Clodronate-Loaded Liposome Treatment Has Site-Specific Skeletal Effects

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Abstract

Ineffective oral wound healing is detrimental to patients' oral health–related quality of life. Delineating the cellular mechanisms involved in optimal healing will elicit better approaches to treating patients with compromised healing. Osteal macrophages have recently emerged as important positive regulators of bone turnover. The contributions of macrophages to long bone healing have been studied, but their role in oral osseous wound healing following tooth extraction is less clear. Clodronate-loaded liposomes were used as a tool to deplete macrophages in C57BL/6J mice and assess oral osseous bone fill after extraction. In addition to macrophage ablation, osteoclast ablation occurred. Interestingly, depletion of macrophages and osteoclasts via clodronate treatment had differential effects based on skeletal location. In the nonwounded tibiae, clodronate treatment significantly increased CD68⁺ cells and decreased F4/80⁺ cells in the marrow, which correlated with increased trabecular bone volume fraction after 7 and 14 d. Serum formation and resorptive markers PINP and TRAcP 5b were decreased as were tibial TRAP⁺ osteoclasts. In healing extraction sockets, clodronate treatment increased extraction socket trabecular bone thickness at 14 d, which correlated with decreased TRAP⁺ osteoclasts and F4/80⁺ macrophages. Conversely, nonwounded maxillary interseptal bone was unaffected by clodronate treatment. Furthermore, the increase in extraction socket bone fill with clodronate was less than the large increase in trabecular bone observed in a nonwounded long bone. These data suggest a temporal and spatial specificity in the roles of macrophages and osteoclasts in normal turnover and healing.

Keywords: wound healing, bone biology, osteoclast(s), immunity, cell biology, macrophages

Introduction

Compromised wound healing in the oral cavity leads to prolonged infection, pain, and overall reduced quality of life. Proper healing of bony defects in the oral cavity from tooth extractions, periodontal procedures, congenital diseases, or surgical reconstruction is crucial to restore function. Compromised immune systems, radiation therapy, or intravenous bisphosphonate cancer therapies can lead to ineffective bone healing in the jaw, causing chronic issues such as osteonecrosis (Politis et al. 2016). Osteonecrosis of the jaw is associated with bisphosphonate use and, for unknown reasons, uniquely affects oral bone versus other skeletal sites. A better understanding of osseous wound healing and how it varies in different skeletal sites will help to abrogate the circumstances of inadequate healing and aid in the design of successful therapeutic strategies.

Extraction sockets are unique locations for healing, as they require bony and soft tissue healing and are exposed to the oral flora. Several cell types are crucial to the osseous healing of extraction sockets, including traditional bone cells (osteoclasts, osteoblasts, osteocytes) and immune cells such as macrophages and neutrophils (Vieira et al. 2015). Macrophages proliferate early during extraction socket healing, yet it is unclear how they contribute to bone formation in the repairing socket. Early in healing, macrophages are proinflammatory and make a phenotypic shift toward anti-inflammatory later in the healing

process (Koh and DiPietro 2011). Altering macrophage presence in extraction sockets will help us understand their specific roles in bone formation during repair.

Clodronate-loaded liposomes are effective tools for macrophage ablation (Frith et al. 2001). Clodronate bisphosphonate is encapsulated into liposomes, which are readily engulfed by macrophages, after which the phagocytic cell machinery degrades the liposome and releases clodronate. Clodronate is converted to a nonhydrolyzable analogue of ATP, resulting in apoptosis of the cell that engulfed the liposome. Mice treated with clodronate-loaded liposomes have depleted macrophages in several tissues in the body, including the bone marrow of long bones (Cho et al. 2014; Soki et al. 2015). The impact of clodronate-loaded liposomes on oral osseous wound healing is

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A supplemental appendix to this article is available online.

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unknown. The aim of this study was to delineate the role of macrophages in oral wound healing of extraction sockets via clodronate treatment.

