RESEARCH ARTICLE

Stimulation of Osteogenic Differentiation in Stromal Cells of Giant Cell Tumour of Bone by Zoledronic Acid

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Abstract

Therapeutic effects of zoledronic acid (ZOL) on giant cell tumour of bone (GCT) have been proven. Apoptosis induction was considered to be one of the mechanisms of ZOL tumour inhibition. In this study, we presented the possibility of an osteogenic differentiation stimulation mechanism of ZOL and further investigated dosage and time effects. We treated stromal cells of GCT (GCTSC) with ZOL for 48 hours at different concentrations (0 μM, 0.01 μM, 0.1 μM, 1 μM, 5 μM, 30 μM) and assessed apoptotic and osteogenic differentiation markers with immunohistochemical techniques and real-time quantitative RT-PCR. Our results suggested that ZOL enhanced mRNA expression of Cbfa-1, osterix and osteocalcin genes with a maximum effect at 1μM in GCTSC. Time course experiments indicated a time dependent osteogenic differentiation effect. In conclusion, ZOL may be considered as an adjuvant in the treatment of GCT not only by inducing apoptosis but also by stimulating osteogenic differentiation of remaining tumor stromal cells after surgery.

Keywords: Giant cell tumour of bone - zoledronic acid - apoptosis - osteogenic differentiation

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Introduction

Giant cell tumor of bone (GCT) is a common benign tumor that comprises 5%-20% of primary bone tumors in adults (Turcotte, 2006). It is characterized by numerous multinucleated giant cells found within the tumor, which are principally responsible for the osteolytic bone destruction. Laboratory studies have shown that the tumor tissue actually contains three typical cellular components: spindle shaped cell (stromal cell), mononuclear cell and multinucleated giant cell (Zheng et al., 2001). Stromal cell (GCTSC) is thought to be the primary neoplastic cells owing to their abilities of stable proliferation in culture and tumor formation in mice (Cowan and Singh, 2013). It expresses differentiation features of mesenchymal lineage and pre-osteoblast phenotype (Werner, 2006). Mononuclear cell expresses CD68-antigen so as to be considered originating from monocytic-histiocytic system (Zheng et al., 2001). It is probably recruited to the tumor site by the stromal cells and fused together to form the multinucleated giant cells (Cowan and Singh, 2013). Multinucleated giant cell is characterized as mature osteoclasts with larger outline and hundreds of nuclei. The osteoclast-like multinucleated giant cells enhance osteolytic bone resorption and cause skeletal-related complications including pathological fractures and and hypercalcaemia. Surgical curettage is the preferred treatment for most GCT patients. But complete resection of the tumor is sometimes difficult to achieve. The local recurrence rate after surgery is relatively high (up to 50%) with rare lung metastasis (Sung et al., 1982). Recurrences may even occur in spite of extensive tumor clearance and repeated surgery or even amputation may be required for tumor eradication (Mendenhall et al., 2006). Many adjuvant treatments including physical methods (blurring, hypothermic or hyperthermic reagents), chemical methods (phenol, hydrogen peroxide) and biologic modalities (bisphosphonates, interferon, denosumab) have been suggested to eliminate microscopic lesions of the disease after surgery so as to reduce the recurrence rate (Gibbs et al., 2005). The benefits gained from adjuvant treatments are various. Biologic modalities have the advantages of targeted and repeated therapy. Clinical studies have already proved that intravenous or peroral administration of bisphosphonates reduce the recurrence of GCT (Arpornchayanon and Leerapun, 2008; Tse et al., 2008; Balke et al., 2010; Nishisho et al., 2011; Gille et al., 2012). Bisphosphonates (BPs) can block the bone resorption activity and promote osteoblast activity. In vitro studies also reveal the mechanism that bisphosphonates exert direct anti-tumor effects by inducing apoptosis of the stromal cells (Chang et al., 2004). However, there are still not many studies investigating the anti-GCT mechanism of bisphosphonates. We used to do experiments to verify the apoptosis inducing mechanism of ZOL on GCT. Moreover, significant calcification around the lesion was observed

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after ZOL adjuvant therapy of GCT (Tse et al., 2008). We think ZOL may also induces osteogenic differentiation on GCTSC. So we did in vitro experiments to find out whether there is potential osteogenic inducing mechanism of ZOL on GCTSC.

