

Expression of Bone Morphogenetic Proteins in Giant Cell Tumor of Bone

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Abstract. *Background:* A giant cell tumor (GCT) of bone is a locally aggressive tumor with a propensity for local recurrence. A characteristic pattern of peripheral bone formation has been described in GCT recurrence in soft tissue, and in some pulmonary metastases from benign GCT. Although the bone formation in GCT is supposedly due to bone morphogenetic proteins (BMPs), the expression pattern of BMPs in GCT has not been well investigated. *Materials and Methods:* The expression of BMPs in GCT tissues, cultured stromal cells from GCT, and osteoclast-like giant cells harvested by laser microdissection (LM), as well as from control osteosarcoma (NOS-1) cells was analyzed using reverse transcriptional-semiquantitative PCR. *Results:* BMP 2, 3, 4, 5 and 6 were expressed in the GCT tissue. The cultured GCT cells expressed BMP 2, 4, 5 and 6. The osteoclast-like giant cells expressed BMP 2, 3, 5 and 6 and BMP 5 was expressed at the highest level. *Conclusion:* Both stromal cells and osteoclast-like cells in GCT expressed several kinds of BMPs.

A giant cell tumor (GCT) of bone is a distinctive locally aggressive neoplasm of undifferentiated cells. The multinucleated osteoclast-like cells apparently result from the fusion of mononuclear cells. Apart from such multinucleated giant cells, there are also two mononuclear

cell types in GCT. The first has a round morphology similar to monocytes. The second cell type is spindle-shaped, fibroblast-like stromal cells (1-3). Cell culture experiments with GCT cells have revealed stromal cells to be the proliferating component of the GCT. The stromal cells probably stimulate monocyte migration into the tumor tissue and enhance their fusion into the osteoclast-like giant cells (4-9). The giant cell itself resembles a normal osteoclast that is able to resorb bone, thus leading to extended osteolysis. Bone morphogenetic proteins (BMPs) are morphogens capable of inducing new cartilage and bone in ectopic sites. The bone forming cells (osteoblasts and osteocytes) produce BMPs (10). The proteins act as autocrine and/or paracrine factors regulating bone growth and remodeling. Recently BMP expression has also been demonstrated in osteoclasts by immunohistochemical analyses. In addition, the bone morphogenetic activity was observed in GCT tissue (11-22). Recurrence in soft tissue of GCT is a rare complication, and the tendency for these lesions to ossify is unexpected, given the lack of significant bone formation in primary or recurrent intraosseous lesions. A characteristic pattern of peripheral bone formation has been described in GCT involving soft tissue recurrence, and in some pulmonary metastases from benign GCT (1-3). This suggests that, in the extraosseous environment, the cells from GCT are able to simulate osteoblastic differentiation and bone formation. In addition, a bioassay for bone formation activity of lyophilized bone tumors has indicated that human GCT has bone morphogenetic activity, and both immunohistochemical and Western blotting studies have revealed the expression of BMP in GCT (23, 28-30). However, the expression pattern of BMPs in GCT has not yet been fully characterized.

This article describes the relationship between the expression of BMPs and the cell types in GCT.

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Key Words: Giant cell tumor, osteoclast-like giant cell, stromal cell, bone morphogenetic protein, laser microdissection.

Table I. PCR primers.

Transcript	Sense primer	Antisense primer
TRAP	5'-AAGGAGGACTACGTGCTCGTGGCCCGGC-3'	3'-TCCACTCAGCACGTAGCCCACGCCGTT-5'
BMP2	5'-TCCTCTCATCAGCCATTGTCCTTC-3'	3'-AGTTACTACACATTCTCATAG-5'
BMP3	5'-TCAAATGAGTTCTTGCCAGGTTATC-3'	3'-CGCCAGGAGATACTCAAGGTAGA-5'
BMP4	5'-ACCTGAGACGGGAAGAA A-3'	3'-TTA AAGAGGAACGAAAAGCA-5'
BMP5	5'-AAGAGGACAAGAAGGACTAAAAATAT-3'	3'-GTAGAGATCCAGCATAAAGAGAGGT-5'
BMP6	5'-CTGGGTATAAGGCACTGGCATG-3'	3'-GTCGTAATCGCTCTACCCAGTCC-5'
BMP7	5'-AGATAGCCATTTCCTCACCG-3'	3'-TGGAGCACCTGATAAACGCT-5'

TRAP: Tartrate-resistant acid phosphatase.

