Stromal Cell Proliferation And Recurrence Of Giant Cell Tumor Of Bone Following Neoadjuvant Denosumab Treatment

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Introduction: Giant Cell Tumor of Bone (GCT) is a primary bone tumor that is treated surgically and in many cases is associated with high morbidity [1]. Until recently, there were no established adjuvant systemic treatment options for patients with extensive or unresectable GCT [2]. GCT consists of osteoclast-like giant cells that express Receptor Activator of Nuclear factor-κB (RANK) and mononuclear stromal cells that express RANK ligand (RANKL). The functional interaction between the stromal-cell derived RANKL and the osteoclast receptor (RANK) results in a steady production of osteoclast-like cells in the tumor that are responsible for resorbing bone [3]. Denosumab is a monoclonal antibody that binds RANKL and directly inhibits osteoclastogenesis [4]. Clinical studies have suggested clinical and histological improvement when denosumab was administered to patients with GCT [5, 6, 7]. Based on our previous report, we have examined the viability of the neoplastic stromal cells from 2 patient samples post-denosumab exposure. Interestingly, one of the two patients suffered a local recurrence at 9 months post-operatively. The objective of this study is to further examine the molecular characteristics of the cells of GCT post denosumab treatment.

Methods: GCT sample collection: Specimens were obtained at the time of biopsy from six patients with histologically confirmed GCT. Two of the GCT patients had been treated with denosumab. Standard paraffin-embedded haematoxylin and eosin (H+E) examination was used for both the biopsy and resection specimens.

Primary cell lines and culture: Primary cell cultures of GCT stromal cells were isolated, characterized and established from the fresh GCT patient tissue at the time of definitive resection as described in our previous studies [8]. Fifth passage cells were used for in vitro experiments.

WST-1 cell proliferation measurement: Mitochondrial dehydrogenase activity, as an indicator of cell number, was assessed by the mitochondrial-dependent reduction of WST-1 to formazan using a colorimeter at indicated time-points over a 2-day time course.

RNA purification, reverse transcription and real-time polymerase chain reaction (PCR): Total RNA was isolated from GCT stromal cells as described in our previous studies [8]. Single-stranded complementary DNA (cDNA) was synthesized from 1.0μg of total RNA and applied to real-time PCR. The expression of RANKL and OPG was analyzed using real-time RT-PCR. PCR experiments were performed in triplicate and included negative no-template controls.

Results: Both GCT post denosumab patient histological specimens showed the absence of giant cells, but with persistent stromal cells (Figure 1). As previously reported, WST-1 cell proliferation studies indicated that GCT stromal cells cultured from clinical specimens exposed to denosumab proliferated at a ~50% slower rate to specimens not exposed to denosumab (Figure 2). Furthermore, the expression of RANKL in post-denosumab GCT specimen was eliminated (Figure 3). In contrast, osteoprotegerin (OPG), the decoy receptor of RANKL, was upregulated over several hundred fold (Figure 3). The recurrence
from patient 1 post-denosumab will be studied and data will be presented from WST-1 cell proliferation studies and RANKL/OPG real-time PCR.

**Discussion:** In contrast to the recent claim that denosumab targets the neoplastic stromal cells of GCT [7], we have confirmed that once the remaining GCT tissue is no longer exposed to denosumab, the stromal cells continue to proliferate, albeit to a lesser degree. They also show complete loss of expression of the osteoclastogenic factor RANKL. Based on the post-denosumab histological specimens reported by the current study and others [5, 7], denosumab appears to be biologically active in inhibiting osteoclastogenesis, which would be expected of a mononclonal antibody that binds RANKL. However, we have shown that once the microenvironment is free of the antibody, the neoplastic cells remain proliferative but do not express RANKL. To further support these findings, one of the denosumab-treated patients suffered an aggressive recurrence. It is clear that treatment with denosumab only addresses an immediate therapeutic need of GCT partially by wiping out the osteoclasts but leaving the neoplastic stromal cells proliferative and active again once denosumab is withdrawn.

**Significance:** The current study represents translational in vitro data on recurrent GCT post denosumab treatment. This is the first report to confirm that denosumab does not eradicate the proliferative characteristics of the neoplastic cells of GCT and therefore tumor progression is likely to follow drug discontinuation. In fact, one patient studied suffered an aggressive local recurrence in the proximal fibula. In cases of GCT in which surgical resection is not feasible and/or long-term pain management is required, denosumab will likely be effective only when systemic drug levels remain at therapeutic levels.

**Figure 1.** (a) Pre-denosumab biopsy specimen with H&E staining showing classic osteoclast-type giant cells (arrow) amid a background of uniform mononuclear stromal cells (*). (b) Post-denosumab operative specimen showing an absence of giant cells with persistence of the mononuclear stromal cells (*).
Figure 2. Proliferation assay on GCT cells with Denosumab treatment compared to 4 untreated GCT samples. Denosumab decreased the growth rate of the GCT stromal cells by approximately 50%.
**Figure 3.** The mRNA expression of OPG and RANKL were determined in GCTs with or without Denosumab treatment. RANKL mRNA expression was eliminated after exposure to Denosumab; however, OPG mRNA increased several hundred fold. RPS18 serves as the internal control gene.