Materials and Methods

Specimens

GCT specimens (4) were collected from patients after operations at Liu Huaqiao Hospital and Nanfang Hospital. This study was conducted in accordance with the declaration of Helsinki. This study was conducted with approval from the Ethics Committee of Guangzhou Liu Hua Qiao Hospital. Written informed consent was obtained from all participants. None of the patients had taken bisphosphonate medication before the operation. The diagnosis was confirmed by routinely prepared paraffin tissue sections of the tumor specimen. GCT tumor samples from freshly harvested surgical specimen were used for primary cell cultures.

Cell cultures

The tumor tissues were freshly chopped in high glucose DMEM (Hyclone) containing 10% FBS (Hyclone, South America), 100 U/ml penicillin, and 100 mg/ml streptomycin. The resultant cell suspension together with small pieces of tissue was transferred to 25-cm^2 flasks for culture at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Half of the culture medium was changed every 3 days with fresh DMEM containing 10% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin. Primary cultures were subcultured and stored in liquid nitrogen for reaching confluence. Primary GCTSC cultures were obtained after the ninth passage (Figure 1), which represent the proliferating homogenous tumor cell population (GCTSC). The purified tumor cells were then used for bisphosphonate treatment and evaluation (Huang et al., 2004). In the following experiment, drug was mixed in culture medium and the concentration was controlled during medium preparation.

Drug Treatments

The GCTSCs were seeded in 6-well plates at a density of 2.5×10^5 cells per well in DMEM/10% FBS overnight. Cells were treated with zoledronate at different concentrations (0 μM, 0.01 μM, 0.1 μM, 1 μM, 5 μM, 30 μM) for 48 hours.

Immunocytochemistry

In the process of cell passage, GCTSCs are equally divided into two groups, blank group and 1 μM zoledronate group. Cells were then cultured for 48 hours on glass coverslips in cell culture wells. Adherent cells were fixed with 4% paraformaldehyde before rinsing in PBS. Endogenous peroxidase activity was then blocked with 0.3% hydrogen peroxide in absolute methanol for 20min. Cell areas were then incubated with 5% normal goat serum in 1% BSA-PBS for 30 min to block nonspecific IgG binding. Thereafter, primary antibodies-rabbit antihuman collagen type I, BSP, osteonectin osteocalcin-were

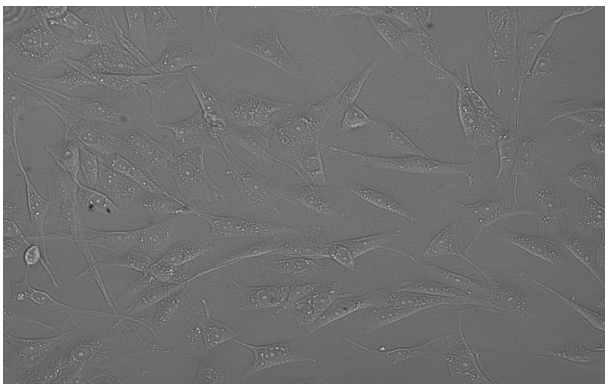

Figure 1. GCTSCs Get Purified after the 9th Passage, Which Represents the Proliferating Homogenous Tumor Cell Population (original magnification: ×200)

applied at 4°C overnight at a dilution of 1:500, except BSP at dilution of 1:300 in 1% BSA–PBS. After stringent washing thrice in PBS, a biotinylated mouse anti-rabbit IgG (diluted 1:200) was used for further incubation and a streptavidin–biotin complex system (ABC regents) with diaminobenzidene (DAB) as chromogen was used for color development. Slides were finally examined under a light microscope.