Materials and Methods

RT-PCR of fresh tumor tissue and cultured cells. Fresh giant cell tumors tissue specimens were obtained from surgical patients who all provided their informed consent. Each tissue specimen was chopped into small pieces and then was placed on dishes containing Roswell Park Memorial Institute tissue culture medium (RPMI) 1640 supplemented with 10% heat-inactive fetal bovine serum, and 300 mg kanamycin sulfate (Wako Tokyo, Japan). These were maintained in a humidified atmosphere of 5% CO₂ in air at 37°C. The fresh frozen giant cell tumor tissue and the spindle-shaped adherent cultured cells after three to five passages (1×10⁶ cells) were harvested for RNA extraction using guanidine isothiocyanate/cesium chloride gradient centrifugation. A human osteosarcoma cell line (NOS 1) which had prominent osteoinductive activity (23) was used as a positive control. The RNA was then reverse transcribed to cDNA using 100 units of Moloney murine leukemia virus reverse transcriptase per reaction with an oligo-dT primer (Promega, Madison, WI USA). The oligonucleotide primers have been described in previous reports (Table I) (23-25). The BMP 2, BMP 5 and BMP 7 primers were designed in house and the specific amplification was confirmed by a direct sequencing analysis using the dideoxy-chain termination method employing an ABI Prism 310 genetic analyzer and Big Dye Terminator cycle sequencing ready reaction kit (Perkin Elmer, Foster City, CA, USA).

Semi-quantitative PCR. The reaction mixture had a total volume of 20 µl containing 2.0 µl 10×PCR buffer (TOYOBO, Osaka Japan), 25 mM MgCl₂, 2 mM deoxynucleotide-triphosphates, 1 µl of each BMP primer and 0.2 µl of β-actin or GAPDH primers. The primer pairs for specific genes and β-actin or glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) were included in the same tube for coamplification. After PCR, the amplified sequences were separated on a 2% agarose gel and visualized by staining with ethidium bromide. The intensity of the bands was measured by NIH Image Ver 1.63 (developed at US National Institutes of Health and available by anonymous FTP at <http://rsb.info.nih.gov/nih-image/download.html>), and the levels of mRNA of BMP 2, BMP 3, BMP 4, BMP 5, BMP 6 and BMP7 relative to the level of GAPDH and β-actin mRNAs were calculated (26).

RT-PCR of giant cells. Frozen sections (5 µm) of fresh giant cell tumor tissues were made and covered mounted on glass slides covered with crosslinked polyethylene (PEN) foil (2.5 µm thick;

Leica Microsystem, Wetzlar, Germany). The sections were fixed with methanol at -20°C for 10 s, and thoroughly air dried. Thereafter, the sections were washed with diethylpyrocarbonate (DEPC)-treated water, stained with 0.05% toluidine blue (TB) solution, (Wako Pure Chemical Industries, Ltd., Osaka, Japan) for 10 s, and then the TB solution was rinsed out with DEPC-treated water, and thereafter the sections were dried. The giant cells were dissected from the frozen sections with a laser microdissection (LMD) system using a 337-nm nitrogen ultraviolet (UV) laser (*Leica* Laser Microdissection System, Leica Microsystems) Figure 1. The dissected giant cells were dropped immediately into a microcentrifuge tube cap filled with 10 µl XB buffer (Picopure RNA Isolation Kit, Arcturus, CA, USA). Over 1,000 giant cells from an individual patient were collected into a 0.5 ml tube, and then the total RNA was extracted using a Picopure RNA Isolation Kit. RT-PCR was performed as described above. Each sample was quantified in triplicate in each of three separate PCR reactions.

Results

The individual tissue samples are described in Table II. The RT-PCR results were expressed as the percentage of mRNA in comparison to two housekeeping genes (β-actin, GAPDH).

