Denosumab Induces Tumor Reduction and Bone Formation in Patients with Giant Cell Tumor of Bone


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Statement of Translational Relevance

Giant cell tumor of bone (GCTB) is a rare, aggressive osteolytic tumor that causes localized pain and impaired mobility and function. Surgery, the definitive therapy for GCT, is often associated with significant recurrence and morbidity including amputation. GCTB is characterized by osteoclast-like giant cells and their precursors that express RANK and mononuclear stromal cells that express RANKL, a key mediator of osteoclast activation. Denosumab binds to RANKL, inhibiting bone destruction and eliminating giant cells. In an open-label phase 2 proof-of-concept study of patients with GCTB, 86% of patients had a tumor response (1). Using histologic analyses from this study, this report explores the mechanism of denosumab’s action in GCTB, supporting continued evaluation of denosumab for treatment of GCTB.
ABSTRACT

Purpose: Giant cell tumor of bone (GCTB) is a locally aggressive, benign osteolytic tumor in which bone destruction is mediated by RANKL. The RANKL inhibitor denosumab is being investigated for treatment of GCTB. We describe histologic analyses of GCTB tumor samples from a phase 2 study of denosumab in GCTB.

Experimental Design: Adult patients with recurrent or unresectable GCTB received subcutaneous denosumab 120 mg every 4 weeks (with additional doses on days 8 and 15). The primary histologic efficacy endpoint was the proportion of patients who had a $\geq 90\%$ elimination of giant cells from their tumor. Baseline and on-study specimens were also evaluated for overall tumor morphology and expression of RANK and RANKL.

Results: Baseline tumor samples were typically composed of densely cellular proliferative RANKL-positive tumor stromal cells, RANK-positive rounded mononuclear cells, abundant RANK-positive tumor giant cells, and areas of scant de novo osteoid matrix and woven bone. In on-study samples from 20 of 20 patients (100%), a decrease of $\geq 90\%$ in tumor giant cells and a reduction in tumor stromal cells were observed. In these analyses, thirteen patients (65%) had an increased proportion of dense fibro-osseous tissue and/or new woven bone, replacing areas of proliferative RANKL positive stromal cells.

Conclusions: Denosumab treatment of patients with GCTB significantly reduced or eliminated RANK-positive tumor giant cells. Denosumab also reduced the relative content of proliferative, densely cellular tumor stromal cells, replacing them with non-proliferative, differentiated, densely woven new bone. Denosumab continues to be studied as a potential treatment for GCTB.
INTRODUCTION

Clinically, giant cell tumor of bone (GCTB) is a benign, locally aggressive, osteolytic tumor that causes significant bone destruction and has a predilection for the epiphyseal/metaphyseal region of long bones and spine (2). Histologically, GCTB is composed of sheets of neoplastic ovoid mononuclear cells with high RANKL expression, RANK-positive mononuclear cells of myeloid lineage, and a randomly distributed population of large RANK-expressing osteoclast-like giant cells (3-14). Many researchers have demonstrated that the giant cell of GCTB is osteoclastic in nature (5, 7, 11) and that the true neoplastic cells are ovoid cells displaying markers of mesenchymal stem cells that have partially differentiated along the osteoblast lineage (15-18). These tumors are frequently described to contain small areas of osteoid matrix deposition, woven bone, and occasionally new bone, which may be reactive tissue at the tumor margin or formed de novo within the tumor (10, 19, 20). The hallmarks of this tumor are its aggressively lytic behavior and the clear role that osteoclast-like tumor giant cells play in the lytic process. Denosumab is a fully human monoclonal antibody that specifically inhibits normal and tumor-associated bone lysis by preventing RANKL-mediated formation and activation of multinucleated osteoclasts or giant cells from RANK-positive mononuclear pre-osteoclasts and macrophages (1, 21). Subcutaneous administration of denosumab has been shown to suppress bone destruction in patients with osteolytic bone disease in multiple myeloma or bone metastases in breast and prostate cancer and other solid tumors (22-24). It was hypothesized that denosumab would inhibit the osteoclast-like giant cells in a similar manner.

An open-label, phase 2, proof-of-concept clinical study was conducted in patients with recurrent or primary unresectable GCTB to evaluate the efficacy of subcutaneous denosumab in reducing tumor-associated bone lysis. The safety and efficacy of denosumab in this study have been described previously (1). This report describes the histologic effects of denosumab
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treatment in GCTB in a subset of 20 patients for whom histologic data were available from baseline and on-study biopsies or resections in the phase 2 study.

