEM (10% FCS; basal media; Fig. 7). However in the presence of basal media supplemented with ascorbate-2-phosphate (100 μg/ml) and dexamethasone (10 nM) at 250 IU/ml IFNα, and 1,000 IU/ml IFNα there was a significant increase in ERα and osteocalcin mRNA expression respectively with no significant effects on alkaline phosphatase mRNA expression (Fig. 7). Examination of BMP-2 mRNA expression, in the same cultures, showed a significant dose-dependent down regulation of BMP-2 mRNA expression in basal media (Fig. 8a) and a dose-dependent up-regulation of BMP-2 mRNA expression in the presence of ascorbate-2-phosphate and dexamethasone which was significant at 100 IU/ml IFNα (Fig. 8b). In the light of the observed susceptibility of the standard housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and α-actin, to IFNα [Virdi et al., 1998] all Northern data were normalised relative to total mRNA as assessed by using an oligo $(dT)_{15–18}$ probe.

DISCUSSION

In the present study IFNα inhibited the proliferation and differentiation of human osteoprogenitors (CFU-F). In primary bone marrow cultures, IFNα inhibited colony formation, cellular proliferation, and basal and dexamethasone-induced alkaline phosphatase specific activities. Northern blot analysis showed significant upregulation of BMP-2, osteocalcin, and ERα mRNA expression in human bone marrow cells cultured in ascorbate/dexamethasone and treated with the higher levels (100–1,000 IU/ml) of IFNα. Furthermore, IFNα addition to human bone marrow cultures resulted in early reduction in the proportion of colonies with HOP-26 immunoreactivity. We have previously shown HOP-26 to be reactive with human osteoprogenitor cells and to be an early marker for osteogenic cells [Joyner et al., 1997] and this suggests that the observed effects of IFNα are on early osteoprogenitors.