The percentages of BMP 2, 3, 4, 5, 6 and 7 mRNA relative to the housekeeping gene transcripts in the control (NOS-1) cells were: BMP 2, 99.6±13.8%; BMP 3, 34.9±4.9%; BMP 4, 47.3±8.9%; BMP 5, 1.8±0.3%; BMP 6, 52.5±2.2% and BMP 7, 56.9±9.9%; Figure 2-A). BMP 2, 3, 4, 5 and 6 were expressed in the GCT tissue (Figure 2-B). A relatively high level of expression of BMP 2, 5 and 6 was detected, and the BMP 5 expression is illustrated in Figure 3A. The cultured cells expressed BMP 2, 4, 5 and 6. A relatively high level expression of BMP 6 was detected. The mean BMP 6 expression in the cultured cells was 33.6% in comparison to the housekeeping gene expression (Figure 2-C). The osteoclast-like multinucleated giant cells expressed BMP 2, 3, 5 and 6. There was a relatively high level of expression of BMP 5 (Figure 3-A) and 6 detected. The mean BMP 5 expression was 62.9% in comparison to the housekeeping genes (Figure 2-D). No BMP 7 expression was detected in the GCT tissue, cultured cells or osteoclast-like giant cells.

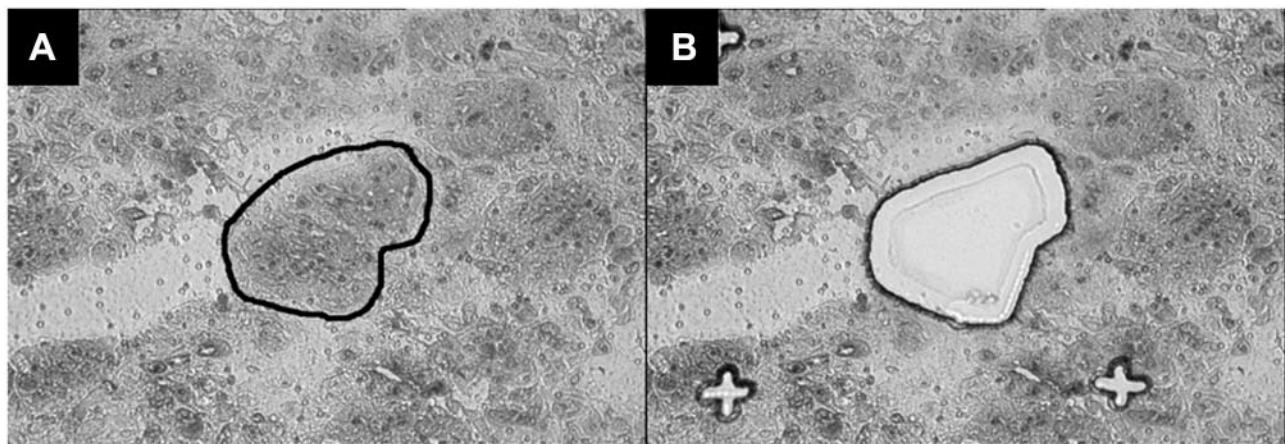


Figure 1. Collection of giant cell using a laser microdissection (LMD) system. A: The giant cell tumor in the frozen section is stained with toluidine blue. B: Laser dissected section.

The expression of tartrate-resistant acid phosphate (TRAP) was highest in the osteoclast-like giant cells (Figures 2-E and 3-B)

Discussion

The considerable level of BMP 2, 5 and 6 expression shown by the human GCT samples in comparison to the NOS-1 cells, indicated that such BMPs may contribute bone morphogenetic activity in human GCT. The relatively high level expression of BMP 2 in the cultured cells and the low level expression of BMP 2 in the osteoclast-like giant cells indicated that BMP 2 is expressed mainly in the stromal cells in GCT. The cultured tumor cells were analyzed after 3 to 5 passages because after three passages, the giant cells and monocytes were eliminated, leaving only stromal cells in the culture. Such stromal cells are thought to be the neoplastic element in GCT, and seem to originate from mesenchymal stem cells. Mesenchymal precursor cells exist in many different areas of the body, and differentiate to form mesenchymal progenitor cells (4-9). The expression of BMP 2 and 6 in the cultured cells from GCT in this study was consistent with previous studies which demonstrated considerable amounts of BMP 2 and 6 in the mesenchymal stem cells (31, 32). BMP 2 and 6 are thought to be the most potent agents for inducing osteoblastic lineage-specific differentiation in mesenchymal progenitor cells (10).

