PTH-RELATED PROTEIN IN GIANT CELL TUMOUR OF BONE

PARATHYROID HORMONE-RELATED PROTEIN IN GIANT CELL TUMOUR OF BONE

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Abstract

Giant cell tumour of bone (GCT) is an aggressive primary bone tumour with an unclear etiology that presents with significant local osteolysis due in part to the accumulation of multinucleated osteoclast-like giant cells. However, it is the neoplastic spindle-like stromal cells within GCT that largely direct the pathogenesis of the tumour. I hypothesize that parathyroid hormone-related protein (PTHrP) is a key mediator within GCT that promotes the characteristic osteolytic phenotype by stimulating both bone resorption and giant cell formation. The work presented in this thesis collectively demonstrates that the stromal cells express PTHrP and its receptor, the parathyroid hormone type 1 receptor (PTH1R), and that PTHrP acts in an autocrine/paracrine manner within the tumour to stimulate expression of factors that promote bone resorption. Data are presented that demonstrate that PTHrP stimulates stromal cell expression of the receptor activator of nuclear factor-κB ligand (RANKL), a known essential regulator of osteoclastogenesis, which results in increased formation of multinucleated cells from murine monocytes. Moreover, the GCT stromal cells express matrix metalloproteinase (MMP)-1 and MMP-13. These results suggest that the stromal cells may participate directly in bone resorption through the degradation of type I collagen, the promotion of osteoclast activity, or through a combination of these elements. PTHrP also regulates the expression of MMP-13 by the stromal cells. Experiments with CD40 ligand show that local factors present within the tumour can influence PTHrP expression by the stromal cells and potentiate its catabolic effects by stimulation of RANKL and MMP-13 expression. Together, this thesis presents evidence that suggests PTHrP is an important

factor in the pathophysiology of GCT by its actions on promoting catabolism within the tumour. The role of PTHrP in normal physiology and the mechanisms of action presented here suggest that research into the effects of PTHrP within GCT may provide invaluable information that enhances our understanding of the biology of this particularly aggressive bone tumour.

Foreword

This thesis is presented as a "sandwich" thesis, composed of three manuscripts that were prepared for publication during the author's Ph.D. candidacy. Two manuscripts have been published (Chapters 2 and 4) and one manuscript is in press (Chapter 3) at the time this thesis was prepared. The manuscripts are presented in separate chapters that include prefaces detailing each author's contributions as well as a description of the overall context wherein the manuscript was prepared. In addition, an introductory chapter that discusses previous research into giant cell tumour of bone (GCT) and other related topics and outlines the overall basis for the thesis is included prior to the manuscripts. A concluding chapter summarizes the thesis and discusses the contributions to the GCT field of study and suggests directions for future research.

References cited within each manuscript are entirely independent and are consistent with the style of each respective journal. Other citations used throughout the thesis appear in the separate References section. Certain differences in spelling between chapters are attributed to journal requirements that use American spelling. Appendices are also included, which describe common materials and methods used during this work, and clarifies the identity of each patient sample used in the preparation of the manuscripts, as each manuscript employed an independent labelling system. Additionally, licence agreements with Elsevier and John Wiley & Sons, Inc. are also included in the appendices.

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This thesis could not have been completed without the support of my family; they provided constant emotional support and understanding — not just during my Ph.D., but

indeed throughout my entire life. Thanks for helping me to pursue my education, for always being there whenever I need you, and for making me the person that I am today.

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List of Abbreviations

α-MEM	α-minimum essential medium
AP-1	activator protein-1
APMA	4-aminophenylmercuric acetate
C/EBPβ	CCAAT/enhancer binding protein β
CaSR	calcium-sensing receptor
Cbfa1	core binding factor alpha 1
CD40L	CD40 ligand
cDNA	complimentary DNA
Ct	cycle threshold
DC-STAMP	dendritic cell-specific transmembrane protein
DEPC	diethylpyrocarbonate
D-MEM	Dulbecco's modified Eagle medium
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GCT	giant cell tumour of bone
hFOB	human fetal osteoblast
HIF	hypoxia-inducible factor
HOS	human osteosarcoma
IgG	immunoglobulin G
IL	interleukin
MCP-1	monocyte chemotactic protein-1
M-CSF	macrophage colony-stimulating factor

MMP	matrix metalloproteinase
mRNA	messenger RNA
NF-κB	nuclear factor κB
OPG	osteoprotegerin
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEA3	polyoma enhancer element A3
PTH	parathyroid hormone
PTH1R	PTH type 1 receptor
PTH2R	PTH type 2 receptor
PTHrP	PTH-related protein
RANK	receptor activator of NF-κB
RANKL	RANK ligand
RNA	ribonucleic acid
Runx2	runt-related transcription factor 2
SDF-1	stromal cell-derived factor-1
SDS-PAGE	sodium dodecyl-sulphate polyacrylamide gel electrophoresis
siRNA	small interfering RNA
TBS-T	Tris-buffered saline with Tween 20
TGF	transforming growth factor
TIMP	tissue inhibitor of metalloproteinases
TIP39	tuberoinfundibular peptide of 39 residues
TNF	tumour necrosis factor
TRAP	tartrate-resistant acid phosphatase

Declaration of Academic Achievement

This thesis is presented as a culmination of three manuscripts – two published papers and a manuscript in press, as follows:

Robert W. Cowan, Isabella W.Y. Mak, Nigel Colterjohn, Gurmit Singh, and Michelle Ghert. 2009. Collagenase expression and activity in the stromal cells from giant cell tumour of bone. *Bone* 44(5): 865-871.

Robert W. Cowan, Gurmit Singh, and Michelle Ghert. 2011. PTHrP increases RANKL expression by stromal cells from giant cell tumor of bone. *J Orthop Res* (In press) doi: 10.1002/jor.22020.

Robert W. Cowan, Michelle Ghert, and Gurmit Singh. 2012. T cells stimulate catabolic gene expression by the stromal cells from giant cell tumor of bone. *Biochem Biophys Res Commun* 419(4): 719-723.

In addition, the author contributed to the following publications:

Michelle Ghert, Nicole Simunovic, **Robert W. Cowan**, Nigel Colterjohn, and Gurmit Singh. 2007. Properties of the stromal cell in giant cell tumor of bone. *Clin Orthop Relat Res* 459: 8-13.

Isabella W.Y. Mak, **Robert W. Cowan**, Snezana Popovic, Nigel Colterjohn, Gurmit Singh, and Michelle Ghert. 2009. Upregulation of MMP-13 via Runx2 in the stromal cell of giant cell tumor of bone. *Bone* 45(2): 377-386.

Alexander Rabinovich, Isabella W.Y. Mak, **Robert W. Cowan**, Robert E. Turcotte, Nigel Colterjohn, Gurmit Singh, and Michelle Ghert. 2009. Matrix metalloproteinase activity in the stromal cell of giant cell tumor of bone. *Open Bone J* 1: 46-52.

Isabella W.Y. Mak, Eric P. Seidlitz, **Robert W. Cowan**, Robert E. Turcotte, Snezana Popovic, William C. Wu, Gurmit Singh, and Michelle Ghert. 2010. Evidence for the role of matrix metalloproteinase-13 in bone resorption by giant cell tumor of bone. *Hum Pathol* 41(9): 1320-1329.

Shalini Singh, Isabella W.Y. Mak, **Robert W. Cowan**, Robert Turcotte, Gurmit Singh, and Michelle Ghert. 2011. The role of TWIST as a regulator in giant cell tumor of bone. *J Cell Biochem* 112(9): 2287-2295.

Isabella W.Y. Mak, **Robert W. Cowan**, Robert E. Turcotte, Gurmit Singh, and Michelle Ghert. 2011. PTHrP induces autocrine/paracrine proliferation of bone tumor cells through inhibition of apoptosis. *PLoS One* 6(5): e19975.

CHAPTER 1

Introduction

Bone physiology

Bone composition

The human skeleton is an intricate arrangement of bones that provides protection and support for vital organs. Bones are complex tissues that serve many functions necessary for human life, and although a significant portion is comprised of inorganic material, bone is actually a dynamic tissue that is continuously undergoing remodelling. The inorganic material consists largely of hydroxyapatite, which are calcium phosphate crystals mostly found on, or within, collagen fibres (as reviewed by Hadjidakis & Androulakis, 2006). Collagen fibres, the major organic component of bone (Dequeker & Merlevede, 1971; Dickerson, 1962; Rogers et al., 1952), largely comprise the bone matrix, and are arranged in highly organized layers that, together with hydroxyapatite, produce a rigid structure that resists compression (as reviewed by Alberts et al., 2002).

Two major types of bone, identical in their chemical composition but differing in their features, are found in the human skeleton: cortical and trabecular. Cortical, or compact bone, is very dense and rigid, and has a slow turnover rate, whereas trabecular bone (also known as cancellous or spongy bone) is more pliable, less dense, and undergoes a higher frequency of remodelling (as reviewed by Hadjidakis & Androulakis, 2006). The distribution of trabecular bone varies within the skeleton, and is mostly found inside the vertebrae, flat bones, and long bones, underneath the calcified cortical bone, which comprises the outer layer (as reviewed by Hadjidakis & Androulakis, 2006). The long bones (bones that are longer than they are wide) include such bones as the metacarpals

and metatarsals of the hands and feet, the femurs and tibias of the legs, and the humeri and radii of the arms.

In addition to the mineral crystals and organic matrix, numerous cells are found within bones. These cells include the bone-forming osteoblasts and the large bone-resorbing osteoclasts. Together, these tightly regulated cells are largely responsible for maintaining and remodelling bone.

Bone remodelling

Osteoblasts are fibroblast-like cells whose function is the creation and maintenance of skeletal tissues. They are responsible for the production of many of the non-collagenous proteins found in the bone matrix, including osteocalcin, osteopontin, osteonectin, and bone sialoprotein (as reviewed by Roach, 1994). Osteoblasts deposit these proteins, together with type I collagen, in what is known as the osteoid, which eventually becomes hard bone matrix after inclusion of hydroxyapatite (as reviewed by Alberts et al., 2002). For this reason, osteoblasts are considered the bone-forming cells. Osteoblasts are derived from multipotent mesenchymal stem cells, and their differentiation is controlled through several stages by growth and transcription factors (as reviewed by Hughes et al., 2006). The runt-related transcription factor 2 (Runx2), originally known as core binding factor alpha 1 (Cbfa1), is a critical element in osteoblast differentiation, as deletion of its gene in mice results in a complete lack of bone formation (Komori et al., 1997). Other important mediators of osteoblast differentiation include osterix and β-catenin (as reviewed by Komori, 2006).

Osteoblasts begin depositing new bone matrix over existing cartilage matrix, which is secreted by chondrocytes (as reviewed by Pazzaglia et al., 2011). Cartilage is relatively simpler than bone, consisting of a flexible uniform matrix that contains the chondrocytes, which become embedded within the matrix. Once trapped within the calcified bone matrix, osteoblasts are unable to divide and subsequently become osteocytes. Osteocytes and chondrocytes occupy small cavities known as lacunae, but unlike chondrocytes, osteocytes are able to maintain communication with other cells through tiny channels known as canaliculi, into which appendage-like portions of the osteocytes project themselves and form gap junctions with other adjacent osteocytes (as reviewed by Burger & Klein-Nulend, 1999). Osteocytes facilitate reactions to mechanical stimuli (Tatsumi et al., 2007), where aspects of the external environment such as gravity, compression, and tension are converted into biochemical responses that help maintain bone homeostasis (as reviewed by Temiyasathit & Jacobs, 2010).

Another important function of osteoblasts is the regulation of osteoclast differentiation (see Figure 1). Osteoclasts, in contrast to osteoblasts, are bone-resorbing cells, and are derived from hematopoietic stem cells (Kurihara et al., 1989; Marks Jr. & Walker, 1981). Osteoblasts regulate osteoclastogenesis by producing several factors, including the macrophage colony-stimulating factor (M-CSF) and the receptor activator of nuclear factor-κB ligand (RANKL). RANKL is a membrane-bound protein, although it can be solubilized through proteolytic cleavage (Hikita et al., 2006; Lynch et al., 2005) or alternative splicing (Ikeda et al., 2001), whereupon it maintains biologic activity (Mizuno et al., 2002). It binds to its receptor, the receptor activator of nuclear factor-κB (RANK),

on monocyte cells to initiate osteoclastogenesis through the nuclear factor-κB pathway (Lacey et al., 1998; Nakagawa et al., 1998; Xing et al., 2002; Yasuda et al., 1998). This process results in the pre-osteoclast monocytes fusing together to form the large, multinucleated osteoclasts, and requires such factors as the recently-discovered dendritic cell-specific transmembrane protein (DC-STAMP) (Yagi et al., 2005). Osteoblasts also produce a soluble decoy receptor for RANKL, osteoprotegerin (OPG), which inhibits osteoclastogenesis (Simonet et al., 1997; Yasuda et al., 1998).

Osteoclasts degrade bone by forming a ruffled membrane in the area facing the bone matrix, creating an isolated environment into which protons supplied by several enzymes, including carbonic anhydrase II, are secreted *via* a vacuolar H^+-ATPase proton pump (as reviewed by Roodman, 2001). Several enzymes are secreted into this acidic environment, including tartrate-resistant acid phosphatase, cathepsin K, and other proteases, which results in the digestion of the collagen fibres and demineralization of hydroxyapatite (as reviewed by Hadjidakis & Androulakis, 2006). Osteoclast activity is regulated by numerous cytokines, growth factors, and hormones, such as calcitonin, which recognizes its receptor on osteoclasts to potently inhibit bone resorption (as reviewed by de Paula & Rosen, 2010). In addition, the vitronectin receptor may be important in detection of bone and initiation of resorption (as reviewed by Chambers & Fuller, 2011).

During bone remodelling, osteoblasts follow osteoclast-mediated bone resorption by migrating to the resorbed area and depositing new bone matrix (as reviewed by Hadjidakis & Androulakis, 2006). The balance between osteoblast and osteoclast activity must therefore be tightly controlled, and irregularities in this balance leads to deformities

and diseases that can compromise the skeleton, such as osteoporosis or osteopetrosis (as reviewed by Feng & McDonald, 2011). Another condition that results in increased bone resorption, giant cell tumour of bone, is the subject of this thesis.

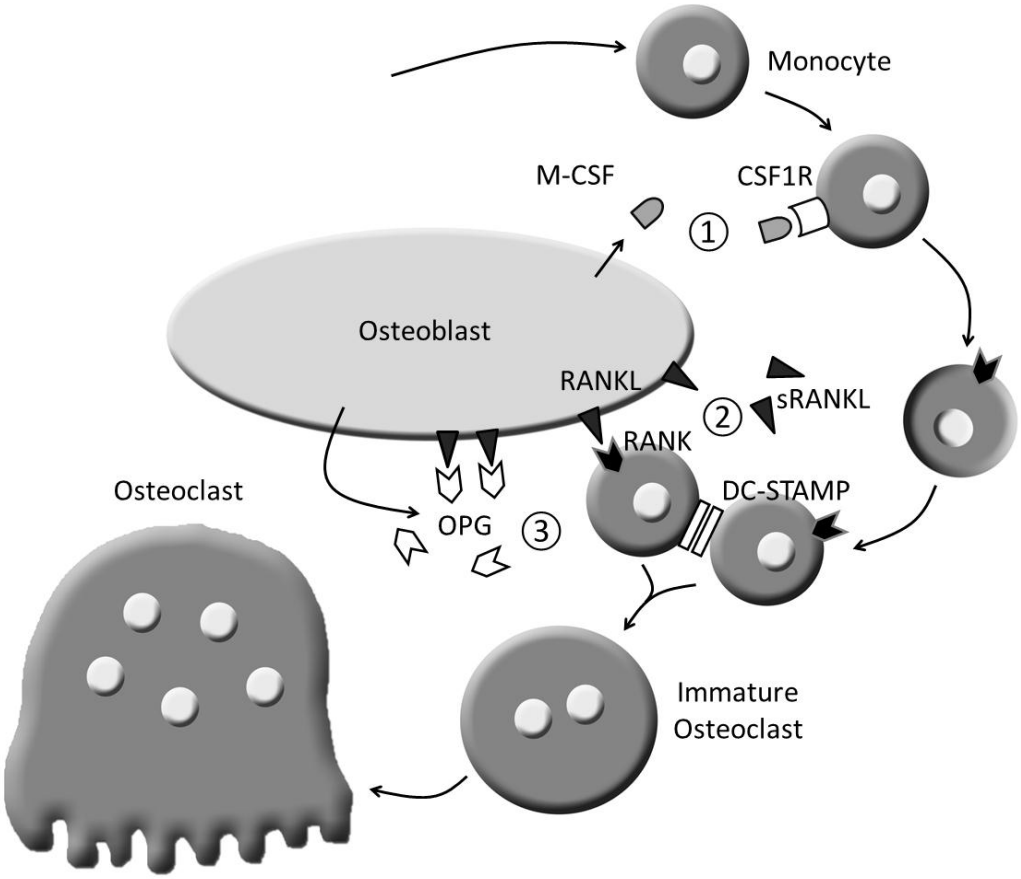

Figure 1 – Diagrammatic representation of osteoclastogenesis. (1) Osteoblasts secrete M-CSF, which stimulates the colony stimulating factor 1 receptor (CSF1R) on monocytes to begin osteoclast differentiation. (2) Osteoblasts produce membrane-bound RANKL, which can be solubilized (sRANKL), and stimulates its receptor, RANK, on monocytes to stimulate their fusion into immature osteoclasts through a process requiring DC-STAMP. (3) Osteoblasts also secrete a soluble decoy receptor for RANKL: OPG.

Giant cell tumour of bone

Clinical presentation and overview

Constituting approximately six percent of all primary bone tumours (Gupta et al., 2008; Unni & Inwards, 2010b), giant cell tumour of bone (GCT) is a prominent lesion with an unclear etiology. Its name derives from the numerous bone-resorbing multinucleated giant cells present within the tumour, which share many features with true osteoclasts, including expression of tartrate-resistant acid phosphatase (Anazawa et al., 2006; Mii et al., 1991), cathepsin K (Drake et al., 1996; Lindeman et al., 2004), and carbonic anhydrase II (Zheng et al., 1993). Giant cells also express many receptors characteristic of osteoclasts, including RANK (Atkins et al., 2006), the calcitonin receptor (Collier et al., 1998), and the vitronectin receptor (Lau et al., 2005; Ohsaki et al., 1992). Moreover, some giant cells show numerous infoldings under electron microscopy that resemble the ruffled membrane of true osteoclasts (Kanehisa et al., 1991; Steiner et al., 1972). Indeed, GCTs were formerly known as "osteoclastomas," (as reviewed by Goldring et al., 1986) and giant cells isolated from these tumours are often used as models for true osteoclasts (Drake et al., 1996; Grano et al., 2000; Knowles & Athanasou, 2009; Zheng et al., 1993). However, the neoplastic components of the tumour are actually the spindle-like stromal cells, which share many properties with immature osteoblasts and can be propagated in culture for experimental analyses (Goldring et al., 1987; Wülling et al., 2003).

GCTs occur predominantly in the long bones of the appendicular skeleton, particularly at the epiphyses (Unni & Inwards, 2010b), but can occur in any other areas of the

skeleton, including the ribs (Kumar et al., 2007) and the skull (Isaacson et al., 2009), as well as in multiple locations simultaneously (Hoch et al., 2006). While small areas of osteoid formation are observed (Bardi et al., 1991), the tumour preferentially results in overall bone loss. GCT often presents with localized pain and swelling, and radiograph imaging of the tumour appears as a solitary osteolytic lesion of varying severity (as reviewed by Gruenwald et al., 2006; Unni & Inwards, 2010b), as demonstrated in Figure 2. Although GCTs are locally aggressive, they are most often benign, and only rarely metastasize to the lungs with a reported incidence rate ranging between two to six percent of cases (Tubbs et al., 1992; Unni & Inwards, 2010b; Viswanathan & Jambhekar, 2010). Pulmonary metastasis from GCT can result in nodule formation, respiratory distress, and even death (Jacopin et al., 2010; Osaka et al., 2004; Tubbs et al., 1992; Unni & Inwards, 2010b). Such cases are often treated with surgical intervention to remove pulmonary nodules (Tubbs et al., 1992). Isolated reports have also detailed cases where GCT has metastasized to other locations, including the breast (Alacacioglu et al., 2006), skin (Tyler et al., 2002), and lymph nodes (Connell et al., 1998). Malignant transformation can also occur in GCT, often, but not exclusively, following radiation treatment, which results in the development of a sarcoma (Unni & Inwards, 2010c).

Clinically, GCTs are typically graded according to the Campanacci method (Campanacci et al., 1987) or the Enneking method (Enneking, 1986), which classify tumours based on radiographic appearance of tumour margins and biological aggressiveness, respectively. However, attempts to correlate *in vitro* data with clinical staging often prove unsuccessful (Gamberi et al., 2004; Si et al., 2003). There is a slight

female predominance in GCT presentation (Balke et al., 2008; Campanacci et al., 1987; Unni & Inwards, 2010b), and the tumour arises most frequently (approximately 85% of cases) in adults between the ages of 20 and 45 (Unni & Inwards, 2010b). Nevertheless, GCT has been reported in both younger (Hoeffel et al., 1996; Puri et al., 2007) and older (García et al., 2006; McCarthy & Weber, 2009) patients. Notably, the South East Asian population has a higher incidence of GCTs than Western populations (Saikia et al., 2011; Sung et al., 1982), and therefore a large quantity of research devoted to GCT arises from that region.

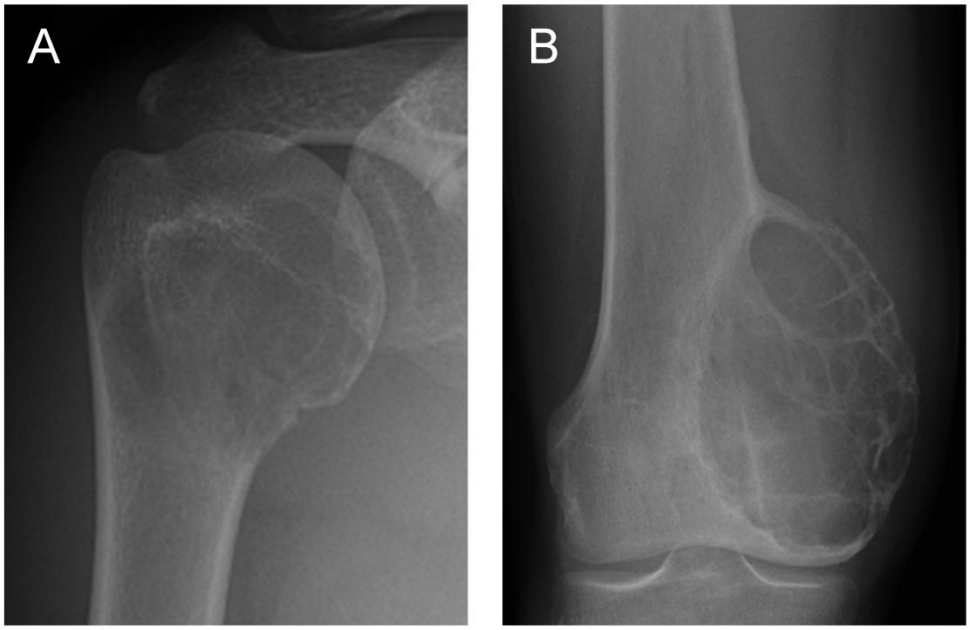

Figure 2 – X-ray images from two patients presenting with GCT. Images show GCTs in the (A) proximal humerus and (B) distal femur.

Treatment

Treatment of GCT usually involves surgical removal of the tumour, accomplished by either intralesional excision or *en bloc* resection. Intralesional excision by curettage is frequently preferred over wide resection, as it spares much of the bone anatomy and often preserves function of any adjacent joints (as reviewed by Errani et al., 2010). However, local recurrence rates following curettage can be high, and are reported to range from approximately 10% to 20% in many recent patient series (Errani et al., 2010; Prosser et al., 2005; Turcotte et al., 2002), or nearly 50% of cases in other series (Becker et al., 2008; Masui et al., 1998). Although wide resection reduces the risk of tumour recurrence compared to curettage (Becker et al., 2008; Chanchairujira et al., 2011; Klenke et al., 2011; Prosser et al., 2005), it is associated with poorer functional outcomes (Errani et al., 2010; Gitelis et al., 1993). Indeed, *en bloc* resection is often reserved for particularly aggressive GCTs or in cases where preservation of joint function is not an issue (as reviewed by Errani et al., 2010). To reduce the risk of local recurrence following curettage, high-speed burring and adjuvant treatments with cytotoxic or other physical agents are often employed to kill residual tumour cells. These agents can include phenol (Dürr et al., 1999; Quint et al., 1998), ethanol (Jones et al., 2006; Lin et al., 2011), hydrogen peroxide (Balke et al., 2008), liquid nitrogen (Malawer et al., 1999), or zinc chloride (Zhen et al., 2004). Such agents were shown to induce a cytotoxic effect on cultured GCT cells (Gortzak et al., 2010). However, the benefits of adjuvant therapies with curettage are controversial in patients, as some reports (Becker et al., 2008; Dürr et al., 1999) suggest reduced rates of recurrence in patients treated with adjuvants compared

to those treated with curettage alone, whereas other reports show no advantages to adjuvant therapies (Klenke et al., 2011; Ruggieri et al., 2010). Polymethylmethacrylate bone cement, which is often applied to fill the cavity produced after removal of the tumour and provide structural support, may also reduce the risk of local recurrence, as reported by several large patient series (Becker et al., 2008; Gaston et al., 2011; Kivioja et al., 2008). However, other reports suggest there is no clinical benefit from the use of cement (Prosser et al., 2005; Turcotte et al., 2002), and the cement itself may have deleterious effects on the patient (as reviewed by Food and Drug Administration, 2002; Gaston et al., 2011). Ultimately, reduced rates of local recurrence may be achievable through experience with treatment of the tumour and improved surgical techniques, as relatively lower rates of local recurrence are reported from single institutions (Blackley et al., 1999; Ghert et al., 2002; Prosser et al., 2005). Nevertheless, the current limitations of GCT surgery necessitate a search for effective alternative treatment options.

Despite the risk of malignant transformation (Unni & Inwards, 2010c), radiotherapy is sometimes performed in cases where surgery is impractical or not desirable. In such instances, GCTs appear moderately radiosensitive when administered total tumour doses of approximately 50 Gy (Caudell et al., 2003; Chakravarti et al., 1999; Ruka et al., 2010). Modern megavoltage units may also reduce the risk of malignant transformation, as compared to radiation administered with older orthovoltage units (Chakravarti et al., 1999; Ruka et al., 2010). However, responses to radiation are slow (Suit & Spiro, 1999), and tumour progression rates, particularly in the axial skeleton, may increase over time (Ruka et al., 2010).

The nature of GCTs, therefore, necessitates a better understanding of its cellular interactions to further improve treatment options. Recent therapeutic strategies target the osteoclast-like giant cells, and the use of bisphosphonates in treating GCT is under investigation. Nitrogen-containing bisphosphonates, such as pamidronate and zoledronic acid, are a class of pharmaceuticals that inhibit osteoclast activity through mechanisms that prevents bone resorption (as reviewed by Kimmel, 2007), induces apoptosis in osteoclasts (Hughes et al., 1995), and also impedes osteoclastogenesis (as reviewed by Rogers et al., 2000). Several *in vitro* studies indicate bisphosphonates induce apoptosis of GCT cells (Chang et al., 2004), not only in the giant cells (Cheng et al., 2003), but in the stromal cells as well (Cheng et al., 2004; Lau et al., 2011). Moreover, zoledronic acid also inhibits giant cell-mediated bone resorption of dentine slices (Balke et al., 2010), and retains biologic activity when mixed with bone cement where it demonstrates a cytotoxic effect on cultured GCT stromal cells (Zwolak et al., 2010). In patients, bisphosphonates were employed as an adjuvant to surgical intervention, and significantly reduced local recurrence rates of the tumour compared to a control group (reducing recurrence in patients from 30% to 4.2%) (Tse et al., 2008). However, histological evidence of an effect on tumours following bisphosphonate treatment did not reveal any differences compared to controls (Tse et al., 2008). In contrast, a recent report showed that locally administered zoledronic acid induced tumour cell death, and this was confirmed by histologic examination in a patient whose tumour was removed by curettage (Nishisho et al., 2011). Bisphosphonate treatment also stabilized tumour size in patients with recurrent, metastatic, or inoperable GCTs (Balke et al., 2010). These studies suggest that

bisphosphonates may be an effective therapy or adjuvant for GCT, although the long-term risks of bisphosphonate use can include both skeletal and non-skeletal complications (as reviewed by Lewiecki, 2011).