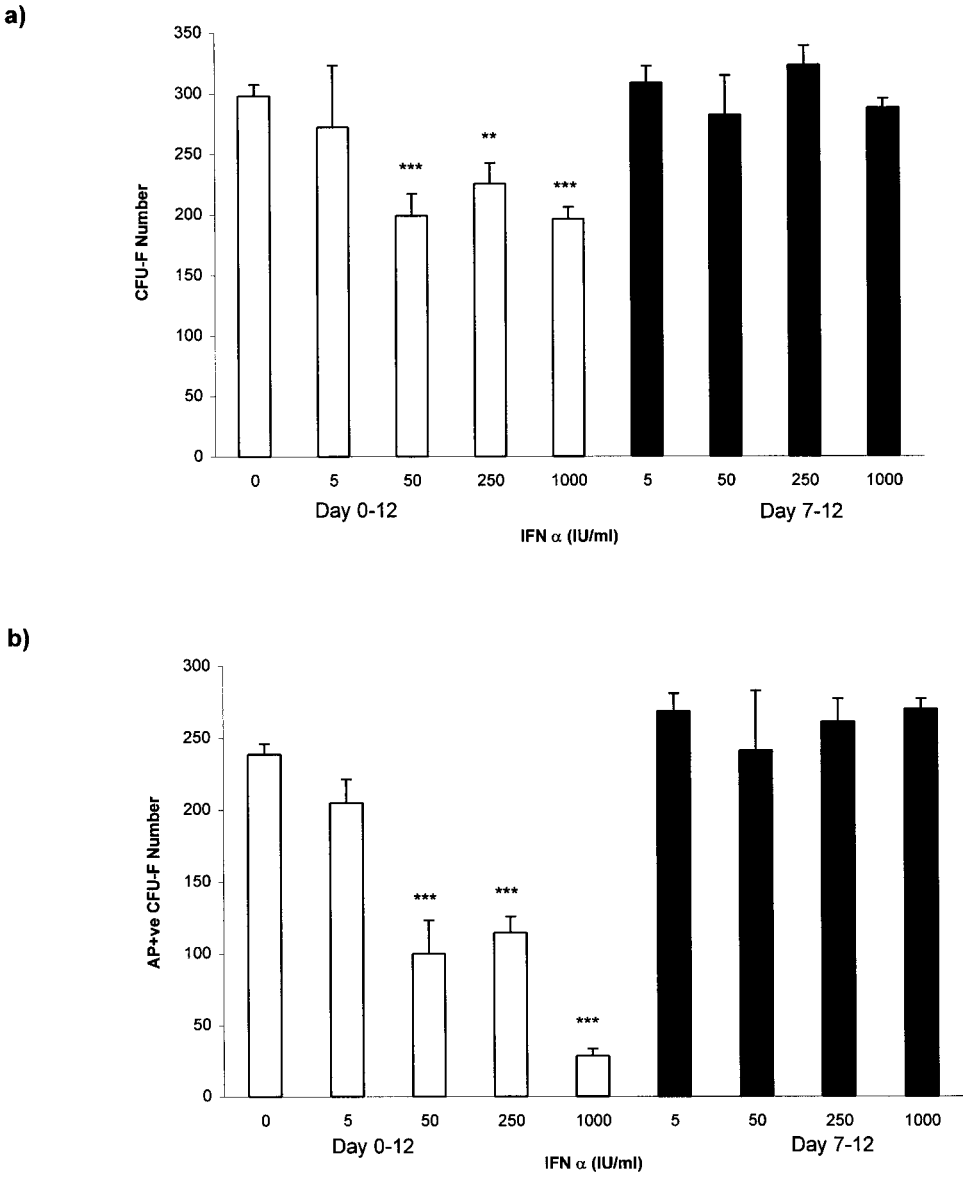

Fig. 4. Variation in **(a)** CFU-F number and **(b)** alkaline phosphatase-positive CFU-F number/25 cm^2 culture flask in human bone marrow cells with exposure to IFNα. Cells cultured in the presence and absence of IFNα in basal media for 0–12 (□) or from days 7–12 (■) as described in Materials and Methods. Each point represents mean ± SEM, n = 4. $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ vs. αMEM/FCS control.

The effects of IFNα in human bone marrow fibroblastic cells differ from the reported effects of IFNα and IFNγ on human bone trabecular bone cells. In our previous studies, IFNα inhibited cell proliferation of bone cells from trabecular bone explant cultures as well as proliferation in a human osteosarcoma cell line [Beresford et al., 1990] although, alkaline phosphatase activity remained unaffected. In contrast, alkaline phosphatase specific activity of human bone marrow osteoprogenitors showed marked sensitivity to IFNα, with inhibition of enzyme activity in short- and long-term cultures as well as a reduction in numbers of alkaline phosphatase-positive CFU-F colonies, although, interestingly, at the time point examined mRNA expression was unaffected. This indicates less utilisation of alkaline phosphatase mRNA in ascorbate-2-phosphate/dexamethasone and basal cultures. A number of observations suggest IFNα acts directly on early osteoprogenitors; i) IFNα reduced HOP- 26 immunoreactivity, ii) IFN_ inhibited CFU-F formation, size, and alkaline phosphatase positive-

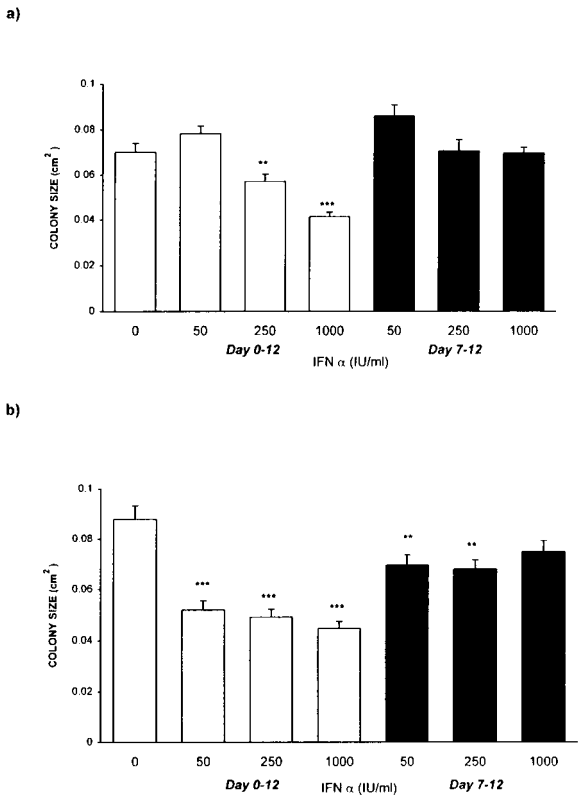

Fig. 5. Effect of IFNα on CFU-F colony size/25 cm^2 culture flask in primary human bone marrow cultures. Cells were incubated from day 0–12 (□) or from days 7–12 (■) with the indicated groups as described in Materials and Methods. **a:** Cultures grown in basal media alone. **b:** Cultures maintained in ascorbate-2-phosphate and dexamethasone. Each point represents mean ± SEM, n = 4. $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ vs. respective control (a) αMEM/FCS (b) ascorbate-2-phosphate/dexamethasone.