The relatively high level expression of BMP 5 in the LMD osteoclast-like giant cells and low level in the cultured cells indicated that BMP 5 is expressed mainly in the giant cells in GCT. A few reports regarding the expression of BMP 5, have indicated that it might play a fully paracrine role in rodent ovarian folliculogenesis, thereby regulating chondrocyte proliferation and differentiation (32-35). Cheng *et al.* showed that BMP 5 exhibited little osteogenic activity in

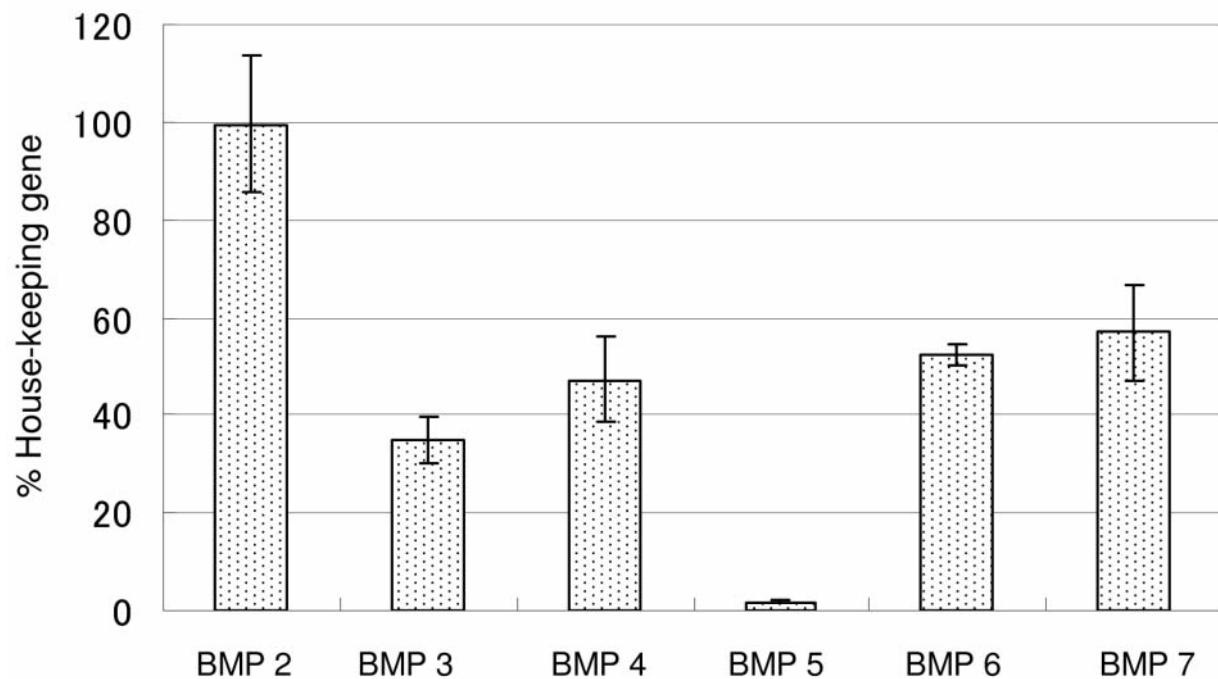
Table II. Characteristics of the RNA samples.

