

Bone-related Growth Factors and Zoledronic Acid Regulate the PTHrP / PTH.1 Receptor Bioregulation Systems in MG-63 Human Osteosarcoma Cells

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Abstract. Bisphosphonates are known to inhibit osteoclast-mediated bone resorption and osteoblast differentiation and are currently used in the treatment of Paget's disease, osteoporosis, metastatic and osteolytic bone disease and hypercalcaemia of malignancy. The parathyroid hormone-related peptide (PTHrP) and type I PTH/PTHrP receptor (PTH.1R) bioregulation systems mediate a wide range of local paracrine/autocrine and intracrine functions in various tissues and modify the actions of pharmaceutical agents on target tissues, both in vivo and in vitro. In addition, bone microenvironment-related growth factors, such as insulin-like growth factor 1 (IGF-1), transforming growth factor beta 1 (TGF beta 1), basic fibroblast growth factor (bFGF) and interleukin 6 (IL-6), can modify the actions of various pharmaceutical agents, including cytotoxic drugs in malignant cell lines. Whether IGF-1, TGF beta 1, bFGF, IL-6 and zoledronic acid affect the expressions of PTHrP and PTH.1R in MG-63 osteoblast-like osteosarcoma cells was investigated in this study. Relative quantitative-PCR (expression at mRNA level) and immunofluorescence analysis (localization of the expression at protein level) were employed to assess PTHrP and PTH.1R expressions. Our data showed that primarily IGF-1, TGF beta 1 and IL-6 (up to 25 ng/ml for 48 h) increased PTHrP mRNA expression and modified its perinuclear localization, while zoledronic acid (up to 100 μ M for 48 h) inhibited cell proliferation and suppressed PTHrP expression in the MG-63 osteosarcoma cells. These growth

factors were incapable of reversing the zoledronic acid decrease of the expression of PTHrP in the MG-63 cells, suggesting that zoledronic acid and the growth factors affect PTHrP expression via an independent intracellular signal transduction pathway in these cells. However, no appreciable modulation of the PTH.1R expression by IGF-1, TGF beta 1, bFGF, IL-6 or zoledronic acid was detected in MG-63 cells. Therefore, we conclude that PTHrP expression possibly mediates the action of bone microenvironment-related growth factors and of zoledronic acid in MG-63 cells.

The parathyroid hormone-related peptide (PTHrP) was originally identified as one of the factors responsible for the syndrome of humoral hypercalcaemia of malignancy (HHM) (1). The physiological functions of PTHrP include the regulation of smooth muscle tone, differentiation and proliferation, transepithelial calcium transport and tissue and organ development (2, 3). However, systemic effects of PTHrP have been reported in various pathophysiological conditions (4). It is interesting to note that neonatal mice, with homozygous ablation of the gene encoding either PTHrP or PTH.1R, die from cardio-myocytic insufficiency at, or just before, birth and exhibit widespread skeletal abnormalities, suggesting an anti-apoptotic effect of PTHrP (5, 6). PTHrP has limited homology with the parathyroid hormone (PTH), however they both bind with identical affinity to the PTH/PTHrP (PTH.1R) G-protein coupled receptor (GPCR). Following its binding to PTH.1R, there is activation of the adenylyl cyclase-protein kinase A (AC-PKA) and phospholipase C-protein kinase C (PLC-PKC) signalling pathways (7-9). The full length of the PTHrP mRNA translational product is a precursor protein, which undergoes alternative splicing of its mRNA to produce 3 different-sized initial translation products that are controlled by 3 different promoters (10, 11). Considerable evidence indicates that PTHrP exerts additional signalling

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as a transcription factor by not being secreted extracellularly but being translocated to the nucleus by importin β , thereby influencing nuclear function directly (12). Mutations of the nuclear localization signal (NLS) abrogated the proliferative effects and nuclear translocation of PTHrP, indicating the involvement of an intracrine mode of action (13). The consequences of this intracrine mode of action are not yet well characterized, but they may modulate processes of vital importance to the cell such as the inhibition of apoptosis (13-15).

A great deal has been learnt in recent years about the regulation of bone cell proliferation and differentiation by pharmaceutical agents, hormones, growth factors and physiological stimuli during skeletal development and growth (16, 17). Specifically, bone is unique in its ability to undergo frequent remodelling under the influence/control of systemic hormones and local growth factors, such as insulin-like growth factor 1 (IGF-1), transforming growth factor beta 1 (TGF beta 1), basic fibroblast growth factor (bFGF) and interleukin 6 (IL-6) (18-20). In this context, bisphosphonates (BPs), which are stable pyrophosphate analogues that distribute mainly to sites of exposed hydroxyapatite, inhibit osteoclast-mediated bone resorption (21). A systematic approach, implicating 7 osteosarcoma cell lines, revealed that pamidronate could act directly on osteosarcoma cells, by inhibiting cell growth in a time- and dose-dependent manner, and decreasing proliferation for up to 73% at 50 μM after 72 h (22). Zoledronic acid is a third-generation nitrogen-containing bisphosphonate, which interferes mainly with the mevalonate pathway and has direct effects on the proliferation and survival of MG-63 osteosarcoma cells *in vitro* (23).