Additionally, a human monoclonal antibody (denosumab) that specifically inhibits RANKL (Bekker et al., 2004) is currently undergoing testing for use in GCT patients (Thomas et al., 2010). In this small open-label phase 2 trial, subcutaneous denosumab injections were well-tolerated, and resulted in near complete elimination of giant cells compared to baseline (≥ 90% reduction) in all twenty patients examined by histology (Thomas et al., 2010). However, in a separate incident, one patient receiving subcutaneous denosumab treatment for a sacral GCT developed osteonecrosis of the jaw (Aghaloo et al., 2010), and the long-term and widespread safety of denosumab treatment must consequently be clearly defined. Further understanding of the etiology and pathophysiology of the tumour may lead to other adjuvants and improved treatment options for patients.

Genetic analyses and cellular interactions

In search of a genetic cause of GCT, numerous karyotyping and other genetic analyses of tumour samples were conducted. Although non-clonal chromosomal aberrations are common within GCT tissues (Bardi et al., 1991; Bridge et al., 1990; Gorunova et al., 2009; Schwartz et al., 1989), few clonal irregularities were discovered. However, clonal alterations may be more prevalent in recurrent GCT as opposed to non-recurrent cases (Moskovszky et al., 2009). Of the non-clonal irregularities, which include insertions, deletions, translocations and other structural or numerical chromosomal rearrangements,

the most prevalent cytogenetic finding in GCTs is telomeric associations. These associations, where two different chromosome arms have fused together at their telomeric ends (resulting in a dicentric chromosome), occur in more than seventy percent of cases (Gorunova et al., 2009), and are also found in isolated cultured stromal cells (Zheng et al., 1999). Several chromosome arms were reported to involve telomeric fusions more often than others, including 11p, 15p, 19q and 20q (Bardi et al., 1991; Bridge et al., 1990; Gorunova et al., 2009; Schwartz et al., 1989). In addition, it was found that telomerase is heterogeneously activated in these tumours (Forsyth et al., 2008), further suggesting that telomeres may participate in the development of GCTs. Indeed, telomere instability may also lead to further loss of genomic integrity (as reviewed by Rodier et al., 2005). However, these cytogenetic abnormalities do not correlate with clinical grading systems (Bridge et al., 1990), and suggest a uniform genetic cause of GCT is unlikely. In actuality, these instabilities may be a symptom of GCT rather than a cause, or more likely, indicate that a variety of genetic aberrations may lead to a general tumour growth with a consistent clinical outcome.

Aside from any possible genetic origin to the tumour, numerous other theories pertaining to the etiology of GCT have been proposed. These include hypotheses that GCT is related to bone injury, as some tumours were reported to arise in areas previously associated with trauma (De Nayer et al., 1987; Langer et al., 1982). Estrogen involvement in GCT was also considered as a cause, although reports of estrogen receptor expression within the tumour have revealed inconsistent findings (Collier et al., 1998; Ishibe et al., 1994; Oursler et al., 1994). Moreover, a more recent report analyzing a

larger sample size of eighty-eight tumours suggests estrogen receptors are detectable by immunohistochemistry in only half of GCTs (Olivera et al., 2002). Therefore, the importance of estrogen in GCTs must still be clarified, although treatment of cells with 17 β-estradiol may stimulate proliferation of the stromal cells (Olivera et al., 2002), and inhibit bone resorption by the giant cells (Olivera et al., 2002; Oursler et al., 1994).

Ultimately, a cause for GCT is likely to reveal itself only through an improved understanding of the pathophysiology of the tumour, and a majority of the basic science investigations of GCT therefore pertain to intercellular interactions. Histologically, GCT is characterized by numerous multinucleated giant cells that are uniformly distributed amongst the spindle-like stromal cells (Figure 3), which are the neoplastic component within the tumour (Goldring et al., 1987; Wülling et al., 2003), as previously mentioned. The stromal cells are the only cells within GCT to express the Ki-67 nuclear proliferation marker (Sulh et al., 1996). These cells show positive expression of bone sialoprotein, osteonectin, osteopontin, osterix, and Runx2 (Ghert et al., 2007; Hasegawa et al., 1993; Huang et al., 2004b; Murata et al., 2005; Robinson et al., 2002; Salerno et al., 2008; Wülling et al., 2003), but only occasional expression of osteocalcin (Ghert et al., 2007; Hasegawa et al., 1993; Robinson et al., 2002), which is often not detectable at the protein level (Huang et al., 2004b; Murata et al., 2005; Wülling et al., 2003), suggesting a mesenchymal lineage and pre-osteoblast phenotype. However, there are conflicting reports as to whether these stromal cells can further differentiate into mature osteoblasts upon stimulation, with one study showing retinoic acid could induce differentiation (Robinson et al., 2002), and another showing specialised media could not (Salerno et al.,

2008). Moreover, another study reports the ability of GCT stromal cells to not only differentiate into osteoblasts, but also into adipocytes and chondrocytes, as determined by the expression of cell-specific products (Wülling et al., 2003). These results may indicate that the stromal cells are populated by cells at multiple stages of differentiation.

In contrast, the giant cells show markers suggesting a hematopoetic lineage (as reviewed by Thomas & Skubitz, 2009). Despite the aforementioned similarities to osteoclasts, giant cells are distinct in that they can be considerably larger, containing hundreds of nuclei (as reviewed by Zheng et al., 2001). Nevertheless, giant cell formation occurs in a similar manner as osteoclastogenesis. Giant cells are formed from RANK-expressing mononuclear osteoclast precursors that are also found within the tumour (Atkins et al., 2006). The spindle-like stromal cells express RANKL and M-CSF (Atkins et al., 2000; Atkins et al., 2001), as well as a variety of other factors that promote osteoclastogenesis. For example, the stromal cell production of chemokines including stromal cell-derived factor-1 (SDF-1) (Liao et al., 2005) and monocyte chemotactic protein-1 (MCP-1) (Zheng et al., 1998) are thought to stimulate the recruitment of mononuclear osteoclast precursors during giant cell formation. Osteoclastogenesis is inhibited by OPG, which is also produced by the stromal cells (Atkins et al., 2001).

Several other aspects of GCT were reported in the literature, and may be important in the pathogenesis of the tumour. For example, investigations into cell cycle regulators show cyclin D1 accumulation within the giant cells (Kandel et al., 2006; Kauzman et al., 2003; Kawaguchi et al., 2004; Matsubayashi et al., 2009) and an absence of cyclin B1 (Kauzman et al., 2003), which mediates the G2 to M transition, indicating that the cell

cycle is arrested within those cells, though the cause of this effect is unclear. However, transforming growth factor (TGF)-β1, which can inhibit osteoclastogenesis (as reviewed by Janssens et al., 2005) is expressed by the stromal cells (Kawaguchi et al., 2004; Zheng et al., 1994), and may affect proliferation of the giant cells. In addition, numerous other proteins have also been considered as diagnostic or prognostic markers for GCT. For example, the significance of p63 expression by the stromal cells (de la Roza, 2011; Dickson et al., 2008; Lee et al., 2008), the role of p53 mutations in clinical outcome (Papanastassiou et al., 2010), or the importance of hypoxia in tumour pathogenesis (Knowles & Athanasou, 2008; Knowles & Athanasou, 2009) were all investigated within the tumour. Moreover, other cells may be involved in the pathogenesis of GCT, such as tumour-infiltrating lymphocytes, which were previously isolated from fresh tissue biopsies (Théoleyre et al., 2005).

Giant cells are the bone-resorbing component of GCT. Although bone resorption is enhanced in the presence of stromal cells (James et al., 1996; Oreffo et al., 1993; Wen et al., 1999), giant cells themselves are capable of independently resorbing dentine slices (Balke et al., 2010; Kanehisa et al., 1991; Ohsaki et al., 1992). The giant cells predominantly express the cysteine proteinase cathepsin K (Drake et al., 1996; Lindeman et al., 2004). However, other proteases, known as matrix metalloproteinases, are also widely expressed by both the neoplastic stromal cells and the giant cells, and may have an important role in the pathophysiology of the tumour.

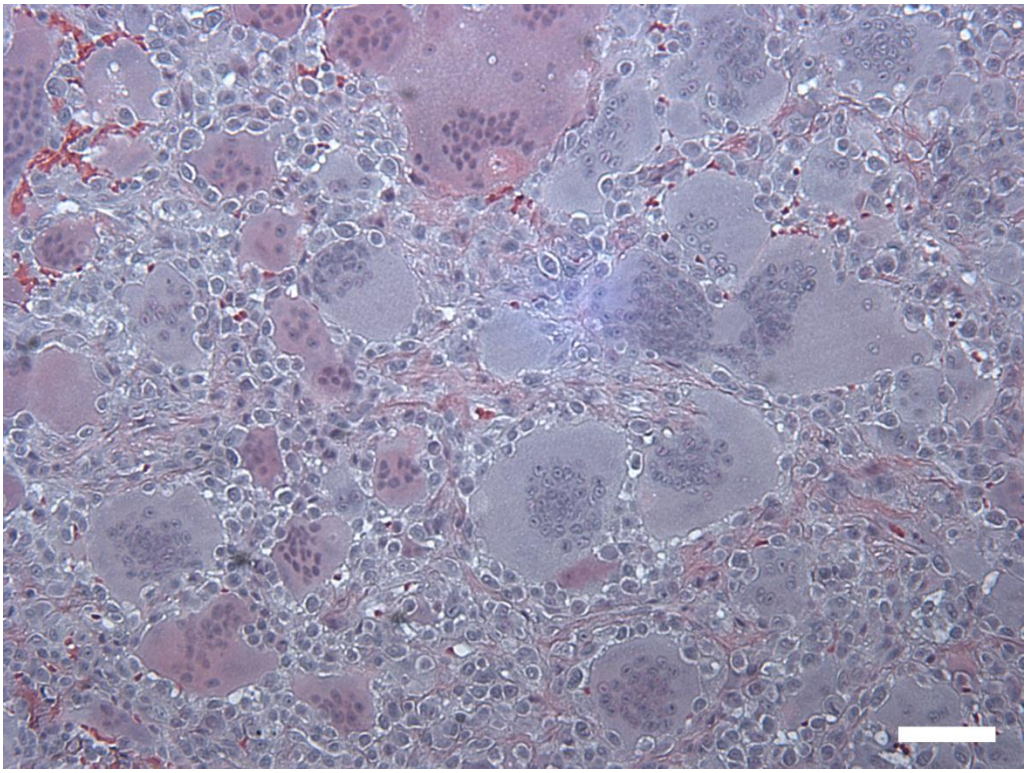

Figure 3 – Representative hematoxylin and eosin stain of paraffin-embedded GCT tissue. The image shows numerous multinucleated giant cells dispersed amongst neoplastic spindle-like stromal cells, monocytes, and other blood cells. Magnification × 100; scale bar = 100 μm.

Matrix metalloproteinases

Overview

Degradation of the extracellular matrix, whose components include collagen, fibronectin and proteoglycans, is largely accomplished by a family of zinc-dependent proteinases that are fittingly named matrix metalloproteinases (MMPs). These enzymes serve to remodel the extracellular matrix in a controlled fashion during processes that include tissue repair and embryonic development, though MMPs are also employed by

many cancers during invasion and metastasis (as reviewed by Ala-aho & Kähäri, 2005). To date, more than twenty human MMPs have been described (as reviewed by Hua et al., 2011), including two MMP-23s that result from a gene duplication (Gururajan et al., 1998). MMPs share several common features, but are classed into subgroups according to their substrate specificity and structure (see Table 1). Both secreted and cell membrane-associated MMPs have been discovered.

All MMPs are produced as inactive zymogens with a pro-peptide domain that inhibits enzyme activity, most often through a conserved cysteine residue that associates with the zinc ion within the catalytic domain (Springman et al., 1990; Van Wart & Birkedal-Hansen, 1990). However, in the case of MMP-23B, a cysteine-rich domain is used in place of the conserved residue (Velasco et al., 1999). These enzymes can be activated *in vitro* by organomercurial compounds such as 4-aminophenylmercuric acetate (APMA), or trypsin (Stetler-Stevenson et al., 1989; Stricklin et al., 1983). Activation of MMPs *in vivo* is achieved through proteolytic cleavage of the pro-peptide domain, or through allosteric interactions that distort the pro-peptide domain away from the active site (as reviewed by Hadler-Olsen et al., 2011). The catalytic domain of each MMP, as mentioned, contains a zinc binding motif, and tightly-bound zinc is a requirement for proper function of the active site (as reviewed by Visse & Nagase, 2003). Another common feature of MMPs, which is absent in only the matrilysins and MMP-23B, is a hemopexin-like domain that is often connected to the catalytic domain by a flexible hinge region. The hemopexin-like domain is involved in numerous protein-protein interactions, having functions in cell migration (Dufour et al., 2008; Dufour et al., 2010), cell signalling (Mantuano et al.,

2008), recognition and binding of substrate (Lauer-Fields et al., 2009), enzyme stability (Mysliwy et al., 2006), and dimerization (Dufour et al., 2010). This domain is also important in the regulation of activity of certain MMPs by tissue inhibitors of metalloproteinases (TIMPs). Specifically, TIMPs are able to form a complex with pro-MMP-2 at its hemopexin-like domain, tethering the zymogen to another, membrane-bound MMP, allowing its activation by other active MMP enzymes (as reviewed by Sato & Takino, 2010).

TIMPs are a family of four secreted proteins that post-translationally regulate MMP activity and share significant sequence homology at the amino acid level. As mentioned, TIMPs may aid in activation of MMPs through interactions at their hemopexin-like domain. However, their primary function is inhibition of active MMPs through non-covalent binding at their active site in a 1:1 stoichiometric ratio (Gomis-Rüth et al., 1997), which also distorts the position of the hemopexin-like domain (Remacle et al., 2011). Collectively, the TIMPs are able to inhibit all known MMPs (as reviewed by Chakraborti et al., 2003). Although traditionally regarded solely as regulators of MMP activity, accumulating evidence suggests TIMPs may have many MMP-independent functions, including induction of apoptosis (Bond et al., 2002), inhibition of cell proliferation (Seo et al., 2003), and stimulation of bone resorption (Sobue et al., 2001).

Although TIMPs are effective MMP inhibitors, the large degree of overlap between MMPs and their substrates requires that the expression of each enzyme be tightly controlled. MMPs are therefore largely regulated at the transcriptional level and their promoters contain numerous *cis*-regulatory elements that permit regulation of gene

expression by various *trans*-activators. Notably, many MMPs contain binding sites within their promoter for activator protein-1 (AP-1) and polyoma enhancer element A3 (PEA3) (as reviewed by Benbow & Brinckerhoff, 1997). However, transcription is complex and is dependent upon many factors. For instance, the distance between the AP-1 and PEA3 binding sites may be short enough to allow their respective transcription factors to act synergistically, as with MMP-1, but the distance may be too great in other MMPs, including MMP-13, to produce a synergistic effect (as reviewed by Sternlicht & Werb, 2001). Moreover, transcription may depend upon several *cis*-regulatory elements at once, such as the requirement for both AP-1 and Runx2 for MMP-13 expression (Jimenez et al., 1999). The composition of the transcription factors themselves is also important. For example, AP-1, which collectively defines a group of homo- and heterodimeric protein complexes composed primarily of members of the Jun and Fos protein families (as reviewed by Hess et al., 2004), can act as either an inducer or a repressor, depending on which of its subunits are present (as reviewed by Clark et al., 2008). These transcription factors, and others, are also influenced by the presence of a wide variety of cytokines and growth factors, where they also act as either stimuli or suppressors (as reviewed by Sternlicht & Werb, 2001). Further, MMP expression may also be regulated by epigenetic factors including methylation and alterations in chromatin structure, which can limit access to the promoter and repress transcription (as reviewed by Yan & Boyd, 2007).

In addition to their role of breaking down proteins within the extracellular matrix, MMPs participate in a diverse list of functions that include inflammation, wound repair,

and embryogenesis (as reviewed by Chakraborti et al., 2003). Moreover, MMPs are collectively capable of degrading or cleaving a large assortment of substrates (as reviewed by Butler & Overall, 2009), signifying their potential importance in modulating numerous signalling pathways.

Matrix metalloproteinases and bone resorption

MMPs are exceptionally important in bone resorption. Whereas cathepsin K functions optimally under the acidic environment produced by the osteoclast (Lecaille et al., 2002), MMPs function best at neutral pH. Nevertheless, several MMPs are associated with osteoclasts, including MMP-9 (Wucherpfennig et al., 1994), and MMP-13 (Nakamura et al., 2004), which associates with the mineralized bone matrix under the ruffled membrane of osteoclasts. Their presence may aid in initiation of bone resorption, as experiments indicate both MMP-9 and MMP-13 are involved in migration of pre-osteoclasts to sites of osteoclast maturation within bone (Blavier & Delaissé, 1995). Indeed, MMPs were also reported to aid in the migration of osteoclasts through collagen (Sato et al., 1998), suggesting they are important in the movement of osteoclasts to new sites of bone resorption. Moreover, collagenase activity encourages osteoclasts themselves to begin resorbing bone, by stimulating formation of the ruffled membrane (Holliday et al., 1997; Holliday et al., 2003).

Considerable evidence suggests that MMPs also aid directly in bone resorption. For example, time-dependent effects on resorption of calvarial bones following inhibition with specific cysteine proteinase or MMP inhibitors show that MMPs participate in the solubilization of matrix proteins following demineralization and subsequent digestion by

the cysteine proteinases (Everts et al., 1998). However, MMPs are not required for osteoclast resorption in long bones (Everts et al., 1999), despite the ability for long bone osteoclasts to compensate for a cathepsin K deficiency with MMPs (Everts et al., 2006). Indeed, osteoclasts act differently at various sites throughout the skeleton, and the proportional involvement of MMPs and cysteine proteinases in resorption differs at specific locations (Shorey et al., 2004). Even so, MMPs participate in the solubilization of collagen during bone resorption, as matrix degradation by osteoclasts is repressed by MMP-specific inhibitors (Everts et al., 1992). MMPs are also involved in bone formation, as bone lining cells must remove residual collagen following resorption by osteoclasts through a process that requires MMPs prior to new matrix deposition (Everts et al., 2002).

MMPs can also modulate bone resorption by affecting osteoclastogenesis. For example, various MMPs were shown to cleave and inactivate the chemokines MCP-1 (McQuibban et al., 2002) and SDF-1 (McQuibban et al., 2001), which help to recruit mononuclear osteoclast precursors. Moreover, MMP-3, MMP-7 and MMP-14 are known to solubilize RANKL, thereby promoting osteoclast formation (Hikita et al., 2006; Lynch et al., 2005). MMPs, including MMP-9 and MMP-13, are also capable of activating TGF-β through proteolytic cleavage of its latent form (Deng et al., 2000; Yu & Stamenkovic, 2000). Therefore, MMPs may regulate osteoclastogenesis through the activation or inactivation of numerous cytokines and growth factors.

Table 1 – Human matrix metalloproteinases (MMPs) listed according to function.

Category	MMP	Name
Collagenases	MMP-1	Interstitial collagenase
	MMP-8	Neutrophil collagenase
	MMP-13	Collagenase 3
Gelatinases	MMP-2	Gelatinase A
	MMP-9	Gelatinase B
Stromelysins	MMP-3	Stromelysin 1
	MMP-10	Stromelysin 2
	MMP-11	Stromelysin 3
Matrilysins	MMP-7	Matrilysin
	MMP-26	Endometase
Membrane-type (MT) MMPs	MMP-14	MT1-MMP
	MMP-15	MT2-MMP
	MMP-16	MT3-MMP
	MMP-17	MT4-MMP
	MMP-24	MT5-MMP
	MMP-25	MT6-MMP
Non-classified	MMP-12	Macrophage elastase
	MMP-19	RASI-1
	MMP-20	Enamelysin
	MMP-21	MMP-21
	MMP-23B	MIFR-1
	MMP-27	MMP-27
	MMP-28	Epilysin

Matrix metalloproteinases in giant cell tumour of bone

Owing to their numerous roles in bone resorption, MMPs understandably feature prominently in GCT research. Several MMPs were previously identified within GCTs, including the gelatinases MMP-2 and MMP-9, which are produced by the stromal cells (Ghert et al., 2007; Rao et al., 1995; Sasaguri et al., 1992; Wucherpfennig et al., 1994) and the giant cells (Kumta et al., 2003; Lindeman et al., 2004; Rao et al., 1995; Ueda et al., 1996; Wucherpfennig et al., 1994), respectively. Microarray analysis confirmed the overexpression of MMP-9 in whole GCT tumour samples compared to various other normal tissues (Skubitz et al., 2004). There is also some evidence that the stromal cells may produce MMP-9 in primary cell cultures (Ghert et al., 2007; Rao et al., 1995), although its expression is decreased or absent upon successive passaging (Rao et al., 1995). However, media from fresh GCT cultures induces MMP-9 expression in late-passaged stromal cells (Rao et al., 1997), and administration of an appropriate stimulus, such as IL-1β or tumour necrosis factor (TNF)-α, which are secreted by the giant cells, can also restore MMP-9 expression by the stromal cells (Rao et al., 1999a; Rao et al., 1999b). These interactions demonstrating the influence of giant cells on stromal cells highlight the reciprocal nature of the GCT cells.

The expression of various other MMPs by GCT cells were also reported, including MMP-1 (Si et al., 2003; Ueda et al., 1996), MMP-3 (Sasaguri et al., 1992; Ueda et al., 1996), MMP-13 (Lindeman et al., 2004; Morgan et al., 2005; Skubitz et al., 2004), and MMP-14 (Lindeman et al., 2004). In addition, the giant cells also express the extracellular matrix metalloproteinase inducer (EMMPRIN) (Si et al., 2003), which can

stimulate MMP production (as reviewed by Gabison et al., 2005). However, the presence or absence of many MMPs in GCTs has not been established. In contrast to MMPs, the expression of TIMPs in GCT has not been extensively investigated, although both TIMP-1 and TIMP-2 were reported to be variably expressed by both the giant cells and stromal cells (Schoedel et al., 1996; Si et al., 2003; Ueda et al., 1996).

The significance of MMPs in the pathogenesis of GCT is unclear. Due to the similarities with normal osteoclasts, it is likely that many of the established functions of MMPs in bone resorption and osteoclastogenesis are also true for the giant cells. MMPs may also have a direct role in bone resorption, as stromal cell-derived gelatinases are capable of proteolytic cleavage of gelatin (Ghert et al., 2007). Also, several reports of co-culture systems suggest that the stromal cells are capable of inducing osteoclast formation from peripheral blood mononuclear cells or the murine monocyte cell line, RAW 264.7, without cell-to-cell contact (Atkins et al., 2006; Huang et al., 2000; Lau et al., 2005; Miyamoto et al., 2000; Nishimura et al., 2005). Therefore, soluble RANKL may be expressed or produced through proteolytic cleavage, as MMP-3 and MMP-14, which solubilize RANKL (Hikita et al., 2006; Lynch et al., 2005), are expressed within the tumour (Lindeman et al., 2004; Sasaguri et al., 1992).

Parathyroid hormone and parathyroid hormone-related protein

Parathyroid hormone

Parathyroid hormone (PTH) is a hormone secreted by the chief cells of the parathyroid glands that helps to maintain serum calcium homeostasis. Calcium, which is involved in multiple biological applications, including cell signalling, muscle contraction, and

enzyme function (as reviewed by Silverthorn, 1998), is predominantly stored in bone as hydroxyapatite. Therefore, to release calcium, PTH stimulates bone resorption by encouraging osteoclastogenesis and osteoclast activity. Specifically, PTH acts on osteoblasts and stromal cells through the PTH type 1 receptor (PTH1R) to stimulate RANKL expression and suppress OPG expression (Fu et al., 2002; Horwood et al., 1998; Huang et al., 2004a; Kondo et al., 2002). Additionally, PTH also stimulates the expression of collagenases in osteoblasts (Hess et al., 2001; Porte et al., 1999; Quinn et al., 1990; Scott et al., 1992). In contrast, calcitonin, a hormone that is secreted by the thyroid glands, potently inhibits osteoclast activity when serum calcium levels are too high, and is particularly involved in regulating calcium homeostasis during periods of calcium stress (as reviewed by de Paula & Rosen, 2010). PTH also has a variety of other functions, both related and unrelated to calcium levels, and its expression is therefore tightly controlled.

One important regulator of PTH production is vitamin D. Specifically, vitamin D_3 is hydroxylated in the liver to form 25-hydroxyvitamin D_3 and then hydroxylated once more in the kidneys to form the biologically active 1,25-dihydroxyvitamin D_3 (as reviewed by Landry et al., 2011), which subsequently binds to the vitamin D receptor and then the promoter region of the PTH gene where it represses transcription (Demay et al., 1992; Russell et al., 1986). Reciprocally, PTH regulates production of 1,25-dihydroxyvitamin D_3, through stimulating synthesis of the renal enzyme responsible for catalyzing the hydroxylation of 25-hydroxyvitamin D_3 (Murayama et al., 1999).

Another regulator of PTH synthesis is calcium itself. However, in contrast to 1,25-dihydroxyvitamin D_3, which inhibits gene transcription, the effects of serum calcium levels on PTH are mediated by proteins that affect mRNA stability (as reviewed by Naveh-Many, 2010). When serum calcium levels are low, multiple binding proteins stabilize the PTH mRNA at a specific *cis*-regulatory element within the 3′ untranslated region (Dinur et al., 2006; Sela-Brown et al., 2000). Alternatively, another protein binds the same *cis*-regulatory element when serum calcium levels are not low, and promotes destabilization of the mRNA transcript, leading to its degradation (Nechama et al., 2008). At present, the specific effects of calcium on these binding proteins are not understood.

Calcium, which is obtained through diet, is absorbed through the intestines, chiefly through active transport requiring 1,25-dihydroxyvitamin D_3 (as reviewed by Fleet & Schoch, 2010). It is excreted by the kidneys in urine, although a majority is reabsorbed into the bloodstream (as reviewed by Boros et al., 2009). PTH affects both of these processes, by directly altering renal reabsorption of calcium through regulation of expression (van Abel et al., 2005) and activation (de Groot et al., 2009) of calcium transporter proteins, and indirectly controlling calcium absorption in the intestines through stimulation of 1,25-dihydroxyvitamin D_3 synthesis (Murayama et al., 1999), as mentioned. Overall changes in serum calcium concentration are detected by the parathyroid glands through the calcium-sensing receptor (CaSR) (Brown et al., 1993), which is activated upon binding to calcium whereupon it prevents secretion of PTH (as reviewed by Peacock, 2010). Conversely, without bound calcium, the CaSR is inactive and PTH secretion proceeds (as reviewed by Peacock, 2010).

PTH also participates in serum phosphate homeostasis, which is also largely stored in bone as hydroxyapatite. Although PTH also releases phosphate *via* its actions on stimulating bone resorption, serum phosphate levels are mostly controlled through excretion and reabsorption by the kidneys. There, PTH exerts an inverse effect on phosphate compared to calcium: whereas PTH increases renal calcium reabsorption, it also acts by decreasing phosphate reabsorption (as reviewed by Biber et al., 2009).

PTH exerts numerous effects on bone cells. However, its actions are complex, and in contrast to its catabolic functions, PTH can also exert anabolic effects. The dissimilar actions are attributable to its mode of delivery: whereas continuous PTH signalling produces catabolic effects, intermittent signalling results in an anabolic response. The stimulatory effect of PTH on bone formation is a result of multiple factors (as reviewed by Aslan et al., 2011), including such aspects as cell cycle arrest (Qin et al., 2005) and prevention of apoptosis (Jilka et al., 1999) in osteoblasts. Furthermore, intermittent PTH acts on osteocytes to reduce expression of sclerostin (Keller & Kneissel, 2005): a protein that inhibits the Wnt signalling pathway involved in osteoblast differentiation (van Bezooijen et al., 2007). Indeed, intermittent PTH administered as daily subcutaneous injections are sometimes employed as a treatment for osteoporosis, where they increase alkaline phosphatase expression and decrease Runx2 and MMP-13 expression in affected women after six months (Zhu et al., 2011). With respect to the catabolic effects of the hormone, continuous stimulation resulting from primary or secondary hyperparathyroidism can result in increased bone resorption, hypercalcemia, and osteolytic lesions known as brown tumours (Unni & Inwards, 2010a).