Case no.	Gender	age (years)	Location	RNA		
				Tissue	Cell	LMD
1	M	34	Lung	Metastasis	+1	-
2	M	39	Sacrum		+2	-
3	F	35	Radius		+3	+3*
4	F	46	Elbow	Soft tissue	+4	+4*
5	M	28	Fibula		+5	+5*
6	M	24	Fibula	Recurrence	+6	+6*
7	M	54	Lumbar spine	L1	+7	+7*
8	F	55	Femur		+8	+8*
9	F	31	Tibia		-	+9
10	M	36	Femur		-	-
						+10

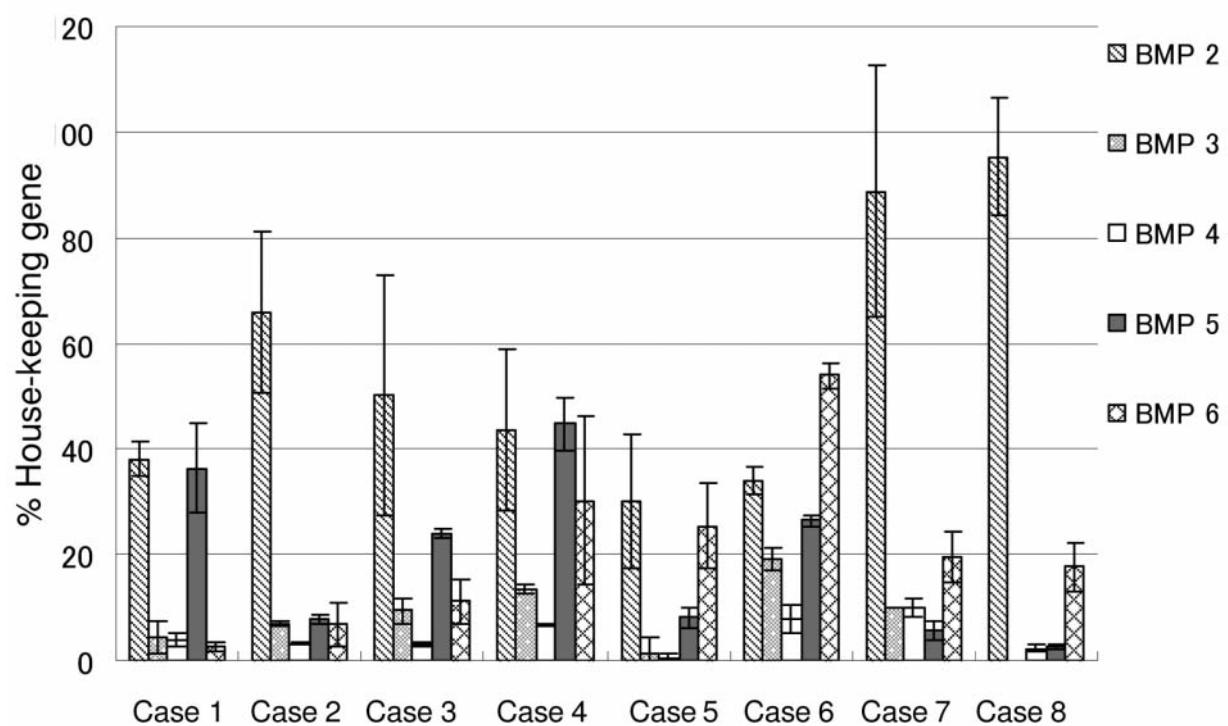
+: mRNA sample analysed, -: not analysed from GCT tissue, *cultured GCT cells, **LMD (laser microdissected) giant cells.

osteoblastic progenitor cell lines, but it did stimulate alkaline phosphatase in relatively mature osteoblasts (10).

The osteoclast-like giant cells also showed considerable expression of BMP 6. Various immunohistochemical studies of BMP expression in osteoclasts have reported that BMP 2, 4 and 7 were expressed in osteoclasts in a rat fracture model (19). BMP 7 was expressed in hamster osteoclasts (14), BMP 2, 3, 4, 5, 6 and 7 were expressed in the rat and human fetal growth plates (12). BMP 2, 4, 6 and 7 were expressed in mouse osteoclasts (18), and BMP 3 and 6 were expressed in human osteophytes, but BMP 2, 5 and 7 were not expressed (22). Because osteoclasts are potentially phagocytotic, it is possible that the BMPs present in the osteoclast cytoplasm may simply be the result of the phagocytosis of BMP-containing bone matrix. To date, there have been few reports of mRNA expression in osteoclasts.



