Tri-iodothyronine regulates the production of interleukin-6 and interleukin-8 in human bone marrow stromal and osteoblast-like cells

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Abstract

Hyperthyroidism is associated with increased bone resorption but the mechanisms by which thyroid hormone (T3) affects bone cell metabolism remain unclear. Recently it has been suggested that T3 stimulates osteoclastic resorption indirectly through the release of soluble mediators from osteoblasts. The aim of the present study was to investigate whether the T3-induced increase in bone resorption could be due to the regulation of cytokine production by human osteoblasts (hOb).

The effects of T3 (1, 10, 100 nM) and IL-1 β (100 U/ml) as the positive control were examined on cytokine protein release and mRNA levels in cultured hOb cell lines (MG63, SaOs-2), primary hOb and human bone marrow stromal (hBMS) cells. T3 increased IL-6 and IL-8 mRNA levels as well as IL-6 and IL-8 protein release into the culture media from MG63 and hBMS cells in a

time- and dose-dependent manner. The maximal effect on protein release in hBMS cells occurred at 24 h with a dose of T3 10 nM (IL-6 5.5 ± 1.1 -fold above controls; IL-8 3.7 ± 0.5 -fold above controls, $P < 0.05$). At the same time, mRNA levels in hBMS cells were increased 6.2 ± 0.8 -fold for IL-6 ($P < 0.05$) and 5.7 ± 0.8 -fold for IL-8 ($P < 0.05$). Similar results were obtained in MG63 cells but no response was seen in SaOs-2 or hOb cells despite measurable basal production. Nor was there detectable regulation of IL-1 β , IL-3, IL-11, IL-4 or granulocyte macrophage-colony stimulating factor by T3 in any cell type.

In conclusion, T3 increases IL-6 and IL-8 production by MG63 and hBMS cells, suggesting that IL-6 and IL-8 may be T3-regulated genes in osteoblasts.

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Introduction

Hyperthyroidism is characterised by an increase in bone turnover accompanied by a negative calcium balance and reduction in bone mineral density (Mosekilde & Melsen 1978). Both histological and biochemical evidence suggests that this is due to stimulation of osteoclastic resorption (Garnero *et al.* 1994, Siddiqi *et al.* 1997). *In vitro* studies with organ cultures of neonatal mouse calvariae and fetal rat limb have also shown direct stimulation of bone resorption by tri-iodothyronine (T3) (Mundy *et al.* 1976, Klaushofer *et al.* 1989). The mechanism for this effect of T3 on bone is not fully understood and in particular T3-responsive genes in bone cells have yet to be identified. Recent reports (Allain *et al.* 1992, Britto *et al.* 1994) have suggested that the effects of T3 on osteoclastic bone resorption are indirect and mediated by osteoblasts. T3 receptors (TR) have been demonstrated in rat, mouse and human osteoblast (hOb) cell lines (Sato *et al.* 1987, Kasono *et al.* 1988, Allain *et al.* 1996) and it is thought that

activation of these osteoblastic TRs results in the release of soluble agents which stimulate osteoclastic bone resorption (Klaushofer *et al.* 1989, Lakatos & Stern 1992, Tarjan & Stern 1995). Since the bone resorbing activity of T3 *in vitro* can be inhibited by cyclosporin A (Lakatos & Stern 1992) and by interferon- γ (Klaushofer *et al.* 1989), cytokines are likely mediators of T3 action.

Several cytokines with osteotrophic effects, such as interleukin-6 (IL-6), IL-8, IL-3, IL-1 β and granulocyte macrophage-colony stimulating factor (GM-CSF), are now known to be released by human osteosarcoma cell lines, hOb and human bone marrow stromal (hBMS) cells (osteoblast progenitor cells) (Chaudhary & Avioli 1994, Passeri *et al.* 1994, Manolagas & Jilka 1995). IL-6 in particular is a potent regulator of osteoclast differentiation and elicits bone resorption both *in vivo* and in *in vitro* models that contain early osteoclast precursors (Ishimi *et al.* 1990, Black *et al.* 1991). More recently, a role for IL-8 in bone metabolism has been shown by the demonstration of expression of IL-8 receptors on osteoclasts (Collin-Osdoby

et al. 1996) and its regulatory effects on osteoclast development and activity (Fuller *et al.* 1995).