All of these aforementioned effects of PTH are mediated through PTH1R. However, PTH is a protein comprising 84 amino acids that is routinely fragmented into multiple sections that are detectable within the bloodstream (as reviewed by Murray et al., 2005). Only the amino-terminal end of PTH can bind PTH1R (Pines et al., 1994), requiring at a minimum, the first 34 amino acids (Potts Jr. et al., 1971), and there is accumulating evidence to suggest another, uncharacterized receptor may exist that recognizes the carboxyl-terminal end of PTH (as reviewed by Murray et al., 2005). This presumed carboxyl-terminal PTH receptor is thought to be primarily expressed by osteocytes, where it may participate in intercellular communication (Divieti et al., 2001). Indeed, carboxyl-terminal and mid-region fragments of PTH are reported to elicit multiple biologic responses, including the stimulation of alkaline phosphatase and osteocalcin expression in osteoblastic cells (Sutherland et al., 1994), and the promotion of apoptosis in osteocytes in the absence of PTH1R (Divieti et al., 2001). Additionally, another receptor, known as the PTH type 2 receptor (PTH2R), is found primarily in the brainstem (Bagó et al., 2008). Although PTH can bind to PTH2R (Usdin et al., 1995), it is not detectable in the brain (as reported by Usdin, 1997), and the natural ligand for PTH2R appears to be a recently-discovered protein known as tuberoinfundibular peptide of 39 residues (TIP39) (Usdin et al., 1999), which may participate in nociceptive signalling (Dobolyi et al., 2002). However, whereas TIP39 interacts with PTH2R, another protein also related to PTH, known as parathyroid hormone-related protein, interacts with PTH1R and shares many properties with PTH.

Parathyroid hormone-related protein

Parathyroid hormone-related protein (PTHrP), as its name implies, shares significant sequence and structural similarities with PTH, but only at its amino-terminal end (Suva et al., 1987). Owing to this similarity, PTHrP also binds to PTH1R (Jüppner et al., 1991), and can elicit many of the same responses as PTH. For example, PTHrP is also able to stimulate renal calcium reabsorption (Syed et al., 2001), and can act as an osteolytic agent by stimulating osteoblastic cells to promote osteoclastogenesis through the expression of factors like RANKL (Fukushima et al., 2005; Guo et al., 2006; Mak et al., 2008) and MCP-1 (Lu et al., 2007). Therefore, the importance of PTHrP is evident from the widespread expression of PTH1R throughout the body, in tissues not regarded as PTH target tissues (Ureña et al., 1993). Indeed, PTHrP is expressed by a variety of tissue types, including placenta, heart, kidney, lung, and skin (as reviewed by Martin et al., 1995). It is produced as one of three isoforms, due to alternative splicing, with lengths totalling 139, 141, and 173 amino acids (as reviewed by Hastings, 2004). Like PTH, PTHrP is also commonly processed into smaller fragments (as reviewed by Hastings, 2004), and mid-region and carboxyl-terminal fragments exert distinct effects from the amino-terminal fragments. For example, carboxyl-terminal PTHrP inhibits osteoclast-mediated bone resorption (Cornish et al., 1997; Fenton et al., 1991). Moreover, PTHrP can also exert intracrine effects due to the presence of a nuclear localization sequence within its mid-region, which permits the energy-dependent uptake of extracellular PTHrP into the nucleus (Henderson et al., 1995; Lam et al., 1999; Lam et al., 2001). There, it

promotes evasion of apoptosis and cellular proliferation in a wide range of cell types (as reviewed by Hastings, 2004).

Although PTHrP has numerous roles, its primary functions may relate to development. In particular, embryonic deletion of PTHrP is lethal, and results in skeletal malformation (Karaplis et al., 1994), as well as improper mammary gland (Wysolmerski et al., 1998) and lung development (Rubin et al., 2004). Correspondingly, PTHrP is found at high levels in fetal tissues (Ferguson II et al., 1992; Moseley et al., 1991). In bone, PTHrP acts on chondrocytes to ensure proper endochondral bone formation. Specifically, as chondrocytes mature, they stop proliferating, become hypertrophic, and osteoblasts subsequently begin ossification (as reviewed by Kronenberg, 2006). Premature cessation of chondrocyte proliferation leads to near complete replacement of cartilage with bone, producing shortened bones (Karaplis et al., 1994). Runx2 is a critical transcription factor in the maturation process (Inada et al., 1999), and PTHrP prevents chondrocyte maturation by decreasing Runx2 expression (Guo et al., 2006) and possibly by targeting the protein for degradation by cyclin-dependent kinases (Zhang et al., 2009). However, PTHrP exerts disparate effects in osteoblasts, where it produces no effect on Runx2 expression following amino-terminal PTHrP stimulation (Guo et al., 2006), or increased Runx2 expression following carboxyl-terminal PTHrP stimulation (Toribio et al., 2010).

In addition to its many actions in normal tissues, PTHrP is often associated with cancers. In fact, the protein was first identified due to its causal role in humoral hypercalcemia of malignancy (Suva et al., 1987): the process whereby certain cancers elicit hypercalcemia without metastasizing to bone. Since its discovery, PTHrP has been

described in numerous cancers, including breast (Southby et al., 1990), lung (Moseley et al., 1987), and kidney (Strewler et al., 1987). Moreover, PTH1R is also prevalent in numerous tumour types (Lupp et al., 2010). The synthesis of PTHrP by cancers is associated with diverse functions that include tumour initiation (Li et al., 2011), promotion of metastasis (Iguchi et al., 1996; Li et al., 2011), and evasion of apoptosis (Massfelder et al., 2004; Talon et al., 2006). In particular, PTHrP mediates osteolysis in breast cancer metastasis to bone (Guise et al., 1996) by stimulating osteoblasts to express RANKL (Thomas et al., 1999).

CD40-CD40L signalling

Overview

Considerable evidence supports the notion that bone cells commonly interact with the immune system. For example, activated B and T cells can express RANKL and stimulate osteoclast formation (Kawai et al., 2006; Weitzmann et al., 2001), while resting T cells, and certain activated T cells, inhibit osteoclastogenesis through the production of multiple cytokines, which stimulate monocyte commitment to other cell lineages (Grcevic et al., 2006; Shinoda et al., 2003). Media conditioned by T cells can also stimulate osteoblasts to express MMP-13 (Rifas & Arackal, 2003). Moreover, osteoclasts themselves serve as antigen-presenting cells that can activate T lymphocytes (Li et al., 2010). Therefore, immune cells may influence multiple aspects of normal and diseased bone physiology.

In particular, activated T cells express CD40 ligand (CD40L) (Armitage et al., 1992), also known as CD154, which is implicated in numerous diseases, including various autoimmune diseases and cancers (as reviewed by Chatzigeorgiou et al., 2009). CD40L

is a membrane-bound protein that can be solubilized by MMP-2 (Choi et al., 2010), and recognizes its receptor, CD40, on a wide variety of cell types that includes B cells, monocytes, macrophages, fibroblasts, and epithelial cells (as reviewed by van Kooten & Banchereau, 2000). Platelets, monocytes, endothelial cells, and other cell populations are also capable of expressing CD40L (as reviewed by Chatzigeorgiou et al., 2009). Although CD40L was shown to bind to several other receptors (as reviewed by Seijkens et al., 2010), it is the CD40-CD40L dyad that has garnered the most attention for its potential as a therapeutic target. For example, many solid tumours express CD40, and CD40L signalling is associated with growth inhibition and induction of apoptosis in tumour cells (as reviewed by Vonderheide, 2007). Alternatively, CD40L potentiates several diseases, including atherosclerosis, where it mediates expression of numerous cytokines and MMPs (as reviewed by Chatzigeorgiou et al., 2009). In normal physiology, CD40L is involved in signal transduction, and is associated with numerous cell-specific functions, including activation of kinases, stimulation of cytokine expression, and induction of cell differentiation (as reviewed by van Kooten & Banchereau, 2000).

CD40L and parathyroid hormone

The immune system may also be important in mediating the effects of PTH and PTHrP in bone. For example, wild type mice administered continuous PTH through an osmotic pump display the characteristic bone resorption of hyperparathyroidism, whereas nude mice, which lack functional T cells, do not respond to PTH treatment (Gao et al., 2008). A similar observation was also noted in athymic mice injected with cells or grafted with

tissue overexpressing PTH, which did not produce a catabolic effect (Hory et al., 2000). Further investigation into the role of T cells in mediating PTH-induced bone resorption in wild type mice revealed CD40L as a critical determinant of the ability of bone marrow stromal cells to respond to PTH (Gao et al., 2008). Indeed, osteoblasts were previously reported to express CD40, where it promoted cell survival upon binding with CD40L (Ahuja et al., 2003). Stimulation of stromal cells with both PTH and CD40L resulted in increased RANKL expression and osteoclastogenesis in co-culture experiments with monocytes (Gao et al., 2008), suggesting CD40L may be an important factor in bone resorption.

Project outline and hypothesis

Rationale

The etiology of GCT remains poorly understood, owing largely to the facts that it is often diagnosed following extensive tumour development and is frequently curable by surgical excision or resection. However, the aggressiveness of the tumour, the potential for metastasis, and the occurrence of GCTs in areas not suitable for surgical intervention necessitate further advances in treatment options that can only arise from a better understanding of the pathogenesis of the tumour. To that end, this thesis will examine factors that affect the core symptom of GCT: bone resorption. Although the giant cells are classically considered the principal bone-resorbing cells within GCT, bone resorption by giant cells is enhanced in the presence of the neoplastic spindle-like stromal cells (James et al., 1996; Oreffo et al., 1993; Wen et al., 1999). Therefore, stromal cells may promote bone resorption by the giant cells *in vitro* through several possible methods.

First, the presence of the stromal cells may promote the accumulation of giant cells, and therefore promote bone resorption by the giant cells alone. Second, the stromal cells may participate in bone resorption, either directly, or by stimulation of giant cell activity. Moreover, the stromal cells may promote bone resorption through any combination of these factors.

Both PTH and PTHrP are factors known to increase bone resorption through promotion of osteoclastogenesis. In fact, hyperparathyroidism can lead to bone lesions called brown tumours that share many radiological and histological features with GCT (as reviewed by Unni & Inwards, 2010a). However, while hyperparathyroidism has systemic effects, GCT is localized. GCT was previously reported to respond to PTH stimulation (Goldring et al., 1977), and although there are no similar reports investigating the effects of PTHrP stimulation, GCT cells may also respond to PTHrP, which stimulates many features characteristic of the tumour, including osteoclastogenesis, bone resorption, and cellular proliferation. Indeed, while PTHrP is widely-recognized as an important mediator of osteolysis from metastasizing tumours, or when secreted systemically at distant sites, there is no reported primary bone neoplasm that is characterized by PTHrP activity. Therefore, this thesis will evaluate whether PTHrP contributes to the pathogenesis of GCT, with a focus on bone resorption.

Hypothesis

PTHrP is a key mediator of GCT physiology, and influences the stromal cells to promote osteoclastogenesis and bone resorption.

Objectives

1. Determine whether GCT stromal cells produce bone-resorbing proteases.
2. Determine whether PTHrP is expressed within GCT, and whether PTHrP stimulates stromal cell-mediated giant cell formation.
3. Determine the effect of PTHrP on the stromal cells' production of bone-resorbing proteases, and whether local factors within the tumour, such as CD40L signalling, stimulate PTHrP expression in GCT.

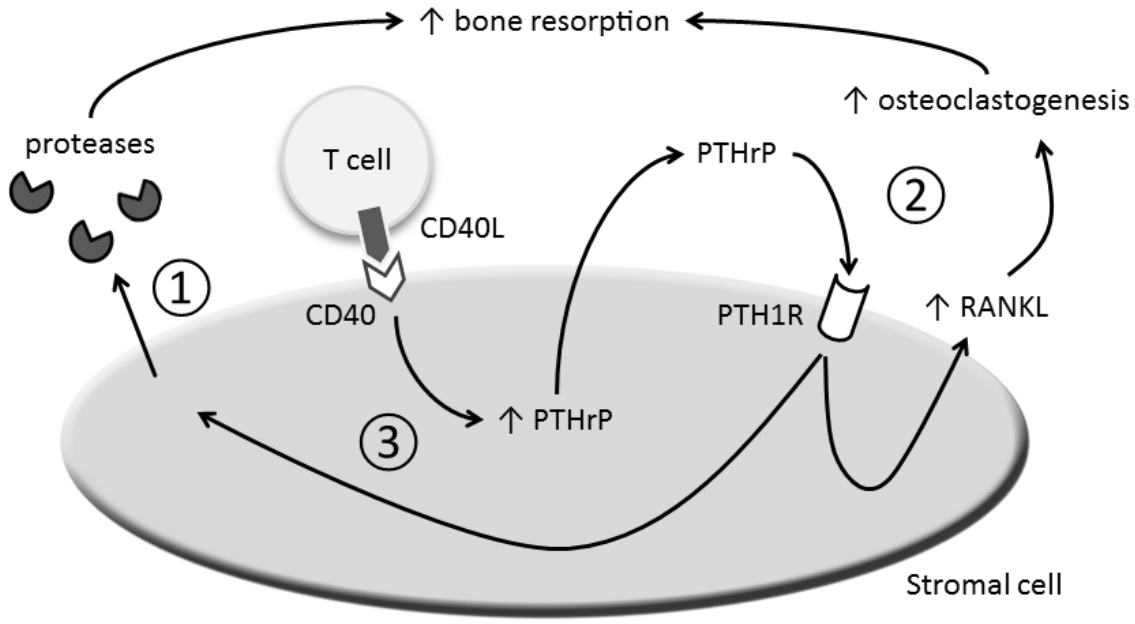

Figure 4 – Diagrammatic representation of the hypothesis. (1) GCT stromal cells produce bone-resorbing proteases. (2) The stromal cells express PTHrP and its receptor, PTH1R, which stimulates RANKL expression and osteoclastogenesis. (3) PTHrP stimulates expression of bone-resorbing proteases and is itself stimulated by local factors within the tumour, such as CD40L.

Thesis outline

The thesis objectives were explored in three manuscripts, and are presented in this work as follows:

Chapter 2: *Collagenase expression and activity in the stromal cells from giant cell tumour of bone.* This paper, published in *Bone* (2009), examines the expression of the bone-resorbing interstitial collagenases by GCT stromal cells and shows the cells express and secrete MMP-1 and MMP-13, but not MMP-8. Activity of the MMPs was confirmed through enzyme-specific assays, which suggests the proteases are sufficient for proteolysis and that the stromal cells may participate in bone resorption by the tumour.

Chapter 3: *PTHrP increases RANKL expression by stromal cells from giant cell tumor of bone.* This paper, accepted on October 31st, 2011 for publication in the *Journal of Orthopaedic Research,* shows that the GCT stromal cells express both PTHrP and its receptor, PTH1R. Stimulation of cultured GCT stromal cells with PTHrP (1-34) significantly increased OPG and RANKL gene expression in a time-dependent manner, which resulted in an increased ability to promote multinucleated TRAP-positive cell formation. These results suggest that PTHrP can participate in the pathology of GCT, and that it may promote giant cell formation within the tumour.

Chapter 4: *T cells stimulate catabolic gene expression by the stromal cells from giant cell tumor of bone.* This paper, published in *Biochemical and Biophysical Research Communications* (2012), shows that PTHrP participates in the

regulation of MMP-13 expression in cultured GCT stromal cells. Moreover, this manuscript identifies expression of CD40L within the tumour and shows that this protein, as well as two T cell lines, stimulates PTHrP expression by the stromal cells. These results show that local factors within the tumour can potentiate the effect of PTHrP in GCT.

CHAPTER 2

Collagenase expression and activity in the stromal cells from giant cell tumour of bone

Robert W. Cowan, Isabella W.Y. Mak,
Nigel Colterjohn, Gurmit Singh, Michelle Ghert
Bone 44(5): 865-871

Preface

GCT is an aggressive tumour, and managing its characteristic bone destruction is of principal concern for both patients and physicians. As indicated in the first objective (Chapter 1), we hypothesized that the stromal cells could either directly contribute to the extensive bone resorption observed within the tumour, or that they facilitated resorption by the giant cells, as prior research demonstrated that bone degradation was enhanced in the presence of stromal cells (James et al., 1996; Oreffo et al., 1993; Wen et al., 1999). We previously investigated the expression of gelatinases within the tumour, and demonstrated that the stromal cells expressed MMPs capable of digesting gelatin (Ghert et al., 2007). Gelatinases alone are not naturally sufficient to independently degrade normal bone (Aimes & Quigley, 1995), and we therefore investigated whether the stromal cells expressed type I collagenases capable of degrading intact fibrillar collagen.

This chapter consists of an author-generated version of the following published article: Robert W. Cowan, Isabella W.Y. Mak, Nigel Colterjohn, Gurmit Singh, and Michelle Ghert (2009) Collagenase expression and activity in the stromal cells from giant cell tumour of bone *Bone* 44(5): 865-871. This article is reprinted with permission from Elsevier (see Appendix B for License Agreement).

I established all the cell lines used for these experiments and performed the analyses by western blotting, multiplex assays and enzyme activity assays. I also assembled the results, generated the figures, and both wrote and revised the manuscript. Isabella W.Y. Mak performed the real-time PCR and immunohistochemistry analyses and reviewed the manuscript. Nigel Colterjohn provided initial intellectual direction for the project.

Gurmit Singh and Michelle Ghert reviewed the manuscript and provided intellectual direction and advice.

This paper establishes the stromal cells as a source of collagenases within GCT. Specifically, we show that both MMP-1 and MMP-13 are expressed by the neoplastic stromal cells, and that these collagenases are sufficient for proteolytic cleavage using enzyme-specific activity assays. These results are consistent with previous microarray (Morgan et al., 2005; Skubitz et al., 2004) and PCR (Lindeman et al., 2004) data from whole GCT that identify MMP-13 as a major protease within the unfractionated tumour, and are significant because they are the first to describe collagenase expression and secretion by isolated GCT stromal cells and to assess their proteolytic activity. This finding has formed the basis for further investigation into the actions of MMP-1 and MMP-13 in GCT, and several potential roles for these enzymes are postulated. In particular, I suggest that the stromal cell-derived collagenases could stimulate the giant cells to begin resorbing bone, in a manner that was observed by Holliday and colleagues (Holliday et al., 1997; Holliday et al., 2003). We therefore examined bone resorption by co-cultures of GCT stromal cells with giant cells on bovine bone slices using MMP-13-specific inhibitors (Mak et al., 2010). Results demonstrated that the inhibitors were capable of reducing the volume of bone resorbed by GCT stromal cells, as well as by unfractionated and giant cell-enriched cultures of GCT cells. These results suggest that MMP-13 may be involved in direct bone resorption by the stromal cells, and possibly in stimulating bone resorption by the giant cells as well. However, further clarification on the significance of MMP-13 in bone resorption by GCT is necessary. Moreover, these

results suggest that any effect of PTHrP on bone resorption within GCT may be mediated by expression of these collagenases, as both PTHrP and PTH are capable of promoting MMP-13 expression (Hess et al., 2001; Ibaragi et al., 2010; Porte et al., 1999).

Title: **Collagenase expression and activity in the stromal cells from giant cell tumour of bone**

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Statement of Ethics: This study has been approved by the Research Ethics Board of McMaster University and Hamilton Health Sciences. Experiments were undertaken with the understanding and written consent of each subject in compliance with the Code of Ethical Principles for Medical Research Involving Human Subjects of the World Medical Association.

† Deceased.

Abstract

The characteristic bone destruction in giant cell tumour of bone (GCT) is largely attributed to the osteoclast-like giant cells. However, experimental analyses of bone resorption by cells from GCT often fail to exclude the neoplastic spindle-like stromal cells, and several studies have demonstrated that bone resorption by GCT cells is increased in the presence of stromal cells. The spindle-like stromal cells from GCT may therefore actively contribute to the bone resorption observed in the tumour. Type I collagen, a major organic constituent of bone, is effectively degraded by three matrix metalloproteinases (MMPs) known as the collagenases: MMP-1, MMP-8 and MMP-13. We established primary cell cultures from nine patients with GCT and the stromal cell populations were isolated in culture. The production of collagenases by primary cultures of GCT stromal cells was determined through real-time PCR, western blot analysis and a multiplex assay system. Results show the cells produce MMP-1 and MMP-13 but not MMP-8. Immunohistochemistry confirmed the presence of MMP-1 and MMP-13 in paraffin-embedded GCT tissue samples. Medium conditioned by the stromal cell cultures was capable of proteolytic activity as determined by MMP-1 and MMP-13-specific standardized enzyme activity assays. The spindle-like stromal cells from GCT may therefore actively participate in the bone destruction that is characteristic of the tumour.

Keywords: giant cell tumour, stromal cells, collagen, osteolysis, matrix metalloproteinases

Introduction

The pathogenesis of giant cell tumour of bone (GCT), a rare primary osteolytic bone tumour, remains a matter of some controversy. The GCT microenvironment is characterized by three cell types; namely, the osteoclast-like multinucleated giant cells [1, 2], the mononuclear CD68-positive round cells of monocytic-macrophage origin [2, 3], and the mononucleated spindle-like stromal cells of mesenchymal origin [4-7]. Although the localized tissue destruction observed in patients with GCT is often attributed to the multinucleated giant cells [8-13], few reports of bone resorption by this tumour have excluded the stromal cells from their analyses, which proliferate rapidly and ultimately dominate the cell population when grown *in vitro*. Indeed, the stromal cells are considered the neoplastic component of the tumour [7], and several studies have reported that overall bone resorption by GCT cells is enhanced by the presence of stromal cells [12, 14, 15]. The stromal cells, therefore, may also actively participate in the bone destruction that is characteristic of GCT.

Previous research has demonstrated that the stromal cell population from GCT produces various matrix metalloproteinases (MMPs) that are known to degrade an assortment of components comprising the extracellular matrix [16-19]. MMPs are a family of zinc-dependent proteases that have been characterized in a multitude of organisms and are prominently involved in such regulatory processes as embryonic development, wound repair, and bone remodeling [20]. Although MMPs share several distinguishing features, they can be classed according to their domain structure and substrate specificity into subgroups that include the collagenases (MMP-1, MMP-8,

MMP-13), the gelatinases (MMP-2, MMP-9), and the stromelysins (MMP-3, MMP-10), among others. Despite a wealth of information concerning MMPs in GCT, there is minimal data on the collagenases.

Collagen is considered the major organic component of normal bone [21-23]. Although several species of collagen have been described, the dominant form found in bone is that of type I collagen, which comprises approximately ninety percent of the entire collagen content [24]. An investigation into the bone degradation that is characteristic of GCT must therefore include an examination of the tumour's ability to degrade type I collagen. Type I collagen consists of three polypeptide chains in the form of $[\alpha 1(I)]_2\alpha 2(I)$ and, in bone, is largely arranged in an insoluble fibrillar structure where it contributes to the structural integrity of the skeleton. However, only certain enzymes are capable of cleaving native fibrillar type I collagen. In this report, we investigated the ability of patient-derived GCT stromal cells to degrade type I collagen by evaluating the production and activity of the three collagenases known to degrade such collagen: MMP-1, MMP-8 and MMP-13.

Materials and Methods

Cells and cell culture

Primary cell cultures were established from biopsy and resection specimens from patients pre-operatively diagnosed with GCT, following patient consent and approval from our institution's Research Ethics Board (Table 1). Diagnoses of GCT were confirmed post-operatively by a qualified pathologist and tumours were graded radiographically according to the Campanacci method of classification [25]. Tissue

samples were macerated with scalpel blades in a sterile glass Petri dish containing Dulbecco's modified Eagle medium (D-MEM) supplemented with 10% fetal bovine serum, 2mM L-glutamine, 100U/mL penicillin, and 100µg/mL streptomycin (Invitrogen Canada/Gibco; Burlington, Ontario, Canada). Aliquots of media containing cells and macerated tissue were passed through a 24-gauge needle and transferred to 25cm^2 vented tissue culture flasks and subsequently maintained at 37˚C in humidified air with 5% CO_2. Following a 24-hour incubation period, the cell culture medium was replaced with fresh, supplemented D-MEM following several washes with phosphate buffered saline (PBS). Thereafter, cell culture medium was replenished every 2 to 3 days. Upon reaching ~80% confluence, cell cultures were digested with a 0.1% trypsin-EDTA solution; cells that easily detached from the flask surface were collected and maintained in supplemented D-MEM. Following several successive passages, the multinucleated giant cells and the CD68-positive monocytes were eliminated from the culture, as we have previously reported [16]. Cells used in experiments were analysed between passages 5 and 17.

Simian virus 40 large T antigen-transfected human fetal osteoblast (hFOB) 1.19 cells were obtained from the American Type Culture Collection (ATCC # CRL-11372; Rockville, Maryland, USA) and maintained in supplemented D-MEM as described for the GCT cells at 34˚C in humidified air containing 5% CO_2. Similarly, human osteosarcoma (HOS) cells were obtained from ATCC (# CRL-1543) and maintained in Eagle's minimum essential medium (ATCC) supplemented with 10% fetal bovine serum, 100U/mL penicillin, and 100µg/mL streptomycin (Invitrogen Canada/Gibco) at 37˚C in humidified air containing 5% CO_2.

Table 1 — Demographic data for GCT primary cell cultures.

Case	Gender	Age	Location	Campanacci Classification
GCT-1	Female	25	Distal femur	Grade II
GCT-2	Male	59	Distal femur	Grade II
GCT-3	Male	41	Distal femur	Grade III
GCT-4	Male	55	Proximal humerus	Grade III
GCT-5	Female	56	Proximal tibia	Grade II
GCT-6	Female	31	Distal ulna	Grade III
GCT-7	Female	47	Distal femur	Grade II
GCT-8	Female	39	Rib	Grade III
GCT-9	Male	20	Distal femur	Grade II

Real-time PCR

Total RNA was isolated from stromal cell cultures lysed with lysis buffer (buffer RLT) containing β-mercaptoethanol using the RNeasy Mini Kit (Qiagen; Mississauga, Ontario, Canada) according to the manufacturer's instructions. Isolated mRNAs were reverse-transcribed into cDNAs using the SuperScript II First-Strand Synthesis System (Invitrogen Canada; Burlington, Ontario, Canada) as per the manufacturer's instructions, and employing oligo-dT probes. The real-time polymerase chain reaction (PCR) was performed on a MiniOpticon Real-Time PCR Detection System (Bio-Rad Laboratories; Mississauga, Ontario, Canada) using the iQ SYBR Green Supermix (Bio-Rad Laboratories), according to the manufacturer's instructions. The reaction was achieved in

a total volume of 20μL, consisting of 1:10 diluted cDNA template (2μL) obtained from RNA (1μg). Amplification was performed using a real-time thermal cycler (Bio-Rad Laboratories) using 40 cycles of 94˚C for 15 seconds, 58-60˚C (based on individual optimization) for 30 seconds, and 72˚C for 30 seconds, with a gradient change in temperature to determine the melting curve of the final PCR products. The gene encoding human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as an internal control. Cycle threshold (Ct) values were established and the relative change in expression from GAPDH was determined according to the $2^{-\Delta\Delta Ct}$ method of analysis and compared to expression by hFOB 1.19 cells.

Primers were designed using the National Center for Biotechnology Information (NCBI)'s GenBank and prepared by Sigma-Genosys Canada (Sigma-Aldrich; Oakville, Ontario, Canada). Sense and anti-sense primers are listed in Table 2.

Western blotting

GCT stromal cells were plated in 55cm^2 Petri dishes and grown to confluence. Cells were scraped and collected in Nonidet P-40 (NP40) lysis buffer (15% NP40, 5M NaCl, 1M Tris pH 7.4, 0.5M EDTA pH 8.0) containing protease inhibitor cocktail tablets (Hoffmann-La Roche; Mississauga, Ontario, Canada) and maintained at 4˚C for 60 minutes. The lysate was centrifuged at 10,000 × *g* for 20 minutes. Protein concentration was determined by the Bradford microassay procedure and 50μg samples were electrophoresed by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) at 90V for 90 minutes, and then transferred to a nitrocellulose membrane using a semi-dry transfer cell (Bio-Rad Laboratories) at 20V for 45 minutes, as per the

Table 2 — Primer information for real-time PCR.