CFU-F number, iii) IFNα was most effective during the first 6 days of culture in contrast to addition during the second stage of culture and, iv) IFNα significantly affected cell proliferation and alkaline phosphatase activity in primary marrow cultures compared to passaged marrow cultures.

Cultures of human primary bone marrow stromal fibroblastic cells used in this study include non-adherent haemopoietic cells which are present until their extensive removal, by vigorous washing, on day 6. Thus it cannot be excluded that the effects of IFNα on stromal progenitors may be mediated, at least in part, indirectly by effects on haematopoietic cells and haematopoietic progenitors. A number of studies have shown that IFNα can inhibit colony formation of haematopoietic progenitor cells, including colony-forming unit—"granulocyte, erythroid, macrophage, megakaryocyte (CFU-GEMM), colony-forming unit-megakaryocyte (CFU-Mk), burst-forming unit-erythroid (BFU-E), and colony-forming unit-granulocyte macrophage (CFU-GM), as well as pluripotent haemopoietic stem cells [Broxmeyer et al., 1983; Mamus et al., 1986; Carlo-Stella et al., 1987; Ganser et al., 1987]. The effects of IFNα on the growth of haematopoietic progenitors are further complicated by observations that the effects of IFNα are also mediated, in part, through modulation of haematopoietic growth factors produced by stromal cells [Broxmeyer et al., 1983; Mamus et al., 1986; Aman et al., 1994, 1996]. The current results indicate osteoprogenitors are similarly affected by this cytokine and the interaction of both haematopoietic and stromal progenitors in mediation of the effects of IFNα awaits further characterisation. In preliminary studies using rabbit and human marrow fibroblasts, Wang et al. [1992] also found IFNα inhibited colony formation of bone marrow fibroblast osteoprogenitors (CFU-F) although the authors did not examine other parameters such as alkaline phosphatase activity, cell proliferation, protein expression, or time of onset of IFNα action.

Bone formation involves the directed differentiation of mesenchymal cells into osteogenic cells, a process subject to regulation by a variety of hormones and factors [Triffitt, 1996; Triffitt and Oreffo, 1999]. BMPs, originally identified as proteins which could induce new cartilage and bone formation in non-bony tissues, are key factors for the differentiation of osteoprogenitors to osteoblasts and chondroblasts during both intramembranous differentiation and endochondral ossification [Yamaguchi et al., 1996; Vukicevic et al., 1989; Hogan, 1996; Mundy, 1996]. Katagiri and coworkers [1990] have shown BMP-2 stimulates osteoblast progenitor cell maturation and the differentiation of undifferentiated C3H10T1/2 cells and, more recently, the differentiation of committed myoblasts into osteoblasts [Komaki et al., 1996]. Our earlier in vivo work indicated a possible role for IFNα in the production of bone-inducing activity by osteosarcoma xenografts [Brosjo et al., 1989; Forster et al., 1989]. In the present studies, the modulation of BMP expression by IFNα, downregulation of BMP-2 mRNA expression in basal conditions and upregulation of BMP-2 mRNA expression in cells treated with ascorbate/dexamethasone suggests a pos-