MATERIALS AND METHODS

Phase 2 clinical study

The clinical study of denosumab in GCTB has been described previously (1). Briefly, in this open-label, single-group study, 37 patients with recurrent or unresectable GCTB were treated with subcutaneous denosumab 120 mg every 28 days, with additional doses on days 8 and 15. The primary efficacy endpoint was the proportion of patients with a tumor response, defined histopathologically as at least 90% elimination of giant cells relative to baseline; or, where giant cells represented < 5% of tumor cells at baseline, complete elimination of giant cells. If histopathologic data were not available, tumor response was defined as a lack of radiologic progression of the target lesion by week 25. Prespecified exploratory analyses included clinical benefit as reported by investigators and subjective evaluation of baseline and on-study tumor tissue specimens for RANKL and RANK expression. Histopathologic evaluations of baseline and on-study tissue specimens from clinical study patients were conducted by a central pathologist who was blinded to the sequence of specimen collection (baseline or on-study).

Patient specimens

For the subset of 20 patients in the phase 2 study from whom histologic specimens were available, we compared baseline and on-study tissue specimens to subjectively evaluate the effects of denosumab treatment on overall tumor morphology. Evaluation included the extent of the tumor section composed of densely cellular ovoid or spindle-shaped mononuclear tumor stromal cells, increased de novo bone matrix, woven bone, or new bone, and the presence of tumor giant cells. Changes in the degree and distribution of expression of RANK, RANKL, Ki67, and activated caspase 3 in the tissue sections and in tumor cells were also noted. Baseline
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biopsy was performed if clinically appropriate; otherwise, the most recent biopsy before
denosumab treatment was used. On-study biopsies or resections were obtained from all patients
between doses 5 and 9 of denosumab (months 3 and 7) unless the procedures were judged to be
clinically unsafe. Up to ten unstained slides of tissue from each biopsy/resection were formalin
fixed and paraffin embedded (FFPE) at clinical sites and sent to the Amgen Tissue Bank.

Tumor morphology

FFPE tissue sections (4–5 μm) were deparaffinized, hydrated, and stained with
hematoxylin and eosin (H&E). Baseline and on-study tissue samples from each patient were
evaluated for changes in overall tissue composition and architecture by light microscopy.
Significant and consistent differences in the content of dense fibro-osseous connective tissue or
new bone between baseline and on-study samples were qualitatively evaluated and described as
increased, decreased, or unchanged relative to baseline. Representative photomicrographs were
obtained.

Expression of RANK and RANKL

Baseline and on-study tissue specimens were evaluated for RANK and RANKL
expression by immunohistochemistry (IHC). Dried tissue sections were incubated at 60° C for 45
minutes or overnight, deparaffinized, hydrated, and prepared by heat retrieval in Diva Decloaker
(Biocare Medical, Concord, CA) or citrate buffer pH 6 (Citra, BioGenex Laboratories Inc,
Fremont, CA ). Immunohistochemistry (IHC) analysis was performed using automated staining
methods (Dako Autostainer: Thermo Scientific, Fremont, California; Dako North America,
Carpinteria, California). Tissue sections were blocked against endogenous proteins and staining
reagents. Tissue sections were stained with a mouse anti-human RANK antibody, Amgen N-2B10.1 (5 μg/ml; Amgen Inc., Seattle, WA) and mouse anti-human RANKL antibody, Amgen
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M366 (0.75 mg/ml; Amgen Inc.) with goat anti-mouse secondary antibody. The specificity of the N-2B10.1 antibody for HuRANK and of the M366 antibody for HuRANKL has been demonstrated in transfected cell lines and in cell lines endogenously expressing the appropriate antigen (25). In these specimens, the pattern of labeling by Western blotting and flow cytometry was concordant with expression observed by IHC in tumor xenograft samples derived from these cell lines. Additionally, no labeling by flow cytometry or IHC was observed in untransfected parental cells or negative control xenografts. An isotype control mouse IgG1 slide was analyzed for each specimen and each antibody. Antibody reactivity was visualized by 3,3’-diaminobenzidine (DAB; Dako, # K3466; Dako North America, Carpinteria, California) enhanced by tyramide amplification (Perkin Elmer, Boston, Massachusetts). The relative degree and distribution of RANK and RANKL-positive cells in the tumor specimen were assessed subjectively for all samples.