RT-PCR

Giant cell tumor stromal cells treated with zoledronate (0 μM, 0.01 μM, 0.1 μM, 1 μM, 5 μM, 30 μM) were harvested for RNA extraction with Tripure reagent after 72h culture. RNA was then reverse transcribed to cDNA using 100 units of M-MLV reverse transcriptase (Roche, Penzberg, Germany) per reaction with an oligo-dT primer (Roche, Penzberg, Germany). Primers designed for PCR using Prier Design software are, respectively, Cbfa-1 (5'ACTGGGCCCTTTTTCAGA3' and 5'GCGGAAGC ATTC-TGGAA3'), osterix (5'CGGGACTCAACAACTC T3' and 5'CCATAGGGGTGTG-TCAT3') and osteocalcin (5'GCCTTTGTGTCCAAGC3' and 5'GGACCCCACAT C-CATAG3'). Housekeeping gene GAPDH (5'GGAGTC AACGGATTTGGT3' and 5'GTGATGGGATTTCCATTG AT3') was used as internal control. For PCR reaction (LightCycler 480), a mastermix of the following reaction components was prepared to the indicated end concentration: 7.5 μl water, 0.25 μl forward and reverse primers (0.5 μM) and 1.0 μl FastStart Universal SYBR Green Master (Roche, Penzberg, Germany). FastStart mastermix (9 μl) was filled in the PCR glass capillaries and 1 μl cDNA (approximately 2 ng) was added as PCR template. Experimental run protocol was used: denaturation program (95°C, 10 min), amplification and quantification program repeated 45 times (for a single fluorescence measurement: 95°C, 10 s; 55°C for Cbfa-1, 60°C for osterix and osteocalcin, 5 s; 72°C, 10 s), melting curve program (60-95°C with a heating rate of 0.1 C/s and a continuous fluorescence measurement) and finally a cooling step to 40°C. After the reaction, identities of PCR products were confirmed by melting curve analysis, which is part of the LightCycler analysis program and agarose gel electrophoresis. Results were analysed using the LightCycler Relative Quantification Software (Roche Diagnostics GmbH). Time course experiments were done

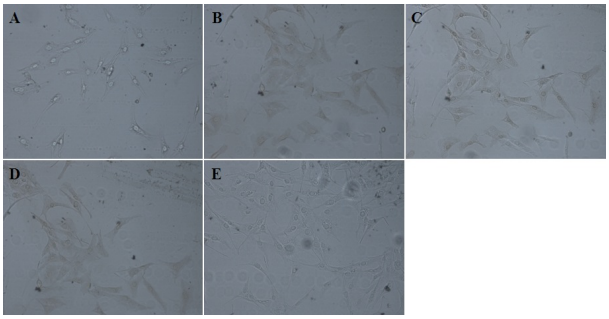

Figure 2. Immunostaining of Collagen Type I (Figure 4A), BSP (Figure 4B), Osteonectin (Figure 4C), Osteocalcin (Figure 4D) in 1 μM Zoledronate Treated GCTSCs and Control Cultures (Figure 4E) (original magnification: ×200)

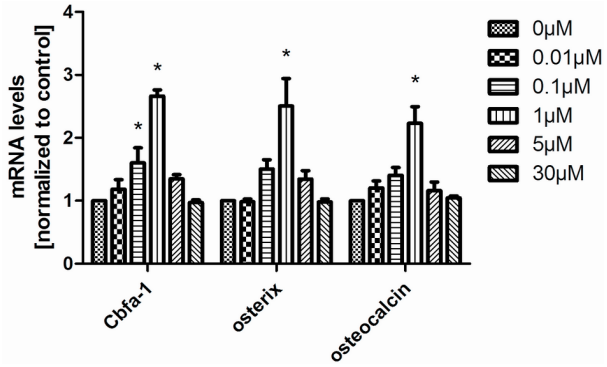

Figure 3. RT-PCR Analysis of Cbfa-1, Osterix and Osteocalcin mRNA Levels Isolated from GCTSCs Cultured for 72 h in the Presence of Various Concentration of Zoledronate. The amplified products are indicated as fold differences with respect to the control cultures (0μM zoledronate). *represents P < 0.05

with 1 μM of zoledronate at different time (0 h, 24 h, 72 h, 168 h). Osteogenic gene mRNA expression was detected in the same method.