The present study was designed to investigate the potential role of cytokines as mediators of T3 action on bone cells. To address this hypothesis we have examined the effects of T3 on medium cytokine concentrations and cytokine gene expression in cultured hOb-like cell lines, primary cultures of hOb and hBMS cells.

Materials and Methods

Cell culture

The hOb-like cell lines MG63 and SaOs-2 were maintained in α -minimum essential medium (Gibco BRL, Life Technologies Ltd, Paisley, Strathclyde, UK) containing 10% FCS (Gibco BRL), penicillin, streptomycin and fungizone (hereafter referred to as medium) at 37 °C in a humidified atmosphere containing 5% CO₂ in air. Cells were sub-cultured twice a week.

hBMS cells were prepared from bone marrow aspirates obtained from patients in remission from haematological malignancies and ethical approval was obtained from the East London and City Health Authority Ethics Committee. A modification of the method described by Haynesworth *et al.* (1992) was used. Briefly, marrow cells were pelleted by centrifugation at 500 *g* for 10 min, resuspended in medium and plated at a density of 2×10^6 cells/75 cm² tissue culture flask. Cells were allowed to attach without disturbance for 7 days. After the attachment period, half the culture medium was replaced with fresh medium and subsequently media changed at 2- to 3-day intervals. At confluence, cells were trypsinised, counted and plated into multi-well culture plates. First passage cells were used in all experiments. Although hBMS cell cultures appear homogenous, they represent not clonal cell lines but multi-colony derived cells. Therefore the cultures exhibit heterogeneity between samples due to potentially differing proportions of one cell type to another (preosteogenic vs preadipogenic vs prechondrogenic). To exclude significant proportions of non-osteoblastic cells, we extensively characterised the phenotype of our cells (see below).

hOb were cultured from bone explants by the method of Robey & Termine (1985) using trabecular bone discarded during orthopaedic surgery from men and women aged between 50 and 85 years. Connective tissue was dissected from bone fragments, which were then washed in PBS, diced into 2–4 mm pieces and placed in tissue culture flasks in medium. Cells growing out from the bone chips were passaged once and grown to confluence. All assays were done on cells trypsinised and subcultured at the end of the first passage.

Characterisation of phenotype

The osteoblastic phenotype of confluent cells from all four sources was confirmed by measuring alkaline phosphatase

(ALP) positivity and 1,25(OH)₂D₃ induction of osteocalcin. ALP staining was confirmed using the ALP substrate 5-bromo 4-chloro 3-indolyl phosphate which gives a blue oxidation product following the addition of nitroblue tetrazolium chloride (Sigma Chemicals, Poole, Dorset, UK). Intense staining was seen with hBMS and SaOs-2 cells, moderate staining with hOb and low intensity staining seen with MG63 cells. Osteocalcin production was determined using the OSTK-PR (CIS Biointernational, Gif-sur-Yvette, Cedex, France) radioimmunoassay. hBMS, SaOs-2 and MG63 cells had only 1,25(OH)₂D₃-dependent osteocalcin secretion, in contrast to hOb cells which had detectable basal osteocalcin secretion. For chondrocyte and adipocyte analyses, cells were washed three times in PBS, followed by fixation with 10% buffered formalin for 5 min. The cultures were then washed with water and stained for 30 min with Alcian blue (10% in 0.1 M HCl) for chondrocytes or overnight with oil red O (0.5% in propylene glycol) for adipocytes. No staining was observed in any of the cell types.