Gene		Primer sequence	GenBank Accession No.	Base Nos.	Size of amplicon
MMP-1	Sense	5'GGACCATGCCATTGAGAAAG3'	NM_002421	461-591	131bp
	Anti-Sense	5'AAGGAGAGTTGTCCCGATGA3'			
MMP-8	Sense	5'ATCTCACAGGGAGAGGCAGA3'	NM_002424	522-607	86bp
	Anti-sense	5'ATTCCATTGGGTCCATCAAA3'			
MMP-13	Sense	5'CTTCCCAACCGTATTGATGC3'	NM_002427	1016-1158	143bp
	Anti-sense	5'TTTGGAAGACCCAGTTCAGA3'			
GAPDH	Sense	5'CATGAGAAGTATGACAACAGCCT3'	NM_002046	511-623	113bp
	Anti-Sense	5'AGTCCTTCCACGATACCAAAGT3'			

manufacturer's instructions. Blots were blocked overnight with 5% skim milk in 1× TBS-T and then incubated with monoclonal anti-human MMP-1 (Calbiochem; Mississauga, Ontario, Canada), MMP-8 or MMP-13 antibodies (R&D Systems; Minneapolis Minnesota, USA) for 3 hours at room temperature. Recombinant protein standards for MMP-8 and MMP-13 (R&D Systems) served as positive controls for those blots, while HOS functioned as a positive control for MMP-1 expression and water served as a negative control for all blots. Blots were subsequently incubated with appropriate secondary antibody and MMP protein was visualized using enhanced chemoluminescence

(ECL) detection (Amersham Biosciences/GE Healthcare Bio-Sciences Inc.; Baie d'Urfé, Quebec, Canada) according to the manufacturer's instructions. Antibodies were removed using stripping buffer (62.5mM Tris-HCl pH 6.8, 2% SDS, 100mM β-mercaptoethanol) at 65˚C for 30 minutes and blots were re-probed with monoclonal anti-actin (MP Biomedicals; Montreal, Quebec, Canada), which served as a loading control.

Multiplex assay

The Fluorokine MAP Multiplex Assay System with Luminex 100 detection equipment (R&D Systems) employs colour-coded microparticles to accurately detect and quantify specific analytes within a medium. The microparticles are equipped with analyte-specific antibodies and are added to a sample of interest where the antibodies bind to their respective substrates. Biotinylated antibodies are subsequently added to the sample and bind the microparticle-affiliated analytes. Lastly, a streptavidin-phycoerythrin conjugate is added to the sample, which binds the biotinylated antibodies. Quantification of specific analytes is achieved using a dual laser approach: one laser is used to determine the specific colours of the microparticle, thereby identifying the substrate, while a second laser determines the amount of bound analyte by assessing the magnitude of the phycoerythrin signal.

GCT stromal cells were grown to confluence in 55cm^2 Petri dishes. Cell lysates and serum-free D-MEM conditioned by stromal cell cultures for 24 hours were collected separately. Total protein content in the lysates was quantified using the Bradford microassay procedure. Additionally, the total number of cells present at the time of the conditioned medium collection was determined by hemocytometer. Simultaneous

quantification of MMP-1, MMP-8 and MMP-13 in the conditioned medium and lysates was achieved on the Multiplex Assay System using Flurokine MAP Human MMP kits (R&D Systems), as per the manufacturer's instructions.

Immunohistochemistry

Paraffin-embedded tissue samples of GCT were cut and mounted onto slides. Tissue sample slides were de-paraffinized in several washes of xylene. Slides were blocked for endogenous peroxidase activity by incubation with 3% hydrogen peroxide for 10 minutes and subsequently washed in 1× TBS-T (Tris-buffered saline with Tween 20) before treatment with 5% normal horse serum for 30 minutes. Next, sample slides were incubated at room temperature for 1 hour in a moist chamber with various dilutions of primary antibodies that included MMP-1 (Calbiochem), MMP-8, and MMP-13 (R&D Systems). Slides were then rinsed three times in TBS-T and incubated for a further 30 minutes at room temperature with a 1:500 dilution of secondary anti-mouse/rabbit/goat immunoglobulin (IgG) (Sigma-Aldrich), as dictated by the primary antibody. Following a further wash with TBS-T and ABC conjugation (Vector Laboratories; Burlington, Ontario, Canada), substrate colour was developed for 2 to 10 minutes at room temperature using a liquid $3,3_0$-diaminobenzidine (DAB) substrate-chromogen solution kit (Dako Canada; Mississauga, Ontario, Canada). Slides were counterstained in hematoxylin, dehydrated in graded ethyl alcohol (70%, 90% and 100%), and mounted in Permount. Preparations without the primary antibody served as negative controls.

Standardized collagenase activity assays

Serum-free D-MEM conditioned by confluent stromal cell cultures for 24 hours were concentrated approximately 40 fold using Amicon Ultra-4 Centrifugal Filter Devices (Millipore; Etobicoke, Ontario, Canada) and analyzed using SensoLyte Plus 520 MMP-1 and SensoLyte Plus 520 MMP-13 Assay Kits (AnaSpec; San Jose, California, USA), as per the manufacturer's instructions. The kits employ 96-well cell culture plates coated with monoclonal anti-human MMP-1 or MMP-13 antibodies that recognize both the latent and active forms of the enzymes. The specificity of the monoclonal antibodies prevents cross-reactivity with other MMPs. Isolated pro-MMP-1 or pro-MMP-13 is subsequently activated by incubation with 4-aminophenylmercuric acetate (APMA) at 37˚C. Proteolytic activity of the enzymes is measured using a fluorescence resonance energy transfer (FRET) peptide containing a quenched fluorophore. Upon cleavage by MMP-1 or MMP-13, fluorescence of the fluorophore was recovered and was measured on a CytoFluor Multi-Well Plate Reader series 4000 (PerSeptive Bio-systems/Applied Biosystems; Streetsville, Ontario, Canada) following an eight-hour incubation period, with an excitation and emission wavelength of 485 ± 20nm and 530 ± 25nm, respectively. Due to equipment design limitations, the contents of each well from the kit's cell culture plate were transferred to a new 96-well cell culture plate immediately prior to quantification.

Statistical analyses

Statistical analyses for the real-time PCR data were performed using the two-sample independent student's *t*-test. The average value of MMP gene expression within each

experiment was expressed relative to the expression of the GAPDH internal control. Results from repeated experiments were subsequently compared to the results of hFOB 1.19 cells and accepted as significant if $P \leq 0.05$.

Results

Collagenase mRNA expression

Amplification of total mRNA isolated from GCT stromal cell lysates and reverse-transcribed into cDNA was evaluated for collagenase expression and quantified by real-time PCR. Results are summarized in Fig. 1. Significantly greater MMP-13 expression was detected in all nine GCT stromal cell cultures when compared to hFOB 1.19 cells and using the two-sample independent student's *t*-test ($P \leq 0.05$). Furthermore, six cell cultures (GCT-1, -2, -3, -5, -8, and -9) similarly expressed MMP-1 mRNA levels that were significantly greater than that of hFOB 1.19 cells, and of those, three cultures (GCT-1, -3, and -9) also expressed significantly greater MMP-8 levels. The hFOB 1.19 cells were employed for comparative reasons due to the mesenchymal origin of the GCT stromal cells [7].

Collagenase protein expression

Western blotting was performed on GCT stromal cell lysates. Both MMP-1 and MMP-13 proteins were detected in all stromal cell lysates as shown in the representative results of Fig. 2. However, MMP-8 was not observed by western blotting, despite positive detection of the control peptide. Band sizes for MMP-1, MMP-8 and MMP-13 corresponded with the anticipated 54, 75 and 55 kDa sizes of the latent enzymes,

respectively. Collagenase protein expression was quantified in a select number of stromal cell lysates, as well as in media conditioned by the cells for 24 hours, using a multiplex assay system, as shown in Table 3. Due to the clinical nature of the cell lines and the variability in available sample sizes, not all cell lines were available at the time of analysis and only the cell lines presented in Table 3 were analyzed. Results are given as total pg/mL for each sample. Data were subsequently normalized to pg of MMP per 100μg total proteins or per 1000 cells for the lysate and conditioned medium samples, respectively. Although overall expression of MMPs in the lysates and subsequent secretion into the conditioned medium varied for each cell line, MMP-8 expression was consistently negligible, whereas MMP-1 and MMP-13 expression was detected in greater quantities.

Immunohistochemistry analyses of GCT

To evaluate whether collagenase expression in GCT was an artifact of cell culturing, immunohistochemical staining of GCT tissue samples with monoclonal anti-human collagenase antibodies was performed. As shown in Fig. 3, results from paraffin-embedded GCT-8 tissue revealed localization of both MMP-1 and MMP-13 in the stromal cell population of GCT. MMP-1 antibody staining was additionally present in many giant cells. However, MMP-8 was not detectable in any of the GCT cell types, despite positive staining in human breast cancer tissue (data not shown), which has previously demonstrated expression of MMP-8 [26]. The collagenase staining pattern observed with GCT-8 was confirmed through analysis of 23 archival GCT specimens obtained through our institution (data not shown).

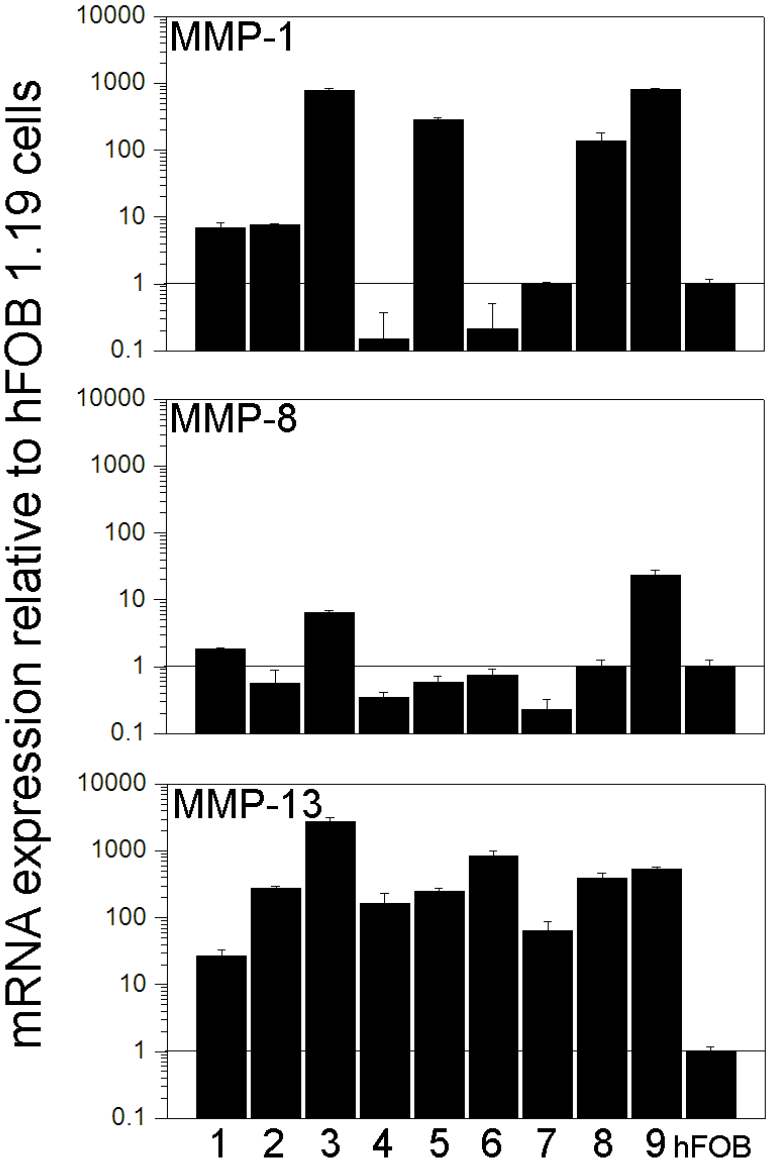

Fig. 1 — Real-time PCR of cDNA from GCT stromal cell lysates showing expression of MMP-1, MMP-8 and MMP-13 relative to hFOB 1.19 cells ("hFOB"). Total cDNA from GCT-1 through 9 are indicated as 1 through 9, respectively. Results are the average of two replicate experiments.

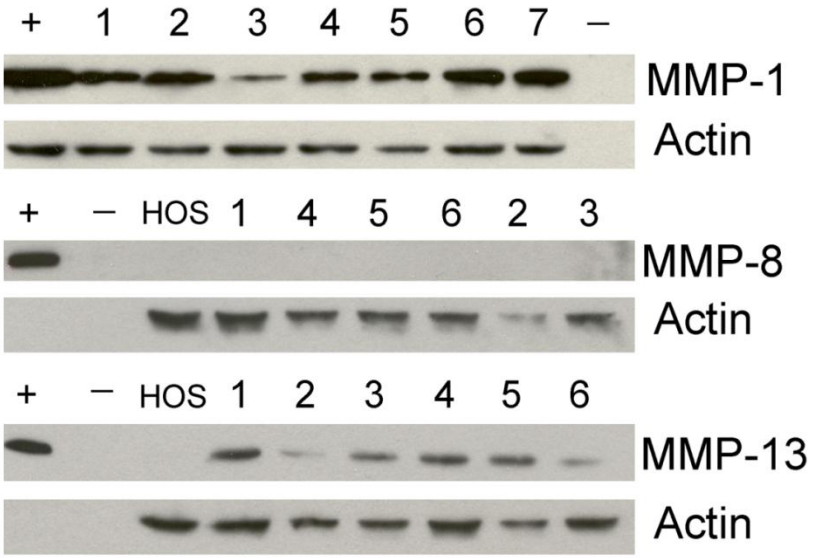

Fig. 2 — Representative western blot analyses of GCT stromal cell lysates for MMP-1, MMP-8 and MMP-13. The blots show protein expression by HOS and GCT stromal cell lines. Corresponding numerical values represent GCT-1 through 9. Controls include serum-free D-MEM (-) and recombinant protein standards for MMP-8 and MMP-13 (+). HOS serves as a positive control for MMP-1. Anti-actin antibodies serve as an internal control. Results are each representative of three independent experiments.

Table 3 — Quantification of MMP-1, MMP-8 and MMP-13 protein levels from GCT stromal cell lysates and conditioned medium by a multiplex assay system.

	MMP-1		**MMP-8**		**MMP-13**	
Conditioned Media	pg/mL	N[a]	pg/mL	N[a]	pg/mL	N[a]
GCT-1	96.00 ± 3.3	1.164	7.33 ± 3.3	0.089	15.83 ± 2.2	0.192
GCT-2	107.67 ± 1.7	1.485	0.17 ± 1.6	0.002	599.33 ± 12.2	8.267
GCT-4	3.33 ± 0.2	0.065	2.00 ± 3.3	0.039	35.33 ± 6.6	0.689
GCT-5	3495.33 ± 105.7	50.841	0.00 ± 2.8	0.000	301.83 ± 41.3	4.390
GCT-7	13.00 ± 0.9	0.067	0.00 ± 0.8	0.000	32.67 ± 2.6	0.168
GCT-9	567.5 ± 18.4	3.266	1.33 ± 1.7	0.008	142.17 ± 6.6	0.818
Lysates	pg/mL	N[b]	pg/mL	N[b]	pg/mL	N[b]
GCT-1	1374.17 ± 165.9	60.0	53.0 ± 6.0	2.3	901.83 ± 124.5	39.4
GCT-2	15830.67 ± 1598.0	1224.7	60.5 ± 1.0	4.7	4185.67 ± 231.6	323.8
GCT-3	13179.33 ± 253.1	1529.7	64.33 ± 3.9	7.5	27984.33 ± 114.6	3248.2
GCT-4	140.5 ± 50.0	11.1	49.0 ± 4.1	3.9	2167.83 ± 55.5	171.7
GCT-5	684.5 ± 33.4	89.6	51.67 ± 2.6	6.8	13887.83 ± 449.7	1818.1
GCT-6	54.5 ± 4.8	3.5	48.33 ± 2.3	3.1	1685.83 ± 21.1	107.2
GCT-7	254.83 ± 13.4	9.4	45.5 ± 1.3	1.7	1358.33 ± 72.9	50.1

a – mean data normalized (N) to pg per 1000 cells

b – mean data normalized to pg per 100 μg total protein

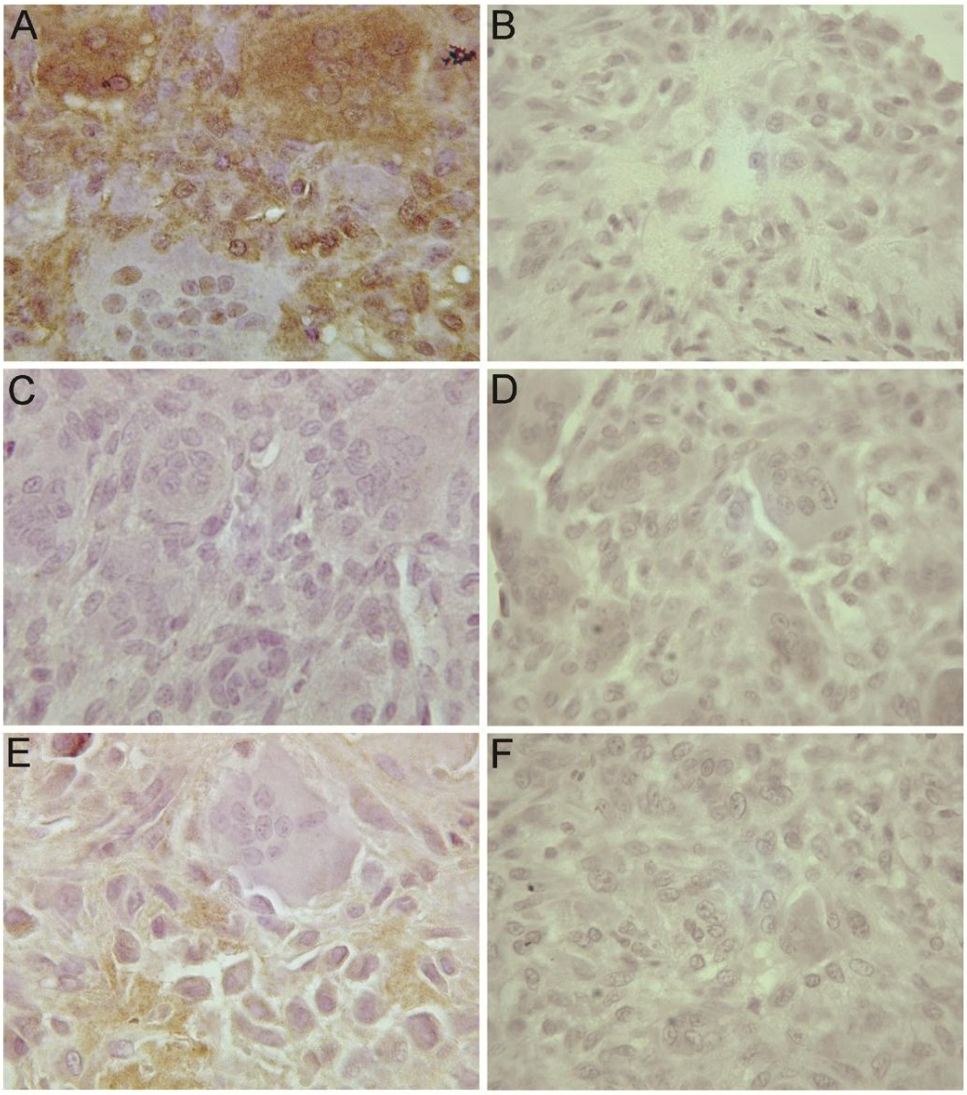

Fig. 3 — Representative immunohistochemistry staining of paraffin-embedded GCT-8 tissue samples with monoclonal anti-human (A) MMP-1 (1:100), (C) MMP-8 (1:50) and (E) MMP-13 (1:50) antibodies. The negative controls for each antibody are shown in (B) for MMP-1, (D) for MMP-8 and (F) for MMP-13. Original magnification × 400.

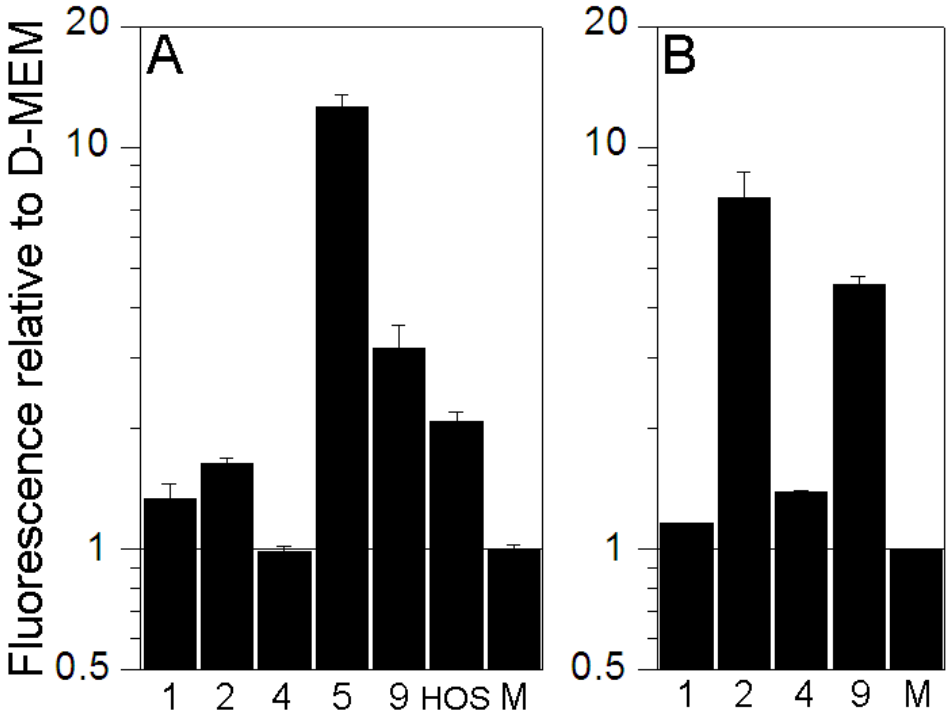

Fig. 4 — Proteolytic activity of (A) MMP-1 and (B) MMP-13 in concentrated media conditioned by HOS or GCT stromal cells for 24 hours, as determined by protease-specific standardized activity assays and relative to serum-free D-MEM ("M"). Quantification was achieved through measurement of fluorescence following incubation with a quenched fluorophore that fluoresced upon proteolytic cleavage by MMP-1 or MMP-13. GCT cell lines tested are indicated numerically and refer to GCT-1, -2, -4, -5, and -9, respectively.

Collagenolytic activity

To confirm the functional abilities of the secreted proteases, enzyme-specific activity assays that utilize a fluorescently-quenched peptide, which fluoresces upon proteolytic cleavage, were employed. Collagenase activity was measured following activation of latent enzymes with APMA. Concentrated media conditioned by stromal cells from selected GCT cell lines showed increased fluorescence relative to media alone using both MMP-1 and MMP-13-specific assays (Fig. 4). Media conditioned by HOS cells, which are known to have MMP-1 activity, are shown for comparison. The conditioned media all presented a range of fluorescent activity that was elevated when compared to serum-free D-MEM. The only exceptions were the GCT-4 samples, which did not show MMP-1 activity (0.99 ± 0.03) despite positive MMP-13 activity (1.39 ± 0.01).

Discussion

A select number of mammalian proteases are capable of cleaving the native fibrillar structure of type I collagen. These enzymes are principally cathepsin K [27], membrane-type 1 MMP (MMP-14) [28], and the interstitial collagenases (MMP-1, MMP-8 and MMP-13); however, gelatinase-A (MMP-2) has also shown a weak affinity for fibrillar collagen [29]. Although we [16], and others [18, 19], have previously reported the production of MMP-2 by the GCT stromal cells, this study focused on the secreted collagenases, which function under physiologic conditions of neutral pH. In contrast, cathepsin K is active within the acidic pH range that is characteristic of an osteoclast environment, and indeed, its production has been associated with the multinucleated giant cells [30-32].

Osteoclasts resorb the bone matrix through the combined actions of vacuolar H^+-ATPase, which demineralizes the crystals of hydroxyapatite [33], and cathepsin K, which largely degrades the organic components of bone [31, 32]. Physiologically, therefore, cathepsin K can be considered the principal protease involved in bone resorption. However, the activity of both enzymes is dependent upon the formation of a ruffled membrane in the osteoclast: a characteristic morphological change comprised of actin rings. Degradation of type I collagen by interstitial collagenases was shown to stimulate the formation of ruffled membranes, thereby inciting osteoclast activity [34, 35]. Osteoclasts themselves do not produce soluble collagenases [34, 36], although MMP-13 is detectable at the resorption zone [37, 38], implying an alternate source for the enzymes such as osteoblasts and other fibroblast-like cells [34].

Here we report the expression of MMP-1 and MMP-13 by the stromal cell population of GCT from nine affected patients. The enzymes were sufficient for proteolytic activity, as determined by standardized activity assays, although stromal cells have previously shown an inability to independently resorb dentine slices [14, 30]. However, the resorptive activity of the osteoclast-like giant cells is enhanced by the presence of stromal cells [12, 14, 15], suggesting that the stromal cells may encourage giant cell activity through the degradation of type I collagen. The stromal cells are the neoplastic element of GCT [7], and have previously shown an ability to stimulate the formation of multinucleated cells from mononuclear cells [6, 39-41]. Moreover, the stromal cells may be at least partly responsible for the initial recruitment of mononuclear osteoclast precursors to the tumour site [42, 43]. Taken together, these results suggest that the

spindle-like stromal cells of GCT largely influence the initiation, propagation and activity of the osteolytic tumour through the formation and activation of the giant cells.

The mRNA expression of each collagenase, as determined by real-time PCR (Fig. 1), largely correspond with the relative protein levels obtained using the multiplex assay system (Table 3). Although elevated MMP-8 mRNA expression was detected in GCT-1, -3 and -9, the results are relative to hFOB 1.19 cells, which are not known to produce MMP-8. Accordingly, all other experimental approaches suggest that MMP-8 expression was consistently negligible in all GCT stromal cell lines. With respect to MMP-1 and MMP-13, the majority of each enzyme was detected in the cell lysates, although it is possible that increased amounts of protein in the surrounding medium are achievable if the incubation period was sustained beyond 24 hours. Indeed, immunohistochemical analyses of GCT tissue revealed considerable positive staining of both MMP-1 and MMP-13 in the stromal cell population (Fig. 3). Regardless, the amount of collagenase secreted into the media was sufficient for proteolytic activity, as determined by the enzyme-specific standardized activity assays (Fig. 4).

Due to the limited availability of some clinical specimens, only a select number of GCT cell lines were available for analysis by the multiplex assay system and the standardized activity assays. Despite this fact, we have demonstrated that MMP-13 is consistently and highly expressed by the spindle-like stromal cell population from GCT. These results expand on previous isolated reports of interstitial collagenase expression in GCT. Microarray analyses of whole GCT samples have demonstrated that MMP-13 is highly expressed by the tumour [44, 45], and real-time PCR analysis of total GCT mRNA

also indicated elevated MMP-13 expression [30]. Few previous reports have examined MMP-1 expression in GCT. Lindeman *et al.* [30] demonstrated no detectable MMP-1 expression by real-time PCR analysis of total GCT mRNA from seven individual tumours. Conversely, we have demonstrated MMP-1 expression by the stromal cells from GCT using a variety of experimental protocols. The variability of MMP-1 expression and activity by these cells suggests that MMP-1 may not be present in all GCTs. However, the consistency of MMP-13 expression by the neoplastic stromal cells warrants further investigation into what role, if any, these collagenases have in the pathogenesis of the tumour.

The variability in collagenase expression amongst the different cell lines is likely a result of differences between patients. Indeed, many reports examining GCT samples from different patients detail differences in the expression of several genes [46]. These differences may be influenced by other factors produced by the stromal cells, including transforming growth factor-β1 (TGF-β1) [47], which has demonstrated the ability to stimulate expression of MMP-13 in renal carcinoma cells [48]. Although few papers have described the production of cytokines by GCT stromal cells, both interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α) are known to stimulate the expression of MMP-1 and MMP-13 expression in osteoarthritis and rheumatoid arthritis [49, 50], while interleukin-17 was shown to induce MMP-13 expression in osteoarthritic chondrocytes [51]. Similar cytokine-mediate mechanisms of stimulating collagenase expression may occur in GCT. Furthermore, it cannot be conclusively ruled out that there may be contaminating cells in the primary cultures, which may influence collagenase expression

by the stromal cells, as the cell lines were established from individual patients. There was no discernable correlation between collagenase expression and aggressiveness of the tumours, as determined by the Campanacci classification. This lack of correlation is not surprising, as many reports have described a similar inability to relate aggressiveness to their findings [46].

In the context of GCT, the role of stromal cell-derived MMP-1 and MMP-13 may be giant cell stimulation, as described above. However, the collagenases themselves may be contributing to bone resorption following demineralization by the giant cells. This intriguing consideration may help account for the extensive bone resorption that is characteristic of GCT in cases where fewer giant cells are present [52, 53]. In point of fact, it is the spindle-like stromal cells, and not the giant cells, that are present at the margins of the tumour where bone resorption occurs [53]. Further, these enzymes may have regulatory functions, as MMP-13 was previously shown to activate other latent MMPs [54] and can be activated by other proteases found in GCT [55].