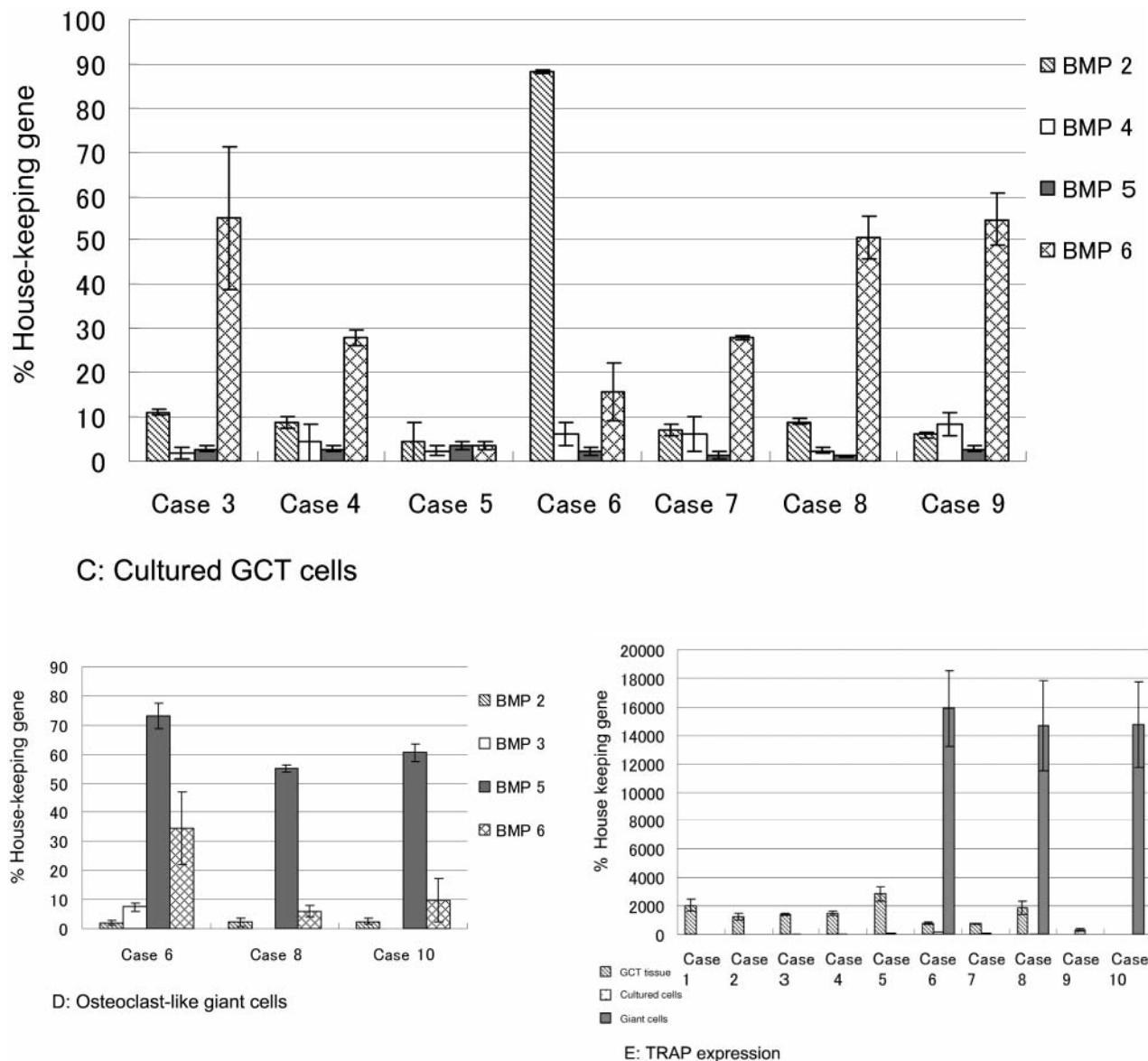
A: Osteosarcoma cell line (NOS-1)



B: GCT tissue

Figure 2. *continued*

Figure 2. continued

Figure 2. BMP expression in osteosarcoma (*NOS-1*) cells (A), GCT tissue (B), cultured GCT cells (C), and osteoclast-like giant cells; (D) TRAP expression in GCT tissue, cultured cells and giant cells (E).