In the present study, we assessed whether the bone microenvironment-related growth factors and zoledronic acid affect PTHrP and/or PTH.1R expression in the MG-63 human osteoblast-like osteosarcoma cell line.

Materials and Methods

The Human MG-63 osteosarcoma cell line was obtained from the American Type Culture Collection (ATCC, Bethesda, MD, USA). This cell line was grown in 75-cm² culture flasks at 37°C in 5% CO₂ using RPMI (Gibco #61870-010), containing 5% fetal bovine serum (FBS, Gibco #310108-165), 100 $\mu\text{U}/\text{ml}$ penicillin/streptomycin (Invitrogen, San Diego, CA, USA #15070-063), 1% L-glutamine (Invitrogen #25030-081) and 1% hepes (Invitrogen #15630-080).

Cell culture conditions. The action of zoledronic acid (Zometa, Novartis Pharmaceutical, Basel, Switzerland) (10-250 μM for 96 h) on the proliferation of MG-63 cells was assessed by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay. Since zoledronic acid acts as a chelating agent on Ca⁺⁺ in cell culture media, the effects of the known chelating agent, EDTA (10-250 μM), on the growth of MG-63 cells were also evaluated under identical experimental conditions.

The action of 25 ng/ml of each of the following growth factors was also assessed: TGF beta 1, IGF-1, IL-6 and bFGF, and zoledronic acid (50-100 μM), on the expression of PTHrP in MG-63 cells. Twenty-four h prior to treatment, the culture media containing 5% FBS was removed and replaced with fresh media containing 0.5% FBS so that the factors already in the serum did not interfere with the experiments.

MTT proliferation assays. This colorimetric assay is used for either proliferation or complement-mediated cytotoxicity assays. MTT is a substance that is cleaved by the active mitochondria of living cells to yield a dark blue formazan product, the intensity of which is proportional to the number of living cells present in a culture. The MG-63 cells were plated in 96-well plates 24 h prior to treatment with the appropriate drug at various concentrations and for different periods of time. After 4 doubling-times of exposure to the drugs, the cells were incubated with 10% MTT (Sigma M-5655) added directly to the medium for 4 h at 37°C. The medium was then aspirated and the cells were dissolved in the organic solvent dimethylsulfoxide (DMSO). The absorbance was determined in a VERSA max microplate reader (Molecular Devices Corp.) at 540 nm and the results are presented as the percent of OD in the treated wells *versus* the controls.

Reverse transcription and relative quantitative-PCR. The direct isolation of polyadenylated RNA was performed with a Fast Track mRNA Isolation kit (Invitrogen) following the manufacturer's recommendations. RNA was measured spectrophotometrically by absorbance at 260 nm, and purity was determined by the ratio of the absorbance at 260/280 (A_{260/280}). An aliquot containing 0.5 μg of mRNA was mixed with 10 pmol of random primers (Invitrogen #48190-011), 0.5 mM dNTPs (Invitrogen #10297018) and was filled up to 12 μl with depc-treated ddH₂O. The reaction was then heated to 65°C for 5 min and quick-chilled on ice water. The RT buffer containing 200 U/ μl of SuperScript II RNase H⁻ Reverse Transcriptase (Invitrogen #18064-014) was then added and the reactants were incubated at 42°C for 50 min and 70°C for 20 min in a 20-ml reaction volume. One microliter of the reverse transcription product (cDNA) was amplified by PCR using the HotStartTaq DNA Polymerase kit (Qiagen #203203).

The 2 primers for PTHrP, which recognize all forms of PTHrP (Genbank Accession Number: M17183), were: forward primer 5'-ACGATGCAGCGGAGACTGGTT-3' and reverse primer 5'-CCCTTCTAGCCCACTCCCAGTCAC-3', which give rise to a 479-bp (exons 5-6) fragment (24). The 2 primers for PTH.1R (Genbank Accession Number: U17418) were: 5'-ACCAATGAG ACTCGTGAA-3' (forward primer) and 5'-AAGGACAGGAA CAGGTGCATG-3' (reverse primer), which give rise to a 167-bp (exons 5-7) fragment (25). The cycle profile was 95°C for 15 min, 34 cycles at 95°C for 30 sec, 56°C for 30 sec, 72°C for 30 sec and a final extension at 72°C for 6 min, and the PCR reaction was realized in a Pharmacid LKB (Gene ATAQ Controller, Pharmacia) PCR cyler. The conditions were determined such that, with a certain number of cycles for each gene, we were consistently within the linear range of detection. For relative quantitative-PCR, a commercially available primer and competitor (2:8 ratio applied) for beta-actin (QuantumRNA beta-actin internal standards kit, Ambion #1720), which give rise to a 294-bp fragment, were employed in order to control the PCR reaction and aliquot loading variability across the samples.

