Author's response to reviews

Title: Host-Derived RANKL is Responsible for Osteolysis in C4-2 Human Prostate Cancer Xenograft Model of Experimental Bone Metastases

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Version: 2 Date: 2 July 2007

Author's response to reviews: see over
We would like to thank the reviewers for their constructive comments, which we addressed as follows:

**Reviewer #1.**

1. **To clearly establish the direct involvement of murine host cells in bone metastases associated osteolysis, the authors should have completed their work by using muRANKL MAb which targets (host) murine osteoblasts to show the decrease of osteolysis. A study by immuno-histochemistry using MAb against RANKL from both origins should also be useful to compare the proportion of RANKL synthesized by host (murine) cells and that synthesized by tumor (human) cells.**

We agree with the reviewer that the ultimate confirmation that murine RANKL is involved in osteolysis associated with C4-2 growth in the bone could involve a neutralizing antibody against murine RANKL. Unfortunately, to our knowledge, there is no such antibody available. Page 16; paragraph 1; line 8-11. There are multiple antibodies available which bind to murine RANKL, but because of the 81% identity between human and mouse RANKL, these antibodies react with RANKL of both origins. However, we have previously demonstrated in an indirect way that RANKL is involved in the increased osteolysis caused by C4-2 cells in the bone (Quinn et al, *Prostate Cancer and Prostatic Diseases*, 2005). In this study we showed that the administration of recombinant OPG inhibited osteolysis and decreased numbers of osteoclasts in tibiae with C4-2 tumors, indicating that RANKL is involved in this process. We have obtained similar results by overexpressing OPG in C4-2 cells (Corey et al, *Cancer Research*, 2005). Page 15; paragraph 1; line 12-14.

We also agree with the reviewer that data showing the alteration of RANKL immunoreactivity using a murine-RANKL-specific antibody would provide additional proof of direct involvement of murine RANKL. Again, we are frustrated by the lack of such an antibody. In our manuscript we present results of immunohistochemistry with an anti-RANKL antibody which recognizes both human and murine RANKL; however, because of the nature of the IHC procedure, the results generated do not provide quantitative information, and small changes which could be biologically significant might not be detected.

We have clarified both of these points in the revised manuscript.

2. **To confirm the author’s hypothesis, co-cultures of human tumor cells with murine osteoblasts should also provide information about the factors synthesized by human prostate cells to induce RANKL expression by murine osteoblasts.**

We agree with the reviewer that *in vitro* experiments are important in understanding the mechanisms of prostate cancer stimulation of osteolysis and the signaling networks involved. A few reports have shown that osteoclastic factors are produced by osteoblasts and that tumor cells can stimulate expression of osteoclastic factors by osteoblasts *in vitro* (Thomas et al, *Endocrinology*, 1999; Barnes et al, *Cancer Res*, 2004, Inoue et al, *Cancer Lett*, 2005; Mori et al, *Bone*, 2007). We have also begun experiments to investigate these effects. However, the interactions between host and tumor cells cannot readily be modeled *in vitro*. *In vivo* experiments are critical to establish biological relevance to human disease. Page 16; paragraph 2; line 6-11 and Page 17; paragraph 2; line 1-2.

3. **I am not convinced by the effectiveness of huRANKL MAb in vivo: why huRANKL is still detected by immuno-histochemistry in C4-2 tumors even after prevention or curative treatment by huRANKL MAb (Fig. 2A)?**

Immunohistochemical detection of RANKL is unrelated to the effectiveness of the huRANKL MAb in inhibiting RANKL activity. 1) Not all neutralizing antibodies decrease the levels of detected protein. Only
some antibodies upon binding to a target protein on the cell surface cause internalization of this protein and target it for degradation. 2) Antibodies bind to specific epitopes. The monoclonal antibody binds to one specific epitope of the protein, while the polyclonal antibody we used for immunohistochemistry contains multiple clones, which recognize multiple epitopes of the protein. Therefore it is possible for two antibodies to bind simultaneously to the same protein, in which case the binding of one antibody does not alter the 3D structure of the protein or sterically hinder binding of the second antibody. This principle is widely used, for example, in ELISA detection of proteins. Therefore the immunohistochemical detection of RANKL does not bear on the effectiveness of the huRANKL MAb. Page 19; paragraph 2; line 1-14.
Reviewer #2

1. The authors should explain why they did not use SCID male mice in Figure 1. In 1A male BDF mice are used to show that huRANKL causes hypercalcemia and that this can be prevented by 10 mg/kg huRANKL MAb. If they know that huRANKL MAb does the same thing in SCID mice, they should cite the data. In 1B they use female BALB/c mice to show that OPG inhibits bone turnover, decreasing TRACP 5b, but that huRANKL MAb does not have the same effect. They are probably correct in concluding that huRANKL MAb performs in a species specific manner, but it would be helpful to know if mouse RANKL MAb decreased TRACP 5b.

The reason for using different types of mice is that the experiments were performed for different purposes. The experiments in the BDF mice were performed during characterization of the huRANKL MAb, to establish that the anti-huRANKL MAb effectively inhibits human RANKL-induced increases in serum ionized calcium levels in vivo, without showing evidence for the inhibition of murine RANKL. This experiment clearly demonstrated that the anti-huRANKL MAb can inhibit huRANKL-induced osteolysis. Once the neutralizing activity and specificity of this antibody in vivo were determined, we used it to investigate the involvement of huRANKL in the stimulation of osteolysis in an animal model of prostate cancer bone metastasis. Using human prostate cancer xenografts in mice requires the use of immune-compromised mice and we have established and characterized C4-2 osseous models with SCID mice previously (Pfitzenmaier et al, JBMR, 2003).

