Journal of Bone Oncology 4 (2015) 59–68

Contents lists available at ScienceDirect

Journal of Bone Oncology

journal homepage: www.elsevier.com/locate/jbo

Research Paper

RANK and RANK ligand expression in primary human osteosarcoma

Daniel Branstetter [a], Kathy Rohrbach [a], Li-Ya Huang [a], Rosalia Soriano [a], Mark Tometsko [b], Michelle Blake [c], Allison P. Jacob [b], William C. Dougall [b,*]

[a] Department of Pathology, Amgen Inc., Seattle, WA, USA
[b] Therapeutic Innovation Unit, Amgen Inc., Seattle, WA, USA
[c] Department of Hematology/Oncology Research, Amgen Inc., Seattle, WA, USA

ARTICLE INFO

Article history:
Received 6 May 2015
Received in revised form
5 June 2015
Accepted 17 June 2015
Available online 29 July 2015

Keywords:
RANK
RANKL
Human osteosarcoma
Antibodies
Protein expression
Immunohistochemistry

ABSTRACT

Receptor activator of nuclear factor kappa-B ligand (RANKL) is an essential mediator of osteoclast formation, function and survival. In patients with solid tumor metastasis to the bone, targeting the bone microenvironment by inhibition of RANKL using denosumab, a fully human monoclonal antibody (mAb) specific to RANKL, has been demonstrated to prevent tumor-induced osteolysis and subsequent skeletal complications. Recently, a prominent functional role for the RANKL pathway has emerged in the primary bone tumor giant cell tumor of bone (GCTB). Expression of both RANKL and RANK is extremely high in GCTB tumors and denosumab treatment was associated with tumor regression and reduced tumor-associated bone lysis in GCTB patients. In order to address the potential role of the RANKL pathway in another primary bone tumor, this study assessed human RANKL and RANK expression in human primary osteosarcoma (OS) using specific mAbs, validated and optimized for immunohistochemistry (IHC) or flow cytometry.

Our results demonstrate RANKL expression was observed in the tumor element in 68% of human OS using IHC. However, the staining intensity was relatively low and only 37% (29/79) of samples exhibited ≥ 10% RANKL positive tumor cells. RANK expression was not observed in OS tumor cells. In contrast, RANK expression was clearly observed in other cells within OS samples, including the myeloid osteoclast precursor compartment, osteoclasts and in giant osteoclast cells. The intensity and frequency of RANKL and RANK staining in OS samples were substantially less than that observed in GCTB samples. The observation that RANKL is expressed in OS cells themselves suggests that these tumors may mediate an osteoclastic response, and anti-RANKL therapy may potentially be protective against bone pathologies in OS. However, the absence of RANK expression in primary human OS cells suggests that any autocrine RANKL/RANK signaling in human OS tumor cells is not operative, and anti-RANKL therapy would not directly affect the tumor.

1. Introduction

Osteosarcoma (OS) is the most common primary malignant tumor of the bone. This neoplasm is defined histologically by osteoid deposition by the malignant mesenchymal cells [1]. In OS, current evidence supports an osteoblastic population as the cell of origin [2], although the distinct histological subtypes (e.g. osteoblastic, fibroblastic, chondroblastic and telangiectactic) indicate potential for heterogeneous origins. In association with varying degrees of bone matrix deposition, OS is characterized by local bone destruction and frequent lung metastasis. Ten-year survival outcomes for patients with localized OS are approximately 65% and outcomes, along with the standard medical treatment, have not changed substantially in recent years. Patients with recurrent OS have a poor prognosis and there is great desire to develop improved therapies [3].