Tumor Proliferation and Apoptosis

To assess evidence of cell proliferation and apoptosis, the baseline and on-study tissue specimens were analyzed for Ki67 and caspase 3 expression. Dried tissue sections were incubated at 60°C for 45 minutes or overnight, deparaffinized, hydrated, and prepared by heat retrieval in Diva Decloaker Solution in Decloaking Chamber (Biocare Medical, Concord, CA). Immunohistochemistry (IHC) was performed using automated staining methods (Labvision Autostainer: Thermo Scientific, Fremont, California; Dako Autostainer: Dako North America). Tissue sections were blocked against endogenous proteins and staining reagents and stained with a rabbit polyclonal Ki-67 antibody, Abcam #ab66155-200 (0.5 μg/ml; Abcam, San Francisco, CA) and caspase 3 antibody, (1:50; Cell Signaling, #9661L, Danvers, MA), followed by anti-
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rabbit secondary antibody, EnVision, Dako #K4003. Antibody reactivity was visualized by DAB.

Archival Specimens

To establish that baseline patient samples from the phase 2 study were morphologically representative of the overall population of GCTB, 40 archival FFPE specimens of primary and recurrent GCTB, unrelated to the patient specimens described above, were stained with H&E. We subjectively evaluated the extent of the tumor section composed of densely cellular ovoid or spindle-shaped mononuclear tumor stromal cells, increased de novo bone matrix, woven bone, or new bone and the presence of tumor giant cells.

Microscopy Methods

Histologic images were photographed using a Nikon Eclipse E600 microscope with a Nikon DXM1200 digital camera. The resulting images were white-balanced using Photoshop CS software; no further image manipulation was employed.

RESULTS

Histopathology results

Among the 37 patients treated with denosumab, 20 patients had baseline and on-study biopsies or histologic specimens from resection. All 20 patients had specimens evaluable for tumor morphology, 19 for the presence of RANKL, and 10 for the presence of RANK. The demographic and disease characteristics in the histologic subset were similar to those in the overall study population (1). Approximately half (55%) of patients were women, and approximately two-thirds were Caucasian; mean age was 33 years (range, 20–59 years) (Table 1). Most (80%) had unresectable disease, either primary or recurrent; 70% had previous surgery (Table 1).
Baseline tumor samples were typically composed of densely cellular RANKL-positive tumor stromal cells and randomly distributed abundant RANK-positive tumor giant cells. Numerous RANK-positive ovoid mononuclear cells were also present. Very sparse focal areas composed of bone matrix, fibro-osseous tissue and woven bone were present in the tumor in 16 of 31 (52%) evaluable baseline samples; paired on-study samples were not available for all baseline samples (Figure 1A, B; Figure 3 A, B; Figure 4 A, C, E; Figure 5 A, C, E).

The 40 archival GCTB samples unrelated to the phase 2 patient samples were similar in morphology to the phase 2 baseline samples in the extent of densely cellular ovoid or spindle-shaped mononuclear tumor stromal cells and the presence of osteolytic tumor giant cells.

Eighteen of 40 (45%) of the archival samples showed small foci of de novo bone matrix, woven bone, or new bone, in a proportion similar to that seen in the phase 2 baseline samples.

As previously reported (1), all 20 evaluable patients showed a decrease in tumor giant cells of ≥ 90% upon histologic analysis of on-study tumor samples. We also observed a marked reduction in the tumor content of densely cellular RANKL-positive tumor stromal cells and increased fibro-osseous tissue and/or new bone that replaced the original RANK-positive tumor cells (Figure 2 A, B, C, D; Figure 3 C, D, E, F, Figure 4 B, D, F; Figure 5 B, D, F). In 20 pairs of patient samples evaluated, 65% of on-study samples showed a marked increase from baseline in the proportion of dense fibro-osseous tissue and/or new bone and a consequent reduction in tumor cellularity.