Cytokine and growth factor response experiments

Cells were plated into multi-well plates at a density of 10 000 cells/cm² in medium. Following overnight incubation, cells were washed in PBS and placed in fresh medium containing charcoal-stripped serum. Twenty four hours later, cells were treated with either 1 nM, 10 nM or 100 nM T3 (Sigma Chemicals), concentrations which correspond to physiological, pathological and pharmacological levels *in vivo*. Control wells were incubated with either no additions (negative control, C) or with 100 U/ml human recombinant IL-1 β (RD Systems Europe Ltd, Abingdon, Oxon, UK) as positive control. Media were analysed for IL-6, IL-1 β , IL-4, GM-CSF, IL-3 and TNF- α at 6 and 24 h and for IL-8 at 3, 6, 8, 24 and 48 h by competitive ELISA assays (Eurogenetics UK Ltd, Middlesex, UK) using human recombinant cytokine or growth factors as standard. The lower limit of detection varied with the cytokine assayed and was 0.2 ng/l for IL-1 β , 1 ng/l for IL-6, IL-8, IL-4 and TNF- α , 7 ng/l for GM-CSF and IL-11 and 31 ng/l for IL-3. The between-batch coefficient of variation of these assays was <13%. Individual experiments were done in duplicate and each experiment was repeated at least three times. Cell extracts were used for mRNA isolation as described below.

mRNA isolation, cDNA synthesis and PCR analysis

mRNA was isolated using the QuickPrep micro mRNA purification kit (Pharmacia Biotech, St Albans, Herts, UK). Briefly, cells were extracted with guanidinium thiocyanate and the extract was incubated with oligo(dT)-cellulose to allow binding with the poly(A) RNA. After washing in low- and high-salt buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA and NaCl (0.1 M or 0.5 M

respectively)), the mRNA was eluted with 10 mM Tris-HCl and 1 mM EDTA and precipitated with glycogen and potassium acetate. The concentration of mRNA was determined by measuring the absorbance at 260 nm. Approximately 1–2 µg mRNA was recovered from each purification and stored at -70°C until use.

A total of 0.2 µg mRNA was sufficient for oligo(deoxythymidine)₁₈-primed cDNA synthesis using the First Strand cDNA Synthesis kit (Pharmacia Biotech). Following heat denaturation at 65°C for 10 min, the mRNA was incubated at 37°C for 1 h in a total reaction volume of 33 µl containing 10 mM Moloney murine leukaemia virus-reverse transcriptase (MMLV-RT), deoxy-NTP, 200 mM DTT and 0.2 µg/µl not1-d(T)₁₈ bifunctional primer. Reverse transcription was also performed in the absence of MMLV-RT. PCR carried out on this RT product yielded no PCR product, confirming no genomic DNA contamination. All cDNA was stored at -70°C until use.

Five microlitres cDNA were used for PCR amplification thus allowing six amplifications to be performed from each sample. For the purposes of semiquantitation and to reduce the variability between samples, PCR components were premixed before addition to individual PCR tubes. The 50 µl reaction mixture contained 0.2 mM dNTP, 5 µl 10-fold concentrated PCR buffer, 5 µl cDNA and 100 pM glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers and either IL-6 or IL-8 primers. The sequences of the primers were specific as confirmed by computer assisted search of GenBank. IL-6 primers (forward primer 5' CTG/CTC/CTG/GTG/TTG/CCT, reverse primer 5' CCT/GCA/GCC/ACT/GGT/TCT) were used to amplify a 511 bp DNA fragment and were designed to span introns in order to avoid false positives from genomic DNA. The IL-8 primers amplifying a DNA fragment of 302 bp were as described by Birch *et al.* (1993). GAPDH, a cell-cycle, stimulation-independent house-keeping gene, was used to normalise mRNA levels. Amplification of human GAPDH mRNA from the cDNA was achieved by using primers from ClonTech (Cambridge, UK) in each PCR and generated a 986 bp fragment. The PCR mixture was heated to 90°C for 3 min and cooled to 84°C before addition of 2.5 U Taq (Boehringer Mannheim, Lewes, East Sussex, UK) followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 45 s. Controls with water replacing template were included in all experiments. PCR products were separated by electrophoresis using 1.6% agarose gels stained with ethidium bromide. HincII-digested DNA was run in parallel as a molecular weight marker.

Method of quantification

All RT reactions used oligo(deoxythymidine)₁₈-primed RNA to minimise the variations in RT efficiency seen

when using specific RT primers. Following optimisation of PCR conditions for each primer pair, comparative kinetic analyses (Noonan *et al.* 1990, Salomon *et al.* 1992) were performed to determine the phase during which there was exponential increase in PCR product before a plateau was reached. It was at this point that the PCR was terminated, allowing semiquantitative data to be obtained.