In summary, we have demonstrated that the stromal cells derived from patients with GCT produce MMP-1 and MMP-13, but not MMP-8, and that the enzymes were sufficient for proteolytic activity *in vitro*. The role of the stromal cell-derived collagenases in GCT remains undetermined, although they may aid in bone resorption either directly, or through the stimulation of giant cell activity. Future study is required to elucidate the mechanism of collagenase expression in GCT and to determine the role of these enzymes *in vivo*.

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CHAPTER 3

PTHrP increases RANKL expression by stromal cells from giant cell tumor of bone

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Preface

Perhaps the most pertinent and least understood aspect of GCT is its origin. GCTs and brown tumours of hyperparathyroidism share many radiologic and histologic features (as reviewed by Unni & Inwards, 2010a), and brown tumours are sometimes mistaken for GCTs (Guliaeva et al., 2009; Vera et al., 2011). I therefore hypothesized that GCT may be related to brown tumours and other giant cell-containing lesions, and investigated the expression of PTHrP within GCT and its potential role in bone resorption. In particular, we examined its effects on giant cell formation, as elevated serum PTH characteristically promotes osteoclastogenesis through the stimulation of RANKL expression (Horwood et al., 1998; Huang et al., 2004a), which is also overexpressed in GCT (Skubitz et al., 2004).

This chapter consists of an author-generated version of an article accepted on October 31st, 2011 for publication in the *Journal of Orthopaedic Research*. This article is reprinted with permission from John Wiley & Sons, Inc. (see Appendix B for License Agreement). Please note that American spelling is used throughout the article, as the journal format dictated. I performed all the experiments, assembled the results, generated the figures, and wrote and revised the manuscript. Gurmit Singh and Michelle Ghert made revisions to the manuscript and provided intellectual direction and advice.

These results demonstrate using immunohistochemistry that PTHrP and its receptor (PTH1R) are expressed within GCT, confirming two isolated reports described previously (Kartsogiannis et al., 1998; Nakashima et al., 2003). However, we have further examined PTHrP and PTH1R expression in isolated stromal cell cultures and show their continued expression *in vitro* through PCR and western blot analyses. These results were also the

first to describe an effect of PTHrP stimulation of GCT stromal cells, which resulted in time-dependent increases in RANKL and OPG gene expression and an increased ability to stimulate multinucleated cell formation from murine monocytes. Moreover, inhibition of PTHrP with purified antiserum decreased RANKL gene expression and thereby revealed the potential importance of PTHrP in the pathophysiology of GCT. These results suggest that PTHrP may influence bone resorption in GCT through promoting increased giant cell formation.

These results also formed the basis for other subsequent investigations into the role of PTHrP in GCT. For example, we found through these experiments that inhibition of PTHrP with purified antiserum prevented GCT stromal cell growth, whereas IgG-matched control antibodies did not prevent growth. This discovery led us to investigate the involvement of PTHrP in cell survival and apoptosis (Mak et al., 2011). Results indicated that PTHrP antiserum induced apoptosis in GCT stromal cells, potentially through a caspase-independent mechanism. Therefore, PTHrP may be inferred to have a protective effect against apoptosis in the stromal cells. This effect has also been described previously in renal carcinomas (Massfelder et al., 2004; Talon et al., 2006). However, further elucidation of a role for PTHrP in proliferation of GCT cells is required.

Title: **PTHrP increases RANKL expression by stromal cells from giant cell tumor of bone**

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Abstract

Giant cell tumor of bone (GCT) presents with numerous osteoclast-like multinucleated giant cells that are principally responsible for the extensive bone resorption by the tumor. Although the precise etiology of GCT remains uncertain, the accumulation of giant cells is partially due to the high expression of the receptor activator of nuclear factor-κB ligand (RANKL) from the neoplastic stromal cells. Here, we have investigated whether parathyroid hormone-related protein (PTHrP) plays a role in the pathogenesis of GCT. Immunohistochemistry results revealed PTHrP expression in the stromal cells of the tumor, and that its receptor, the parathyroid hormone type 1 receptor (PTH1R), is expressed by both the stromal cells and giant cells. PCR and Western blot analyses confirmed the expression of PTHrP and PTH1R by isolated stromal cells from five patients presenting with GCT. Treatment of GCT stromal cells with varying concentrations of PTHrP (1-34) significantly increased both RANKL gene expression and the number of multinucleated cells formed from RAW 264.7 cells in co-culture experiments, whereas inhibition of PTHrP with a neutralizing antibody decreased RANKL gene expression. These results suggest that PTHrP is expressed within GCT by the stromal cells and can contribute to the abundant RANKL expression and giant cell formation within the tumor.

Keywords: giant cell tumor; osteoclastogenesis; PTHrP; RANKL; OPG

Introduction

Giant cell tumor of bone (GCT), an aggressive primary osteolytic bone tumor, is composed of three cell types: the multinucleated osteoclast-like giant cells; the mononuclear cells of monocytic-macrophage origin; and the mesenchymal spindle-like stromal cells. Giant cell formation is thought to occur in a similar manner as osteoclastogenesis, where osteoblasts express the receptor activator of nuclear factor-κB ligand (RANKL), which stimulates its receptor, RANK, on osteoclast precursor cells and initiates their fusion into osteoclasts [1,2]. In GCT, it is the neoplastic stromal cells that are known to express RANKL [3,4], and are thought to stimulate giant cell formation from the RANK-expressing monocytic cells [5]. In fact, GCT stromal cells were previously shown to induce osteoclast-like cells from human blood monocytes [4,6,7] and the murine myeloid cell line, RAW 264.7 [8].

Parathyroid hormone-related protein (PTHrP) is a small protein known to play a pivotal role in skeletal development [9] that is also expressed by a variety of cancer [10-13] and normal cells [13,14]. It shares structural similarities with parathyroid hormone (PTH) and recognizes the same receptor, the PTH type 1 receptor (PTH1R) [15]. PTH is known to stimulate RANKL expression in osteoblasts [16,17], and indeed, hyperparathyroidism increases the number of osteoclasts formed in bone lesions known as brown tumors. Brown tumors present with similar clinical features as GCTs [18-22], but are considered less infiltrative and are discernible by elevated PTH levels. PTHrP itself is known to increase bone resorption when secreted systemically by malignant tumors [10,23], and was previously shown to stimulate the formation of osteoclast-like

cells to the same degree as PTH in mouse bone marrow cultures [24]. GCT is known to respond to PTH stimulation [25-29], suggesting GCT may also respond to PTHrP.

Several other giant cell-containing lesions were reported to express PTHrP, including giant cell tumor of tendon sheath [30], and giant cell granulomas of the jaw [31]. Therefore, despite several clear differences between GCT and brown tumors, such as the systemic nature of hyperparathyroidism, we postulated that there may be some commonalities amongst these lesions, and that PTHrP may act locally within GCT and contribute to its phenotype. Specifically, we investigated whether PTHrP has any effect on the formation of osteoclast-like giant cells in GCT.

Methods

Cells and cell culture

Primary cell cultures were established from five patients presenting with GCT, following patient consent and approval of our institution's Research Ethics Board (Table 1). GCT stromal cells were isolated in culture as previously described [32]. All other cell lines were obtained from the American Type Culture Collection, Manassas, VA: 786-0 renal cell adenocarcinoma cells (ATCC# CRL-1932); MRC-5 normal lung fibroblasts (ATCC# CCL-171); and the mouse RAW 264.7 monocytes (ATCC# TIB-71). Cells were maintained in medium supplemented with 10% fetal bovine serum, 2mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin (Invitrogen Canada/Gibco, Burlington, ON, Canada) at 37ºC in humidified air with 5% CO_2. GCT-5 and MRC-5 cells were maintained in RPMI-1640, while α-minimum essential media (α-MEM) was used for RAW 264.7 cells. All other cell lines used Dulbecco's modified Eagle medium

(D-MEM). GCT stromal cell cultures between passages 5 and 10 were used for all experiments. The 786-0 cells serve as positive controls for PTHrP and PTH1R in PCR and Western blot analyses. RAW 264.7 monocytes were used in the osteoclastogenesis assays, and MRC-5 fibroblasts were assayed by Western blotting for comparison with GCT-5 cells.

Table 1 – Details of GCT patient specimens.

Case	Gender	Age	Location
GCT-1	Male	58	Proximal phalanx
GCT-2	Female	26	Proximal tibia
GCT-3	Female	31	Distal ulna
GCT-4	Female	34	Tibia
GCT-5	Male	23	Lung

Immunohistochemistry

Formalin-fixed paraffin-embedded GCT tissue samples were cut and mounted onto slides, washed in xylene and ethanol to remove paraffin, and subsequently treated with either 0.02 U/mL neuraminidase (EMD Chemicals, Mississauga, ON, Canada) for 60 min to reveal PTHrP epitopes, or 10mM citrate buffer, pH 6.0, at 95ºC for 30 min to reveal PTH1R epitopes. Endogenous peroxidase activity was blocked with 3% H_2O_2 for 5 min then washed with TBS-T and treated with 5% normal goat serum for 20 min. Overnight incubation of slides at 4ºC with 10 µg/mL rabbit polyclonal anti-PTHrP (Abcam #

ab16827) or 20 µg/mL rabbit polyclonal anti-PTH1R (Abcam # ab13078, Cambridge, MA) antibodies was followed by 30 min room temperature incubation with secondary antibody and 30 min treatment with ABC solution (Vector Laboratories, Burlington, ON, Canada), as per the manufacturer's instructions. Substrate color was developed using a Nova Red Kit and slides were counterstained with hematoxylin, dehydrated in graded ethanol and mounted in Permount. Sections of paraffin-embedded tissue samples from human renal carcinoma and normal human hippocampus (US Biomax, Inc., Rockville, MD) were similarly treated for comparison.

PCR

GCT stromal cells and 786-0 cells were grown to confluence in 55 cm^2 Petri dishes. RNA was collected using the TRIzol (Invitrogen Canada/Gibco) collection method and 1 µg total RNA was reverse-transcribed into cDNA using Superscript III (Invitrogen Canada/Gibco), according to the manufacturer's instructions. Previously published primer sequences for total PTHrP, as well as the 139, 141 and 173 amino acid isoforms were used (Table 2). Primer sequences for PTH1R were obtained from the PrimerBank (# 4506271a3). Amplification was performed using an MJ Mini Personal Thermal Cycler (Bio-Rad Laboratories, Mississauga, ON, Canada) and the following protocol: 94ºC for 3 min, followed by 40 cycles of 94ºC for 30 s, 55ºC for 30 s, 72ºC for 60 s, and a final 10 min extension at 72ºC. Samples were separated on a 2% agarose gel and visualized using ethidium bromide on a Molecular Imager Gel Doc XR System (Bio-Rad Laboratories).

Table 2 – PCR primer information. PTHrP primers employed in PCR experiments used a common sense (S) primer, and different anti-sense (A) primers to amplify different products, as indicated. PTH1R primers are also listed. GAPDH primers serve as an internal control.

Gene	S/A	Primer Sequence (5′→3′)	Product	Size of product (bp)	Ref.
PTHrP	S	CGGTGTTCCTGCTGAGCTA			[44]
PTHrP	A	TGCGATCAGATGGTGAAGGA	Total PTHrP	161	[44]
PTHrP	A	CACAATCGATAGAGATAC	139aa isoform	603	[45]
PTHrP	A	CAGAATCCTGCAATATGTCC	141aa isoform	543	[45]
PTHrP	A	GAGATCATTAGTTGCATATG	173aa isoform	565	[45]
PTH1R	S	CTCCGGGAACAAAAAGTGGAT			
PTH1R	A	CTGAGACCTCGGTGTATGGTG		236	[46,47]
GAPDH	S	AACTTTGGTATCGTGGAAGGA			
GAPDH	A	CAGTAGAGGCAGGGATGATG		136	

Western blotting

Cells were plated in 55 cm^2 Petri dishes and grown to confluence. Cells were scraped and collected in lysis buffer composed of 50mM Tris pH 7.4, 0.4M NaCl, 5mM EDTA pH 8.0, and 1% NP-40 and contained protease inhibitor cocktail tablets (Hoffmann-La Roche, Mississauga, ON, Canada). Normal murine brain homogenates from Balb/c nude mice were prepared using tissue from our institutional tumor bank by macerating 35 mg

tissue in 350 µL of the lysis buffer described above. Protein concentration was determined by the Bradford microassay procedure and 35 or 100 µg samples were electrophoresed by 10% or 15% SDS-PAGE for PTH1R and PTHrP blots, respectively. Samples were transferred to a nitrocellulose membrane using transfer buffer containing 40% methanol at 15 V for 5 h for PTHrP blots, or 20% methanol at 100 V for 1 h for PTH1R blots, and then blocked overnight with 5% skim milk in TBS-T at 4ºC. Membranes were probed with 1:100 rabbit polyclonal anti-PTHrP (Abcam # ab16827 and ab40642) or 1:2000 rabbit polyclonal anti-PTH1R (Abcam # ab75150) for 3 h at room temperature. Goat anti-rabbit IgG-HRP secondary antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were employed and PTHrP and PTH1R protein was visualized using enhanced chemoluminescence detection (Amersham Biosciences/GE Healthcare Bio-Sciences Inc., Baie d'Urfé, QC, Canada) according to the manufacturer's instructions. Blots were re-probed with monoclonal anti-actin (MP Biomedicals, Solon, OH), which served as a loading control.

Real-time PCR

GCT stromal cells (1.5×10^6 cells) were seeded in 55 cm^2 Petri dishes and treated with 0, 1, 10 or 100 nM PTHrP (1-34) (Bachem) prepared in supplemented D-MEM for specified intervals. Alternatively, cells were treated with 5 or 10 µg/mL rabbit anti-PTHrP (1-34) (Bachem, Torrance, CA) or 5 or 10 µg/mL rabbit IgG control (Genscript, Piscataway, NJ) for 24 h. RNA was collected and reverse-transcribed as described above. Gene expression was analyzed by real-time PCR on a MiniOpticon System (Bio-Rad Laboratories) using Express SYBR Green (Invitrogen Canada/Gibco) and the primer pairs

listed in Table 3. The reaction amplified 1 µg of total RNA and was achieved in a volume of 20 µL using the following protocol: 50ºC for 2 min, then 95ºC for 2 min, followed by 40 cycles of 95ºC for 15 s and 60ºC for 60 s, with a gradient change in temperature from 60 to 95ºC to determine the melting curve of the final products. Gene expression was quantified according to the comparative threshold cycle method (ΔΔCt). Results are pooled from three to four separate experiments.

Table 3 – Real-time PCR primer information. RANKL gene expression analyses employed the TAF10 housekeeping gene, whereas the RNA polymerase II polypeptide A (POLR2A) gene served as a normalization control for OPG gene expression. Sense (S) and anti-sense (A) primers are shown.

Gene		Primer Sequence (5′→3′)	GenBank accession no.	Bases	Size of product (bp)
RANKL	S	TATGCCAACATTTGCTTTCG	NM 003701	518-744	227
	A	TCGATGCTGATTTCCTCTCC			
OPG	S	CAGTGTCTTTGGTCTCCTGCT	NM 002546	535-772	238
	A	GCTGTGTTGCCGTTTTATCC			
POLR2A	S	GGGTGCTGAGTGAGAAGGAC	NM 000937	4420-4557	138
	A	AGCCATCAAAGGAGATGACG			
TAF10	S	GAAGATTACACGCCTACGATCC	NM 006284	370-475	106
	A	CAGCTAAGGAGATGAGCCGAA			

Osteoclastogenesis assays

RAW 264.7 cells were seeded in 24-well plates at a density of 10^5 cells per well. GCT stromal cells were subsequently seeded in α-MEM within 0.4 μm cell culture inserts (BD Biosciences, Mississauga, ON, Canada) overlaying the RAW 264.7 cells at a density of 2×10^5 cells per insert. The plates were maintained at 37ºC in humidified air containing 5% CO_2, and after cell adherence, the media was removed and replaced with α-MEM containing 0, 10 or 100nM PTHrP (1-34) (Bachem). Cell culture medium was removed and refreshed every second day. Following 10 days of incubation, the cell culture inserts were discarded, and RAW 264.7 cells were fixed and stained for tartrate-resistant acid phosphatase (TRAP) activity using the Acid Phosphatase, Leukocyte (TRAP) Kit (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada), as per the manufacturer's instructions. Cells were visualized at 100× magnification using a light microscope. Electronic images of five pre-determined areas per well were obtained and multinucleated TRAP-positive cells containing three or more nuclei were counted in each image.

Statistical analyses

Statistical analyses for the real-time PCR and osteoclastogenesis assays were performed using the two-sample independent Student's *t*-test. Results of treated cells were compared to the control and accepted as significant if $P \leq 0.05$.

Results

PTHrP expression in GCT

To confirm the presence of PTHrP and PTH1R in GCT, immunohistochemistry was performed on paraffin-embedded GCT tissue samples (Fig. 1). Results revealed localization of PTHrP predominantly within the stromal cells of GCT. However, several giant cells also stained positive, in either the cytoplasm or nuclei. Both the giant cells and the stromal cells stained positive for PTH1R expression. Results were consistent for all GCT tissue samples tested (GCT-5 tissue was not formalin fixed at the time of sample collection, and was therefore not tested using immunohistochemistry). Renal carcinoma tissues showed positive expression of both PTHrP and its receptor, whereas normal adult hippocampus tissue revealed no expression of either protein.

Additionally, we assessed gene expression of PTH1R, as well as total PTHrP and its three human isoforms from cDNA generated using RNA isolated from GCT stromal cells and 786-0 cells, which are known to express all three PTHrP isoforms [33]. Positive PTHrP and PTH1R gene expression in all cell lines is shown in Figure 2. GCT stromal cell lysates were assessed by Western blotting and PTHrP was detected as a band with a molecular size of approximately 18-19 kDa (Fig. 3). Antibodies that recognize either the amino-terminal region (amino acids 1-35) responsible for stimulation of PTH1R, or the 70-89 amino acid midregion that does not cross-react with PTH were employed. PTHrP protein was detected in all GCT patient samples tested, including the metastasized sample collected from a lung. Renal cell adenocarcinoma cells also expressed PTHrP, as expected [34]. Normal fetal lung fibroblasts are also shown for comparison, and show

positive expression of PTHrP, which is widely expressed in lungs during development and first trimester lung mesenchyme was previously shown to express PTHrP mRNA [14]. GCT stromal cell lysates were also assayed for PTH1R expression by Western blotting. Results revealed multiple bands consistent with structural analyses indicating PTH1R exists primarily as a dimer, but becomes dissociated by ligand binding to become monomeric [35,36]. The positive control 786-0 cells showed a band at approximately 56 kDa, which is consistent with the manufacturer's information and is the monomeric form of PTH1R. The 786-0 cells also showed another more intense band at approximately 90-100 kDa, which corresponds to the hypothesis that PTH1R exists as a dimer [36]. PTH1R expression was also observed in all GCT cell lines, whereas mouse brain homogenates did not show positive expression of PTH1R.

PTHrP increases GCT stromal cell RANKL expression

Treatment of GCT-2 cells with varying concentrations of PTHrP (1-34) resulted in increased RANKL gene expression compared to control as early as 6 h after administration of the peptide (Fig. 4). The largest increase in RANKL gene expression, compared to control, occurred 16 h post-treatment, with both 10 nM ($P = 0.0048$) and 100 nM ($P = 0.0414$) PTHrP (1-34). OPG, which was previously shown to inhibit osteoclastogenesis in GCT [37], was also significantly increased at 6 h (1 nM: $P = 0.0312$; 10 nM: $P = 0.0174$), when compared to control. Indeed, peptide treatment initially increased OPG gene expression prior to an eventual decrease in expression, and analysis of the RANKL:OPG ratio suggests a decrease in factors that support osteoclastogenesis at 1 and 6 h following treatment, while prolonged treatment with

PTHrP for 16 and 24 h favors osteoclastogenesis. Moreover, inhibition of PTHrP with neutralizing antibodies for 24 h resulted in decreased RANKL gene expression compared to control (Table 4).

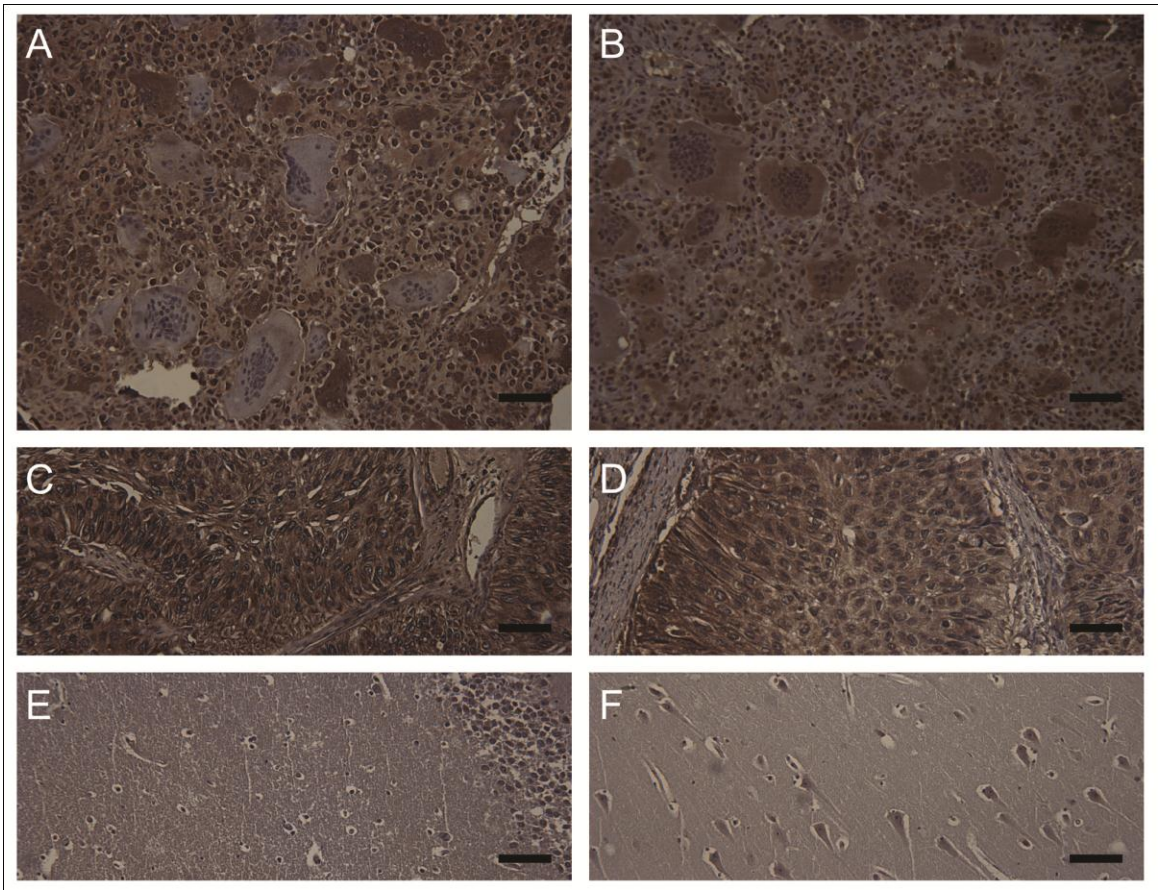

Figure 1 – PTHrP and PTH1R expression in paraffin-embedded GCT tissue samples. Representative immunohistochemistry staining results of GCT-1 (A,B), renal carcinoma (C,D) and normal hippocampus (E,F) tissue with polyclonal anti-PTHrP (A,C,E) or anti-PTH1R (B,D,F) antibodies. Magnification ×100; scale bar = 100 µm.

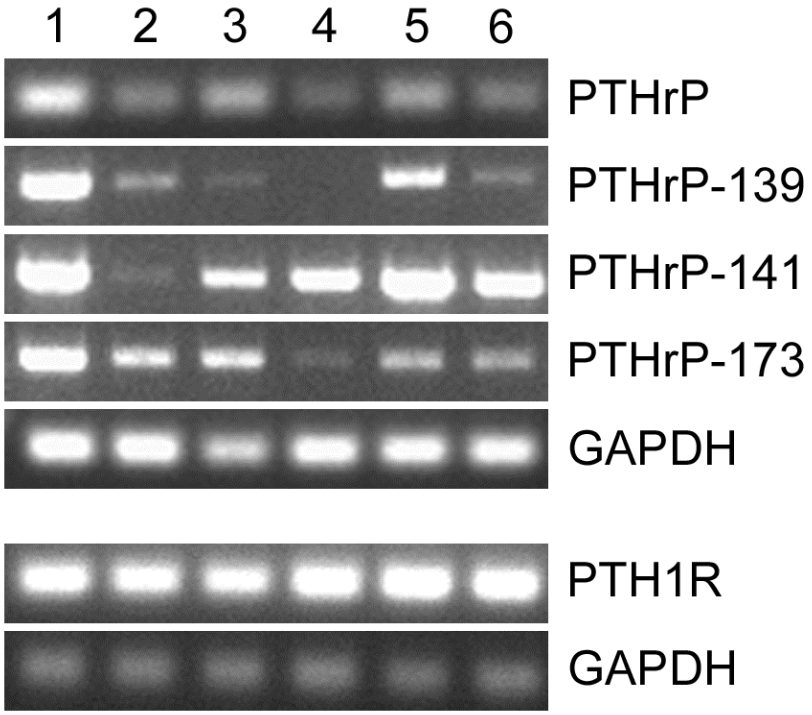

Figure 2 – PTHrP isoform and PTH1R gene expression in 786-0 and GCT cells. PCR results showing the 139 aa, 141 aa, and 173 aa PTHrP isoforms individually, or total PTHrP (top), as well as PTH1R gene expression in 786-0 (lane 1) and GCT-1 through 5 (lanes 2-6, respectively). GAPDH expression is shown for comparison.

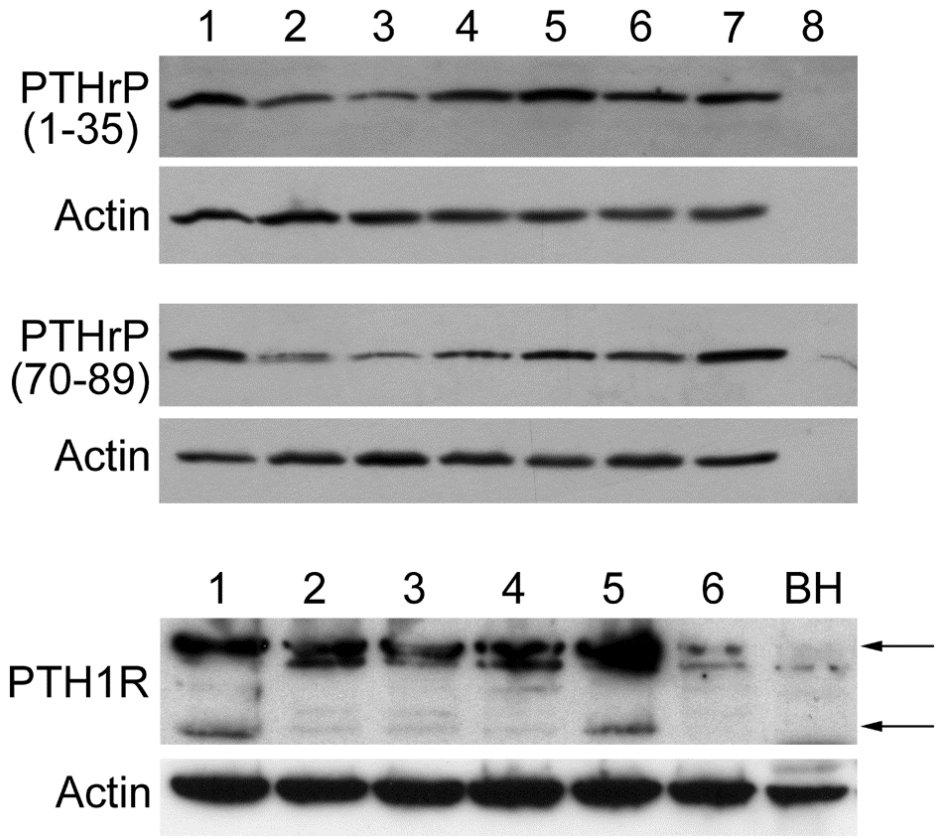

Figure 3 – Western blot analyses of PTHrP and PTH1R expression in GCT stromal cell lysates. Representative results from antibodies recognizing PTHrP (1-35), PTHrP (70-89), and PTH1R are shown for GCT stromal cell lines 1 through 5 (lanes 2-6, respectively). PTHrP and PTH1R expression in 786-0 (lane 1) lysates are also shown. For comparison, MRC-5 (lane 7) lysates and no cell lysate (lane 8) are shown for PTHrP blots, whereas mouse brain homogenates (BH) are shown for PTH1R. Actin serves as a loading control. Arrows indicate PTH1R.

