Journal of Bone Oncology 2 (2013) 95–104

Contents lists available at ScienceDirect

Journal of Bone Oncology

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

Research Article

# Osteoprotegerin inhibits bone resorption and prevents tumor development in a xenogenic model of Ewing's sarcoma by inhibiting RANKL

Gaëlle Picarda [a,b], Etienne Matous [a,b], Jérôme Amiaud [a,b], Céline Charrier [a,b], François Lamoureux [a,b], Marie-Françoise Heymann [a,b,c], Franck Tirode [d], Bruno Pitard [e], Valérie Trichet [a,b], Dominique Heymann [a,b], Françoise Redini [a,b,*]

[a] INSERM, Equipe Ligue Contre le Cancer 2012, UMR-957, Nantes F-44035, France
[b] Université de Nantes, EA3822, Faculté de Médecine, Laboratoire de physiopathologie de la résorption osseuse et thérapie des tumeurs osseuses primitives, Nantes F-44035, France
[c] Service d'anatomo-pathologie, CHU Hôtel Dieu, Nantes F-44035, France
[d] INSERM UMR830, Institut Curie, 26 rue d'Ulm, Paris F-75006, France
[e] INSERM UMR1087, IRT-UN, Quai Moncousu, Nantes F-44035, France

ARTICLE INFO

*Article history:*
Received 22 March 2013
Received in revised form
29 April 2013
Accepted 29 April 2013
Available online 20 May 2013

*Keywords:* Ewing's sarcoma
Osteoprotegerin
RANKL
Bone resorption
TRAIL

ABSTRACT

Ewing's sarcoma (ES) associated with high osyeolytic lesions typically arises in the bones of children and adolescents. The development of multi-disciplinary therapy has increased current long-term survival rates to greater than 50% but only 20% for high risk group patients (relapse, metastases, *etc.*). Among new therapeutic approaches, osteoprotegerin (OPG), an anti-bone resorption molecule may represent a promising candidate to inhibit RANKL-mediated osteolytic component of ES and consequently to limit the tumor development.

Xenogenic orthotopic models of Ewing's sarcoma were induced by intra-osseous injection of human TC-71 ES cells. OPG was administered *in vivo* by non-viral gene transfer using an amphiphilic non ionic block copolymer. ES bearing mice were assigned to controls (no treatment, synthetic vector alone or F68/empty pcDNA3.1 plasmid) and hOPG treated groups. A substantial but not significant inhibition of tumor development was observed in the hOPG group as compared to control groups. Marked bone lesions were revealed by micro-computed tomography analyses in control groups whereas a normal bone micro-architecture was preserved in the hOPG treated group. RANKL over-expressed in ES animal model was expressed by tumor cells rather than by host cells. However, TRAIL present in the tumor microenvironment may interfere with OPG effect on tumor development and bone remodeling via RANKL inhibition.

In conclusion, the use of a xenogenic model of Ewing's sarcoma allowed discriminating between the tumor and host cells responsible for the elevation of RANKL production observed in this tumor and demonstrated the relevance of blocking RANKL by OPG as a promising therapy in ES.



## 1. Introduction

Ewing's sarcoma (ES) is a high-grade neoplasm representing the second most common primary bone malignancy in both children and adults. With a peak incidence at 15 years, this disease accounts for 2% of childhood cancers [1–3]. ES is defined as a bone tumor which may occur at any site within the skeleton but preferentially affects the trunk and the diaphysis of long bones. Less commonly, it arises in extraskeletal soft tissues (15%). It is characterized by a rapid tumor growth and extensive bone destruction that can result in bone pain and pathological fracture. A particularity of ES tumors is the occurrence of a typical chromosomal translocation that fuses the EWS gene on chromosome 22q12 to a member of the ETS transcription gene family, most commonly to Fli-1 on 11q24 ( > 90% of cases) [4,5]. This translocation leads to the production of an aberrant transcription factor that promotes tumorigenicity [6–8]. Due to progress in surgery and chemotherapy, survival rates have increased from less than 10% to 55–60% for patients presenting local disease [9]. However, the survival rates decrease to 15–25% when metastases are detected at diagnosis, or for patient presenting resistance to treatment or relapsed disease. Moreover, a survival plateau seems to have been reached with conventional therapies. Accordingly, new therapeutic approaches should be actively explored, especially for high-risk patients, to increase long-term survival by