Materials/Methods

Animals

Mice were maintained in accordance with institutional animal care and use guidelines, and experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Michigan. Animals were housed at a density of 4 or 5 mice per cage in specific pathogen-free conditions. Six-week-old male C57BL/6J mice (Jackson Laboratories) were anesthetized (ketamine/xylazine; 90 mg/kg, 5 mg/kg, intraperitoneal) and adequate anesthesia verified. Mice were given buprenorphine analgesic (0.05 to 1.0 mg/kg, subcutaneous) as directed by the Institutional Animal Care and Use Committee. The mandible was retracted and left and right maxillary first molars extracted. Animals were assigned numeric IDs and randomized into treatment groups. Phosphate-buffered saline (PBS)- or clodronate-loaded liposomes (5 mg/mL; clodronateliposomes.com) were administered locally (2 μ L) to extraction sockets following hemostasis and allowed to settle in the socket for 5 min, in addition to daily intraperitoneal injections at a dose of 10 μ L/g from day 0 to 6 and 6 μ L/g from day 7 to 13, similar to a previously published regimen (n = 10 per group per time point; Alexander et al. 2011). Mice were monitored throughout the experiment and euthanized at day 7 or 14 following tooth extraction, and tissues were analyzed in a blinded manner. Time points were selected due to the high presence of macrophages early in oral wound repair and according to previously published studies detailing the typical healing window for rodent extraction sockets (Kuroshima, Entezami, et al. 2014; Kuroshima, Mecano, et al. 2014; Tanoue et al. 2015). These methods are in compliance with the ARRIVE guidelines.

In Vitro Osteoblast Differentiation

Preosteoblasts (MC3T3-E1) were treated with PBS- or clodronate-loaded liposomes according to previously published protocols (Okada et al. 2018). Protocol details are in the Appendix.

Serum ELISAs

Blood was harvested at the time of euthanasia via intracardiac puncture under anesthesia (ketamine/xylazine), allowed to coagulate for 30 min at room temperature, spun down at 8,000 rpm for 10 min, and serum collected and stored at -80°C until use. Enzyme immunoassays were used to measure serum concentrations of tartrate-resistant acid phosphatase form 5b (TRAcP 5b) and propeptide of type I procollagen (P1NP) according to the manufacturer's instructions (IDS) and measured with an EZ Read 400 microplate reader with Galapagos software (Biochrom US).

Flow Cytometry Analysis

Bone marrow was isolated from tibiae via flushing in flow cytometry staining buffer (1 $\times$ PBS with 2% fetal bovine serum, 0.5mM EDTA), red blood cells lysed with 1 $\times$ ACK, and 10^6 cells stained with anti-mouse F4/80 (APC, A3-1; Abcam) and anti-mouse CD68 (FITC, FA-11; Biolegend). Isotype controls were used to confirm antibody specificity. Flow analysis was performed with a BD FACs Aria II (BD Biosciences).

Micro-computed tomography

Tibiae and maxillae were harvested at sacrifice (7 or 14 d), fixed in 10% neutral buffered formalin 24 to 48 h at 4°C , and then stored in 70% ethanol. Samples were scanned by micro-computed tomography (12- μ m voxel size, 70 kVp; μ CT-100, Scanco) as described (Sinder et al. 2017) and following established guidelines (Bouxsein et al. 2010). Detailed methods are included in the Appendix.

Histomorphometry

Maxillae and tibiae were fixed in 10% neutral buffered formalin for 24 to 48 h at 4°C , decalcified in 14% EDTA for 10 to 20 d, embedded in paraffin, and sectioned at 5 μ m. A transverse section of the maxilla was cut to view bilateral extraction sockets or interradicular bone and stained with hematoxylin and eosin, tartrate-resistant acid phosphatase (TRAP; 387A, Sigma), or F4/80. TRAP+ osteoclast quantification in tibiae was performed as described (Sinder et al. 2017; Michalski et al. 2018) and according to the histomorphometric analysis standards of the American Society for Bone and Mineral Research (Dempster et al. 2013). Detailed methods are in the Appendix.