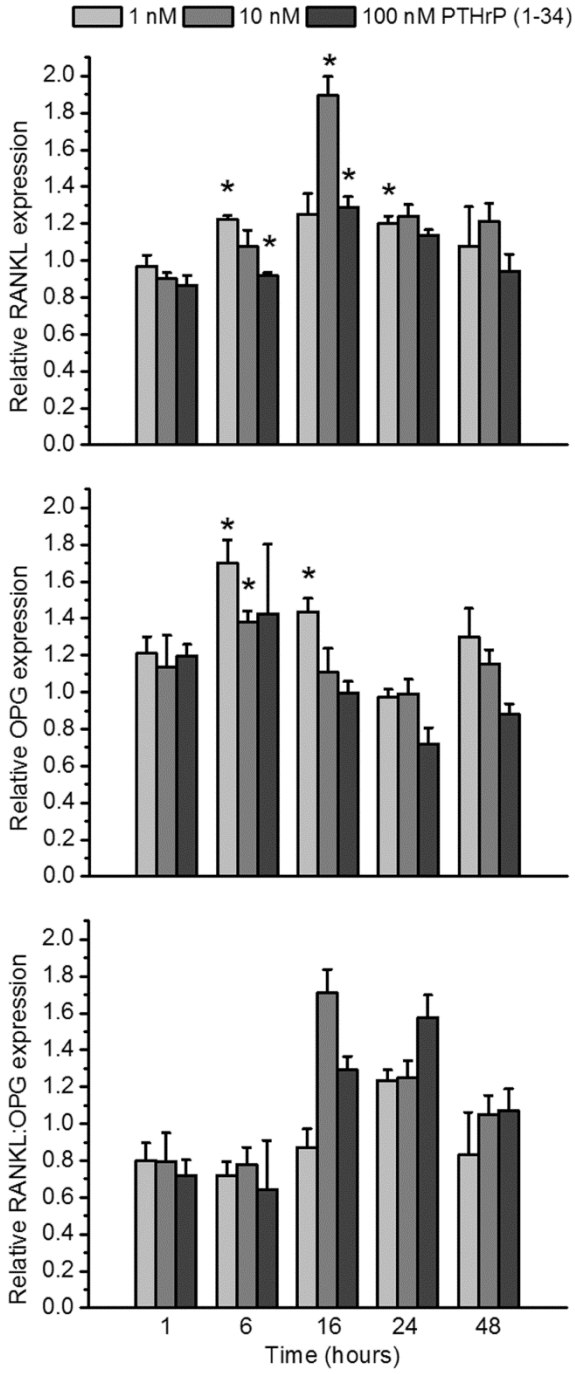

Figure 4 – Relative mRNA expression of GCT-2 cells treated with PTHrP (1-34). RANKL and OPG gene expression are shown at specific time intervals following administration of the peptide. Results are relative to untreated controls (=1). RANKL and OPG mRNA expression significantly different from control by the two-sample independent t-test are indicated (*). Error bars indicate standard error.

Table 4 – Relative mRNA expression of GCT-2 cells treated with PTHrP (1-34) purified rabbit antiserum. RANKL gene expression from GCT stromal cells treated with 5 or 10 µg/mL anti-PTHrP for 24 h relative to cells treated with IgG-matched control antibodies.

Treatment	Relative RANKL gene expression	*P* value
5 µg/mL anti-PTHrP	0.90 ± 0.004	0.0044
10 µg/mL anti-PTHrP	0.77 ± 0.022	0.0041

PTHrP stimulates GCT stromal cell-induced multinucleated cell formation

To functionally test whether the observed differences in gene expression translate into perceivable differences in osteoclastogenesis, GCT stromal cells were seeded in cell culture inserts overlaying the murine monocytic cell line RAW 264.7 and treated with PTHrP (1-34). The stromal cells induced a significantly increased number of TRAP-positive cell formation from the RAW 264.7 cells when either 10 nM ($P = 0.0146$) or 100 nM ($P = 0.0117$) PTHrP (1-34) was administered, compared to untreated controls (Fig. 5). However, no significant difference was detected between PTHrP-treated cells.

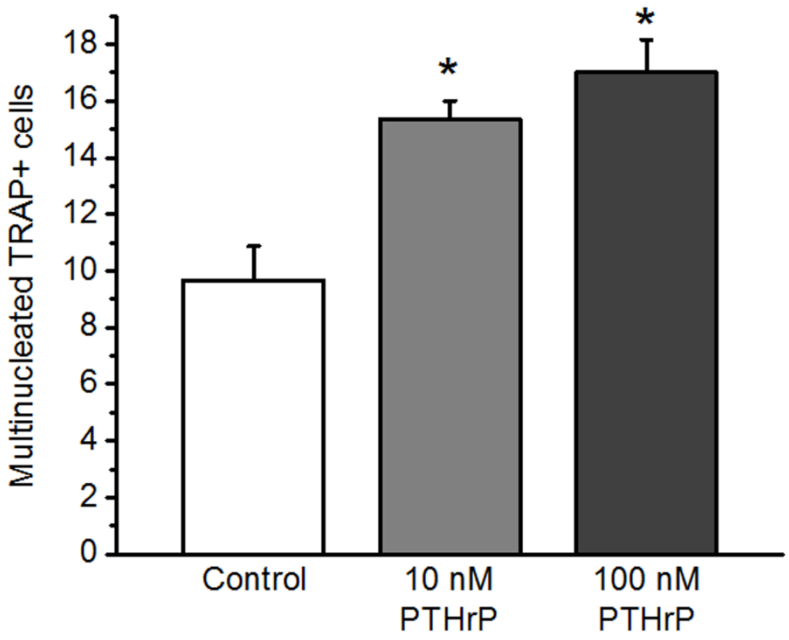

Figure 5 – GCT stromal cell-induced multinucleated cell formation. GCT stromal cells treated with PTHrP (1-34) increased the number of multinucleated TRAP-positive cells formed from RAW 264.7 cells in co-culture experiments. Cells from five pre-determined areas in each well were counted in three experiments. PTHrP (1-34) treatment of RAW 264.7 cells alone did not induce multinucleated TRAP-positive cell formation. Both co-cultures treated with PTHrP were significantly different from control by the two-sample independent t-test (*). Error bars indicate standard error.

Discussion

Immunohistochemistry analyses of paraffin-embedded GCT samples revealed strong PTHrP expression in the stroma of GCT (Fig.1). Other isolated reports have examined PTHrP expression in GCT. Kartsogiannis et al. [38] examined PTHrP expression in osteoclasts and found similar results to ours, where giant cells from GCT showed the presence of PTHrP in either the cytoplasm or the nucleus, or not at all. Nakashima et al. [39] similarly focused on the giant cells, showing positive expression for both PTHrP and PTH1R. However, both reports incidentally reveal strong PTHrP expression by the mononuclear stromal cells. Here, we have focused on the neoplastic stromal cells, and show through PCR analyses the transcription of all three PTHrP isoforms (Fig. 2). However, Western blot analyses showed only a single band (Fig. 3), suggesting not all isoforms are translated into proteins, or are not detectable by the antibodies employed. Future analysis using a more sensitive protocol may clarify which isoforms are expressed as a protein by the GCT stromal cells. Nevertheless, these combined results support and expand on the previously-mentioned studies indicating GCT stromal cells express PTHrP.

Both the stroma and the osteoclast-like giant cells showed strong expression of PTH1R. PTH1R expression by isolated GCT stromal cells was confirmed through PCR and Western blot analyses. These results correspond with previous studies showing various responses by GCT to PTH stimulation [25-29], which implied the presence of PTH1R. The expression of PTH1R by the giant cells is in line with the results discussed earlier [39], and suggests the giant cells themselves may respond to PTHrP stimulation. Normal osteoclasts produced from peripheral blood monocytes were shown to express

PTH1R and respond directly to PTH by increasing bone resorption activity [40]. Indeed, PTHrP may contribute to bone resorption in GCT, as PTHrP was previously shown to stimulate bone resorption by osteoclasts isolated from rats [41], and media conditioned by the PTHrP-producing breast cancer cell line MDA-231 also stimulated bone resorption by giant cells obtained from GCT [42].

We hypothesized that the stromal cell-derived PTHrP could act in an autocrine manner to stimulate RANKL expression. RANKL is characteristically overexpressed in GCT [43], and is thought to be largely responsible for the accumulation of giant cells within the tumor. Treatment of GCT stromal cells with the amino-terminal fragment of PTHrP responsible for stimulating PTH1R revealed an increase of RANKL gene expression (Fig. 4). Further examination of OPG indicated an initial increase of gene expression following administration of the peptide. However, prolonged treatment with PTHrP favored signals that promote osteoclastogenesis and this may be representative of PTHrP signaling within GCT. Indeed, inhibition of stromal cell-derived PTHrP with neutralizing antibodies resulted in decreased RANKL mRNA expression (Table 4).

To determine whether the observed changes in gene expression would correlate with increased osteoclastogenesis, we analyzed co-cultures of GCT stromal cells with RAW 264.7 cells. Following administration of the PTHrP (1-34) peptide, RAW 264.7 cells formed a significantly greater number of TRAP-positive multinucleated cells. These results suggest that PTHrP could similarly stimulate monocytes within the tumor to form giant cells, thereby contributing to the pathogenesis of the tumor. Furthermore, the ability of the stromal cells to stimulate multinucleated cell formation through a cell culture insert

suggests that soluble RANKL is expressed by the stromal cells, which corresponds with previous studies [4-8].

In summary, we have shown the expression of both PTHrP and PTH1R by the GCT stromal cells. Moreover, we have demonstrated an effect of PTHrP treatment on these cells, which resulted in time-dependent increases in RANKL and OPG gene expression as well as an increased ability to promote multinucleated cell formation from RAW 264.7 cells. Furthermore, inhibition of PTHrP decreased mRNA expression of RANKL. Clearly, there are other factors than PTHrP involved in giant cell formation as PTH alone induces true osteoclasts and not giant cells. Additionally, systemic PTHrP leads to hypercalcemia, which is not present in patients with GCT. This may result from differences in PTHrP expression: whereas GCT may produce a constant, minimal level of PTHrP directly within the bone that results in a localized effect, other tumors secrete enough PTHrP to significantly raise the overall serum concentration resulting in a larger systemic effect. Moreover, GCT patients typically do not present with other complications that could affect normal clearance of calcium. We propose that PTHrP may act locally within GCT and play an important role in the pathogenesis of the tumor.

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CHAPTER 4

T cells stimulate catabolic gene expression by the stromal cells from giant cell tumor of bone

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Preface

The discovery that GCT stromal cells not only produce PTHrP, but respond to it as well, suggest it may be an important factor in bone resorption by the tumour through stimulation of giant cell formation. Moreover, the stromal cells may directly influence bone resorption, due to their expression of MMP-1 and MMP-13, as discussed in Chapter 2, and further explored in our laboratory (Mak et al., 2010). However, any effect of PTHrP on collagenase expression remained unknown, and we therefore investigated the role of PTHrP on MMP-13 expression by GCT stromal cells. Previous experiments in our lab, which may support a role for PTHrP in MMP-13 expression, showed Runx2, a known essential transcription factor for MMP-13 (Jimenez et al., 1999), was required for its expression in GCT stromal cells (Mak et al., 2009). PTH is known to stimulate MMP-13 expression through a process that involves both Runx2 and AP-1 (Hess et al., 2001; Porte et al., 1999), which is a required transcription factor for MMP-1. Therefore, PTHrP may similarly affect MMP-13 expression within the tumour.

Interstitial collagenase activity is required for PTH-induced osteoclast-mediated bone resorption (Zhao et al., 1999; Chiusaroli et al., 2003). Moreover, reports also suggest that T cells may be required for PTH-induced bone resorption (Gao et al., 2008; Hory et al., 2000). Therefore, we investigated whether T cells could contribute to the catabolic effects of PTHrP in GCT.

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Biophysical Research Communications 419(4): 719-723. This article is reprinted with permission from Elsevier (see Appendix B for Licence Agreement). Please note that American spelling is used throughout the article, as the journal format dictated. I performed all of the experiments, assembled the results, generated the figures and wrote the manuscript. Michelle Ghert and Gurmit Singh made revisions to the manuscript and provided intellectual direction and assistance.

These results follow directly from the results of the previous chapter, and show that PTHrP (1-34) stimulates MMP-13 gene expression by cultured GCT stromal cells. Inhibition of PTHrP with neutralizing antibodies significantly reduced MMP-13 gene expression compared to IgG-matched control antibodies. Moreover, T cell lines, and a protein expressed by activated T cells, CD40L, also stimulate expression of PTHrP, RANKL, and MMP-13 genes. Indeed, the CD40 receptor is expressed by both stromal cells and giant cells, and the CD40L gene was detected in whole GCT tissues but not in isolated stromal cell cultures. These results suggest that locally-produced factors can influence PTHrP expression by GCT stromal cells, thereby potentiating its catabolic effects within the tumour. These are the first results to examine the influence of T cells on GCT stromal cells, and also the first to report expression of CD40 and CD40L within the tumour. The presence of these two proteins suggests that CD40-CD40L signalling, and the influence of T cells overall, may represent an important factor in the pathogenesis of GCT, particularly with respect to the actions of PTHrP.

Title: **T cells stimulate catabolic gene expression by the stromal cells from giant cell tumor of bone**

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Abstract

The factors that promote the localized bone resorption by giant cell tumor of bone (GCT) are not fully understood. We investigated whether T cells could contribute to bone resorption by stimulating expression of genes for parathyroid hormone-related protein (PTHrP), matrix metalloproteinase (MMP)-13, and the receptor activator of nuclear-factor κB ligand (RANKL). Two cell lines, Jurkat clone E6-1 and D1.1, were co-cultured with isolated GCT stromal cells. Real-time PCR analyses demonstrated a significant increase of all three genes following 48 h incubation, and PTHrP and MMP-13 gene expression was also increased at 24 h. Further, we examined the expression of CD40 ligand (CD40L), a protein expressed by activated T cells, and its receptor, CD40, in GCT. Immunohistochemistry results revealed expression of the CD40 receptor in both the stromal cells and giant cells of the tumor. RNA collected from whole GCT tissues showed expression of *CD40LG*, which was absent in cultured stromal cells, which suggests that CD40L is expressed within GCT. Stimulation of GCT stromal cells with CD40L significantly increased expression of the PTHrP and MMP-13 genes. Moreover, we show that inhibition of PTHrP with neutralizing antibodies significantly decreased *MMP13* expression by the stromal cells compared to IgG-matched controls, whereas stimulation with PTHrP (1-34) increased MMP-13 gene expression. These results suggest that T cells may potentiate the catabolic effect of GCT.

Keywords: giant cell tumor; CD40; CD40L; PTHrP; MMP-13; RANKL

Introduction

Deciphering the mechanisms leading to the significant localized bone resorption that is characteristic of giant cell tumor of bone (GCT) is critical for developing new treatment options for this aggressive tumor. GCT is a heterogeneous tumor that is primarily composed of osteoclast-like multinucleated giant cells, spindle-like stromal cells, and mononuclear cells of monocyte-macrophage origin. However, previous research has revealed that tumor-infiltrating lymphocytes are also present in fresh GCT biopsies [1]. Indeed, multiple studies suggest that T cells may participate in the pathogenesis of GCT. Specifically, Muscolo and Ayerza showed that GCT patients' lymphocytes were stimulated *in vitro* by their own tumor cells in co-culture experiments, thus demonstrating an immune response to GCT antigens [2]. Moreover, osteoclasts themselves serve as antigen-presenting cells that can activate T lymphocytes [3].

We previously reported that the neoplastic GCT stromal cells express parathyroid hormone-related protein (PTHrP), which stimulates expression of the receptor activator of nuclear factor-κB ligand (RANKL) by these cells [4]. The stromal cells also express elevated levels of matrix metalloproteinase (MMP)-13 [5-8], which is a known stimulator of bone resorption and osteoclast differentiation [9;10]. We hypothesized that PTHrP plays an important role in the pathogenesis of GCT by acting locally to stimulate osteoclastogenesis and other factors such as proliferation and bone resorption. Indeed, PTHrP and parathyroid hormone (PTH) elicit many similar responses through the PTH type 1 receptor (PTH1R), including stimulation of MMP-13 expression [10-13]. However, it is not known whether PTHrP stimulates MMP-13 expression in GCT.

Additionally, conflicting reports in the literature suggest that T cells may [14;15] or may not [16;17] be required to stimulate a catabolic response to PTH and PTHrP. Therefore, we investigated whether T cells could potentiate the catabolic effect of PTHrP in GCT by examining expression of the genes encoding PTHrP, RANKL, and MMP-13.

Materials and Methods

Cells and cell culture

Stromal cells were isolated in culture from tissues as previously described from patients presenting with GCT [5], following patient consent and approval from our institution's Research Ethics Board. Primary cell lines were maintained in Dulbecco's modified Eagle medium (D-MEM) supplemented with 10% fetal bovine serum, 2mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin (Invitrogen Canada/Gibco) at 37ºC in humidified air containing 5% CO_2. GCT cells were assayed between passages 5 and 10, to ensure no residual giant cells or monocytes were present in the cultures. Jurkat clone E6-1 and D1.1 cells were obtained from the American Type Culture Collection (ATCC # TIB-152 and CRL-10915, respectively), and maintained in RPMI-1640 medium (ATCC), supplemented as described above, and containing 10mM HEPES.

Immunohistochemistry

Fresh GCT tissues were formalin-fixed and embedded in paraffin, then cut and mounted onto slides. Paraffin was removed by several washes in xylene, and tissues were rehydrated in graded ethanol. Heat-induced epitope retrieval using 10mM citrate buffer, pH 6.0, was performed at 95ºC for 30 min in a rice cooker. After cooling, slides were

incubated with 3% H_2O_2 for 5 min to block endogenous peroxidase activity then washed with Tris-buffered saline with Tween 20 (TBS-T). Tissues were incubated with 5% normal goat serum for 20 min, and then 20 µg/mL rabbit polyclonal anti-CD40 (Abcam # ab58391) was applied for overnight incubation at 4ºC. Secondary antibody was subsequently applied for 30 min, and then slides were treated with Vectastain ABC reagent (Vector Laboratories) for 30 min, as per the manufacturer's instructions. Substrate color was developed using a Nova Red Kit (Vector Laboratories) as per the instructions. Tissues were counterstained with hematoxylin, dehydrated in graded ethanol, and mounted in Permount. Sections of paraffin-embedded tissue from normal human hippocampus (US Biomax, Inc.) were also tested for comparison.

PCR

RNA was collected from cells or tissue using the TRIzol (Invitrogen Canada/Gibco) collection method and cDNA was prepared from 1µg RNA using Superscript III (Invitrogen Canada/Gibco), as per the manufacturer's instructions. An MJ Mini Personal Thermal Cycler (Bio-Rad Laboratories) was utilized for amplification according to the following protocol: 94ºC for 3 min followed by 35 cycles of 94ºC for 30 s, 55ºC for 30 s, and 72ºC for 60 s, and then a final 10 min extension at 72ºC. Samples were electrophoresed on a 1% agarose gel and visualized on a Molecular Imager Gel Doc XR System (Bio-Rad Laboratories) using ethidium bromide. Primer sequences for CD40 and CD40 ligand (CD40L) genes were obtained from the PrimerBank [18;19] (IDs: 23312371a3 and 58331233b2, respectively). *CD40*: 5′-AGACACCTGGAACAGAGAGAC-3′ (forward) and 5′-

GCAGATGACACATTGGAGAAGAA-3′ (reverse); *CD40LG*: 5′-GAGCAACAACTTGGTAACCCT-3′ (forward) and 5′-GGCTGGCTATAAATGGAGCTTG-3′ (reverse); *GAPDH* (GenBank ID: NM_002046): 5′-AACTTTGGTATCGTGGAAGGA-3′ (forward) and 5′-CAGTAGAGGCAGGGATGATG-3′ (reverse).

Real-time PCR

GCT stromal cells (1.5×10^6 cells) were seeded in 55 cm^2 Petri dishes and treated with the following, as indicated: 0, 10 or 100 ng/mL CD40L protein (Abcam # ab51956) for 16 h; 0 or 10 nM PTHrP (1-34) (Bachem) for 16 h; 5 or 10 µg/mL rabbit anti-PTHrP (1-34) (Bachem) or 5 or 10 µg/mL rabbit IgG control (Genscript) for 24 h; or co-cultured with 2×10^5 or 6×10^5 Jurkat clone E6-1 or D1.1 cells for 24 and 48 h. As T cell lines are suspension cells, they are easily removed from the Petri dishes following several washes with phosphate buffered saline (PBS) and gentle agitation at the time of RNA collection. RNA was collected from GCT stromal cells following the specified treatment interval and reverse-transcribed as described above. Gene expression was determined by a MiniOpticon System (Bio-Rad Laboratories) using Express SYBR GreenER qPCR Supermix Universal (Invitrogen Canada/Gibco) and the following protocol: 50ºC for 2 min, then 95ºC for 2 min, followed by 40 cycles of 95ºC for 15 s and 60ºC for 60 s, with a gradient change in temperature from 60 to 95ºC for melt curve analysis. Gene expression was quantified according to the comparative threshold cycle method ($\Delta\Delta C_t$) and combined results from triplicate experiments are presented. Expected product sizes were confirmed by agarose gel electrophoresis. Primer sequences for the *MMP13* and *TAF10*

genes were obtained from the PrimerBank [18;19] (IDs: 4505209a1 and 5454106a2, respectively). *MMP13*: 5′-TTTCAACGGACCCATACAGTTTG-3′ (forward) and 5′-CATGACGCGAACAATACGGTTA-3′ (reverse); *TAF10*: 5′-GAAGATTACACGCCTACGATCC-3′ (forward) and 5′-CAGCTAAGGAGATGAGCCGAA-3′ (reverse). Primers detecting total PTHrP were previously reported [20], and other sequences for genes encoding RANKL (*TNFSF11*) and phosphoglycerate kinase 1 (*PGK1*) were also employed. *PTHLH*: 5′-CGGTGTTCCTGCTGAGCTA-3′ (forward) and 5′-TGCGATCAGATGGTGAAGGA-3′ (reverse); *TNFSF11* (GenBank ID: NM_003701): 5′-TATGCCAACATTTGCTTTCG-3′ (forward) and 5′-TCGATGCTGATTTCCTCTCC-3′ (reverse); *PGK1* (GenBank ID: NM_000291): 5′-AAGCTGGACGTTAAAGGGAAGCG-3′ (forward) and 5′-GACTACCGACTTGGCTCCATT-3′ (reverse). *MMP13* and *PTHLH* expression were normalized to *PGK1* expression, whereas *TNFSF11* employed *TAF10* as a housekeeping gene.

Statistical analyses

Real-time PCR results were analyzed by the two-sample independent Student's *t*-test. Results of treated cells were compared to the control and accepted as significant if $P \leq 0.05$.

Results

T cells stimulate gene expression by GCT stromal cells

T cells express a variety of factors that promote osteoclastogenesis and bone resorption, such as tumor necrosis factor, numerous interleukins, interferon-γ, and CD40L. T lymphocytes isolated from fresh GCT biopsies [1] may therefore represent a contributing factor to the extensive bone resorption that is characteristic of the tumor. To determine whether T cells could affect isolated GCT stromal cells, we assayed tumor cell gene expression by real-time PCR following co-culture with Jurkat clone E6-1 or D1.1 cell lines (Fig. 1). Both T cell lines significantly increased PTHrP gene expression at their lower concentration at either 24 (D1.1: $P = 0.002$) or 48 h (Jurkat: $P = 0.00003$). Moreover, the T cell lines also increased MMP-13 gene expression, which resulted in a significant and sustained increase in expression compared to controls. Jurkat clone E6-1 cells also significantly increased RANKL gene expression by the stromal cells after prolonged exposure for 48 h ($P = 0.03$). Conversely, T cells at the higher concentration initially decreased RANKL gene expression by the stromal cells at 24 h, although prolonged co-cultures showed a trend toward elevated expression at 48 h.

CD40 and CD40L signaling in GCT

PTH-induced catabolism is inhibited in mice lacking functional T cells [14;15]. Gao *et al.* reported that the T cell-expressed CD40L was critical for PTH-induced bone resorption and acted by stimulating its receptor, CD40, on osteoblastic cells, thereby priming them to respond to PTH [14]. Therefore, given that PTHrP is expressed in GCT by the stromal cells [4], and responded to T cells in the co-culture experiments, we sought

to identify if CD40 and CD40L are also expressed within the tumor. Immunohistochemistry analyses of GCT tissues revealed expression of the CD40 receptor on both the osteoclast-like giant cells and the spindle-like stromal cells (Fig. 2A). CD40 expression on these cell types is consistent with previous studies that show monocytes, macrophages [21] and osteoblasts [22] can express this receptor. For comparison, neurons also showed positive expression of CD40, whereas surrounding hippocampus tissue did not (Fig. 2B), as expected [23]. Expression of the receptor is maintained in isolated GCT stromal cells, as indicated by agarose gel electrophoresis (Fig. 2C). Importantly, these results also demonstrate that *CD40LG* is expressed within the unfractionated tumor tissue, but not expressed when isolated GCT stromal cells are grown in culture. The T cell lines, Jurkat clone E6-1 and D1.1 also expressed CD40L, as expected [24].

As CD40L is expressed within GCT, but absent in isolated stromal cell cultures, we examined the effects of CD40L stimulation on gene expression by stromal cells (Fig. 3). Results were obtained 16 h following introduction of CD40L, based on our previous results showing a maximal effect of PTHrP (1-34) stimulation at that time point [4]. Consistent with our previous results, PTHrP (1-34) alone increased RANKL gene expression compared to control ($P = 0.04$). Moreover, PTHrP also significantly increased PTHrP ($P = 0.0002$) and MMP-13 ($P = 0.01$) gene expression. Incubation with CD40L also significantly increased PTHrP and MMP-13 gene expression compared to control. However, despite the increased expression by either PTHrP (1-34) or CD40L alone, no significant synergistic effect was noted following stimulation with both proteins at the

tested interval. CD40L did not independently increase RANKL gene expression, and the significant increase detected following incubation with both proteins may be the result of PTHrP itself.

PTHrP regulates MMP-13 expression in GCT

Expression of MMP-13 is elevated in cultured GCT stromal cells [5]. The further stimulation of *MMP13* by PTHrP (1-34) (Fig. 3) suggested that the increased gene expression may be related to autocrine signaling by the stromal cell-derived PTHrP. Therefore, to confirm the importance of PTHrP in MMP-13 expression by GCT stromal cells, we evaluated gene expression following inhibition of PTHrP with neutralizing antiserum (Fig. 4). Results revealed a significant decrease in *MMP13* expression compared to isotype controls at both concentrations tested (5 μg/mL: $P = 0.00002$; 10 μg/mL: $P = 0.000004$). However, no significant difference between concentrations was observed.