TRAP is known as a specific marker for osteoclast (37, 38). The present study confirmed the overexpression of TRAP in the LMD osteoclast-like giant cells, which demonstrated the reliability of the LMD technique and the osteoclastic nature of the osteoclast-like giant cells in GCT, in line with the almost complete osteoclastic phenotype previously demonstrated in GCT giant cells (3-7, 36). Therefore, osteoclasts themselves probably produce various types of BMPs, including BMP 2, 3, 5 and 6.

There is ample evidence that bone formation is coupled to bone resorption (coupling phenomenon). The stimulation of bone resorption by agents such as prostaglandin E and parathyroid hormone is associated with increased bone formation. The mechanism whereby bone resorption facilitates bone formation is unknown. A local coupling factor linking bone resorption to subsequent bone formation may be the key regulator of the remodeling process. The bone matrix is a source of growth factors including BMPs, transforming growth

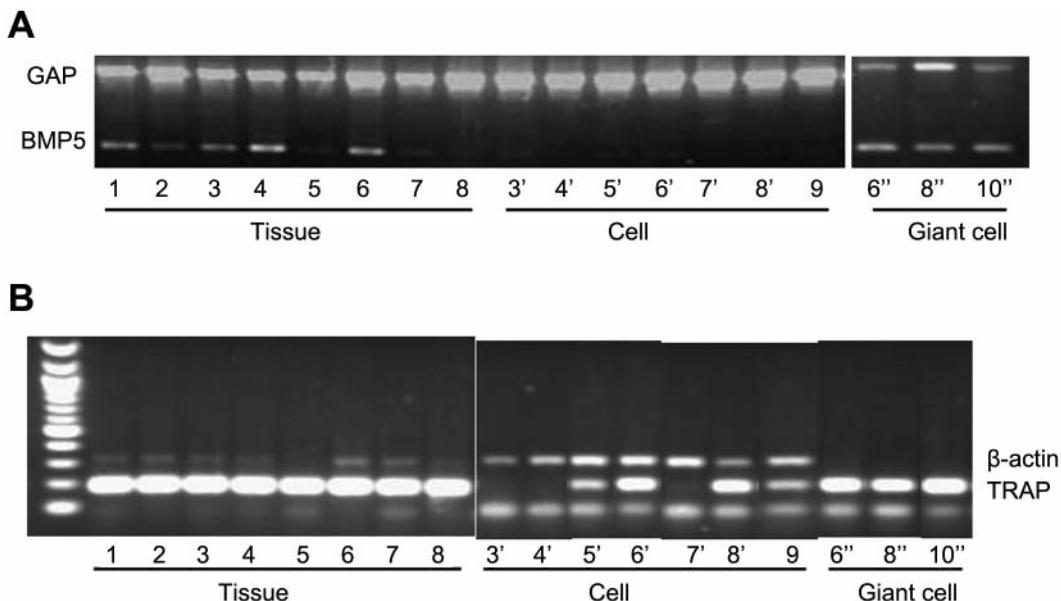


Figure 3. RT-PCR of GCT tissue, GCT cultured cells and LMD giant cells. A: BMP 5 in comparison to GAPDH. B: TRAP in comparison to β -actin. The numbers refer to the samples in Table II.

factors, insulin-like growth factors and fibroblast growth factors and it has been suggested that growth factors are released from the matrix during bone resorption by osteoclasts (37-39). In addition, a recent study indicated that osteoclast themselves could be the source of activity that contributes to the fine control that is a feature of the coupling phenomenon (38). A high level expression of BMP 5 and 6 in osteoclast-like giant cells in GCT may also support this hypothesis.

In conclusion, GCT tissue expresses BMP 2, 3, 4, 5 and 6 and cultured stromal cells express a high level of BMP 2 and 6. Purified LMD osteoclast-like giant cells also expressed BMP 5 and 6.

Acknowledgements

We gratefully acknowledge Y Tanaka, K Tanaka, and H Akazawa for their valuable help and technical assistance.

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*Received August 6, 2008**Revised December 6, 2008**Accepted February 13, 2009*