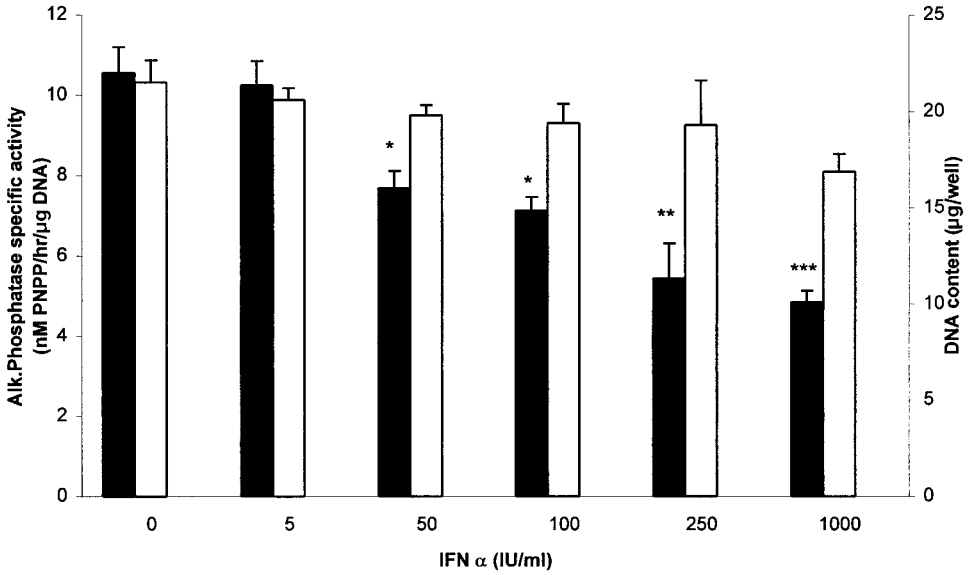

Fig. 6. Effect of IFNα on alkaline phosphatase specific activity (■) and DNA content (□) in passaged human bone marrow fibroblastic cells (passage 3) cultured in basal media. Cells plated at 2×10^4/well and incubated for 7 days with the indicated groups (mean ± SEM, n = 4). *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$ vs. αMEM/FCS control.

sible role for this cytokine in the regulation of bone formation dependent on the local microenvironment. This is further indicated by the upregulation of ERα and osteocalcin mRNA observed under these culture conditions in this study. In addition, the change in BMP mRNA expression with culture in ascorbate and dexamethasone suggests IFNα may function in part through upregulation of BMP expression.

The suppression of myelopoiesis by IFNα has resulted in its use for a variety of myeloproliferative diseases including chronic myelogenous leukaemia, polycythemia vera, and essential thrombocytosis [Giles et al., 1988; Silver, 1990; Gutterman, 1994]. In myelofibrosis with myeloid metaplasia (MMM) a disease characterised by marrow fibrosis and extramedullary haematopoiesis, treatment with IFNα results in significant suppression of circulating colony-forming unit (CFU-GEMM), CFU-Mk, BFU-E, and CFU-GM [Carlo-Stella et al., 1987]. Recently, Tiefenthaler and colleagues [1997] reported that IFNα and IFNω (interferon omega 1) produced a dose-dependent inhibition of colony formation for erythroid (BFU-E, CFU-E) and granulocyte-macrophage (CFU-GM) progenitors from both normal donors and patients with chronic myelogenous leukemia. IFNα may suppress myelopoiesis and thrombopoiesis by inhibiting the proliferation and differentiation of osteoblasts and osteoblast progenitors including the CFU-F. An interesting case report from Lehmann and colleagues [1996] has shown that severe osteoporosis due to Systemic Mast Cell disease, a clonal disorder derived from early pluripotent stem cells of the bone marrow, can be treated successfully with IFNα. However, in the report, the modulation of bone formation could only be assessed by trabecular bone mineral density, limiting interpretation of the therapeutic efficacy of IFNα in osteoporosis. With the known inhibitory effects of IFN's on osteoclast formation and activity [Takahashi et al., 1986; Gowen et al., 1986], on normal human bone cells [Beresford et al., 1990], and our present results on the modulation of marrow osteoprogenitor differentiation and activity, production of interferon within the bone microenvironment in metabolic bone diseases characterised by high bone turnover may provide a mechanism to protect bone volume.

In conclusion, the current results demonstrate that IFNα inhibits human osteoprogenitor cell proliferation, fibroblastic colony formation and alkaline phosphatase activity and modulates BMP-2 mRNA expression within these cells. These results suggest a role for IFNα in local bone turnover through the specific modulation of osteoprogenitor cell proliferation.