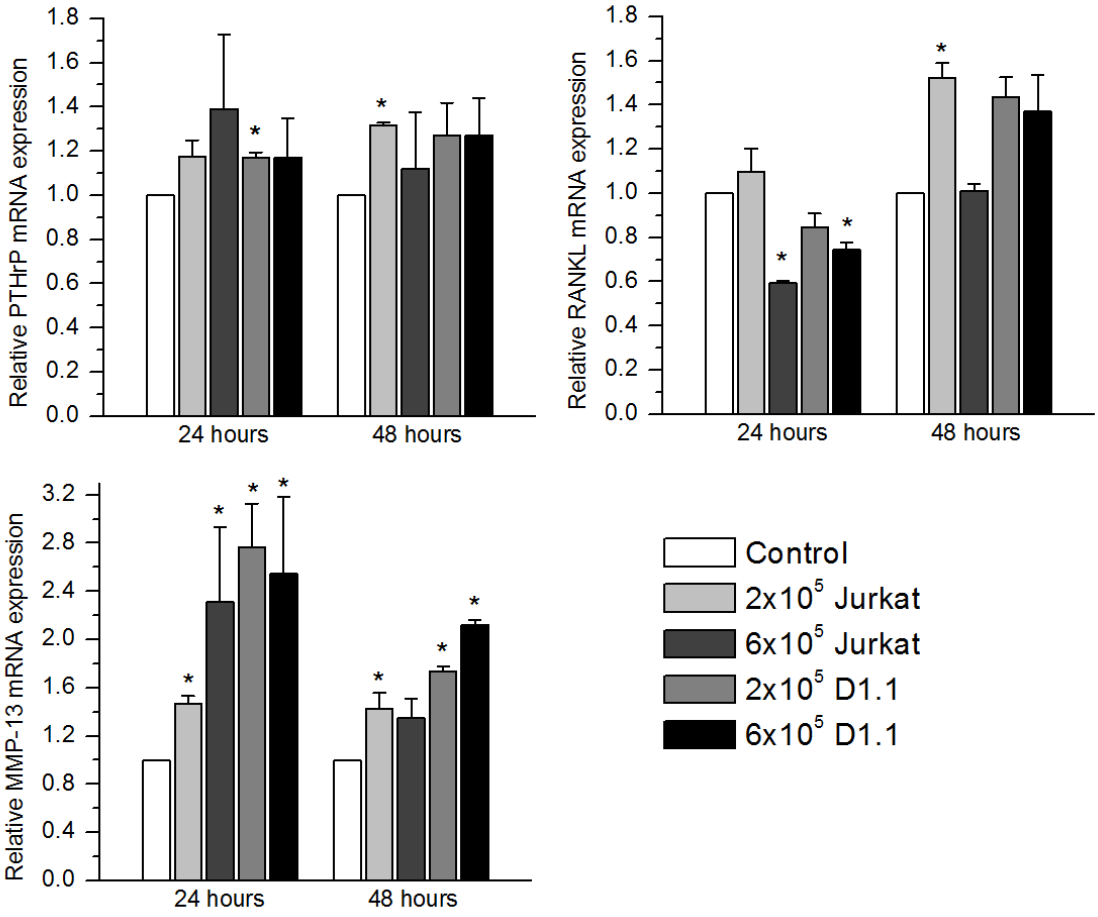

Fig. 1. T cell lines stimulate gene expression by GCT stromal cells. Stromal cells were co-cultured with Jurkat clone E6-1 or D1.1 cells for 24 and 48 h. Expression of genes encoding PTHrP (*PTHLH*), RANKL (*TNFSF11*), and MMP-13 (*MMP13*) by stromal cells were compared to untreated controls. Results are pooled from triplicate experiments and expression significantly different from control ($P \leq 0.05$) by the two-sample independent Student's *t*-test are indicated (*). Error bars indicate standard error.

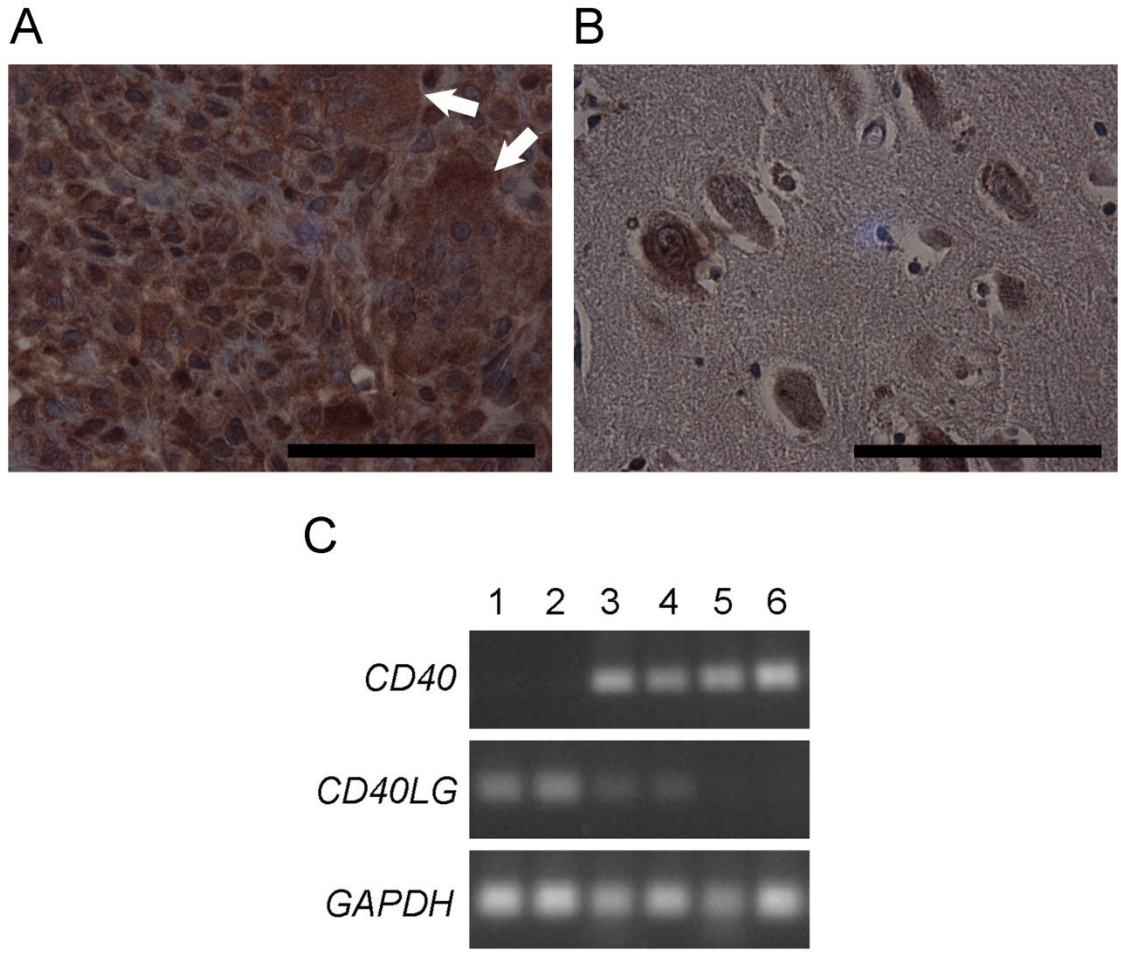

Fig. 2. CD40 and CD40L expression in GCT. (A-B) Representative immunohistochemistry results show positive CD40 expression in the giant cells (arrows) and stromal cells of GCT (A) and neurons from normal hippocampus tissue (B); scale bar = 100 µm. (C) Agarose gel showing *CD40* and *CD40LG* expression in Jurkat clone E6-1 (lane 1) and D1.1 (lane 2) cells, as well as whole GCT tissues (lanes 3-4) and isolated GCT stromal cell lines (lanes 5-6) from separate patients. Expression of *GAPDH* is shown for comparison.

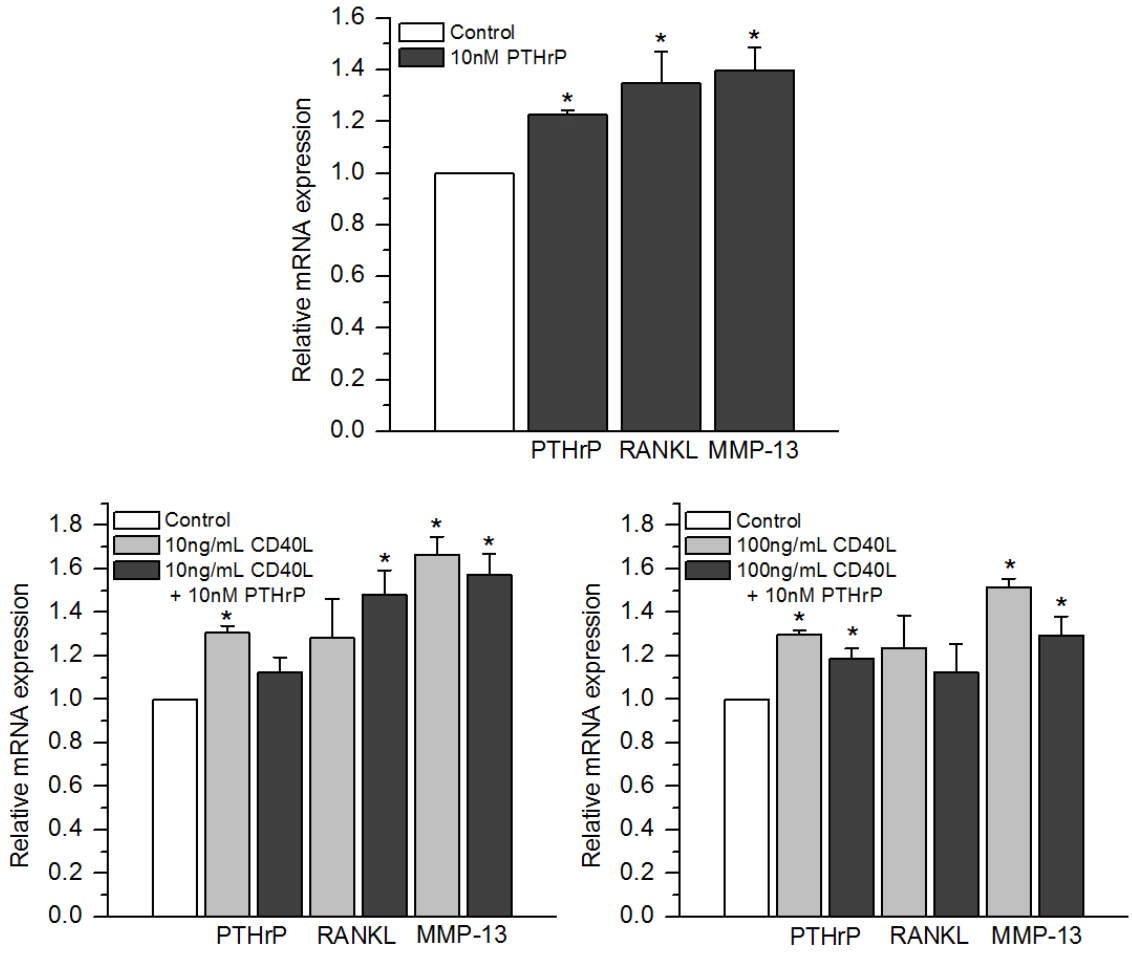

Fig. 3. CD40L stimulation of cultured GCT stromal cells. Cells were treated as indicated with CD40L and PTHrP (1-34) for 16 h. Expression of genes encoding PTHrP (*PTHLH*), RANKL (*TNFSF11*) and MMP-13 (*MMP13*) by treated cells were compared to untreated control by real-time PCR analyses. Pooled results from triplicate experiments are displayed and expression significantly different from control ($P \leq 0.05$) by the two-sample independent Student's *t*-test are indicated (*). Error bars indicate standard error.

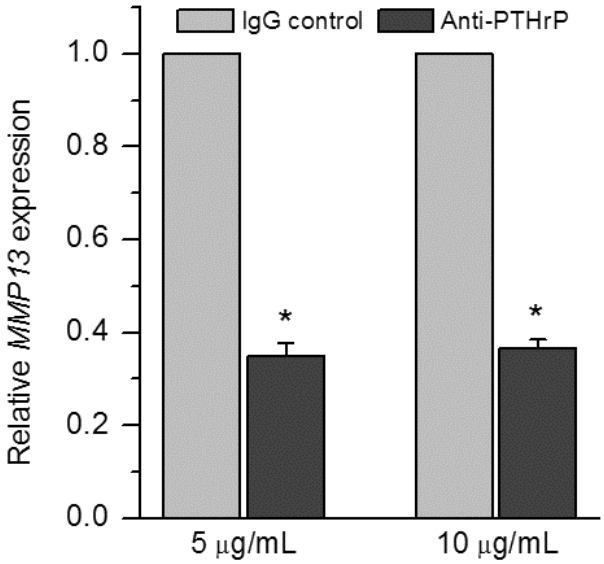

Fig. 4. PTHrP neutralization decreases *MMP13* expression in GCT stromal cells. *MMP13* expression from isolated GCT stromal cells treated with 5 or 10 μg/mL rabbit anti-PTHrP for 24 h relative to cells treated with rabbit IgG control antibodies. Results are significantly different ($P \leq 0.05$) by the two-sample independent Student's *t*-test (*) and are pooled from triplicate experiments. Error bars indicate standard error.

Discussion

Extensive bone resorption is a characteristic feature of GCT. However, the mechanisms leading to the significant osteolysis by this tumor are poorly defined. Although the osteoclast-like giant cells are considered the principal bone-resorbing cells within the tumor, we hypothesize that PTHrP locally stimulates resorption by increasing expression of factors such as RANKL and MMP-13 by the stromal cells. Indeed, stromal cells enhance the resorptive capability of GCT cells [25-27], and both RANKL and

MMP-13 are associated with increased bone resorption: RANKL is a known essential mediator of osteoclastogenesis [28], and MMP-13 is involved in both osteoclast differentiation and activity [10]. Several reports suggest that T cells may be required to elicit a catabolic response to PTH [14;15], which is structurally related to PTHrP at the amino-terminal end responsible for stimulation of their shared receptor, PTH1R [20;29].

Here, we have investigated whether T cells stimulate the catabolic effect of PTHrP in GCT. To our knowledge, we are the first to report an effect of T cells on GCT stromal cells, which resulted in the significant stimulation of expression of genes encoding PTHrP, RANKL, and MMP-13 (Fig. 1). The most pronounced effect was on *MMP13*, where both cell lines significantly increased expression following 24 h, which remained significantly elevated compared to control at 48 h. Indeed, media conditioned by activated T cells was similarly shown to stimulate MMP-13 expression by osteoblasts [30]. Moreover, we examined the role of CD40L signaling in GCT. CD40L is a protein implicated in the pathogenesis of many diseases [31], and is constitutively expressed by the T cell lines employed, although its expression is reportedly higher in D1.1 cells [24]. The co-culture results therefore correspond with a potential role for CD40L in gene expression, as D1.1 cells produced an elevated or faster effect on gene expression compared to Jurkat clone E6-1 cells. As such, we investigated whether CD40L could replicate the results of co-culture experiments.

The CD40 receptor was expressed by both the stromal cells and giant cells from GCT (Fig. 2A). Moreover, *CD40* expression was maintained in culture by isolated stromal cells (Fig. 2C). Conversely, *CD40LG* was expressed within whole GCT tissues, but

absent in culture. We hypothesized that the CD40L present within GCT is found on activated T lymphocytes, as tumor-infiltrating lymphocytes were previously isolated from fresh GCT biopsies [1]. However, other cells within the tumor could be the source of CD40L, such as platelets [32], or the giant cells themselves. Indeed, macrophages were previously reported to express CD40L [21]. Nevertheless, we show for the first time that *CD40LG* is expressed within GCT, and *in vitro* stimulation of stromal cells with CD40L increases expression of the genes encoding PTHrP and MMP-13 (Fig. 3). CD40L is also known to stimulate other factors involved in osteoclastogenesis, and may therefore have additional roles in the pathogenesis of GCT. For example, CD40L is reported to stimulate expression of RANK in dendritic cells [33] and dendritic cell-specific transmembrane protein (DC-STAMP) in peripheral blood monocytes [34]. DC-STAMP is considered required for monocyte fusion and osteoclastogenesis [35] and is found in GCT [36]. Although CD40L did not independently stimulate RANKL gene expression, it did increase PTHrP gene expression. Therefore, a delayed increase in RANKL gene expression may result from increased PTHrP, and future analyses should investigate expression at intervals longer than 16 h. Indeed, these results may suggest that the observed effects of T cells and CD40L on RANKL gene expression by the stromal cells are mediated by PTHrP, as our previous results indicated that PTHrP initially decreases RANKL gene expression prior to increasing its expression [4]. Therefore, the initial significant decrease in RANKL gene expression following co-culture with T cells may result from CD40L stimulation of PTHrP expression. Correspondingly, prolonged co-

culture with T cells for 48 h increased RANKL gene expression compared to control, although the results were only significant for Jurkat clone E6-1 cells.

These results show that T cells can stimulate gene expression by cultured GCT stromal cells, and a potential mechanism may be through CD40L signaling, which similarly stimulated expression of the genes for PTHrP and MMP-13. Given the role of these proteins in osteoclastogenesis and bone resorption, these results suggest that the involvement of T cells should be considered in the osteolytic pathogenesis of GCT, and further investigation into the participation of T cells within the tumor is required. Moreover, we show that PTHrP stimulates *MMP13* expression by the stromal cells, and neutralization of PTHrP with antibodies significantly decreased expression of the protease compared to control. Therefore, PTHrP itself may play an important part in promoting osteoclastogenesis and bone resorption within GCT by stimulating expression of RANKL and MMP-13.

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CHAPTER 5

Discussion and Summary

Discussion

Improved treatment options for patients with giant cell tumour of bone (GCT) can only be developed with a better understanding of the cellular interactions that dictate its pathophysiology. To that end, this thesis explores the mechanisms that lead to bone resorption by the tumour. The significant local osteolysis caused by GCT is largely attributable to the multinucleated osteoclast-like giant cells. However, the bone-resorbing potential of the tumour is enhanced by the presence of the neoplastic component, the spindle-like stromal cells (James et al., 1996; Oreffo et al., 1993; Wen et al., 1999). GCT shares numerous properties with brown tumours of hyperparathyroidism. Namely, both tumours appear as local osteolytic lesions, and result in the accumulation of bone-resorbing osteoclasts or osteoclast-like giant cells. Despite these similarities, the tumours differ in that brown tumours are considered less infiltrative and arise from systemic increases in serum parathyroid hormone (PTH), which can induce widespread bone resorption and hypercalcemia, whereas GCT results in localized bone destruction without hypercalcemia. Therefore, local factors within the tumour likely promote osteolysis. As the etiology of GCT is not established, I hypothesized that parathyroid hormone-related protein (PTHrP) could largely influence the pathogenesis of the tumour in a similar fashion as hyperparathyroidism promotes brown tumours. Indeed, other giant cell-containing lesions express PTHrP, such as giant cell tumour of tendon sheath (Nakashima et al., 1996) and giant cell granulomas of the jaw (Houpis et al., 2010). Moreover, PTHrP is also associated with giant cell formation in trophoblast cell lines, where they downregulate cyclin B1 and upregulate cyclin D1 expression (El-Hashash et al., 2005), a

characteristic also noted in giant cells from GCT (Kauzman et al., 2003). Therefore, given the osteolytic nature of GCT, we investigated whether PTHrP promotes bone resorption within the tumour.

The spindle-like stromal cells express bone-resorbing proteases

The stromal cells are largely responsible for the accumulation of giant cells within GCT, due to their production of a variety of pro-osteoclastogenic factors. Nevertheless, the enhanced bone-resorption observed in co-culture analyses may not result simply from an increase in the number of giant cells; the stromal cells themselves may promote bone resorption by the giant cells, or directly resorb bone. To determine the effect of stromal cells in bone resorption, we investigated whether isolated stromal cells are capable of directly degrading bone by examining the expression of proteases capable of degrading type I collagen: the major component of the organic matrix. Previous investigations have concluded that the stromal cells do not produce cathepsins, which are instead produced by the giant cells (Drake et al., 1996; Lindeman et al., 2004). Moreover, gelatinase expression by the stromal cells has been extensively investigated, and although matrix metalloproteinase (MMP)-2 can degrade type I collagen, its concentration must be artificially high to achieve degradation (Aimes & Quigley, 1995). We therefore focused on the expression of the interstitial collagenases: MMP-1, MMP-8, and MMP-13. As demonstrated in Chapter 2, we determined that isolated GCT stromal cells express and secrete MMP-1 and MMP-13, but not MMP-8. These results imitate conditions within the unfractionated tumour, as immunohistochemistry analyses revealed positive expression of both MMP-1 and MMP-13 in the stroma. Moreover, numerous giant cells

showed positive MMP-1 staining, which is in accordance with a previously-reported finding (Ueda et al., 1996). However, osteoclasts are not known to express collagenases (Fuller & Chambers, 1995; Holliday et al., 1997), and further study is necessary to determine whether MMP-1 is merely associating with the giant cells or are indeed expressed by these cells.

Both MMP-1 and MMP-13 were sufficient for proteolytic activity, as determined by standardized activity assays. These enzymes may therefore contribute to bone resorption by the tumour. Indeed, collagenase activity is required for PTH-induced bone resorption in mice, where it stimulates osteoclast activity (Zhao et al., 1999; Chiusaroli et al., 2003), and osteoblasts were previously shown to independently resorb bone matrix after osteoclast-mediated resorption in the lacunae in an MMP-dependent manner (Parikka et al., 2005). Following these results, we therefore investigated whether inhibition of MMP-13 could prevent *in vitro* resorption of bovine bone slices by co-cultures of stromal cells and giant cells (Mak et al., 2010). Results indicated that inhibitors specific for MMP-13 could decrease resorption by both isolated stromal cells and co-cultures of stromal cells with giant cells. These results suggest that MMP-13 can directly resorb bone, and may stimulate giant cell-mediated resorption. However, MMP-13 inhibition also decreased resorption by the giant cell-enriched fraction, which may be due to residual stromal cells present in the assay, but nonetheless indicate that further clarification into the role of MMP-13 in resorption by the tumour is warranted. For example, although stromal cell-derived collagenases may stimulate resorption by giant cells, an evaluation of any giant cell-independent resorption by the MMPs could be established through experiments

determining proteolytic cleavage products of soluble type I collagen. Specifically, interstitial collagenases uniquely cleave type I collagen into ¾ amino-terminal and ¼ carboxyl-terminal fragments, which are detectable by SDS-PAGE (Birkedal-Hansen, 1987; Garnero et al., 1998; Ohuchi et al., 1997). In addition, MMP-1 and MMP-13 may produce other effects in GCT that are unrelated to bone resorption. For example, both of these enzymes affect osteoblast differentiation by modulating expression of Runx2, osterix, and other osteoblastic markers (Hayami et al., 2008).

Parathyroid hormone-related protein expression in giant cell tumour of bone

The GCT stromal cells express PTHrP, as demonstrated in Chapter 3 using multiple techniques including established primers from the literature. Moreover, these results are corroborated by two previous reports that examined PTHrP expression in human osteoclasts using immunohistochemistry (Kartsogiannis et al., 1998; Nakashima et al., 2003). The stromal cells also express the PTH type 1 receptor (PTH1R) and responded to PTHrP (1-34) treatment by stimulating gene expression. Expression of the receptor was expected, as PTH1R is widely expressed (Ureña et al., 1993) and GCT stromal cells were previously reported to respond to PTH (Goldring et al., 1977). Although the role of the amino-terminal PTHrP was investigated in this work, mid-region PTHrP was also detected by western blotting, and future experiments could examine the role of mid-region and carboxyl-terminal PTHrP in GCT. Moreover, karyotype analyses reveal that the stromal cells themselves are not a homogeneous population (Bardi et al., 1991; Gorunova et al., 2009), and it would be interesting to investigate whether genetically aberrant stromal cells or healthy stromal cells express PTHrP. Specifically, PTHrP is

involved in fracture healing (Okazaki et al., 2003), and the healthy stromal cells may be responding to the mutated cells by expressing PTHrP.

Interestingly, the presence of receptors for PTHrP on the giant cells, as determined by immunohistochemistry and confirmed in another report (Nakashima et al., 2003), suggests PTHrP may influence these cells as well. As discussed in Chapter 3, PTH reportedly increased bone resorption by osteoclasts generated from peripheral blood monocytes that expressed PTH1R (Dempster et al., 2005). Therefore, PTHrP may directly influence the giant cells by stimulating bone resorption, although early studies on giant cells and bone resorption with PTH may suggest otherwise, as no significant effect of the hormone on bone resorption by isolated giant cells was observed (Chambers et al., 1985). Nevertheless, PTHrP may affect giant cells through some other undetermined mechanisms, and the role of giant cells as the primary resorbing cells within the extensively osteolytic tumour suggests that any such relationship between PTHrP and the giant cells warrants further investigation.

In Chapter 4, local factors present within GCT, particularly CD40 ligand (CD40L), were shown to stimulate PTHrP gene expression. CD40L is expressed by activated T cells (Armitage et al., 1992), and indeed, two T cell lines that constitutively express CD40L (Choi et al., 2008) also increased expression of the PTHrP gene. Future experiments could examine the significance of CD40L-mediated stimulation of PTHrP *in vitro* using small interfering RNA (siRNA), which could be employed to knock down expression of the CD40 receptor by GCT stromal cells. Gene expression following co-culture with T cell lines could then be assayed to determine whether T cell-mediated gene

stimulation acts *via* CD40-CD40L signalling or some other mechanism. Moreover, siRNA directed against PTH1R would elucidate whether stimulation of genes for the receptor activator of nuclear-factor κB ligand (RANKL) and MMP-13 by CD40L act directly through CD40 stimulation, or indirectly *via* increasing PTHrP expression.

Aside from CD40L signalling, other factors may influence the expression of PTHrP within GCT. For example, hypoxia-inducible factor (HIF)-2α, which is expressed in GCT (Knowles & Athanasou, 2008), increases PTHrP expression in multiple cancer cell lines (Manisterski et al., 2010). Moreover, hypoxia may promote osteoclastogenesis and giant cell activity in GCT (Knowles & Athanasou, 2008; Knowles & Athanasou, 2009), which may be mediated in part by increased PTHrP expression. Therefore, a variety of factors may converge within the tumour to potentiate the effects of PTHrP by the stromal cells and lead to the development of the characteristic GCT phenotype. Indeed, it would be greatly beneficial to quantify the local expression of PTHrP from newly-isolated tumour biopsies and compare levels to a natural control, as the characteristics from the tumour *in vivo* are undoubtedly different than those found in cell culture. An exploration as to whether PTHrP expression correlates with clinical grading may also provide insight into the significance of PTHrP in the tumour. These experiments could further clarify whether PTHrP is involved in the pathogenesis of GCT.

Parathyroid hormone-related protein stimulates stromal cell-mediated giant cell formation

Expression of PTHrP, like PTH, can result in increased bone resorption (Guise et al., 1996; Suva et al., 1987) and is often associated with cancers (Moseley et al., 1987;

Southby et al., 1990; Strewler et al., 1987). Stimulation of cultured GCT stromal cells with PTHrP (1-34) resulted in time-dependent increases in RANKL and osteoprotegerin (OPG) gene expression and an increased ability to promote multinucleated cell formation *in vitro*. These results confirm the ability of the stromal cells to respond to PTHrP and suggest PTHrP may stimulate bone resorption in GCT through the promotion of osteoclast-like giant cell formation by increasing RANKL expression. Indeed, inhibition of PTHrP with neutralizing antibodies significantly decreased expression of the RANKL gene compared to control. However, the decreased expression was not as pronounced as the decrease observed with *MMP13*. This suggests that PTHrP may directly stimulate MMP-13 expression in GCT, whereas other additional factors likely contribute to RANKL expression.

Determining the mechanisms leading to increased RANKL expression could provide new therapeutic targets to complement the monoclonal RANKL antibody already under review in a clinical trial (Thomas et al., 2010). As an example, it would be beneficial to determine whether PTHrP has any effect on the CCAAT/enhancer binding protein β (C/EBPβ) transcription factor, which is known to induce RANKL expression in GCT (Ng et al., 2010), and was also shown to cooperate with Runx2 to induce MMP-13 expression in chondrocytes (Hirata et al., 2011). Indeed, PTHrP may stimulate C/EBPβ expression in GCT, as the transcription factor was previously found to be induced by PTH in osteoblasts (Dhawan et al., 2005). Moreover, the effect of PTHrP on stimulating osteoclastogenesis may be multifaceted. For example, PTHrP was previously shown to stimulate osteoblasts to secrete monocyte chemotactic protein-1 (MCP-1) (Lu et al.,

2007) and PTH stimulates stromal cell-derived factor-1 (SDF-1) (Jung et al., 2006), which are both expressed by the GCT stromal cells (Liao et al., 2005; Zheng et al., 1998). Therefore, future studies could evaluate the effect of PTHrP on these and other factors that affect osteoclastogenesis in GCT.

Stromal cell-derived matrix metalloproteinase-13 expression is regulated by parathyroid hormone-related protein

Although MMP-1 is detectable in whole GCT by PCR (Si et al., 2003) or ELISAs (Ueda et al., 1996), it is inconsistently identified in mononuclear and giant cells by immunohistochemistry (Ueda et al., 1996). Additionally, Lindeman *et al.* detected no MMP-1 using real-time PCR in whole GCT from seven patients (Lindeman et al., 2004). Therefore, due to the variability in MMP-1 expression noted in our own experiments and reported elsewhere, we focused on MMP-13 as a characteristic protease consistently expressed by GCT stromal cells. PTHrP affects osteoclastogenesis in GCT by modulating RANKL and OPG gene expression. These results suggest that PTHrP could stimulate bone resorption through increasing giant cell populations. However, the stromal cell production of collagenases and subsequent experiments investigating bone resorption by these cells (Mak et al., 2010) prompted an exploration of the ability of PTHrP to directly alter the expression of bone-resorbing proteases. Indeed, PTHrP was previously shown to stimulate osteoclast-mediated bone resorption in co-culture systems by stimulating osteoblasts (Evely et al., 1991). Moreover, inhibition of interstitial collagenase activity prevents PTH-induced bone resorption by the osteoclasts (Zhao et al., 1999; Chiusaroli et al., 2003).