Statistical analysis

Results are expressed as the mean±the standard error of the mean (SEM). The significance of gene regulation of Cbfa-1, osterix and osteocalcin in GCTBSC were summarized using one-way ANOVA, SPSS statistical software for Windows. The LSD test was performed to determine statistical significance. A *P*-value of 0.05 was considered as statistical significance.

Results

The expression of preosteoblast markers with immunocytochemical study indicates osteogenic property. In this study, preosteoblast markers, collagen type I, BSP and osteonectin, osteocalcin, were detected. The intensity and extent of immunostaining were found more obvious in zoledronate treated group (Figure 2). Osteogenic genes such as Cbfa-1 and osterix are specifically activated during osteoblast differentiation. Moreover osteocalcin gene is a differentiated osteoblast marker. By using real-time quantitative RT-PCR, we evaluated the mRNA expression of Cbfa-1, osterix and osteocalcin gene. After zoledronate

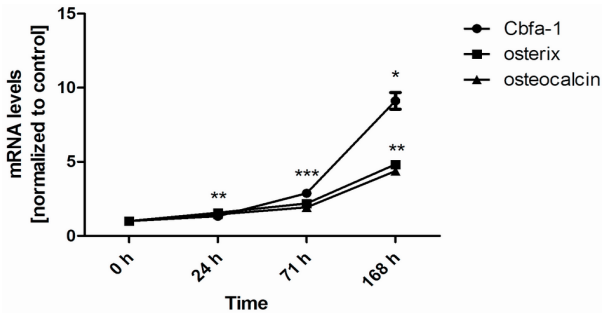

Figure 4. RT-PCR Analysis of Cbfa-1, Osterix and Osteocalcin mRNA Levels Isolated from GCTSCs at Different Time Points. The amplified products are indicated as fold differences with respect to the control cultures (0 h zoledronate treatment). *represents *P* < 0.05

treatment, the GCTSC varied in their individual response in different groups. The levels of mRNA were increased. Cbfa-1 mRNA was increased significantly at 0.1 μM and 1μM zoledronate compared to the control group (*P* < 0.05). Notably, upregulation of Cbfa-1, osterix and osteocalcin mRNA levels are most prominent in 1μM zoledronate group (*P* < 0.01) (Figure 3). Time course experiments were performed to further characterize the effect of zoledronate on mRNA expression of osteogenic genes. The GCTSC were treated with 1μM of zoledronate at different time (0 h, 24 h, 72 h, and 168 h). The mRNA expression was steadily increased over time (Figure 4). Osterix and osteocalcin mRNA levels were higher than the control group with significant difference at all measuring points (*P* < 0.05). Zoledronate significantly increased Cbfa-1 mRNA at 72 h (*P* < 0.001), and the induced mRNA levels were upgraded by 9 fold after 168 h.

Discussion

Giant cell tumor of bone (GCT) is one of the most common benign bone tumors. Operative removal of the tumor lesion is usually easy to achieve. But how to reduce the local recurrence of GCT is an urgent problem to be solved. Previous researches have made some progress and less local recurrence of the disease with various adjuvant therapies have been reported (Szendroi et al., 2004). Recently, the anti-osteolytic and anti-tumor function of bisphosphonates by inhibition of farnesylation and geranylgeranylation of RAS related proteins has attracted much attention. The potential of bisphosphonates in killing tumor cells and relieve the clinical symptoms of GCT has been observed (Cheng et al., 2004; Tse et al., 2008). However, there was little evidence to reveal the therapeutic mechanism of bisphosphonate as an adjuvant in GCT.

