Journal of Cranio-Maxillo-Facial Surgery 44 (2016) 1387–1394

Contents lists available at ScienceDirect

Journal of Cranio-Maxillo-Facial Surgery

journal homepage: www.jcmfs.com

Severe compromise of preosteoblasts in a surgical mouse model of bisphosphonate-associated osteonecrosis of the jaw

Luis A. Córdova [a, b, c, *], Florian Guilbaud [a, b], Jérôme Amiaud [a, b], Séverine Battaglia [a, b], Céline Charrier [a, b], Frédéric Lezot [a, b], Benoît Piot [d, e], Françoise Redini [a, b], Dominique Heymann [a, b, e, f]

[a] INSERM, UMR 957, Equipe Ligue Contre le Cancer 2012, 1 rue Gaston Veil, Nantes Cedex 1, 44035, Nantes, France
[b] University of Nantes, Nantes Atlantique Universities, Pathophysiology of Bone Resorption and Therapy of Primary Bone Tumours Laboratory, 1 rue Gaston Veil, Nantes Cedex 1, 44035, Nantes, France
[c] Department of Oral and Maxillofacial Surgery, San Borja Arriarán University Hospital – Faculty of Dentistry, University of Chile, Sergio Livingstone Polhammer 943, Independencia, Santiago, Chile
[d] Department of Stomatology and Maxillofacial Surgery, Nantes University Hospital, 1 Place Alexis-Ricordeau, 44093, Nantes Cedex 1, France
[e] Nantes University Hospital, 1 Place Alexis-Ricordeau, 44093, Nantes Cedex 1, France
[f] Department of Oncology and Metabolism, Medical School, Beech Hill Road, S10 2RX, Sheffield, UK

ARTICLE INFO

Article history:
Paper received 14 February 2016
Accepted 15 July 2016
Available online 22 July 2016

Keywords:
Osteonecrosis of the jaw
Zoledronic acid
Osteoclast
Osteoblast
Alveolar bone
Basic multicellular unit

ABSTRACT

Objectives: The effect of amino-bisphosphonates on osteoblastic lineage and its potential contribution to the pathogenesis of bisphosphonate-associated osteonecrosis of the jaw (BONJ) remain controversial. We assessed the effects of zoledronic acid (ZOL) on bone and vascular cells of the alveolar socket using a mouse model of BONJ.
Material and methods: Thirty-two mice were treated twice a week with either 100 μg/kg of ZOL or saline for 12 weeks. The first left maxillary molar was extracted at the third week. Alveolar sockets were assessed at both 3 weeks (intermediate) and 9 weeks (long-term) after molar extraction by semi-quantitative histomorphometry for empty lacunae, preosteoblasts (Osterix), osteoclasts (TRAP), and pericyte-like cells (CD146). Also, the bone microarchitecture was assessed by micro-CT.
Results: Osteonecrotic-like lesions were observed in 21% of mice. Moreover, a decreased number of preosteoblasts contrasted with the increased number of osteoclasts at both time points. In addition, osteoclasts display multinucleation and detachment from the endosteal surface. Furthermore, the number of pericyte-like cells increased at the intermediate time point. The alveolar bone mass increased exclusively with long-term ZOL treatment.
Conclusion: The severe imbalance between bone-forming cells and bone-resorbing cells shown in this study could contribute to the pathogenesis of BONJ.