In solid tumors which have metastasized to bone or in giant cell tumor of bone (GCTB), lytic bone destruction is mediated by osteoclasts. Osteoclasts are highly specialized cells derived from the monocyte/macrophage lineage necessary for the degradation

Abbreviations: APC, allophycocyanin; ATCC, American Type Culture Collection; cDNA, complementary deoxyribonucleic acid; ELISA, enzyme linked immunosorbent assay; FACS, fluorescence-activated cell sorting; FBS, fetal bovine serum; FFPE, formalin-fixed, paraffin-embedded; GCTB, giant cell tumor of bone; IgG1, immunoglobulin G1; IHC, immunohistochemistry; ISH, in situ hybridization; LN, lymph node; mAb, monoclonal antibody; mRNA, messenger ribonucleic acid; OS, osteosarcoma; RANK, receptor activator of nuclear factor kappa-B; RANKL, receptor activator of nuclear factor kappa-B ligand; RNA, ribonucleic acid; RT-PCR, reverse transcriptase polymerase chain reaction

* Corresponding author. Fax: +1 206 217 0494.
E-mail address: dougallw@amgen.com (W.C. Dougall).

http://dx.doi.org/10.1016/j.jbo.2015.06.002
2212-1374/

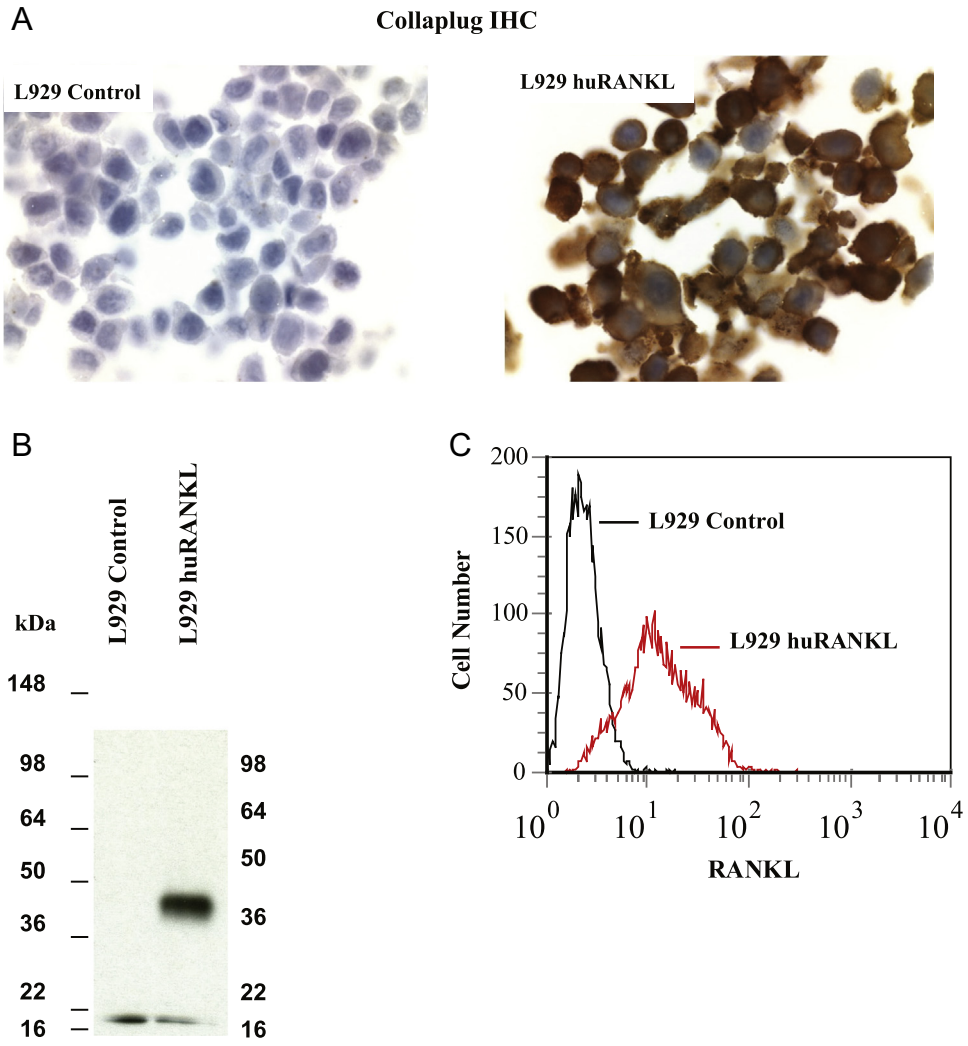

Fig. 1. huRANKL antibody validation for IHC methods. (A) Anti-huRANKL mAb M366 IHC reveals specific signal in mouse L929 cells transduced with huRANKL cDNA (L929 huRANKL), but not parental L929 cells (L929 control). (B) Similarly, analysis of dissociated proteins on western blot using mAb 366 of L929 huRANKL cells detected a protein of approximately 45 kDa, the predicted size for full-length human RANKL [28]. Positions of molecular weight markers are illustrated on left (kDa). (C) Determination of RANKL cell surface protein expression on L929 cells was performed using flow cytometry. Expression of RANKL was detected using the M366 mAb and a goat antimouse secondary antibody conjugated to APC. M366 staining on huRANKL transduced L929 cells is indicated with the red line and on parental L929 cells (L929 control) a solid black line. The M366 anti-huRANKL antibody detects a signal by IHC, western blot, and flow cytometry specifically in L929 cells transduced with huRANKL and not parental L929 cells. APC, allophycocyanin; cDNA, complementary deoxyribonucleic acid; IHC, immunohistochemistry; mAb, monoclonal antibody; RANKL, receptor activator of nuclear factor kappa-B ligand.