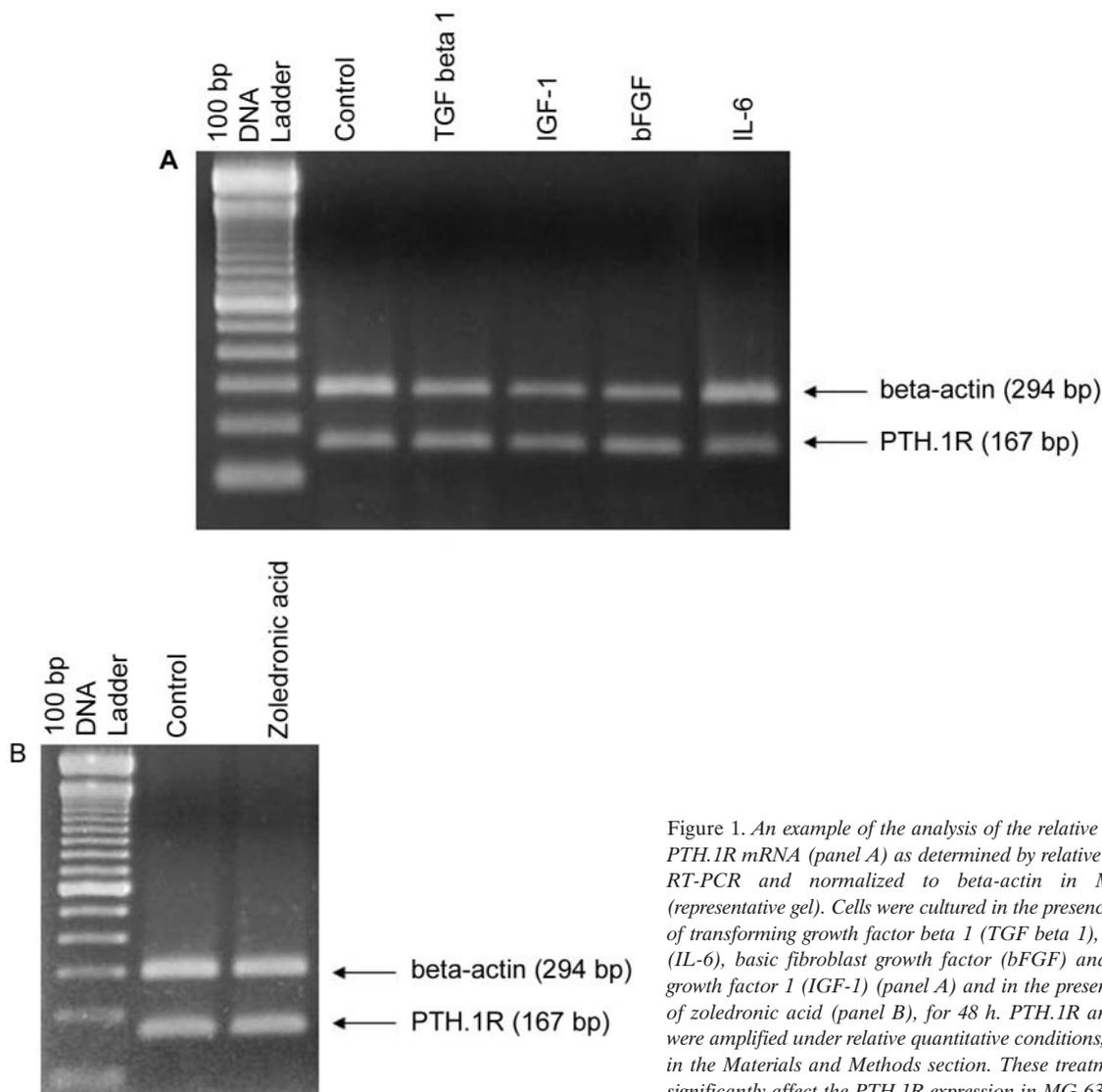


Figure 1. An example of the analysis of the relative expression of *PTH.1R* mRNA (panel A) as determined by relative quantitative-RT-PCR and normalized to beta-actin in MG-63 cells (representative gel). Cells were cultured in the presence of 25 ng/ml of transforming growth factor beta 1 (TGF beta 1), interleukin 6 (IL-6), basic fibroblast growth factor (bFGF) and insulin-like growth factor 1 (IGF-1) (panel A) and in the presence of 50 μ M of zoledronic acid (panel B), for 48 h. *PTH.1R* and beta-actin were amplified under relative quantitative conditions, as described in the Materials and Methods section. These treatments did not significantly affect the *PTH.1R* expression in MG-63 cells.