Statistical Analysis

Statistical analyses were performed by 1-way analysis of variance and Tukey's post hoc test to compare ≥ 3 groups (PBS-liposome vs. clodronate-liposome treatments and between time points) with a significance of $P < 0.05$. Data are presented as mean $\pm$ SD. Power analysis details are in the Appendix.

Results

Clodronate-Loaded Liposomes Depleted Bone Marrow F4/80+ Macrophages

To assess the effect of phagocyte depletion on extraction socket healing, maxillary M1 molars were extracted bilaterally, and mice were treated with a one-time intradefect application of control PBS- or clodronate-loaded liposomes in addition to daily intraperitoneal injections of PBS- or clodronate-loaded liposomes (Fig. 1). Treatment with clodronate-loaded liposomes resulted in body weight reductions as compared with PBS-loaded liposomes after 7 d of treatment, whereas no changes

Figure 1. Experimental model. Six-week-old male C57BL/6J mice were anesthetized with ketamine and xylazine, the mandible retracted and maxillary M1 molars extracted bilaterally. Mice received local delivery of clodronate- or phosphate-buffered saline (PBS)-loaded liposomes (2 µL at a concentration of 5 mg/mL) in extraction sockets at time of surgery. Intraperitoneal injections of clodronate- or PBS-loaded liposomes were given daily at a dose of 10 µL/g (at a concentration of 5 mg/mL) from days 0 to 6 and 6 µL/g from days 7 to 13. Mice were sacrificed at day 7 or 14 after surgery and tissues harvested for serum biomarkers, bone marrow flow cytometric analysis (FACs), micro-computed tomography (micro-CT), and histomorphometric analysis. Images on right depict regions defined for micro-CT analyses. The red box shown on the left tibia shows the trabecular region of interest, and the red box on the right demonstrates the region of interest in the midshaft of the tibia used for cortical bone analysis. The yellow dotted line on the day 0 maxilla shows the slice orientation for micro-CT analysis of extraction sockets. Extraction sockets were defined in the maxillary micro-CT slices according to socket morphology and as shown in the bottom right (green outline). Scale bar = 1.0 mm, $n = 8$ to 10 per group.

were seen at 14 d (Fig. 2A). Spleen weights were reduced in clodronate-treated mice after 14 d of treatment (Fig. 2B). Flow cytometric analysis of long bone marrow showed that clodronate treatment decreased F4/80+ macrophages (Fig. 2C). In contrast, the percentage of CD68^{hi} cell populations was increased with clodronate treatment as compared with control PBS (Fig. 2D, E). F4/80+ cell depletion was confirmed by immunohistochemistry of tibial sections (Fig. 2F, Appendix Fig. 1).

Clodronate Treated Mice Have Increased Tibial Trabecular Bone Associated with Decreased Resorption

The effects of clodronate on the appendicular skeleton were determined. Clodronate treatment resulted in dramatic increases in trabecular bone in the proximal tibia as early as 7 d after treatment and further increased trabecular bone after 14 d (Fig. 3A–F). Trabecular bone tissue mineral density (TMD) was decreased with clodronate treatment (Fig. 3E), which shows that although there was increased bone, the average density of the bone in the clodronate-treated mice was lower than that in PBS-treated mice. Cortical bone thickness was unchanged in clodronate-treated mice at both time points (Fig. 3G). The serum formation marker PINP and the serum resorptive marker TRAcP 5b were significantly reduced in clodronate-treated mice at 14 d (Fig. 3H, I). Given the reduction in serum TRAcP 5b, the increased bone phenotype could have been largely due to alterations in osteoclast number and/or activity. To further assess the effect of clodronate on osteoclasts, TRAP+ cells were quantified in tibial histology sections. Clodronate treatment decreased TRAP+ osteoclasts in tibial sections at day 7 and 14 (Fig. 3J–L). Clodronate treatment