of the organic and inorganic matrices of bone. Receptor activator of nuclear factor kappa-B ligand (RANKL), a tumor necrosis factor ligand superfamily member, is essential for the formation, activation, and function of osteoclasts. RANKL is expressed by cells of the osteoblast lineage in the bone stroma as well as osteocytes and acts via a paracrine mechanism, binding to its cognate receptor RANK expressed on osteoclasts and osteoclast precursors [4]. Denosumab, a fully human monoclonal antibody (mAb) specific to RANKL, inhibits osteoclastogenesis and osteoclast-mediated bone destruction. In clinical studies, denosumab reduced tumor-induced bone resorption and skeletal complications of metastatic bone disease [5–7], including delaying the development of bone metastasis in men with castrate-resistant prostate cancer [8,9].

Osteoclasts have been observed in OS at sites of bone resorption, either at the tumor/bone interface or within the tumor tissue at sites of neoplastic osteoid [10]. Cortical destruction and extension of the tumor mass into the soft tissue is frequently evident in OS patients, suggesting involvement of osteoclasts in associated bone pathologies. The human OS cell line SaOS-2 has been shown to support osteoclastogenesis via RANKL production on the surface of OS cells [11] and RANKL expression has been reported on primary feline, canine [12], and human OS cells [13] with variable frequency. In preclinical studies, animal models of OS have also indicated that RANKL levels increase in tumor-involved bone [14,15]. Pharmacologic inhibition of RANKL has been shown to prevent increased osteolysis, reduce skeletal tumor growth and reduce lung metastases (often associated with an increased survival) in these models [16–18]. Osteoclast inhibition, achieved with either RANKL blockade or bisphosphonates, results in similar antitumor and bone-protective effects in these models [19]. These studies support the notion that RANKL, produced within the reactive bone stroma and potentially within OS cells themselves, contributes to OS-mediated bone degradation/lysis. In addition to potential alterations in RANKL in OS, RANK expression has been reported in mouse and human OS cell lines [20,21] and in primary human OS [20,22]. The prevalence of RANKL and RANK expression, as well as any associated prognostic significance, varies considerably in these published reports of human OS.

In a recent characterization of another primary bone tumor, GCTB, we and others have confirmed significant RANKL expression