The PCR products were run on a 1.5% agarose gel and the bands were visualized by ethidium bromide staining and photographed by a DC290 Zoom digital camera. DNA molecular weight markers (100-bp ladder, Invitrogen #15628-050) were included on each gel in order to confirm the PCR product sizes. Each PCR was performed at least in triplicate and the results were reproducible. Data analysis was performed by the Image analysis software (Kodak Electrophoresis documentation and analysis system-EDAS 290). The normalization to the housekeeping gene beta-actin enabled the standardization of data to permit the differentiation between real (biological) variations in gene expression levels and variations due to the measurement process. The identity of the products was confirmed by dideoxy sequencing.

Immunofluorescence labelling. To assess the expression and to detect the localization of PTHrP, cells grown on Lab-Tek chamber slides (Nalge Nunc, Naperville, IL, USA) were treated with the

growth factors or zoledronic acid for 48 h. After being washed with cold phosphate-buffered saline (PBS), the cells were fixed in methanol for 10 min at -20°C , air-dried, washed again with PBS, incubated in 3% H_2O_2 in methanol for 10 min, incubated in 50 mM ammonium chloride in PBS for 15 min at room temperature and then washed with PBS. After blocking non-specific protein binding by incubation with blocking serum (Ultra Vision Detection System Anti-Polyvalent, HRP, Lab Vision Corporation #TP-060-HL) for 20 min at room temperature and washing with PBS, the cells were incubated with the primary antibody (mouse monoclonal IgG; Oncogene Research Products #GF08) for 60 min at room temperature. This antibody reacts with amino acids 38-64 of human PTHrP and shows no cross-reactivity with human PTH. The control cells received no primary antibody. After washing again with cold PBS, the cells were treated with fluorescein isothiocyanate (FITC)-labelled goat anti-mouse secondary antibody (sc-2010) for 60 min at room temperature, washed again with PBS, mounted using Aqua