* Correspondence to: INSERM UMR957, Faculté de Médecine, 1 rue Gaston Veil, 44 035 Nantes Cedex 1, France. Tel.: +33 240 412842; fax: +33 240 412860.
*E-mail address:* francoise.redini@univ-nantes.fr (F. Redini).

2212-1374/$ - see front matter © 2013 Elsevier GmbH. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
http://dx.doi.org/10.1016/j.jbo.2013.04.004

decreasing metastases development and preventing drug resistance.

ES is characterized by extensive bone destruction due to osteolysis. Ewing's sarcoma cells are unable to directly degrade the bone matrix and accordingly, osteoclast activation and subsequent bone resorption might be responsible for the clinical features of bone destruction [10]. Indeed, as demonstrated for bone metastases [11], a vicious cycle between bone cells (osteoclasts and osteoblasts) and tumor cells occurs during the development of tumor in bone site. Therefore, targeting the osteoclasts may represent a promising adjuvant strategy for the treatment of bone tumors. Among the factors involved in the regulation of bone remodeling, the molecular triad osteoprotegerin (OPG)/receptor activator of NF-kB (RANK)/RANK Ligand (RANKL) is strongly implicated [12,13]. Osteoclast differentiation and activation is mainly mediated by RANKL, a cytokine member of the tumor necrosis factor (TNF) superfamily (TNFSF11) that binds to its receptor RANK at the surface of osteoclasts [14]. OPG (TNFRSF11B) is a soluble protein that acts as a decoy receptor for RANKL inhibiting osteoclast formation, function and survival by preventing the binding of RANKL to RANK [15]. Transgenic mice overexpressing OPG exhibit an osteopetrotic phenotype, whereas OPG-knockout mice have severe osteoporosis [16,17]. The OPG/RANKL/RANK system is also involved in various pathologies associated with tumors in bone [18,19]. Therefore, OPG has demonstrated increased interest as a therapeutic strategy in malignant bone pathologies associated with osteolytic lesions [20,21]. Concerning primary bone tumors, the inhibition of RANKL activity by OPG induced a significant therapeutic effect on bone lesion and tumor development in two preclinical models of osteosarcoma in mice (POS-1) and in rats (OSRGa) [22]. This effect was also confirmed by using the soluble form of the RANKL receptor, RANK-Fc [15] or by the RNA interference strategy targeting RANKL [23]. In addition, OPG is also able to bind to the TNF Related Apoptosis Inducing Ligand (TRAIL), another member of the TNF superfamily (TNFSF10) [23], thereby limiting its ability to induce apoptosis in tumor cells. It has been even reported that OPG acts as a pro-tumoral factor in some cancer cell lines *in vitro* [24–26]. In addition, Taylor et al. previously reported that the expression of RANKL in Ewing's sarcoma cell lines and tissues could support osteoclast activation [10]. Therefore, targeting this cytokine with OPG may represent a promising therapeutic option.

The aim of this study was to determine the therapeutic relevance of blocking RANKL in Ewing's sarcoma by using OPG administered by non-viral gene transfer approaches in two models of human Ewing sarcoma in immunodeficient mice. OPG was administered using amphiphilic polymers constituted by blocks of poly(ethylene oxide) and of poly(propylene oxide) as previously reported for osteosarcoma preclinical studies [22]. These synthetic vectors have been used with high efficiency for *in vivo* gene transfer in various organs including skeletal and cardiac muscles [27,28] and in lungs [29]. Intramuscular injections of these synthetic vectors led to the synthesis of proteins for local benefit such as dystrophin or of systemic erythropoietin [30].