Treatment of cultured GCT stromal cells with PTHrP (1-34) yielded a significant increase in *MMP13* expression. Additionally, inhibition of PTHrP with neutralizing antibodies resulted in a marked reduction in *MMP13* expression compared to control. These results suggest that PTHrP can influence MMP-13 expression in GCT, and may stimulate bone resorption by acting on the stromal cells. Together with the previous results, the work presented in this thesis strongly supports a role for PTHrP in mediating bone resorption in GCT through stimulation of RANKL and MMP-13 gene expression by the stromal cells. Future studies should evaluate the contribution of PTHrP in mediating tumour cell-induced bone resorption in isolated stromal cell and giant cell populations, as well as in co-cultures of all GCT cell types.

Future Directions

Several important questions should be addressed in future work examining the contribution of PTHrP to the pathogenesis of GCT. As mentioned, the influence of PTHrP on bone resorption by GCT cells requires further investigation, as PTHrP may enhance bone resorption by GCT co-cultures. Furthermore, inhibition of MMP-13 may decrease any effect of PTHrP-induced bone resorption and provide insight into the contribution of stromal cell-derived interstitial collagenases in bone resorption by the tumour. It would also be worthwhile to investigate the role of MMP-1 in resorption. Additionally, the presence of PTH1R on giant cells may also permit these cells to respond directly to PTHrP by enhancing bone resorption, and the significance of these receptors on giant cells should be determined. On a broader scope, PTHrP may also affect GCT cells in manners unrelated to bone resorption, such as through the promotion of cell

survival or by affecting cell cycle progression in giant cells, and these aspects should be investigated. Perhaps most critically, the significance of PTHrP in GCT should be determined through quantification of PTHrP from fresh tumour biopsies. Locally elevated PTHrP concentrations may signify the importance of this protein in the pathogenesis of the tumour, and solidify the basis for examination of the effects of PTHrP in GCT.

Summary

The central hypothesis to this thesis is that PTHrP is a key mediator of GCT physiology, and influences the stromal cells to promote osteoclastogenesis and bone resorption (see Figure 1). The work presented here shows that the spindle-like stromal cells express the interstitial collagenases, MMP-1 and MMP-13, but not MMP-8. Additionally, the stromal cells express PTHrP, which was confirmed by multiple assays. Moreover, both the stromal cells and giant cells express PTH1R. Stimulation of cultured GCT stromal cells with PTHrP (1-34) significantly increased MMP-13 gene expression, as well as RANKL and OPG gene expression in a time-dependent manner. The stromal cells also increased tartrate-resistant acid phosphatase-positive multinucleated cell formation from murine monocytes upon stimulation with PTHrP (1-34). Therefore, PTHrP may promote bone resorption in GCT by encouraging giant cell formation and by stimulating expression of MMP-13 by the stromal cells. Additionally, expression of PTHrP itself is stimulated *in vitro* by other factors present within the tumour, such as T cells. Indeed, experiments revealed gene expression of the T cell protein CD40L in whole GCT tissues, but that it is absent from cultured stromal cells. However, both the

stromal cells and giant cells expressed the CD40 receptor. Stimulation with CD40L increased both PTHrP and MMP-13 gene expression. These results suggest that local factors can potentiate the expression of PTHrP in GCT, and that PTHrP contributes to the osteolytic pathology of the tumour. Specifically, we have identified a local signal in GCT that can stimulate bone resorption through the promotion of osteoclastogenesis and by augmenting the expression of bone-resorbing proteases. This work therefore supports the hypothesis that PTHrP is an important factor in the pathogenesis of GCT and further characterization of the role of PTHrP within the tumour may lead to improved treatment options for GCT patients. For example, targeted inhibition of PTHrP or its targets may provide a useful alternative or adjuvant therapy by limiting or reducing the significant bone resorption caused by the tumour. Moreover, understanding the contribution of PTHrP to the pathogenesis of GCT may provide important insight into the physiology of other giant cell-containing lesions.

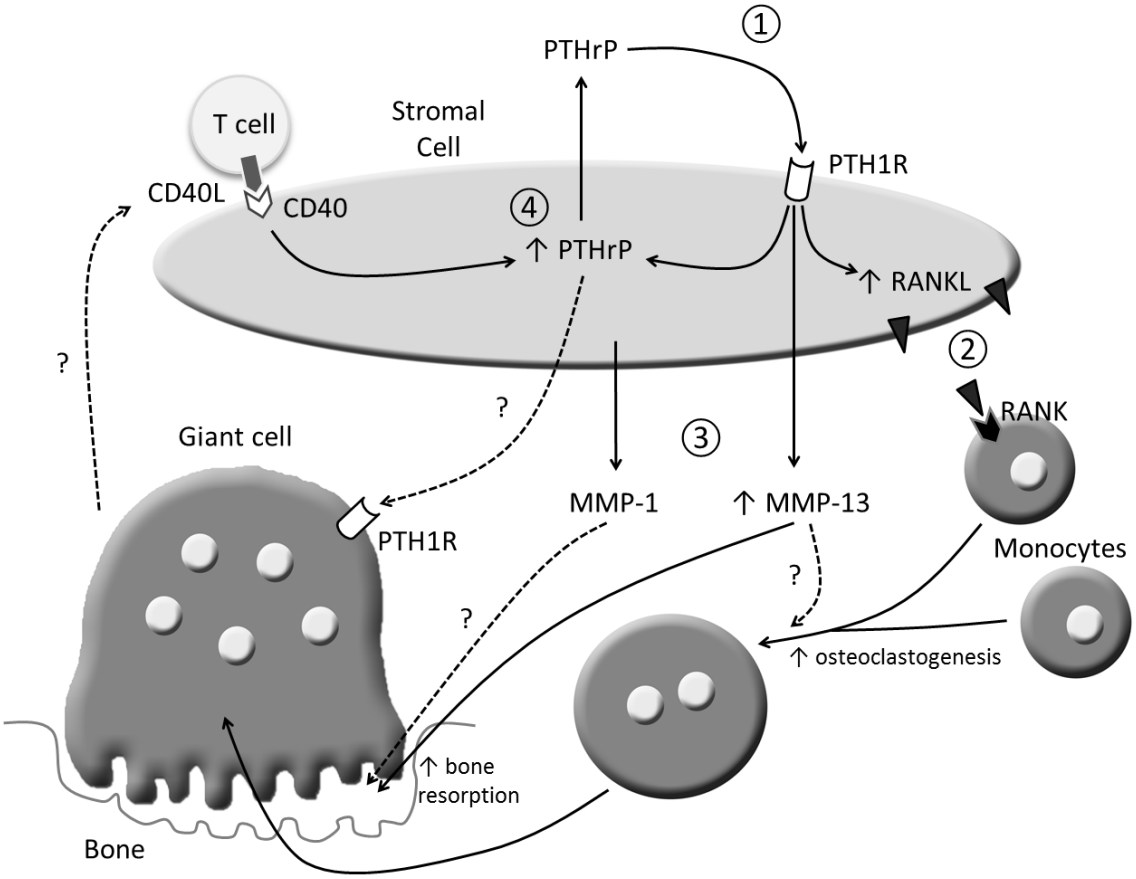

Figure 1 – Diagrammatic summary of results. (1) PTHrP is expressed by the GCT stromal cells, which acts *via* its receptor, PTH1R. (2) PTHrP stimulates RANKL expression, which binds to RANK on monocytes to promote mononuclear cell fusion leading to giant cells. (3) PTHrP also stimulates MMP-13 expression, which increases bone resorption either directly or by increasing giant cell activity. MMP-1 is also produced by the stromal cells, and may similarly promote resorption. (4) PTHrP expression is increased by other local factors within the tumour, including by PTHrP itself, and through CD40L signalling. Unresolved factors are indicated with a dotted line.

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APPENDIX A

Materials and Methods

Overview

This section describes several common materials and methods in greater detail than was permissible in each chapter. On several occasions, step-by-step protocols are listed.

Giant cell tumour cell culture and tissues

Commercially-available cell lines established from giant cell tumour of bone (GCT) are not readily available. Although the establishment of a few cell lines were reported in the literature, only one cell line derived from the lung metastatic site is currently available for purchase through the American Type Culture Collection (ATCC # TIB-223). Cells are therefore obtained as primary cultures. In this thesis, tumour samples were obtained with informed patient consent and approval of McMaster University's Faculty of Health Sciences Research Ethics Board during surgery at the Juravinski Hospital and Cancer Centre or St. Joseph's Healthcare Hamilton. On several occasions, and also with patient consent, tumours were obtained from McGill University and sent *via* courier mail to our laboratory for processing. Fresh tumour was either collected in formalin (for fixation and paraffin-embedding), or in culture medium.

Tumour samples were macerated with scalpel blades in a sterile Petri dish containing culture medium to disperse the cells. Aliquots of media containing cells and small pieces of tissue were drawn through a 24-gauge needle and transferred to vented tissue culture flasks (or Petri dishes) for cell culture. Flasks were placed in incubators maintained at 37ºC in humidified air containing 5% CO_2. Following a 24-hour incubation period, which allows the cells to adhere to the flask, the cultures were washed several times with phosphate buffered saline (PBS) and replenished with fresh culture medium to eliminate

red blood cells and other non-adherent cells. At this stage, both giant cells and spindle-like stromal cells are present within the flask. Brief trypsinization dislodges the stromal cells only, whereas prolonged trypsinization dislodges both cell types, thereby allowing fractionation of cultures into stromal cell and giant cell-enriched fractions. Giant cells do not persist in culture, and following several successive passages, are eliminated from the culture, as widely reported in the literature, and confirmed in our laboratory (Ghert et al., 2007). Stromal cell cultures were typically used between the fifth and tenth passage.

Cells were maintained in Dulbecco's modified Eagle medium (D-MEM) supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin (Invitrogen Canada/Gibco; Burlington, ON), unless otherwise indicated. Cell cultures were assigned an in-house laboratory label in the order that they arrived for processing, and were given a new label in each chapter, as needed (Table 1).

Table 1 – GCT tissue samples and cell lines from each chapter are indicated together with their corresponding in-house laboratory label.

In-house label	Chapter 1	Chapter 2	Chapter 3	Chapter 4
GCT-1		GCT-1		
GCT-2		GCT-2		
GCT-3	Figure 3B	GCT-3		
GCT-6	Figure 3A	GCT-4		
GCT-7		GCT-5		
GCT-9		GCT-6	GCT-3	
GCT-10		GCT-7		
GCT-11		GCT-8		
GCT-12		GCT-9		
GCT-14			GCT-5	
GCT-23	Figure 4		GCT-1	Fig. 2A,C#5,3
GCT-24			GCT-2	Fig. 1,2C#6,4
GCT-26				Fig. 2C#4
GCT-28			GCT-4	
GCT-41				Fig. 2C#3

Immunohistochemistry

Tissue processing and paraffin embedding

Formalin-fixed GCT tissues were transferred to histologic cassettes and processed by a Shandon Citadel 2000 tissue processor according to the following protocol: 30 minutes each of PBS and 50% ethanol, then 60 minutes each of 70%, 90%, and 100% ethanol, then two further incubations in 100% ethanol for 2 hours each, followed by washes in xylene (60 minutes, then two 90-minute incubations), and finally two incubations in paraffin wax (2 and 3 hours each). Tissues were subsequently embedded in paraffin wax using a Tissue-Tek II embedding machine. Sections of tissue (5µm thick) were cut using a microtome and placed on slides for analysis.

Reagents

<u>50mM sodium phosphate buffer, pH 6.0:</u>
Solution A (0.2 M NaH_2PO_4):
Dissolve 2.78g sodium dihydrogen orthophosphate (monobasic) into 100mL water.

Solution B (0.2M Na_2HPO_4):
Dissolve 5.36g sodium phosphate dibasic heptahydrate into 100mL water.

Mix 87.7mL Solution A with 12.3mL Solution B. Add 100mL water and mix to get 0.1M sodium phosphate buffer, pH 6.0. Add another 200mL water to get 400mL of final solution.

<u>0.02U/mL neuraminidase:</u>
Dilute stock (5U/mL) neuraminidase in 50mM sodium phosphate buffer, pH 6.0 for final concentration

<u>10mM citrate buffer, pH 6.0:</u>
Dilute 2.941 g tri-sodium citrate in ~950mL water. Adjust pH to 6.0 with HCl.

Immunohistochemistry protocol

1. Wash slides by submerging in xylene three times for 5 minutes each to remove paraffin.
2. Rinse slides in 100% ethanol until clear, then 70% ethanol until clear, then 50% ethanol until clear, then water until clear.
3. Perform epitope retrieval.
    a. For heat-induced epitope retrieval, incubate slides in 10mM citrate buffer, pH 6.0 at 95ºC in rice steamer for 30 minutes. Let cool in same solution for another 30 minutes.
    b. For enzymatic epitope retrieval, add approximately 100µL enzyme solution to each slide (enough to cover tissue). Let sit at room temperature for 60 minutes.
4. Rinse slides with TBS-T for 5 minutes on slide chamber.
5. Block endogenous peroxidase activity with 3% H_2O_2 (prepared in TBS-T) for 5 minutes.
6. Rinse slides for 2 minutes with TBS-T. Repeat rinse.
7. Prepare normal goat serum (500µL normal goat serum in 9.5mL TBS-T). Incubate slides with approximately 100µL normal goat serum solution for 20 minutes.
8. Prepare primary antibody at desired concentration in normal goat serum (see antibody-specific suggestions, or try a range of dilutions such as 1:50 and 1:100).
9. Add primary antibody (approximately 100µL) to slides. (Slides without primary antibody are left with normal goat serum alone.) Let sit at room temperature for 60 minutes, or let sit overnight at 4ºC.
10. Rinse slides with TBS-T for 2 minutes. Repeat rinse.
11. Prepare secondary antibody (1:250 dilution) in TBS-T, as dictated by the primary antibody's species of origin, and raised in goat (otherwise must change normal goat serum at step #7 to appropriate serum).
12. Add secondary antibody (approximately 100µL) to slides. Let sit at room temperature for 30 minutes.
13. Prepare Vectastain ABC as per the manufacturer's instructions. Let sit for 30 minutes.
14. Rinse slides with TBS-T for 2 minutes. Repeat rinse.
15. Add ABC solution to slides. Let sit for 30 minutes.
16. Rinse slides with TBS-T for 5 minutes.
17. Rinse slides with distilled water.

18. Prepare Nova Red Kit, as per the manufacturer's instructions. Add approximately 100µL to each slide and monitor under light microscope. Rinse slides with gently running tap water for 5 minutes when done (maximum development time of 10 minutes).
19. Counterstain tissues in hematoxylin for 90 seconds.
20. Rinse slides twice in warm tap water for 5 minutes each to develop hematoxlin.
21. Rinse slides in 95% ethanol until clear, twice. Rinse slides in 100% ethanol until clear, then wash slides in xylene until clear, twice.
22. Mount coverslips on slides using Permount. Let slides dry overnight.

Hematoxylin and eosin stain protocol

1. Wash slides by submerging in xylene three times for 5 minutes each to remove paraffin.
2. Rinse slides in 100% ethanol until clear, then 70% ethanol until clear, then 50% ethanol until clear, then water until clear.
3. Stain tissues in hematoxylin for 3 minutes.
4. Rinse slides in water. Repeat rinse.
5. Immerse slides in saturated lithium carbonate for 10 seconds.
6. Rinse slides in water.
7. Stain tissues in eosin for 45 seconds.
8. Rinse slides in 95% ethanol, then two washes of 100% ethanol, then rinse with xylene until clear, twice.
9. Mount coverslips on slides using Permount. Let slides dry overnight.

Protein protocols

Cell lysate collection

Cells were seeded in 55cm^2 Petri dishes. Media was removed by aspiration, and cells were washed twice with cold PBS. Cells were collected in 1mL cold PBS in 1.5mL Eppendorf tubes and centrifuged for 5 minutes at 10,000×*g* and 4ºC. The supernatant was removed and the pellet was resuspended in 100µL lysis buffer, prepared as described below. Tubes were placed on a rocker at 4ºC for 60 minutes, then the lysate was

centrifuged again for 5 minutes at 10,000×*g* and 4ºC. The supernatant was collected and transferred to a new Eppendorf tube, labelled, and stored at -80ºC.

Reagents

Lysis buffer:
Combine the following in a 500mL reagent bottle:
16.7mL 15% NP-40
20.0mL 5M NaCl
12.5mL 1M Tris pH 7.4
2.5mL 0.5M EDTA pH 8.0

Bring to 250mL volume with water. Store at 4ºC. Immediately prior to use, remove 10mL to centrifuge tube and add one protease inhibitor tablet (Roche).

Bio-Rad protein assay

Protein concentration in whole cell lysates was determined by the Bio-Rad protein assay. A 5µL volume of cell lysate was diluted in 45µL water. In addition, 1mg/mL albumin from bovine serum (BSA) stock solution was prepared as a standard and was diluted with water into 50µL volumes containing final concentrations of 0, 0.05, 0.1, 0.2, 0.3, 0.4, and 0.5mg/mL BSA. Samples and standards were mixed and 10µL were added in triplicate wells to a 96-well plate and diluted further with 10µL water. Finally, a 200µL volume of Bio-Rad Protein Assay Solution (containing 1mL Bio-Rad Protein Assay Dye Reagent and 4mL water) was added to each well. Bubbles were popped with a micropipette tip, and plates were assayed at 570nm on a Bio-Tek Powerwave XS Spectrophotometer.

Spectrophotometry results are exported to Microsoft Excel, and a standard curve is prepared from the mean values of the triplicate standard data points. A linear relationship

between the standards should result, and protein concentration of lysate is determined from the resulting trend line.

Western blotting

Separating and stacking gels for sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) were prepared using the volumes listed in Table 2. Both 10% ammonium persulfate (APS) and TEMED were added immediately prior to pouring gel solution in apparatus.

Table 2 – Recipe for SDS-PAGE preparation.

Component	**Separating gel**		**Stacking gel**
	10%	**15%**	
30% acrylamide/bis 29:1	3.33 mL	5 mL	0.75 mL
Water	4.2 mL	2.3 mL	3.0 mL
Buffer A (1.5M Tris, pH 8.8)	2.5 mL	2.5 mL	0 mL
Buffer B (0.5M Tris, pH 6.8)	0 mL	0 mL	1.25 mL
20% SDS	50 µL	50 µL	25 µL
10% APS	100 µL	100 µL	50 µL
TEMED	5 µL	5 µL	7.5 µL

Equal amounts of protein (determined by the Bio-Rad protein assay) were diluted in water to identical volumes and SDS loading buffer containing 0.2% 2-mercaptoethanol was added to each sample. Proteins were denatured by heating at 95ºC for 5 minutes prior to loading samples in the gel. Samples were electrophoresed at 100V. Next, proteins were transferred to a nitrocellulose membrane by electrophoresis and incubated

overnight at 4ºC in TBS-T containing 5% skim milk to block non-specific binding of antibodies. Following overnight incubation, primary antibodies were diluted in TBS-T containing 5% skim milk and added to the membrane for 3-hours at room temperature. Primary antibodies were subsequently removed and the membrane was rinsed with TBS-T. Multiple washes of the membrane in TBS-T followed, for a cumulative duration of 30 minutes. Appropriate horseradish peroxidase-linked IgG secondary antibodies were diluted in TBS-T containing 5% skim milk and added to the membrane for 1-hour room temperature incubation. Proteins were visualized using enhanced chemoluminescence detection (Amersham Biosciences/GE Healthcare Bio-sciences Inc.) according to the manufacturer's instructions and developed on Kodak Biomax XAR film (Kodak Canada).

Multiplex assay system

Quantification of MMPs with the Bio-Plex assay was performed on the Fluorokine MAP Multiplex Assay System with Luminex 100 detection equipment (R&D Systems) according to the protocol described for the Fluorokine MAP: Human MMP MultiAnalyte Profiling Base Kit (R&D Systems). Reagents and standard dilutions were prepared as dictated by the protocol and 35µL samples of cell culture media were diluted with 140 µL calibrator diluent. Lysate preparations were not diluted with calibrator diluent. Samples were assayed according to the following method:

1. Pre-wet filter-bottomed plate by adding 100 µL wash buffer to each well to be used. Liquid removed by using vacuum manifold set at 2″. Remnants blotted dry with Kimwipe.
2. Microparticle mixture resuspended and 50 µL added to each well (done quickly with lights on).

3. Added 50 µL standard or sample per well. Covered plate with foil sealer and incubated at room temperature for 2 hours on horizontal orbital microplate shaker set at 500 ± 50 rpm. During incubation, prepared biotinylated antibodies by combining 50 µL each microparticle concentrate (for MMP-1, MMP-8, and MMP-13) with 5 mL microparticle diluent. Also prepared streptavidin-phycoerythrin conjugate by diluting the 100× stock solution in wash buffer.
4. After 2 hour incubation, removed liquid using vacuum manifold as described above. Washed each well with 100 µL wash buffer and removed liquid again. Blotted dry on Kimwipe. Wash was repeated three times.
5. Added 50 µL of biotin antibody cocktail to each well. Covered wells again with new foil sealer and incubated at room temperature for 1 hour on the shaker at 500 ± 50 rpm. During incubation, calibrated Bio-Plex machine.
6. Following 1 hour incubation, repeated washes as described in step #4.
7. Added 50 µL of streptavidin-phycoerythrin to each well. Covered with new foil sealer and incubated at room temperature for 30 minutes on shaker set at 500 ± 50 rpm.
8. Repeated washes described in step #4.
9. Resuspended microparticles by adding 100 µL of wash buffer to each well. Incubated on shaker for 2 minutes at room temperature and 500 ± 50 rpm.
10. Fluorescence was measured on Bio-Plex machine.

RNA protocols

RNA isolation

RNA was collected from cells seeded in 55cm^2 Petri dishes, using the following protocol:

1. Aspirate media from dish. Wash cells twice with PBS. Completely aspirate PBS.
2. Add 1mL TRIzol to each dish and collect cells with cell scraper into 1.5mL Eppendorf tubes. (Can place in -80ºC freezer at this point until ready to proceed.)
3. Add 200µL chloroform to each tube. Vortex solution vigorously and then centrifuge tubes at 12,000×*g* for 15 minutes at 4ºC.
4. Remove clear supernatant (the RNA) to new 1.5mL Eppendorf tubes, being careful not to collect white mid-layer (the DNA). Add 500µL cold isopropanol to precipitate RNA. Let tubes sit at room temperature for 5 minutes, then store at -20ºC for 2-6 hours.

5. Following incubation, centrifuge tubes for 30 minutes at 12,000×*g* and 4ºC.
6. Dump isopropanol into new 1.5mL Eppendorf tube. Wash remaining RNA pellet in 1mL 75% ethanol (prepared in DEPC-treated water).
    - Note: If no RNA pellet is visible at this stage, centrifuge the new tubes containing isopropanol again for 30 minutes at 12,000×*g* and 4ºC to obtain RNA pellet.
7. Centrifuge tubes at 7500×*g* for 6 minutes at 4ºC. Remove ethanol carefully using micropipette. Let remaining ethanol evaporate in laminar flow hood until pellets are dry.
8. Resuspend RNA pellets in 50µL DEPC-treated water. Heat samples at 60ºC for 10 minutes. Store RNA at -80ºC, or proceed to DNase treatment and RNA quantification.

DNase treatment and RNA quantification

1. Use Ambion DNA-free treatment on RNA samples to remove DNA. RNA should be kept on ice unless otherwise stated. Add 1/10 volume buffer to each tube ($\therefore$5µL).
2. Add 1µL DNase to each tube. Heat tubes in waterbath at 37ºC for 25-30 minutes.
3. Add 5µL DNase inhibitor to each tube to stop reaction.
4. Centrifuge tubes at 13,000×*g* for 1 minute at 4ºC. Remove clear supernatant to new tube.
5. Add 50µL 1M Tris pH 7.5 to 10mL DEPC-treated water in 15mL centrifuge tube. Mix well, and then prepare new Eppendorf tubes to quantify RNA, and add 2µL RNA to 98µL of newly-prepared solution.
6. Measure UV absorbance of RNA dilutions at 260nm and 280nm on a spectrophotometer.
7. Calculate the RNA concentration (in µg/mL) using the following formula:

$$[\text{RNA}] = \text{Absorbance}_{(260\text{nm})} \times 40\mu\text{g/mL} \times \text{dilution factor (}i.e.\text{ 50)}$$

cDNA synthesis

RNA was reverse-transcribed into cDNA with Superscript III (Invitrogen Canada/Gibco), as per the manufacturer's instructions. Specifically, 1µg RNA was diluted with DEPC-treated water into a total volume of 8µL per sample. Next, 1µL each of 10mM dNTP mix and Oligo$(dT)_{20}$ were added to the reaction tube. Samples were

briefly centrifuged, and then heated at 65ºC for 5 minutes on an MJ Mini Personal Thermal Cycler (Bio-Rad Laboratories), and then transferred to ice. A master mix was prepared for all samples that included (per sample) 2µL 10× RT buffer, 4µL 25mM $MgCl_2$, 2µL DTT, 1µL RNase OUT, and 1µL Superscript III. A 10µL aliquot of the master mix was added to each reaction tube. Samples were briefly centrifuged then returned to the cycler for incubation at 50ºC for 50 minutes, followed by 85ºC for 5 minutes, and then cooling to 4ºC. RNase H (1µL) was added to each reaction tube and samples were heated to 37ºC for 20 minutes to remove residual RNA. Samples were processed immediately by PCR or real-time PCR.

PCR

Amplification of cDNA (prepared as described above) was performed in a 100µL volume containing the following reagents: 10µL 10×PCR buffer, 2µL 10mM dNTP mix, 3µL 50mM $MgCl_2$, 2.5µL each 10µM primer, 0.5µL Taq polymerase (Invitrogen Canada/Gibco), 75.5µL DEPC-treated water, and 4µL cDNA. Master mixes were prepared for each primer pair and 96µL aliquots were transferred to reaction tubes for addition of cDNA. Products were amplified on an MJ Mini Personal Thermal Cycler (Bio-Rad Laboratories) according to the following protocol: 94ºC for 3 minutes, followed by 35-40 cycles of 94ºC for 30 seconds, 55ºC for 30 seconds, and 72ºC for 60 seconds, with a final 72ºC extension for 10 minutes and cooling to 4ºC. Products were diluted in loading buffer and electrophoresed on 1% or 2% agarose gels containing 0.5µg/mL ethidium bromide. Bands were compared to ladder to determine product size, and target genes were compared to housekeeping gene amplification.

Real-time PCR

Real-time PCR reactions were performed using a 20µL total volume containing 10µL Express SYBR GreenER Universal (Invitrogen Canada/Gibco), 7.2µL DEPC-treated water, 0.4µL each 10µM primer, and 2µL cDNA (prepared as described above). Master mixes were prepared for each primer pair and 18µL aliquots were transferred to reaction tubes for addition of cDNA. Appropriate housekeeping genes were determined by establishing comparable amplification efficiencies for serially-diluted cDNA. Products were amplified on a MiniOpticon System (Bio-Rad Laboratories) according to the following protocol: 50ºC for 2 minutes, then 95ºC for 2 minutes, followed by 40 cycles of 95ºC for 15 seconds and 60ºC for 60 seconds, with a gradient change in temperature from 60 to 95ºC to determine the melting curve of the final products. Gene expression was quantified according to the comparative threshold cycle method ($\Delta\Delta C_t$).

Osteoclastogenesis assay

Co-cultures were established by seeding 10^5 RAW 264.7 cells per well in a 24-well plate, and seeding 2×10^5 GCT stromal cells in 0.4-µm cell culture inserts (BD Biosciences) that sit atop the RAW 264.7 cells. The cells were seeded in α-minimum essential media (α-MEM) supplemented with 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin (Invitrogen Canada/Gibco), which were found to promote osteoclast growth to a greater degree than other cell culture mediums after troubleshooting, and the plate was placed in an incubator at 37ºC in humidified air containing 5% CO_2. After permitting the cells to adhere for 4 hours, cell culture medium was gently removed using a micropipette (to reduce disturbance of cells) and replaced with new α-MEM

supplemented as described above and containing 0, 10, or 100nM PTHrP (1-34) (Bachem). Plates were returned to the incubator, and thereafter cell culture medium was renewed every second day. On the tenth day of incubation, the cell culture inserts were discarded and the RAW 264.7 cells were fixed and stained for tartrate-resistant acid phosphatase (TRAP) activity with the Acid Phosphatase, Leukocyte (TRAP) Kit (Sigma-Aldrich Canada Ltd.), according to the protocol. TRAP-positive cells containing three or more nuclei were counted using a light microscope at 100× magnification at five consistent locations in each well.

APPENDIX B

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