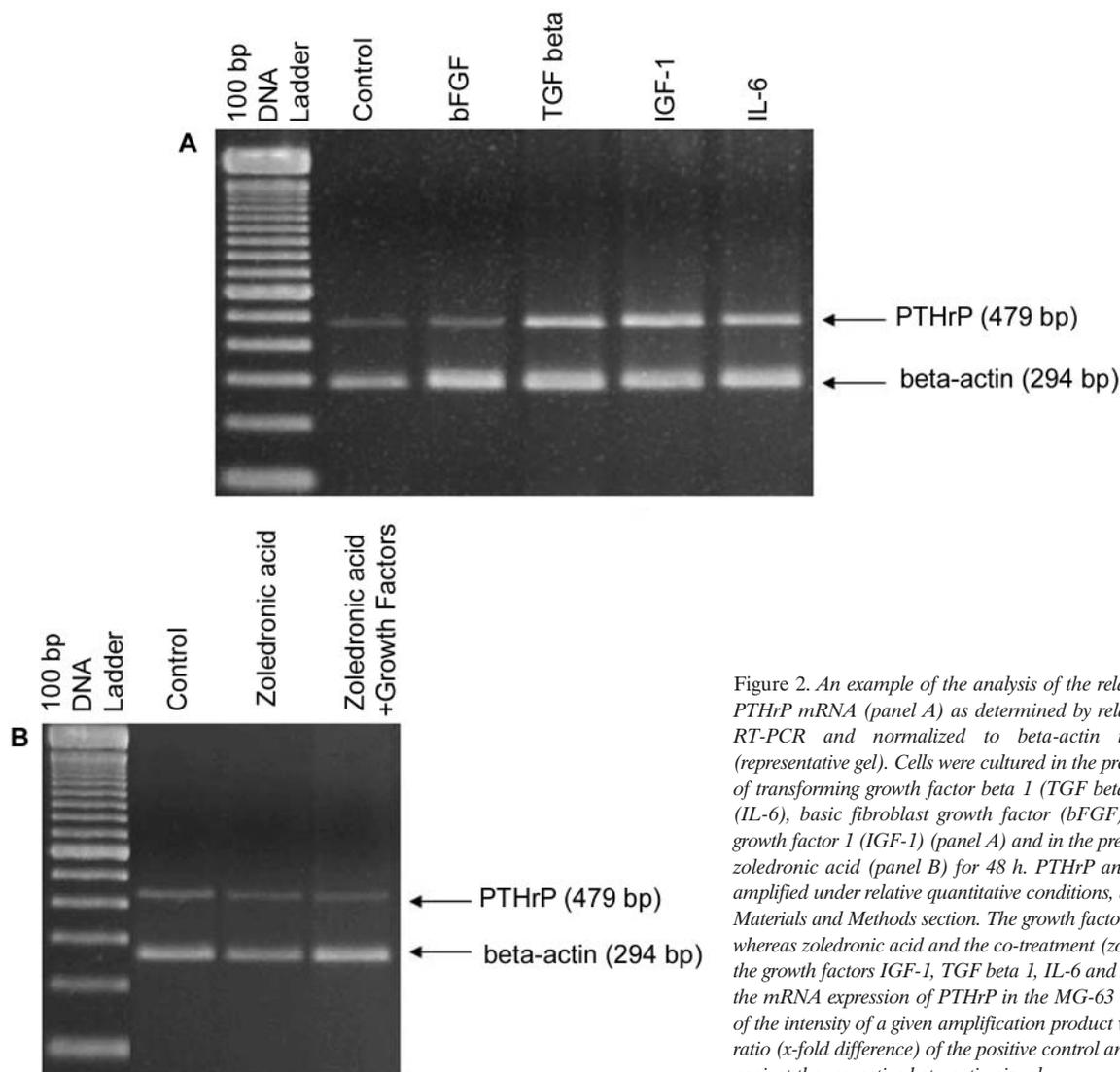


Figure 2. An example of the analysis of the relative expression of PTHrP mRNA (panel A) as determined by relative quantitative-RT-PCR and normalized to beta-actin in MG-63 cells (representative gel). Cells were cultured in the presence of 25 ng/ml of transforming growth factor beta 1 (TGF beta 1), interleukin 6 (IL-6), basic fibroblast growth factor (bFGF) and insulin-like growth factor 1 (IGF-1) (panel A) and in the presence of 50 μ M of zoledronic acid (panel B) for 48 h. PTHrP and beta-actin were amplified under relative quantitative conditions, as described in the Materials and Methods section. The growth factors alone increased, whereas zoledronic acid and the co-treatment (zoledronic acid plus the growth factors IGF-1, TGF beta 1, IL-6 and bFGF) decreased, the mRNA expression of PTHrP in the MG-63 cells. The analysis of the intensity of a given amplification product was expressed as a ratio (x-fold difference) of the positive control and was normalized against the respective beta-actin signal.

Poly Mount (Polysciences, Inc. #18606) and analyzed using an Olympus fluorescence microscope (Olympus Corp., Melville, NY, USA) with a wide-band green filter.

Results

Confirmation of the expressions of PTHrP and PTH.1R. Initially, the mRNA expressions of PTH.1R (Figure 1, panel A) and PTHrP (Figure 2, panel A) were confirmed by RT-PCR in MG-63 cells. The sequencing analysis confirmed the nature of the amplified PCR products in the cells (data not shown). In addition, PTHrP expression was confirmed in the MG-63 cells by immunofluorescence (Figure 3).

The effects of bone-related growth factors and zoledronic acid on the proliferation of MG-63 cells. The action of 25 ng/ml

of the growth factors on the proliferation of MG-63 cells was evaluated by the MTT assay: IGF-1 provoked an increase of 20% and bFGF an increase of 15%, while TGF beta 1 caused a decrease of 40% and IL-6 a decrease of 20% of the cell proliferation; these effects correspond to the maximal action on the proliferation of MG-63 cells when tested in a large scale of concentrations (25 pg up to 50 ng/ml) (data not shown).

Increasing concentrations of zoledronic acid (10-250 μ M) produced a dose-dependent inhibition of the growth of MG-63 cells after 96 h of continuous exposure (maximal inhibition: 60%), whereas 96 h of continuous exposure to EDTA had no effect on cell proliferation. The inhibition of MG-63 cell growth was evident at concentrations of zoledronic acid as low as 30 μ M (Figure 4), suggesting that