## 2. Materials and methods

### 2.1. In vivo experiments

#### 2.1.1. Plasmid constructs

The pcDNA3.1.3-hOPG1-194 contains the cDNA coding for the truncated form of OPG (1–194) cloned using the pcDNA™ 3.3-TOPO® TA cloning® Kit (Invitrogen) according to manufacturer's recommendations, the empty pcDNA3.1 plasmid (Invitrogen) being used as a control.

#### 2.1.2. Xenograft models of human Ewing's sarcoma

All procedures involving mice were conducted in accordance with the institutional guidelines of the French Ethical Committee (CEEA.PdL.06, protocol number 2010.23). Four-week-old male athymic mice purchased from Harlan were housed in the Experimental Therapeutic Unit at the Faculty of Medicine of Nantes (France). The TC-71 ES model was induced by transplantation of a fragment of tumor ($2 \times 2 \times 2$ mm$^3$) in close contact with the tibia, resulting from the initial injection of $2 \times 10^6$ TC-71 ES cells next to the tibia. To confirm the effects of OPG, another Ewing's sarcoma model was developed, induced by i.m. injection of $2 \times 10^6$ human A-673 ES cells in close contact with the tibia, leading to a rapidly growing tumor in soft tissue with secondary contiguous bone invasion. Mice were anesthetized by inhalation of a combination of isoflurane/air (1.5%, 1 L/min) and buprenorphine was given by sc injection during the protocol (0.05 mg/kg; Temgesic®, Schering-Plough).

#### 2.1.3. Synthetic gene transfer

The synthetic vector used in this study (named F68) belongs to the Lutrol family of vectors, non ionic block copolymers of poly (ethyleneoxide)$_{75}$-poly(propyleneoxide)$_{30}$-poly(ethyleneoxide)$_{75}$ generously provided by Dr. Bruno Pitard (INSERM UMR1087, Nantes, France) [30]. Stock solutions were prepared at 6% (w/v) in water and stored at 4 °C. Formulations of DNA with block copolymers were prepared by equivolumetric mixing block copolymers in water and DNA solution at the desired concentration (50 μg/muscle).

#### 2.1.4. Experimental protocol

Groups of 6–8 mice were assigned as control vectors (F68/pcDNA3.1 alone) and hOPG1-194 (F68/pcDNA3.1-OPG1-194). F68 alone or associated with the empty vector pcDNA3.1 does not affect tumor development as compared to non-treated mice that develop the Ewing sarcoma model (data not shown). Mice were anesthetized by inhalation of a combination of isoflurane/air (1.5%, 1 L/min) and the F68/DNA formulations were injected into both tibial anterior muscles once a week. Because the transgene expression is optimal seven days after injection of the DNA formulations, the treatment began 7 days before Ewing's sarcoma implantation as a preventive treatment, up to 21 days post-implantation. The truncated form of OPG was chosen in accordance to previous results obtained by our group in osteosarcoma models, showing that the biological activity of the complete OPG isoform may be limited by interaction with proteoglycans present in the extracellular matrix, inhibiting OPG biological availability [31]. The Ewing sarcoma model was induced by tumor fragment transplantation or tumor cell injection as described above. The tumor volume was calculated by using the formula $L \times l^2/2$, where $L$ and $l$ are the longest and the smallest perpendicular diameter, respectively. Treatment continued until each animal showed signs of morbidity, which included cachexia or respiratory distress, at which point they were sacrificed by cervical dislocation or by $CO_2$ inhalation. The mice were also sacrificed for ethical reasons when the tumor volume exceeded 3000 mm$^3$. Lung tumor dissemination was assessed at necropsy. The tumor-bearing hind limb was dissected and kept in 10% paraformaldehyde for radiography, micro-computed tomography (micro-CT) and histological analyses.

#### 2.1.5. Micro-computed tomography (micro-CT) analysis

Analyses of bone micro-architecture were performed using a Skyscan 1076 *in vivo* micro-CT scanner (Skyscan, Kontich, Belgium). Tests were performed after sacrifice on tibias for each treatment group. All tibias were scanned using the same parameters (pixel size 18 μm, 50 kV, 0.5-mm Al filter, 10 min of scanning).