reduced the number of osteoblasts per bone surface and the percentage of bone surface covered by osteoblasts but did not affect the number of cells embedded in bone (osteocytes) per total bone area (Appendix Fig. 2A–K). In vitro treatment of preosteoblasts (MC3T3-E1) with clodronate showed that high concentrations of clodronate (0.5 to 1.0 mg/mL) significantly increased *Bglap* expression as compared with untreated controls, suggesting a potential direct effect of clodronate on osteoblast maturation (Appendix Fig. 3).

Bone Fill following Tooth Extraction Was Unchanged in Clodronate-Loaded Liposome Treated Mice

Maxillary M1 molars were extracted and sockets analyzed for bone fill at 7 and 14 d postsurgery via micro-computed tomography analysis. Clodronate treatment had no effect on trabecular bone parameters in the extraction site at 7 d of treatment (Fig. 4A–G), with the exception of trabecular TMD (Tb.TMD), which was significantly decreased with clodronate treatment. After 14 d of daily treatment with clodronate- or PBS-loaded liposomes, clodronate-treated mice displayed a trend of increased extraction socket BV/TV as compared with PBS-treated mice (Fig. 4A). Trabecular thickness was significantly increased in clodronate-treated animals as compared with PBS at day 14 (Fig. 4D). Given the changes seen in extraction socket bone fill, nonwounded bone in the maxilla was analyzed to determine the effects of clodronate on bone in other oral sites. Trabecular bone volume was measured between the maxillary M2 and M3 molars (Fig. 4H). No significant differences were seen in nonwounded oral bone volume after 7 or 14 d of treatment with clodronate-loaded liposomes.

Figure 2. Clodronate-treated mice have decreased spleen weight and altered bone marrow macrophage populations. **(A)** Body weight was measured at the time of sacrifice. Clodronate-treated mice had lower body weights at 7 d, whereas mice sacrificed at 14 d after surgery showed no differences in body weight. **(B)** Spleens were harvested and weighed at time of sacrifice. Spleen weight was reduced for clodronate-treated mice at day 14. **(C–E)** Bone marrow was flushed from tibiae at the time of sacrifice and stained for flow cytometric analysis of F4/80+ cells (macrophages) and CD68^{hi} cells (monocytes, dendritic). Clodronate-loaded liposome-treated mice displayed decreased F4/80+ and increased CD68^{hi} marrow populations as compared with PBS-control mice. **(F)** Paraffin-embedded tibiae were stained for F4/80 (DAB, brown), and percentage of F4/80+ area was quantified. $n = 8$ to 10 per group. * $P < 0.05$. ** $P < 0.01$. *** $P < 0.001$. Data are mean $\pm$ SD. CLOD, clodronate; PBS, phosphate-buffered saline.

Treatment with Clodronate-Loaded Liposomes Decreased F4/80+ Cells in Extraction Sockets

Mice treated with clodronate- or PBS-loaded liposomes were assessed for TRAP+ (osteoclasts) and F4/80+ (macrophages) cells in the maxillary extraction sockets at 7 and 14 d after surgery. TRAP+ (red/purple) multinucleated osteoclasts were increased in extraction sockets at 14 d as compared with 7 d in PBS-treated mice (Fig. 5A, B, Appendix Fig. 4A). Osteoclast number per bone surface was unchanged at days 7 and 14; however, TRAP+ osteoclast surface per bone surface showed a trend of a decrease at 14 d after extraction in the clodronate-treated mice as compared with controls. F4/80+ (brown) macrophages were present in the healing extraction socket at 7 and 14 d after extraction and were decreased with clodronate

treatment relative to PBS at 14 d (Fig. 5C, D, Appendix Fig. 4B). Osteoblasts and osteocytes in extraction sockets were unchanged with clodronate treatment, and osteocyte numbers per bone area were decreased in PBS- and clodronate-treated animals at day 14 as compared with day 7 (Appendix Fig. 5A–I).