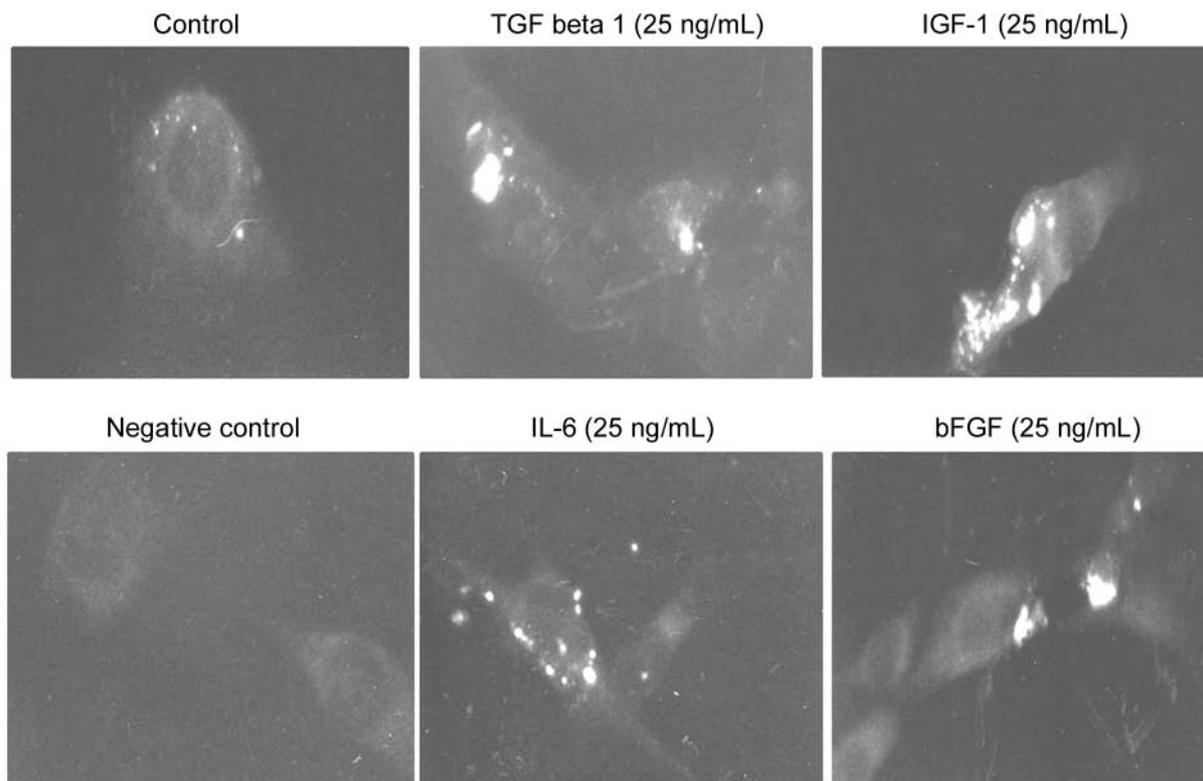


Figure 3. Example of PTHrP expression in MG-63 cells, by immunofluorescence, displaying the effects of the exogenous administration of 25 ng/ml of transforming growth factor beta 1 (TGF beta 1), insulin-like growth factor 1 (IGF-1), interleukin 6 (IL-6) and basic fibroblast growth factor (bFGF) on intracellular PTHrP expression and localization. Note that the control cells showed significantly less intense staining than cells treated with the growth factors, whereas a specific PTHrP distribution was depicted at the perinuclear zone (Magnification: x630).

it can inhibit the growth of MG-63 cells by a mechanism independent of Ca^{++} chelation.

Bone-related growth factors and PTH.1R mRNA expression. The PTH.1R mRNA expression was measured in MG-63 cells after their exposure to 25 ng/ml of IGF-1, 25 ng/ml of TGF beta 1, 25 ng/ml of IL-6 or 25 ng/ml of bFGF (final concentration; 48-h exposure; dose representing the maximal effective dose in a cell proliferation assay). These bone microenvironment-related growth factors did not significantly affect the PTH.1R mRNA expression in the MG-63 cells, as determined by relative quantitative-PCR normalized to beta-actin in triplicate experiments (Figure 1, panel A).

Bone-related growth factors and PTHrP mRNA expression. The effects of the growth factors on the PTHrP mRNA expression were assessed and IGF-1, TGF beta 1 and IL-6 were found to enhance PTHrP expression, while bFGF showed minimal effects. Under conditions identical with

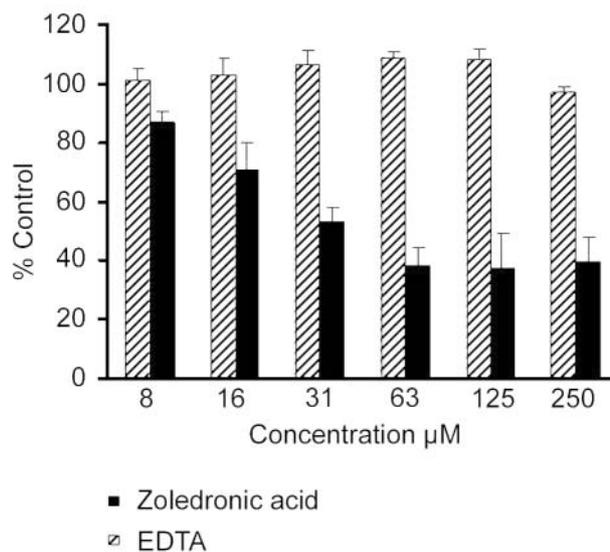


Figure 4. Dose-response curves for the continuous administration (96 h) of zoledronic acid (10-250 μM) and EDTA (10-250 μM) on MG-63 cells, as assessed by the MTT assay. The results are expressed as a percentage of controls.

those used for the PTH.1R mRNA experiments, 25 ng/ml of TGF beta 1, IGF-1 and IL-6 increased the PTHrP mRNA expression from 1.4- up to 1.9-fold, as determined by relative quantitative-PCR normalized to beta-actin in the MG-63 cells (Figure 2, panel A). The fold change increase of PTHrP vs. beta-actin in triplicate experiments was as follows: TGF beta 1 (1.4, 1.5, 1.9), IGF-1 (1.5, 1.4, 1.4), IL-6 (1.4, 1.5, 1.6) and bFGF (1.0, 1.1, 1.2).