The gels were photographed on a UV light box with Polaroid type 665 film to yield a negative. Using whole-band analysis software (BioImage Computer Systems, Crewe, Chester, UK), each negative was scanned, the image digitised and the optical density of each PCR generated band measured. This optical density was in turn a measure of the quantity of PCR product (Nakayama *et al.* 1992). The value for each band was expressed as a ratio to the quantity of GAPDH. The coefficients of variation for repeated measurements were less than 20%. The specificity of the amplified bands was validated by sequencing of the PCR product using Taq cycling with fluorescent labelled dye terminators.

Statistical analysis

Results are expressed as means $\pm$ S.E.M. for each experimental group and were compared by one-way ANOVA. When the *F* statistic was significant ($P < 0.05$), the analysis was continued using Fisher's multiple comparisons test.

Results

Cytokine response studies

Both of the hOb-like cell lines (MG63 and SaOs-2), the hOb and the hBMS cells released IL-6 and IL-8 basally. However, the constitutive expression and the response to T3 varied with the cell type. Higher basal release of IL-6 and IL-8 was present in hOb (IL-6: 6 h 1063 ± 111.2 ng/l, 24 h 4463 ± 540 ng/l; IL-8: 6 h 752 ± 98.3 ng/l, 24 h 2326 ± 221 ng/l) and hBMS cells (IL-6: 6 h $24\,900 \pm 6300$ ng/l, 24 h $75\,100 \pm 203$ ng/l; IL-8: 6 h $48\,100 \pm 9300$ ng/l, 24 h $107\,200 \pm 50\,100$ ng/l) compared with the osteosarcoma cell lines MG63 (IL-6: 6 h 204.8 ± 41.4 ng/l, 24 h 211.8 ± 59.6 ng/l; IL-8: 6 h 21.0 ± 6.7 ng/l, 24 h 38.86 ± 9.1 ng/l) and SaOs-2 (IL-6 and IL-8 $< 1-3$ ng/l).

The levels in hOb and SaOs-2 did not rise further following 24 h incubation with T3, although both cells did respond to the positive control IL-1 β by 24 h (hOb: IL-6 8809 ± 203 ng/l, $P < 0.05$; IL-8 4032 ± 193 ng/l, $P < 0.05$; SaOs-2: IL-6 21.13 ± 5.6 ng/l; IL-8 15.02 ± 5.3 ng/l). In contrast MG63 and hBMS cells responded in a dose- (Fig. 1) and time-dependent (Fig. 2) manner to both T3 and IL-1 β , with an increase in the release of IL-6 and IL-8. The maximum response of 5.5-fold above control wells was reached at 24 h with pathological levels

Figure 1 Dose effect of T3 on IL-6 production from MG63 and hBMS cells. Cells were cultured in charcoal-stripped medium for 24 h prior to treatment with either no stimulant (C), T3 1, 10 or 100 nM or IL-1 β 100 U/l. Media was assayed 24 h later for IL-6 by ELISA and results are expressed as the fold increase above IL-6 production in unstimulated wells for both MG63 (211.8 ± 59.6 ng/l) and hBMS cells ($75\,100 \pm 203$ ng/l). Each bar represents the mean $\pm$ S.E.M. for three experiments done in duplicate. * $P < 0.05$, ** $P < 0.01$ vs C.

of T3 (10 nM) and increasing the T3 concentration had no further effect. Similarly, IL-8 was produced both constitutively and on stimulation with T3 in MG63 and hBMS cells (Figs 3 and 4) when a maximum 3.8- and 3.7-fold rise was seen in MG63 and hBMS cells respectively at 24 h. Interestingly, MG63 showed a significant response with T3 1 nM, but hBMS cells were stimulated only at T3 10 nM, at which concentration, IL-6 and IL-8 levels approached that of the positive control IL-1 β (Figs 1 and 3).

The release of IL-1 β , TNF- α , IL-4, IL-3 and GM-CSF was not detected either basally or on stimulation with T3 in any cell type studied. IL-11 was detectable basally in hOb (50.0 ± 11.2 ng/l) and hBMS cells (331 ± 23.6 ng/l) but was unresponsive to T3 stimulation at any of the concentrations used.