Discussion

Macrophages are positive regulators of bone, yet the precise mechanisms by which they exert these effects are currently unknown (Michalski and McCauley 2017). The clodronate-loaded liposome macrophage depletion model results in increased bone volume in the appendicular skeleton, which is associated with F4/80-positive macrophage cell depletion and

an increase in CD68⁺ cells in the bone marrow (Cho et al. 2014; Soki et al. 2015). Widespread depletion of monocytes/macrophages, including CD68⁺ and F4/80⁺ cells, via the MAFIA mouse model resulted in overall decreased bone volume (Chang et al. 2008; Alexander et al. 2011; Cho et al. 2014;

Raggatt et al. 2014; Vi et al. 2015). These contrasting phenotypes in which CD68⁺ cells are either high (clodronate) or low (MAFIA) suggest that CD68⁺ macrophage populations are positive regulators of bone homeostasis as compared with the F4/80⁺ population. Given that the CD68 cell surface marker

Figure 4. Clodronate treatment does not affect extraction socket bone fill. Maxillae were harvested at time of sacrifice and analyzed via micro-computed tomography. (A–E) Extraction socket bone fill was unchanged after 7 d of treatment with clodronate, whereas clodronate liposome treatment showed a trend of increased extraction socket bone fill after 14 d as compared with PBS-treated mice due to significantly increased trabecular thickness. (F, G) Representative 3- and 2-dimensional images of bone fill in extraction socket after 14 d of healing. (H) Interseptal bone volume fraction was measured between M2 and M3 molars in the maxilla at days 7 and 14 with no statistically significant changes with clodronate treatment (representative image at right). $n = 8$ to 10 per group. $^{*}P < 0.01$. Data are mean $\pm$ SD. CLOD, clodronate; PBS, phosphate-buffered saline.

was reported to detect macrophages, dendritic cells, and osteoclasts, it is possible that CD68⁺ dendritic cells are targeted to the sites of macrophage depletion and wounded bone, resulting in an upregulation in CD68⁺ cells. Additionally, macrophages and dendritic cells can differentiate into osteoclasts. Since clodronate mainly acts on macrophages, dendritic cells may mobilize to these sites of reduced osteoclast precursors to replenish the loss of osteoclasts. Further studies to target macrophage population subsets for ablation are needed to delineate the contributions of each cell type to homeostatic bone turnover as well as healing.

Clodronate liposome treatment reduced osteoclasts in the long bone and showed a trend of decreased osteoclasts in extraction sockets. Osteoclasts were shown to uptake clodronate liposomes, leading to their reduced differentiation and activity (Frith et al. 2001). Depletion of osteoclast precursor cells could explain the reduction in osteoclasts. However, the

typical life span of an osteoclast is 2 wk (Manolagas 2000), and if precursors were the only cells depleted by clodronate liposomes, it would be expected that the effect on osteoclasts would be greater in the marrow at 14 d of treatment as compared with 7 d. Because of the large decrease in osteoclasts seen after 7 d of treatment, there may be a combinatorial effect of clodronate directly on osteoclasts as well as on their precursors.