Bone-related growth factors and localization of PTHrP. The expression and localization of PTHrP in MG-63 cells was analysed by immunofluorescence analysis, which has an apparently higher sensitivity than immunocytochemical detection and provides significant data on the localization of PTHrP-specific staining. Given that PTHrP, via its nuclear localization signal, can act as a transcription factor and that the nuclear localization of PTHrP is related to the peptide's anti-apoptotic properties, the presence of PTHrP in the cytoplasm and/or the nucleus was investigated before and after treatment with the growth factors.

Indeed, it was observed that the bone-related growth factors, such as TGF beta 1, IGF-1, IL-6 and bFGF, enhanced the PTHrP staining in MG-63 cells, showing a particular increase of its perinuclear distribution (Figure 3).

Zoledronic acid and PTH.1R mRNA expression. Based on the biological effects of zoledronic acid on the proliferation of the MG-63 cells, the PTH.1R mRNA expression in the cells after their exposure to 50 μ M of zoledronic acid (final concentration; 48 h; dose representing the maximal effective dose in a cell proliferation assay) was analysed. Zoledronic acid did not alter the PTH.1R expression in triplicate experiments (Figure 1 panel B).

Zoledronic acid and PTHrP expression. We investigated whether 50 μ M of zoledronic acid had a direct effect on the expression of PTHrP in MG-63 cells, when treated for 48 h. Indeed, our analysis revealed that zoledronic acid attenuated the PTHrP mRNA expression of MG-63 cells (Figure 2, panel B) by 0.73-, 0.74- and 0.8- fold change decrease, respectively, (PTHrP vs. beta-actin) in 3 experiments. However, this effect was neither time-dependent (24 h vs. 48 h) (data not shown) nor dose-dependent (50 μ M vs. 100 μ M) (Figure 5).

In the immunofluorescence experiments, the control cells (which received no treatment) showed more intense staining than those cells treated with 50 μ M zoledronic acid for 48 h. Such analysis indicates that zoledronic acid suppressed both the cytoplasmic and perinuclear localization of the PTHrP staining in the MG-63 cells (Figure 6).

Bone-related growth factors and the zoledronic acid-induced decrease in PTHrP expression. The effect of the co-treatment

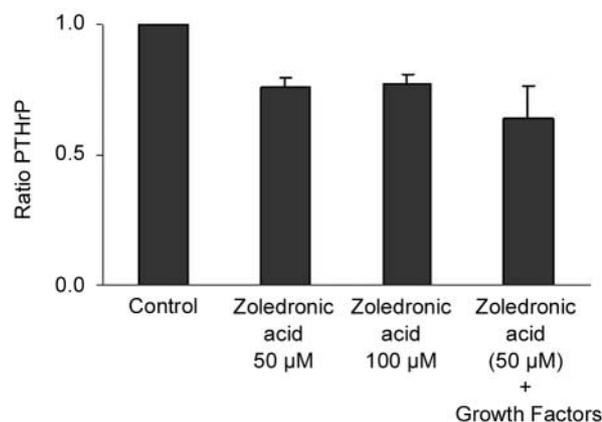


Figure 5. Effect (48 h) of zoledronic acid and of the co-treatment (zoledronic acid plus the growth factors IGF-1, TGF beta 1, IL-6 and bFGF) on the relative expression of PTHrP mRNA (ratio), as determined by relative quantitative-RT-PCR and normalized to beta-actin in MG-63 cells, using RT-PCR methods as described in the Materials and Methods section.

of the MG-63 cells with 50 μ M of zoledronic acid and 25 ng/ml of the growth factors TGF beta 1, IGF-1, IL-6 and bFGF for 48 h was evaluated. These growth factors, which increased the PTHrP expression when alone (Figure 2, panel A), were incapable of reversing the decrease in the expression of PTHrP produced by zoledronic acid after 48 h of co-treatment (Figure 5). Our analysis revealed that zoledronic acid still attenuated the PTHrP mRNA expression of MG-63 cells by a 0.64-fold change decrease (PTHrP vs. beta-actin) in 3 experiments.

Discussion

Osteosarcoma is a mesenchymally-derived, high-grade bone sarcoma. The first choice is chemotherapy, employing multiple anticancer drugs such as doxorubicin, cisplatin and etoposide, which are used either as single agents or in combination. However, despite significant improvements in patient survival and treatment of the primary tumour, some groups of patients who present with metastatic disease, particularly osteolytic bone metastases, as well as patients with tumours that recur after treatment, continue to have a poor prognosis (26-28). Therefore, there is a pressing need to develop new and alternative approaches to the current treatment of osteosarcomas.

Recently, bone microenvironment-related growth factors, such as IGF-1, TGF beta 1, bFGF, IL-6 and PTHrP, have been found to act as survival factors on metastatic prostate and breast cancer cells, thereby inhibiting chemotherapy apoptosis, *in vitro* (29-31). For example, bone-related growth factors have been widely viewed as key players in tumour resistance against anticancer therapies in metastatic cancer.