Effects of T3 on IL-6 and IL-8 mRNA expression in MG63 and hBMS cells

To study whether cytokine protein release was related to mRNA expression in the cells, RT-PCR was performed on the cDNA extracted from cells treated with or without T3 and IL-1 β . Cytokine mRNA expression was not investigated in hOb or SaOs-2 as cytokine release from these cells had been unaffected by T3 stimulation. To provide a semiquantitative assessment of mRNA expres-

Figure 2 Time course effect of T3 on production of IL-6. MG63 and hBMS cells were cultured for 24 h in charcoal-stripped medium prior to stimulation with either no treatment (open bar) or T3 10 nM (solid bar). Media was assayed by ELISA for IL-6 at 6 h and 24 h and results are expressed as the fold increase above IL-6 production in unstimulated wells for both MG63 (6 h 204.8 ± 41.4 ng/l, 24 h 211.8 ± 59.6 ng/l) and hBMS cells (6 h $24\,900 \pm 6300$ ng/l, 24 h $75\,100 \pm 203$ ng/l). Each bar represents the mean $\pm$ S.E.M. for three experiments done in duplicate. * $P < 0.05$ compared with untreated wells.

sion, standardised PCR protocols were established as described in the Methods section. Figures 5 and 6 indicate the results of ratios of expression of IL-6 and IL-8 respectively to GAPDH mRNA in MG63 and hBMS cells. These ratios are expressed as fold increases relative to control samples.

Although IL-6 and IL-8 mRNA were detectable at 6 h, no response was seen to T3 in either MG63 or hBMS cells (data not shown). In MG63 cells after 24 h, there was a dose-dependent rise in IL-6 (1.6 ± 0.5 -fold rise, n.s., T3 1 nM) which reached significance with T3 10 nM (4.8 ± 0.8 -fold rise, $P < 0.05$). A similar dose-dependent rise in IL-6 mRNA expression was observed in hBMS cells with T3 1 nM and T3 10 nM (1.9 ± 0.5 -fold rise (n.s.) and 6.2 ± 0.8 -fold rise ($P < 0.05$) respectively. GAPDH transcripts were unchanged by any treatment.

A similar induction in IL-8 mRNA expression was seen in MG63 and hBMS cells. At 24 h in MG63 cells there was a significant 4.3 ± 0.6 -fold rise ($P < 0.05$) in IL-8 mRNA with T3 1 nM which increased to 5.4 ± 0.9 -fold ($P < 0.05$) with T3 at 10 nM. Similar responses were seen in hBMS cells with a 2.8 ± 0.5 - (n.s.) and 5.7 ± 0.8 -fold rise ($P < 0.05$) with T3 1 nM and 10 nM respectively.

In both MG63 and hBMS cells, IL-1 β (100 U/l) was used as a positive control for induction of IL-6 and IL-8 mRNA expression and at 24 h caused a 8.2 ± 1.2 - and 7.3 ± 1.1 -fold rise ($P < 0.01$) respectively in

Figure 3 Dose effect of T3 on IL-8 production from MG63 and hBMS cells. Cells were cultured in charcoal-stripped medium for 24 h prior to treatment with either no stimulant (C), T3 1 or 10 nM or IL-1 β 100 U/l. Media was assayed 24 h later for IL-8 by ELISA and results are expressed as the fold increase above IL-8 production in unstimulated wells for both MG63 (38.86 ± 9.1 ng/l) and hBMS cells ($107\,200 \pm 50\,100$ ng/l). Each bar represents the mean $\pm$ S.E.M. for three experiments done in duplicate. * $P < 0.05$, ** $P < 0.01$ vs C.

IL-6 mRNA and a 7.8 ± 1.2 - and 7.4 ± 1.1 -fold rise ($P < 0.01$) in IL-8 mRNA respectively.