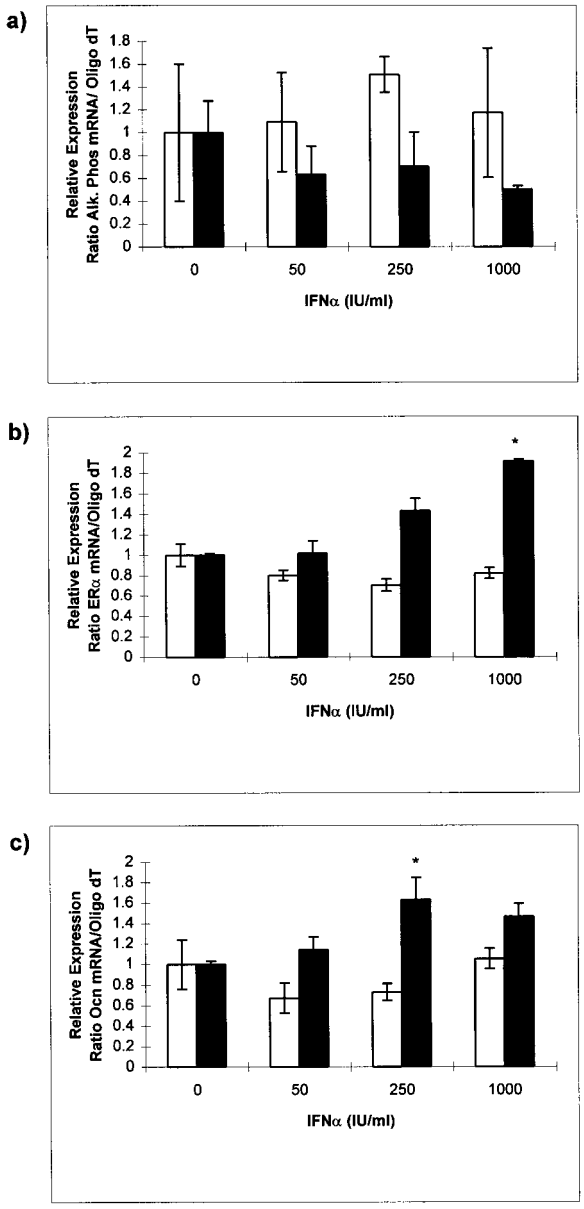

Fig. 7. Modulation of alkaline phosphatase **(a)**, ERα **(b)**, and osteocalcin **(c)** mRNA expression as assessed by Northern blot analysis in primary human bone marrow fibroblastic cells cultured in basal media (□) or ascorbate-2-phosphate and dexamethasone (■) and treated with IFNα (50–1,000 IU/ml)for 21 days. Each point represents the mean ± SD from triplicate samples except IFNα 1,000 IU/ml, which represents the average and range from paired samples. *$P \leq 0.05$ vs. ascorbate-2-phosphate/dexamethasone control.

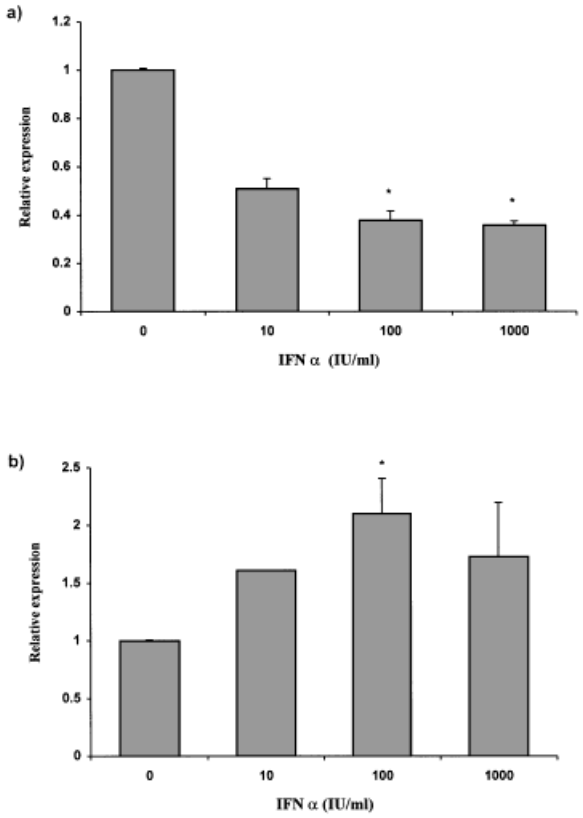

Fig. 8. Modulation of BMP-2 mRNA expression as assessed by Northern analysis in primary human bone marrow cultures treated with IFNα for 21 days **(a)** cultures maintained in basal media, **(b)** cultures maintained in ascorbate-2-phosphate and dexamethasone. Each point represents the average and range from paired samples.*$P \leq 0.05$ vs. respective control (a) αMEM/FCS (b) ascorbate-2-phosphate/dexamethasone.

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