After treatment, small islands of residual tumor tissue remained, composed of tumor stromal cells expressing significant levels of RANKL and mononuclear cells expressing RANK, while tumor giant cells were typically absent (Figure 2 A, B, C; Figure 3 C, D; Figure 4 D, F; Figure 5 D, F). In baseline samples, Ki67-positive tumor stromal cells were observed, but tumor
giant cells were not Ki67-positive. In on-study samples, the proportion of Ki67-positive cells was reduced; however, the residual areas of densely cellular stromal cells contained a similar proportion of Ki67-positive cells to that found in baseline samples (Online Supplementary Data Figure S1 A and B). The proportion of activated caspase 3 was very low in both baseline and on-study samples and was not changed by treatment at the time of on-study sample collection 3–7 months after the start of treatment.

Clinical benefit

Nineteen of the 20 patients in the histologic subset had baseline and on-study investigator assessments of clinical benefit. Of these patients, 17 (90%) were judged by investigators at various stages of treatment to have derived clinical benefit from denosumab (e.g., improved functional status or reduced pain). Of the 13 patients in this analysis for whom histologic evidence of new bone formation was observed, investigators reported clinical benefit for 11 patients and no clinical benefit for two. Seven patients with no histologic evidence of new bone formation also experienced clinical benefit. These results should be interpreted with caution; the study population was small and the study was not statistically powered to test these correlations. The condition of patients at baseline varied considerably, and the timing of histologic samples was independent of the reporting of clinical benefit.

DISCUSSION

This report summarizes the results of histologic analysis of tissue specimens from patients with GCTB, providing evidence that the fully human monoclonal antibody denosumab significantly reduces or eliminates RANK-positive tumor giant cells, reduces the relative proportion of proliferative, densely cellular tumor stroma, and promotes the formation of differentiated bone tissue.
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Some aspects of the histogenesis of GCTB have been previously characterized. GCTB contains three histologically different cell types: round to oval polygonal or elongated mononuclear stromal tumor cells, a minor component of reactive rounded mononuclear cells of monocyte lineage representing precursors of the giant cells, and evenly scattered giant cells. In many publications, the mononuclear stromal cells are reported to overexpress RANKL and are thought to be the true neoplastic components of GCTB (3, 8, 11, 14). These transformed mononuclear stromal cells have been shown to express many characteristics of mesenchymal stem cells that have differentiated along the osteoblast lineage but not to a completely differentiated osteoblast phenotype, with minimal expression of such factors as osteocalcin and alkaline phosphatase (15-18, 26, 27). They also show cytogenic markers indicating transformation, including telomere associations, but fail to show consistent chromosomal changes and are reported to be polyclonal (28-31). Interestingly, GCTB tumors are occasionally reported to contain scant focal areas of osteoid matrix and woven bone that is present within the tumor and separate from the tumor margins, in an incidence ranging from 22%–52% (10, 19, 20, 32). Our own analysis of 31 baseline samples from the phase 2 study and an archival set of 40 GCTB samples revealed a similar pattern in 45–52% of the tumors evaluated, although localization of the biopsy to the tumor margin was not established.

Within the tumor environment, numerous soluble factors that are chemotactic for myelomonocytic cells such as SDF-1, MIP-1α, and csf-1 have been identified (3, 12, 33-35). It is thought that the expression of these factors results in the accumulation of RANK-positive monocyctic cells of bone marrow origin expressing a pre-osteoclastic phenotype (3-5, 8, 9, 13, 14). Signalling by RANKL on tumor stromal cells activates RANK on the pre-osteoclasts, inducing giant cell formation and resulting in the destructive osteolysis of GCTB. Coculture of
GCTB stromal cells with circulating mononuclear cells has been shown to induce osteolytic giant cells in vitro (32, 36-38).

The importance of the RANK/RANKL signaling pathway in GCTB is thought to represent a neoplastic analogue to normal bone remodeling in which the RANKL-positive osteoblast and RANK-positive osteoclasts are coupled to result in regulated bone deposition and removal. In normal bone, this coupling is regulated as the bone architecture is modified during growth and changing stresses (39). Inhibition of normal bone remodeling by interfering with this pathway has been clearly established (40, 41). The destructive osteolysis that is characteristic of GCTB, where dysregulated stimulation of osteoclastic bone remodeling is observed, is also dependent on this pathway. In cultures of GCTB, it has been shown that the activation of monocytes to form osteoclasts can be inhibited by osteoprotegerin (OPG), a soluble decoy receptor for RANKL (42). The results of the GCTB phase 2 study presented here also clearly demonstrate that the anti-RANKL antibody denosumab is clinically active in altering the osteolytic effect of GCTB (1).