1. Introduction

Bisphosphonate-associated osteonecrosis of the jaw (BONJ) is characterized by the persistent jaw bone exposure (>8 weeks) after a surgical procedure in patients with a history of use of bisphosphonates and without previous exposure to head and neck radiotherapy (Ruggiero et al., 2009). The long-term use of intravenous third-generation amino-bisphosphonates (risedronate and zoledronic acid [ZOL]), the most powerful antiresorptive agents, is considered a critical risk factor related to the development of BONJ (Wessel et al., 2008; Basso et al., 2013). The pathogenesis of BONJ

* Corresponding author. Division of Plastic & Reconstructive Surgery, Stanford School of Medicine, 257 Campus Dr, GK211, Stanford, CA 94035, United States. Fax: +1 650 736 4374.
E-mail addresses: lcordova@stanford.edu (L.A. Córdova), florian.guilbaud@univ-nantes.fr (F. Guilbaud), jerome.amiaud@univ-nantes.fr (J. Amiaud), severine.battaglia@univ-nantes.fr (S. Battaglia), celine.charrier@univ-nantes.fr (C. Charrier), frederic-lezot@univ-nantes.fr (F. Lezot), benoit.piot@chu-nantes.fr (B. Piot), francoise.redini@univ-nantes.fr (F. Redini), dominique.heymann@sheffield.ac.uk (D. Heymann).

http://dx.doi.org/10.1016/j.jcms.2016.07.015
1010-5182/

remains unknown and several hypothesis have been proposed; nevertheless, the suppression of bone remodeling induced by bisphosphonates seems to be the most consistent with their intrinsic mechanism of action (Mawardi et al., 2011; R. H. Kim et al., 2011; Allen and Burr, 2009).

Bone remodeling is the coupled process initiated by osteoclastic bone resorption followed by osteoblastic new bone formation (Natalie A. Sims and Martin, 2014). This process occurs in the entire skeleton throughout life and it takes place in the basic multicellular units (BMUs) of cortical and trabecular bone (Natalie A. Sims and Martin, 2014). Tight control of bone remodeling in each BMU is essential for maintaining normal bone mass. This control is regulated by dynamic interactions between the cellular components and coupling factors released during bone resorption (N. A. Sims and Ng, 2014). The former includes osteoclast precursor and mature osteoclasts, osteoblastic lineage, endothelial cells and pericytes, macrophages and dendritic cells (Natalie A. Sims and Martin, 2014). On the other hand, the coupling factors are protein molecules released during the osteoclasts differentiation: cardiotrophin 1, sphingosine-1-phosphate (S1P), bone morphogenetic protein (BMP)-6 and Wnt10b, collagen triple helix repeat containing 1 (CTHRC1) and Sema4D. Also, the coupling factors include bone matrix proteins released during bone resorption: insulin growth factor (IGF)-1 and transforming growth factor (TGF)-β (N. A. Sims and Ng, 2014).

The clinical and preclinical benefits of blocking osteoclast differentiation and activity with subsequent increase of bone density using amino-bisphosphonates have been extensively reported (D Heymann, 2010; Le Goff et al., 2010; D Heymann et al., 2005). However, their effects on the osteoblastic lineage remain poorly understood (Sakagami et al., 2005). Human biopsies show that the terminal stage of bisphosphonate-associated osteonecrotic lesions (bone sequestra) is characterized by the absence of the endosteal osteoblasts, empty osteocyte lacunae and damage in the canalicular system (Lesclous et al., 2009). These findings confirm the compromise of the entire osteoblastic lineage including preosteoblasts, osteoblasts, and osteocytes (Koch et al., 2011; Manzano-Moreno et al., 2015). On the other hand, *in vitro* studies report cytotoxic effects of bisphosphonates on osteoblastic cells, decreasing their viability and osteogenic ability in a dose-dependent manner (Pozzi et al., 2009; Basso et al., 2013). Therefore, the understanding of the effect of amino-bisphosphonates on both osteoblastic lineage and bone remodeling in *in vivo* models is a crucial step to further understand the pathogenesis of BONJ. We postulated that osteoblastic cells are sensitive to the effect of amino-bisphosphonates after a surgical stimulus in alveolar bone. The aim of this study was thus to assess – at the cellular level – the intermediate and long-term effects of clinically relevant high doses of ZOL on the bone and vascular cell components of alveolar socket BMU using a surgical mouse model for BONJ.