We are unaware of any literature indicating that bone remodeling is different between wild-type and SCID mice. Therefore we believe that this fact does not negate our results. We have added explanation of this point to the revised manuscript. Page 7; paragraph 2; Line 3-4. Page 8; paragraph 2; line 1-6.

As discussed above in the response to the Reviewer #1, we do not have access to a specific anti-mouse RANKL antibody with neutralizing properties. Page 16; paragraph 1; line 8-11. Therefore we could not test whether using such an antibody would decrease TRACP 5b. However, the data showing that OPG decreases TRACP 5b levels are indicative of the involvement of RANKL inhibition in this effect, since the only known biological function of OPG is as a decoy receptor in the RANKL pathway.

2. The nice data in Figure 2 show RANKL is highly expressed in the C4-2 tumor, and that huRANKL MAb did not prevent tumor growth/establishment as assessed by PSA. It would have been helpful to show that OPG did or did not have an effect on this particular tumor, similar to the partial effect of OPG in another CaP line they have studied (Kiefer 2004), and that Zhang et al 2001 have shown in a related tumor line (C4-2B). At the very least they should comment on their experience.

We agree with the reviewer that it is important to show that RANKL is involved in the osteolysis induced by these particular prostate cancer cells, and studies with OPG would provide such results. We apologize for the fact that this point was not made clearer in our manuscript. We have performed studies using C4-2 cells growing in the bone and shown that administration of recombinant OPG inhibited the osteolysis associated with growth of these C4-2 cells in the bone (Quinn et al, Prostate Cancer and Prostatic Diseases, 2005). We have also reported that overexpression of OPG in C4-2 cells had similar effects (Corey et al, Cancer Research, 2005). We explain this issue in detail in the revised manuscript. Page 15; paragraph 1; line 12-14.

3. The data in Figure 3 shows that tumor growth and bony destruction caused by C4-2 intratibial injection is not prevented by huRANKL MAb. These data are matched by the histology in Table 1. The difference in osteoclast number in the treatment group may not be biologically significant.
We agree with the reviewer that because of the anarchic nature of tumor effects on the bone, the alterations in osteoclast numbers may not be biologically relevant. We have added this statement to the revised manuscript. Page 13; paragraph 2; line 11-15.

4. *It is unclear what conclusions they can make with the data in figure 4. They do not shown a true control for either TRACP 5b or for calcium, i.e., a age/sex/strain matched mouse without a tumor – is that because the TRACP 5b and calcium in the tumored “control” is unchanged. Furthermore, if osteolysis can occur in the absence of increased TRACP 5b (and/or calcium), bone destruction seen in fig 3 may simply not depend on RANKL from the host or the tumor might the skeleton simply fail in the face of a rapidly expanding lesion? It may be that the authors could argue this point, especially if they have data showing that OPG could prevent the bone destruction. The second possibility, which they discuss on page 18, is that the huRANKL MAb might not work very well. If the huRANKL MAb did not “demonstrate strong immunoreactivity…on the C4-2 cells”, was this the right approach?*

The results in Figure 4 show evaluation of the effect, or lack of it, of administration of huRANKL MAb on levels of TRACP 5b in mice bearing C4-2 tumors in the tibiae. In this study we focused on evaluation of effects of the inhibitory huRANKL MAb on osteolysis associated with growth of the tumor in the bone environment, and therefore we have not included a “true control” which would show levels of TRACP 5b in mice without the tumors. We agree with the reviewer that it is possible that levels of TRACP 5b are not different in normal mice vs. mice bearing C4-2 tumors in the tibiae. The reason for such results might be that The TRACP 5b and calcium levels may not have changed significantly after administration of huRANKL MAb because the tumor growth affects only small portion of the skeleton and therefore increases in systemic levels of markers of bone destruction might be too low to be detected. This stands in contrast to our studies involving administration of recombinant human RANKL, which would affect the whole skeleton and exhibit systemic effects. Based on this comment we have reworded our discussion of these results to reflect this possibility. Page 18; paragraph 2; line 1-11.

As stated above, we have shown previously that OPG can inhibit osteolysis associated with C4-2 growth in the bone, indicating the involvement of RANKL in this process. Page 15; paragraph 1; line 12-14.

The reviewer expresses doubt that the approach we have chosen to evaluate involvement of human RANKL is the right one, based on the results showing the lack of RANKL immunoreactivity when huRANKL MAb was used under immunohistochemistry conditions.

Not all antibodies recognize the targeted protein after formalin fixation and embedding in paraffin. Therefore the lack of immunoreactivity does not necessarily mean the absence of the target protein. In this particular case, because we have shown expression of RANKL in C4-2 cells in the tibiae using other antibodies, as well as RANKL expression in cells grown in vitro, we have concluded that the lack of immunoreactivity of RANKL under IHC conditions with the huRANKL MAb is due to its inability to recognize the protein in paraffin-embedded tissues. We believe that our selection of this model for our studies is justified for following reasons: 1) we have shown previously that their growth in the bone results in increases in bone destruction; 2) these cells express RANKL; and 3) RANKL is involved in this process. Page 19; paragraph 2; line 1-14.