Bisphosphonates (BPs) are analogues of endogenous pyrophosphonate with chemical compounds of P-C-P structure. They are generally divided into N-containing (nitrogenous) bisphosphonates and non-N-containing bisphosphonates. In vivo, bisphosphonates have high affinity to hydroxyapatite on the bone surface and are delivered preferentially to mineral components of increased bone formation or resorption (Boonekamp et al., 1986). Nitrogenous bisphosphonates can inhibit the synthesis of farnesyl diphosphate in the cells, and decrease

its downstream metabolite geranylgeranyl diphosphate. Geranylgeranyl diphosphate exerts prenylation of small GTP (guanosinetriphosphate) ases, which play an important role in many signaling pathways regulating cell processes of osteoclast, tumor cell and other cells (Luckman et al., 1998). Moreover, high concentration of bisphosphonates inhibit the proteolytic activity of tumor-cell-derived MMP (metalloproteinase)-2 and MMP-9 by inhibiting the zinc-dependent proteolytic activity of matrix MMPs, a family of proteolytic enzymes essential for the degradation of extracellular matrix proteins, invasion, and migration (Boissier et al., 2000). Bisphosphonates (BPs) have been widely used in clinics preferentially to reduce the skeletal complications in benign and malignant bone diseases associated with enhanced osteolytic bone resorption (Reid et al., 2002). Bisphosphonates block osteoclasts, thus disrupt bone resorption and inhibit bone loss so as to relieve osteoporosis. Systemically administered bisphosphonate has been shown to reduce skeletal-related event (SRE), including pathological fractures (non-vertebral, vertebral, combined), radiotherapy, spinal cord compression, orthopaedic surgery, and hypercalcaemia, in association with malignancy (eg. myeloma and breast carcinoma) (Chang et al., 2004). Local administrated bisphosphonate with eluted bone cement is also demonstrated effective in growth inhibition on cell lines from giant cell tumor of bone, myeloma and renal cell carcinoma (Zwolak et al., 2010).

Most histologic and pathologic researches support that the stromal cells is the neoplastic components among the three constituents of GCT. Previous studies revealed that bisphosphonates can exert a significant apoptosis inductive function on GCT stromal cells with a dose depentdent manner (Cheng et al., 2004), While they also inhibit adipogenic differentiation in bone marrow stromal cells via ERK and JNK activation (Fu et al., 2008) and stimulate osteogenesis and bone regeneration of human adipose-derived stem cells (hADSC) at a low concentration about 1-10μM (Wang et al., 2010). These results demonstrated bisphosphonates a potent tumor cytotoxic and osteo-inductive agent at different concentration. There were clues that GCT stromal cells might be derived from an osteoblastic lineage and retain the ability to differentiate into osteoblast after certain stimulation (Huang et al., 2004). It is reasonable to consider that bisphosphonates may have the ability to induce the osteogenic differentiation of GCT stromal cell other than apoptosis.

Zoledronate is one of the latest bisphosphonate drugs. From the result of our research, zoledronate induced apoptosis of GCTSC in a dose-dependent manner which is consistent with prior researches. The immunocytochemical staining results indicates the simulated secretion of preosteoblast markers, collagen type I, BSP osteonectin, osteocalcin in GCTSC with zoledronate treatment. Compared with controlled group, the mRNA expression of Cbfa-1, osterix and osteocalcin gene is the most remarkable at 1 μM zoledronate. Time course experiments indicated that the longer treatment time was, the more obvious of the osteogenic effect appeared. It is poossible that bisphosphonate may have a sustaining adjuvant effect for GCT. Early period high concentration bisphosphonate induce apoptosis of the tumor cells. And long term low concentration adminstration of bisphosphonate may induce osteogenic differentiation of remaining tumor cells and promote the osteo-reconstruction after surgical excision of GCT.

Putative osteogenic inducing effect of bisphosphonates on GCTSC may be conducive the adjuvant therapy of GCT after the surgery. However, the osteogenic mechanism needs further investigation and the clinical value is worthy of further observation.

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