2. Material and methods

2.1. Animals, drug administration and surgical procedure

Thirty-two C57BL/6 male mice (Janvier, Le Genest-Saint Isle, France) aged 10 weeks were randomly divided into two groups and treated intra-peritoneally (i.p.) with either 100 μg/kg of ZOL (kindly provided by Novartis, Switzerland) (experimental group; n = 16) or saline solution (control group; n = 16) twice a week for 12 weeks (Supplementary Appendix 1). The drug tolerance of the mice was assessed daily by clinical examination. The total dose of ZOL administered was the equivalent of a lifetime dose of the drug over 4 years of therapy in a 70 kg adult multiple myeloma patients (Pozzi et al., 2009). At the end of the third week, the first left maxillary molar was surgically extracted from all the animals (Supplementary Appendix 1). After 6 weeks of treatment with ZOL (or saline solution), and 3 weeks after the molar extraction, 50 % of the animals were sacrificed to assess the situation at an intermediate time point (the equivalent of 2 years according to Pozzi et al., 2009). The remaining 50 % of the animals was sacrificed at the end of the protocol, after 12 weeks of treatment with ZOL (or saline solution) (the equivalent of 4 years according to Pozzi et al., 2009) and 9 weeks after the molar extraction, for long-term assessment.

2.2. Histology analysis

Harvested maxillae were fixed in 4% buffered formaldehyde for 48 h and then decalcified with 4.13% ethylenediaminetetraacetic acid (EDTA) and 0.2% paraformaldehyde in phosphate-buffered saline (PBS) for 96 h using the KOS microwave histostation (Milestone, Kalamazoo, MI, USA) before embedding in paraffin. Two 4 μm-thick sagittal sections were obtained from 2 levels of the alveolar socket site (each one separated by 50 μm). All slides were stained with Masson trichrome to assess the bone matrix and empty lacunae in both, bone sequestra and submucosal bone. Furthermore, all slides were stained with tartrate-resistant acid phosphatase (TRAP) to identify osteoclasts (Supplementary Appendix 1). The immunostaining for osteoblastic cells was performed using rabbit monoclonal anti-osterix antibody (1/800; Abcam). The immunostaining of the pericytes was carried out using rabbit monoclonal anti-CD146 antibody (1/200; Abcam).

Histological images were acquired using a NanoZoomer 2.0-RS slide scanner (Hamamatsu, Japan). The region of interest (ROI) corresponded to a rectangular area of alveolar bone comprising the full length of the alveolar socket. Static histomorphometric analysis of the number of empty lacunae, percentage of osteoclasts (Gobin et al., 2014a; Gobin et al., 2014b; Lamoureux et al., 2014), number of osterix and $CD146^+$ cells in their defined ROIs, were performed using ImageJ software (NIH, Bethesda, MD, USA).

2.3. Micro-computed tomography assessment

The analysis of alveolar bone microarchitecture was performed at the time of necropsy (6 and 12 weeks) using the high-resolution X-ray micro-computed tomography (micro-CT) system for small-animal imaging SkyScan-1076 (SkyScan, Kontich, Belgium) (Supplementary Appendix 1). The assessment of alveolar bone density was performed by measuring the mineralized bone detected within the VOI (Bone Volume; BV) and expressed in cubic millimeters (mm^3).

2.4. Statistical analysis

All analyses were performed using GraphPad InStat Version 3.02 software (GraphPad Software, La Jolla, CA, USA). The histological and micro-CT results were analyzed by comparisons between experimental and controls groups with unpaired parametric two-tailed t-test. Results were considered significant at p-value < 0.05.

3. Results

3.1. Zoledronic acid and molar extraction induce clinical osteonecrotic-like changes in alveolar bone

A 12-week administration of high doses of ZOL was well tolerated by all mice demonstrated by their conservation of body weight (data not shown). In addition, 21 % of the ZOL-treated mice exhibited osteonecrotic-like changes, characterized by both exposed and necrotic bone (sequestra) in the operative site at the