Surprisingly, histological comparison of baseline and on-study samples of GCTB from this study also showed that the classic architecture of GCTB, consisting of proliferative densely cellular stromal cells, RANK positive mononuclear cells, and tumor giant cells, was substantially changed by treatment. In the on-study samples, a marked increase in non-proliferative osteoid matrix, woven bone and new bone was present with only focal areas of proliferative RANKL-positive tumor stromal cells and RANK-positive mononuclear cells, and with greatly reduced numbers of tumor giant cells. This indicates that the tumor cell component was induced to differentiate towards a non-proliferative osteoblastic phenotype not typical of untreated tumors, with a marked reduction in osteolysis at the tumor margins. It is possible that these observations
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reflect a changing balance of bone formation/resorption in favor of bone formation, and that the histologic findings are a manifestation of this change in bone metabolism. The reports that GCTB stromal cells express many markers of the osteoblastic lineage but minimally express the late stage markers of osteocalcin and alkaline phosphatase (15, 26) suggest that these neoplastic cells are either incapable of further differentiation, lack critical differentiation factors, or are being inhibited from differentiation by factors in the tumor environment. However, results from in vitro cultures of GCTB stromal cells indicate that they are capable of differentiating into a mature osteoblast phenotype when separated from the osteoclastic component and following treatment with factors such as bone morphogenic protein (BMP). In addition, GCTB stromal cells can form mature bone when implanted into immunologically deficient mice (43). We report here, as others have, that limited de novo bone matrix formation is observed in primary GCTB and also in GCTB that has metastasized to the lung (10, 19, 20, 32, 44).

In normal osteoblast-osteoclast coupling, the activated osteoclast typically releases factors that stimulate bone deposition (39-44). Since denosumab treatment significantly reduces or eliminates osteoclasts, we would not expect the increased bone matrix and new bone to be observed. These observations indicate that the microenvironment within GCTB decouples the interaction of the tumor stromal cells and tumor giant cells, resulting in very limited bone formation and marked stimulation of osteolysis.

It is thought that the RANK/RANKL pathway is the final common signal for osteoclast formation from monocytes/macrophages. In contrast, numerous factors have been described that can either stimulate or inhibit osteoblast maturation such as BMPs and the Ephrin/Eph bidirectional signaling between osteoblast and osteoclast (44-49). The results of the current study indicate that inhibiting RANKL signaling in GCTB induces bone formation by tumor stromal...
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cells either indirectly by eliminating factors associated with tumor giant cells or by direct action on the tumor stromal cell to form bone. Since neither published reports nor our analyses provide evidence of RANK expression on the tumor stromal cell, we would conclude that the increased bone formation induced by denosumab is an indirect mechanism.

Treatment of GCTB by local curettage or resection, accompanied in some cases by local adjuvant treatments (2, 6, 7, 10-12) and by systemic treatment with interferon-α2a or nitrogen-containing bisphosphonates, has been previously reviewed (6, 11). Bisphosphonates are reported to cause increased apoptosis of GCTB stromal cells and tumor giant cells. In one study, no change in the morphological structure of the tumor was observed; however, several patients showed an increase in radiological density and bone mineral content (50).

The striking differentiation of the densely cellular tumor tissue into more normal woven bone and connective tissue components posed a limitation in the current study; this beneficial but unexpected change made it difficult to accurately define the region of interest that represented what had been the extent of pretreatment tumor when examining the on-study samples. For this reason, the magnitude of decrease in RANK/RANKL or Ki67 expression and the reduction in the tumor content of tumor stromal cells or the increase in fibro-osseous tissue and/or new bone were reported semi-quantitatively. However, examination of the typical histologic changes shown in Figures 1 through 5 and Supplementary Online Figure 1 shows striking differences between the baseline samples and the on-study samples. These observed histologic changes are consistent with the radiologic changes reported previously (1), showing a clear effect of treatment. We have also recently observed similar changes in pulmonary metastases of GCTB after treatment with denosumab (data not shown).
In the phase 2 study reported here, denosumab treatment caused a marked reduction in tumor giant cells and significant histological evidence of treatment-induced differentiation of highly cellular proliferative tumor stromal cells to non-proliferative osteoid bone matrix, woven bone and mature bone. These findings are consistent with the tendency of baseline tumors to show small foci of bone matrix before treatment and the published data indicating that the neoplastic stroma of GCTB can be induced to form bone when the tumor microenvironment is altered. These results confirm and explain the radiologic tumor response previously reported in this study with denosumab (1). Treatment of GCTB with denosumab offers a novel and effective treatment option for this aggressive tumor.