Discussion

The results of the present study demonstrate that T3 increases IL-6 and IL-8 production from both hBMS cells and the human osteosarcoma cell line MG63 cell in a dose- and time-dependent fashion. This stimulated production of IL-6 and IL-8 occurred in the absence of effects on T3 on IL-1 β release, suggesting that, in these human cells, T3 is not potentiating the stimulatory effects of IL-1 β as previously proposed in studies using fetal rat limb bone cultures (Tarjan & Stern 1995). Our *in vitro* evidence suggests that T3 interacts directly with osteoblasts, stimulating them to produce IL-6 and IL-8, thus providing a mechanistic paradigm whereby T3 could modulate bone resorption. Consistent with this hypothesis are recently reported studies *in vivo* documenting high levels of serum IL-6 and soluble IL-6 receptor in patients with hyperthyroidism (Salvi *et al.* 1996, Lakatos *et al.* 1997).

hBMS cells have been previously reported to produce IL-6 and IL-8 in response to stimulation with a number of factors including parathyroid hormone (PTH) (Kim

Figure 4 Time course effect of T3 on production of IL-8. MG63 and hBMS cells were cultured for 24 h in charcoal-stripped medium prior to stimulation with either no treatment (open bar) or T3 10 nM (solid bar). Media was assayed by ELISA for IL-8 at 6 and 24 h and results are expressed as the fold increase above IL-8 production in unstimulated wells for both MG63 (6 h 21.0 ± 6.7 ng/l, 24 h 38.86 ± 9.1 ng/l) and hBMS cells (6 h $48\,100 \pm 9300$ ng/l, 24 h $107\,200 \pm 50\,100$ ng/l). Each bar represents the mean $\pm$ S.E.M. for three experiments done in duplicate. * $P < 0.05$ compared with untreated wells.

et al. 1997), IL-1 and tumour necrosis factor (TNF)- α (Chaudhary & Avioli 1994). Both PTH and IL-1 were shown to cause an approximately 2-fold increase in IL-6 levels (Kim *et al.* 1997) after 24 h of incubation. In our studies, IL-1 β (at an equivalent dose of 100 U/ml) produced a 5.5-fold increase in IL-6, an increase that was matched by T3 at a concentration of 10 nM. Interestingly, stimulated levels of IL-6 produced in response to T3 in both types of cells were in the range (1–10 ng/ml) shown previously to promote bone resorption (Roodman 1992).

hBMS cells are reported as having low basal levels of IL-8 which can be dramatically increased by both IL-1 β and TNF- α (Chaudhary & Avioli 1994). We found similar low basal levels of IL-8 in MG63 cells but higher levels in hBMS cells.

Basal mRNA expression of IL-6 and IL-8 mRNA has been demonstrated in hOb and hBMS cells and regulation of gene expression in response to glucocorticoids, IL-1 and growth hormone (GH) (Littlewood *et al.* 1991a, Chaudhary & Avioli 1996, Swolin & Ohlsson 1996) has been documented. We have now shown T3-dependent upregulation of IL-6 and IL-8 mRNA expression in MG63 and hBMS. These additional data would suggest that the increased output of IL-6 and IL-8 may occur as a result of effects of T3 on IL-6 and IL-8 gene expression. mRNA levels at 24 h were higher than at 6 h, which correlates well with the peak release of IL-6 and IL-8 into the culture media. This induction of mRNA could be due

Figure 5 RT-PCR was performed on mRNA isolated from MG63 and hBMS cells demonstrating the presence of mRNAs encoding IL-6 and GAPDH in cells treated for 24 h with either no stimulant (C), 1 nM T3, 10 nM T3 or IL-1 β 100 U. Products were run on 1.6% agarose gel and stained with ethidium bromide. The corresponding histograms show the ratio of the densitometric values of IL-6 to GAPDH transcripts relative to the unstimulated control, C. Each bar represents the mean $\pm$ S.E.M. for three experiments. * $P < 0.05$, ** $P < 0.01$ relative to C.

to either an upregulation of gene transcription or to mRNA stabilisation. The latter mechanism is a particular feature of cytokine genes as many contain a reiterated AU motif in the 3' untranslated region which mediates rapid mRNA decay (Wilson & Treisman 1988). In addition to a possible post-transcriptional effect, the absence of an early mRNA response leads us to speculate that this effect of T3 on IL-6 and IL-8 maybe indirect and indeed there is no immediately recognisable T3 response element in the IL-6 or IL-8 promoter. Both these possibilities need to be pursued in future studies.