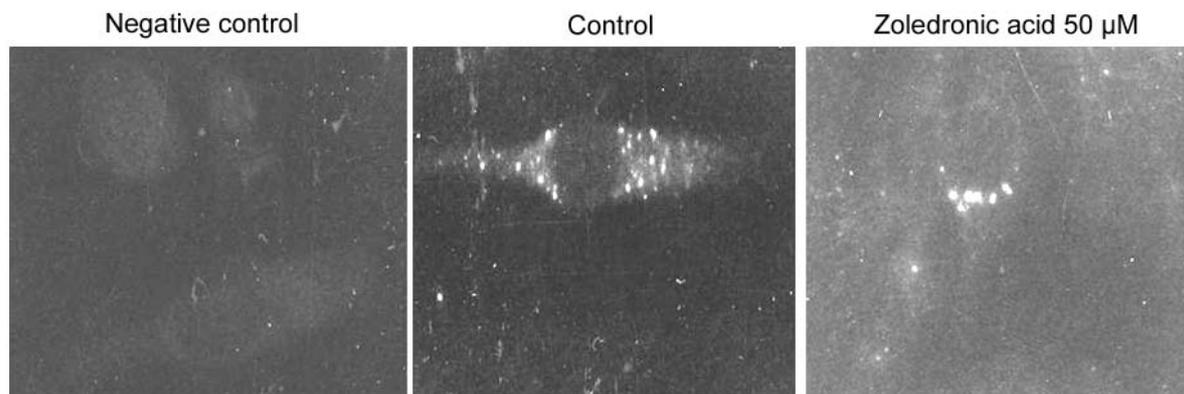


Figure 6. An example of the PTHrP expression in MG-63 cells in response to zoledronic acid (50 μ M) by immunofluorescence, as described in the Materials and Methods section. Zoledronic acid suppressed the expression of PTHrP as compared to the controls (Magnification: $\times 630$).

We, therefore, studied whether PTHrP expression, a well known systemic and local regulator of osteosarcoma cells (32-34), is modulated by bone microenvironment-related growth factors, thereby mediating their action on the human MG-63 osteoblast-like osteosarcoma cell line. Our results indicated that TGF beta 1, IL-6 and IGF-1 enhanced the expression of PTHrP in MG-63 cells. However, these growth factors did not affect the expression of PTH.1R, suggesting that the bone microenvironment-related growth factors modulate PTHrP activity directly *via* their regulation of PTHrP transcription. In addition, our experiments showed that these growth factors affected the intracellular trafficking of PTHrP from the cytoplasm to the nucleus, since an increasing perinuclear localization of PTHrP was detected in the MG-63 cells. Consequently, the intracrine action of PTHrP, known to be associated with the inhibition of apoptosis (14, 15, 35), is increased significantly by the bone microenvironment-related growth factors in MG-63 cells. It is, therefore, conceivable that PTHrP mediates, at least in part, the survival activity of these growth factors in osteosarcoma cells.

Bisphosphonates, currently used in the treatment of Paget's disease, osteoporosis, metastatic and osteolytic bone disease and hypercalcaemia of malignancy (36, 37), are specifically internalized into osteoclasts (38) and lead to the inhibition of cell function because of changes in the cytoskeleton and loss of the ruffled border, as well as apoptosis (39). It is widely recognized that bisphosphonates inhibit bone resorption by suppressing the action of osteoclasts through antagonizing the mevalonate pathway, thereby reducing osteolytic bone metastases derived from different cancers, *i.e.* breast carcinoma and multiple myeloma (39, 40). Our results indicated that zoledronic acid (a third-generation bisphosphonate) attenuated the expression of PTHrP, while it did not affect the expression

of PTH.1R. Since PTHrP transcription and its perinuclear localization can be attenuated by zoledronic acid, it is conceivable that such compounds can attenuate the intracrine action of PTHrP in osteosarcoma cells.

In the past, the development of anticancer drugs traditionally relied on *in vitro* tests aimed almost exclusively at assessing the potential of direct killing or growth inhibition of cancer cell lines. However, accumulating evidence indicates that the *in vivo* response of malignant cells to anticancer therapies is mostly influenced by the host-tissue microenvironment in which cancer cells, particularly metastatic cancer cells, reside (30, 31). It has also been shown that host-tissue (bone) growth factors, present in the microenvironment of osteoblastic lesions of prostate cancer patients with bony metastasis, cannot neutralize the anticancer actions of zoledronic acid on metastatic cancer cells (41). Given that, in our experiments, the bone-related growth factors were not capable of neutralizing the effect of zoledronic acid on PTHrP expression, a known anti-apoptotic pathway, we suggest that zoledronic acid may prevent the optimization of MG-63 osteosarcoma cell survival. Conceivably, zoledronic acid could potentiate the pro-apoptotic action of cytotoxic drugs in osteosarcomas.

In conclusion, our data suggest that bone microenvironment-related growth factors and zoledronic acid can differentially modulate PTHrP expression in MG-63 cells. Further investigation of the exact role of PTHrP on osteosarcoma cells may contribute to the design of a more effective adjuvant treatment for osteosarcoma patients.

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