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Table 1. Demographic and Disease Characteristics: Histology Subset

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<th>Characteristic</th>
<th>Denosumab-treated patients (N=20)</th>
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<td>Female sex, n (%)</td>
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<td>GCTB disease type, n (%)</td>
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<td>Anatomic site of biopsy/resection, n (%)</td>
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* Unresectable disease includes disease for which resection could not be done without nerve damage or substantial impairment of joint function

† Indicates the number of patients for whom the biopsy/resection site were identified.
Figure Legends

Figure 1. Baseline H&E-stained specimen (Subject 1). A) Densely cellular tumor with numerous tumor giant cells surrounded by abundant spindle-shaped tumor stromal cells; B) Areas of increased bone matrix and woven bone (arrows).

Figure 2. On-study H&E-stained specimen (Subject 1). A, B) Low magnification fields showing transition from residual densely cellular (DC) tumor cell areas to fibro-osseous intercellular areas and new bone (NB); C) Higher magnification of residual densely cellular tumor; D) Higher magnification showing replacement of tumor with trabeculae of woven bone and more normal new bone.

Figure 3. RANKL and RANK stains (Subject 1). A) Baseline section: numerous RANKL-positive tumor stromal cells surrounding RANKL-negative tumor giant cells; B) Baseline section: RANK-positive tumor giant cells surrounded by RANK-negative tumor stromal cells; C) On-study section: residual area of dense RANKL-positive tumor stromal cells; D) On-study section: residual densely cellular tumor cells with numerous RANK-positive mononuclear cells but no RANK-positive tumor giant cells; E) On-study section: area where densely cellular tumor is replaced by trabeculae of woven bone and more mature new bone which contain a small number of RANKL-positive stromal cells; F) On-study section: area where densely cellular tumor cells are replaced by trabeculae of woven bone and more mature new bone with no RANK-positive cells remaining.
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**Figure 4.** H&E, RANKL, and RANK stains (Subject 2). A) Baseline section: densely cellular tumor with numerous tumor giant cells surrounded by abundant spindle-shaped tumor stromal cells; B) On-study section: transition from residual densely cellular tumor cell areas to fibro-osseous intercellular areas and new bone; C) Baseline section: numerous RANKL-positive tumor stromal cells surrounding RANKL-negative tumor giant cells; D) On-study section: residual area of RANKL-positive densely tumor stromal cells separated by wide areas of poorly mineralized fibro-osseous matrix; E) Baseline section: RANK-positive tumor giant cells surrounded by RANK-negative tumor stromal cells; F) On-study section: residual densely cellular tumor cells with numerous RANK-positive mononuclear cells, but no RANK-positive tumor giant cells separated by wide areas of poorly mineralized fibro-osseous matrix.

**Figure 5.** H&E, RANKL, and RANK stains (Subject 3): A) Baseline section: densely cellular tumor with numerous tumor giant cells surrounded by abundant spindle-shaped tumor stromal cells; B) On-study section: transition from abundant residual densely cellular tumor cell areas to scant regions of fibro-osseous intercellular areas; C) Baseline section: numerous RANKL-positive tumor stromal cells surrounding RANKL-negative tumor giant cells; D) On-study section: abundant residual area of dense RANKL-positive tumor stromal cells separated by scant areas of poorly mineralized fibro-osseous matrix; E) Baseline section: RANK-positive tumor giant cells surrounded by RANK-negative tumor stromal cells; F) On-study section: abundant residual densely cellular tumor cells with numerous RANK-positive mononuclear cells but no RANK-positive tumor giant cells separated by wide areas of poorly mineralized fibro-osseous matrix.
Figure 1

A

B
Figure 3

Baseline

A

B

On-study

C

D

E

F
Figure 4

A B
Baseline

C D
On-study

E F

50 µm  50 µm  50 µm  50 µm  50 µm  50 µm
Figure 5

A Baseline  

B On-study  

C  

D  

E  

F
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