Although there was an increase in bone mass in the appendicular skeleton, serum bone formation marker PINP was decreased. This could be attributed to a reduction in osteal macrophages or due to a coupling response in which decreased osteoclast signaling feeds back to decrease osteoblast formation and activity. Clodronate treatment reduced the number of osteoblasts per bone surface in the tibia but did not affect the osteoblast numbers in the extraction sockets. In vitro differentiation of osteoblast precursor cells showed an increase in osteoblast maturation gene signature with clodronate treatment

Figure 5. Clodronate treatment decreases F4/80-positive cells in extraction sockets. **(A, B)** Maxillae sections were stained for TRAP, and TRAP+ multinucleated osteoclast numbers (N.Oc/BS) and surface (Oc.S/BS) were measured. Clodronate treatment significantly reduced Oc.S/BS at day 14 after treatment. **(C, D)** Paraffin-embedded maxillae were sectioned and stained for F4/80. F4/80+ cells were quantified in extraction sockets. Clodronate ablated F4/80+ macrophages in the extraction sockets at days 7 and 14 postextraction. $n = 8$ to 10 per group. * $P < 0.05$. Data are mean $\pm$ SD. CLOD, clodronate; PBS, phosphate-buffered saline.

at high doses but did not affect early differentiation markers. These data suggest that clodronate affects osteoblast differentiation and should be further explored.

Interestingly, we observed a decrease in TMD in the tibiae and extraction socket with clodronate-loaded liposome treatment. One factor that could drive this change in TMD is a difference in the rate of mineralization. While clodronate liposomes may not directly interact with the mineralization process in a significant way, they did inhibit macrophages, which have a known role in regulating bone mineralization (Chang et al. 2008; Alexander et al. 2011; Raggatt et al. 2014). Specifically, macrophages were shown to secrete osteogenic factors such as TGF- β 1 (Michalski et al. 2016), oncostatin M (Guihard et al. 2012), and BMP-2 (Champagne et al. 2002), which could affect mineralization. Additionally, if clodronate liposome treatment preserved regions of bone that are naturally less mineralized by preventing resorption, this could explain the observed decrease in TMD.

Mice treated with clodronate-loaded liposomes had significantly increased trabecular bone in the tibiae. The trabecular bone volume fraction was increased 81% and 152% as compared with PBS treatment at days 7 and 14, respectively. Alternatively, while oral extraction socket bone fill trended toward an increase with clodronate treatment at day 14 following surgery, the increase in extraction socket bone fill was only 7%. Additionally, nonwounded oral bone displayed no changes when treated with clodronate-loaded liposomes. This suggests that macrophage and osteoclast depletion via clodronate-loaded liposomes has site-specific skeletal effects as well as differential effects on healing versus homeostatic turnover.

Recently, Viniegra et al. (2018) showed that systemic clodronate liposome treatment prevented bone resorption but impaired regeneration in a ligature-induced periodontitis model in part due to a reduction in osteoclasts. The clodronate-mediated decrease in osteoclasts in the long bones was more substantial than the osteoclast decrease seen in the oral

extraction sockets, and steady-state oral bone had relatively few residing osteoclasts (Appendix Fig. 6). Additionally, steady-state long bones had a higher number of osteoclasts per bone surface (4.9 osteoclasts/mm) as compared with wounded oral bone (day 7: 1.6 osteoclasts/mm, day 14: 3.4 osteoclasts/mm). This is supported by the literature, which demonstrates higher numbers of osteoclasts in the rat tibia as compared with mandible (Chaichanasakul et al. 2014).

The differences observed in the long bone site versus the oral cavity may be due to many factors, including differences in the cell populations in the marrow in these sites, origin of these cells, mechanical stimulation, amount of cortical versus trabecular bone, and exposure to different environmental cues. Bone marrow stromal cells from the mandible were shown to differentiate faster and mineralize more than such cells from long bone sites (Akintoye et al. 2006; Aghaloo et al. 2010). Additionally, osteoclasts from the mandible and tibia have similar resorptive activity, but osteoclasts from the mandible differentiate faster than those from the long bone (Azari et al. 2011; de Souza Faroni et al. 2011; Chaichanasakul et al. 2014). Furthermore, although these cells look similar histologically (Leucht et al. 2008), they arise from different origins during embryonic development (Chai and Maxson 2006), and they are formed by different ossification processes: intramembranous ossification (maxilla and mandible) versus endochondral ossification (long bones; Chai et al. 2000).