Basal levels of IL-6 and IL-8 in hOb were comparable with previously reported levels of 120–2000 pg/ml which are seen in trabecular bone explants (Chaudhary *et al.* 1992) but, as demonstrated for PTH and 1,25(OH) $_2$ D $_3$ (Littlewood *et al.* 1991a), did not rise further with T3. Moreover, IL-6 does not stimulate bone resorption in these cells (Littlewood *et al.* 1991b) arguing against a role for these cells in osteotropic hormone-induced bone

resorption. SaOs-2 cells did not respond either to T3 stimulation. This difference in responsiveness between the cell types studied is intriguing and the explanation may lie in the expression of TRs in osteoblasts with MG63 and hBMS cells having higher levels of expression. Allain *et al.* (1996) have recently demonstrated the presence of TR β and TR α 2 in MG63 cells but similar data on TR expression in hBMS cells is currently not available.

An alternative explanation may be related to the phenotypic characteristics of the four cell types studied. It is now increasingly accepted that, when compared with hOb, hBMS cells represent an earlier stage in the osteoblast life cycle (Rickard *et al.* 1996) and that they act as the source of the osteoprogenitor cells from which the renewal of the osteoblast population at the bone surface is considered to occur via differentiation along the osteoblast lineage (Wlodarski 1990). The differences between MG63 and SaOs-2 cells are less clear. MG63 cells are thought to exhibit an earlier osteoblast phenotype based primarily on

Figure 6 RT-PCR was performed on mRNA isolated from MG63 and hBMS cells demonstrating the presence of mRNAs encoding IL-8 and GAPDH in cells treated for 24 h with either no stimulant (C), 1 nM T3, 10 nM T3 or IL-1 β 100 U. Products were run on 1.6% agarose gel and stained with ethidium bromide. The corresponding histograms show the ratio of the densitometric values of IL-8 to GAPDH transcripts relative to the unstimulated control, C. Each bar represents the mean $\pm$ S.E.M. for three experiments. * $P < 0.05$, ** $P < 0.01$ relative to C.

ALP expression, which is relatively less than SaOs-2 and which has prompted some authors (Chaudhary & Avioli 1994, Cheng *et al.* 1994) to draw parallels between them and hBMS cells. Whether osteoblast maturation, as represented by phenotype, alters the expression or function of TRs is currently not known.

The bone marrow microenvironment contains various interleukins which, in synergy with IL-6, stimulate osteoclast precursor proliferation, differentiation and recruitment (Manolagas & Jilka 1995). IL-8, also present in bone marrow, regulates osteoclastogenesis and promotes pseudopodial motility and cytoplasmic spreading (Fuller *et al.* 1995). Thus, a role for hBMS cells in bone remodelling is now well appreciated and our data would suggest that they may be the site of T3 action.

Despite the powerful role of TNF- α (Johnson *et al.* 1989) in bone resorption, we failed to demonstrate any basal or stimulated production of TNF- α in either cell line. Other authors have found a lack of response of TNF- α to IL-1 β , PTH and 1,25(OH) $_2$ D $_3$ (Gowen *et al.* 1990)

suggesting that it is not the primary cytokine by which osteotrophic hormones mediate bone resorption. Similarly, IL-3, GM-CSF and IL-4, which are known to be produced by osteoblasts (Lorenzo *et al.* 1987, Felix *et al.* 1988, Watanabe *et al.* 1990) and play a part in bone turnover, do not appear to be regulated by T3.

Since cellular heterogeneity is an inherent problem in bone marrow cell cultures, there remains a possibility that the T3-responsive cells were non-osteoblastic. We think this unlikely in our cultures since the majority of the hBMS cells were ALP positive and we did not observe chondrocytes or adipocytes in our culture system. Monoclonal antibodies such as STRO-1, which identifies early stromal cell osteoprogenitors (Simmons & Torok-Storb 1991), are potentially useful in isolating purer preparations of stromal cells but also recognise erythroid progenitors, and little is known about the antigenic epitope they recognise.

In conclusion, we have examined the effects of T3 on cytokine production from hOb and osteoblast-like cells.

We have shown that T3 regulates both the production and mRNA expression of IL-6 and IL-8 in hBMS and MG63 cells but not in hOb or SaOs-2 cells. Future studies will need to examine whether these cytokines mediate T3-induced bone resorption.

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