It is important to note some limitations of our study. Oral bone is largely cortical in nature, and analysis of nonwounded interseptal bone showed no changes with clodronate treatment. This was similar to the lack of effect seen in the cortical bone of the tibia, suggesting that clodronate may act similarly on cortical bone. The low amount of trabecular bone in maxilla makes it difficult to make a comparison to trabecular bone in the tibia. Additionally, we made comparisons of newly formed trabecular bone in extraction sockets to nonwounded tibial trabecular bone. Clodronate-liposome treatment was investigated by others in the context of long bone injury healing. Treatment with clodronate-loaded liposomes significantly decreased new bone formation in a tibial injury model (Alexander et al. 2011; Sandberg et al. 2017) and in femoral fracture healing (Lin and O'Connor 2016; Schlundt et al. 2018), further supporting site-specific responses to clodronate treatment.

To better understand how the changes seen in extraction socket bone fill are mediated, it is helpful to understand the sequence of events in extraction socket healing. Early in the healing of a mouse extraction socket (days 0 to 5), inflammatory cells and mesenchymal stem cells migrate and proliferate (Korpi et al. 2009), which is associated with an increase in inflammatory chemokines (Lin et al. 2011). Around day 3 after extraction, fibroblasts proliferate and osteoblasts differentiate to aid in collagen synthesis and bone formation, respectively. The levels of inflammatory cells and fibroblasts peak at around day 7, and osteoblasts peak at day 14 (Vieira et al. 2015). Osteoclasts differentiate and begin to remodel the new bone around day 14. This sequence of events leads to a rapid increase in bone tissue from day 5 to 14, at which time

bone fill levels off as osteoclast remodeling takes over. The effects of clodronate on extraction socket bone fill may be related to the drug distribution during the early and late macrophage and osteoclast proliferation in extraction sockets.

Clodronate is a bisphosphonate, and bisphosphonates have been studied in the context of extraction socket healing due to their association with osteonecrotic lesions in the oral cavity. Bisphosphonate-related osteonecrosis uniquely affects the bones of the oral cavity, which rightly poses questions regarding how these therapeutics target different skeletal sites. Empty osteocyte lacunae are characteristic of necrotic bone following bisphosphonate treatment (Kuroshima, Entezami, et al. 2014; Kuroshima, Mecano, et al. 2014). In the current study, treatment with clodronate-loaded liposomes did not alter the presence of osteocytes in lacunae. Additionally, zoledronic acid treatment increases extraction socket bone fill (Allen et al. 2011; Kuroshima, Mecano, et al. 2014), typically to a greater extent than that seen in the present study. Although the decreased osteoclasts explains an overall increase in bone, the percentage increase in bone with clodronate treatment may be less than that seen with other bisphosphonates due to the additional ablation of F4/80+ macrophage populations by clodronate. Decreasing F4/80+ cells that support osteoblast bone formation may compromise the ability of antiresorptive bisphosphonate to increase bone mass.

The extent to which clodronate exerted its effect on bone was variable according to the location, type of bone, and whether the bone was under normal homeostatic turnover or new bone was being generated in response to a wound. This study highlights the importance of understanding what cell populations may be potential targets when a drug's ability to aid in osseous healing is assessed. Further insight into the temporal and spatial specificities of osteal macrophages and osteoclasts will ultimately lead to better methods to therapeutically target these cells.

Author Contributions

M.N. Michalski, contributed to conception, design, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript; L.E. Zweifler, B.P. Sinder, A.J. Koh, contributed to conception, design, data acquisition and interpretation, critically revised the manuscript; J. Yamashita, contributed to design, data analysis and interpretation, critically revised the manuscript; H. Roca, contributed to conception, data analysis and interpretation, critically revised the manuscript; L.K. McCauley, contributed to conception, design, data analysis and interpretation, drafted and critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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