

Diverse Osteoclastogenesis of Bone Marrow From Mandible Versus Long Bone

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Background: Mandibles (MB) and maxillae possess unique metabolic and functional properties and demonstrate discrete responses to homeostatic, mechanical, hormonal, and developmental stimuli. Osteogenic potential of bone marrow stromal cells (BMSCs) differs between MB versus long bones (LB). Furthermore, MB- versus LB-derived osteoclasts (OCs) have disparate functional properties. This study explores the osteoclastogenic potential of rat MB versus LB marrow in vitro and in vivo under basal and stimulated conditions.

Methods: Bone marrow from rat MB and LB was cultured in osteoblastic or osteoclastic differentiation media. Tartrate-resistant acid phosphatase (TRAP) staining, resorption pit assays, and real-time polymerase chain reaction were performed. Additionally, osmotic mini-pumps were implanted in animals, mandibles and tibiae were isolated, and multinucleated cells (MNCs) were measured.

Results: MB versus LB marrow cultures that were differentiated with receptor activator of nuclear factor- κ B ligand (RANKL) and macrophage colony-stimulating factor produced more TRAP⁺ MNCs and greater resorptive area. To explore MB versus LB BMSC-supported osteoclastogenesis, confluent BMSCs were cultured with parathyroid hormone (PTH), 1,25-dihydroxyvitamin D₃ (1,25D₃), or PTH+1,25D₃. 1,25D₃- or PTH+1,25D₃-treated LB BMSCs expressed significantly higher RANKL and lower osteoprotegerin (OPG) mRNA and increased RANKL:OPG ratio. When whole marrow was cultured with PTH+1,25D₃, more TRAP⁺ MNCs were seen in LB versus MB cultures. Ultimately, rats were infused with PTH+1,25D₃, and MB versus tibia MNCs were measured. Hormonal stimulation increased osteoclastogenesis in both MB and tibiae. However, higher TRAP⁺ MNC numbers were observed in tibiae versus MB under basal and hormonal stimulation.

Conclusion: Collectively, these data illustrate differences of both osteoclastogenic potential and OC numbers of MB versus LB marrow. *J Periodontol* 2014;85:829-836.

KEY WORDS

Bone marrow; hormones; mandible; osteoclasts; stromal cells; tibia.

Similar to other craniofacial bones, the mandible (MB) and maxilla display developmental, functional, and homeostatic properties distinct from the appendicular skeleton. The jaws arise from neural crest cells of neuroectoderm rather than mesoderm¹ and are formed primarily by intramembranous as opposed to endochondral ossification.² 1,25-dihydroxyvitamin D₃ (1,25D₃) and parathyroid hormone (PTH) knockout mice demonstrate that MB mineralization is affected by 1,25D₃ deficiency but remains unaltered by abolishment of PTH. In contrast, long bones (LB) show effects in loss of both hormones.³ Similarly, in ovariectomized and malnutrition rodent models, the MB loses significantly less bone than the proximal tibia.⁴ Skeletal diseases only affecting the jaws, such as periodontitis,⁵ cherubism,⁶ hyperparathyroid jaw tumor syndrome,⁷ and bisphosphonate-related osteonecrosis of the jaws (ONJ),⁸ further support distinctive MB homeostasis.

An increased osteogenic potential of rodent and human MB versus LB bone marrow stromal cells (BMSCs) both in vitro and in vivo has been described.⁹⁻¹¹ Furthermore, human mandibular or maxillary BMSCs show enhanced response to osteogenic differentiation factors and bone morphogenetic protein 2 compared with cells derived from iliac crest.^{10,11}

Osteoclasts (OCs) are multinucleated, bone-resorbing cells whose differentiation

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and maturation requires macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor- κ B ligand (RANKL), which are among the cytokines supplied by BMSCs.¹² Bone site-specific phenotypic and functional differences of osteoclasts have been proposed.¹³ OCs from calvariae versus LB show differential usage of proteinases^{14,15} and expression levels of the enzyme tartrate-resistant acid phosphatase (TRAP).^{15,16} In addition, murine jaw and LB marrows have different osteoclastic potential in the presence of M-CSF and RANKL stimulation and exhibit distinctive shape and response to culturing substrates.^{17,18}

BMSCs are important regulators of osteoclastogenesis. BMSCs produce osteoprotegerin (OPG), an osteoclast decoy receptor that competes with RANK for RANKL binding.¹⁹ Therefore, the RANKL:OPG ratio pivotally determines the direction of osteoclastogenesis. RANKL and OPG expression is modulated by local cytokines and systemic hormones such as PTH and 1,25D₃.^{20,21} Interestingly, the basal RANKL:OPG ratio is higher in mouse jaw versus LB cultures, suggesting differential ability of the marrow environment to support osteoclastogenesis.¹⁸ However, differential ability of MB versus LB BMSCs to support osteoclastogenesis in stimulated conditions is not well understood.

The authors hypothesize that the jaws have distinct basal and induced osteoclastogenic potential compared with the other skeletal sites. Such differential osteoclastogenic response could underlie, at least in part, the pathophysiologic mechanisms of diseases unique to the jaws. This study explores the osteoclastogenic potential of rat MB versus LB marrow in vitro and in vivo under basal and hormone treatment. To the best of the authors' knowledge, this is the first report investigating MB marrow osteoclastogenesis under stimulated conditions. These data support differences of both osteoclastogenic potential and OC numbers of MB versus LB marrow.

MATERIALS AND METHODS

Isolation and Culture of Mandible and LB Marrow Cells

Animal approval and surgical procedures conformed to guidelines by the University of California–Los Angeles Chancellor's Animal Research Committee. MB and LB marrow cells were isolated from 1-month-old male Sprague-Dawley rats,[§] as previously described.⁹ Harvested whole marrow was pooled in a single suspension, and red blood cells were lysed by red blood cell lysis solution.^{||}

For BMSC cultures, cells were cultured at a density of 1×10^6 cells/mL (5.3×10^5 cells/cm²) in α -minimal essential medium (α -MEM)[¶] supplemented with 10% FBS and 1% antibiotics (100 U/mL penicillin and 100

μ g/mL streptomycin). After 6 days and upon confluence, suspension cells were discarded. Adherent BMSCs were cultured in fresh osteogenic differentiation medium (α -MEM + 10% FBS with 50 μ g/ μ L ascorbic acid and 4 mM β -glycerophosphate) with the addition of vehicle, PTH (10 nM), or 1,25D₃ (10 nM), which was replaced every 3 to 4 days. For whole marrow experiments, cells were cultured without the removal of suspension cells, and half of the media were replaced every 3 to 4 days.

Osteoclastogenesis

Bone marrow cell suspensions in media supplemented with 25 ng/mL rat M-CSF[#] were plated in 100-mm culture dishes overnight. Non-adherent cells were plated at 1.5×10^5 cells/100 μ L in osteoclastogenic medium (α -MEM + 10% FBS, 50 ng/mL M-CSF, 80 ng/mL sRANKL),** which was replaced every 2 to 3 days. After 6 days, cells were fixed and stained for TRAP activity using the leukocyte acid phosphatase kit. TRAP⁺ multinucleated cells (≥ 3 nuclei) were counted under light microscope.

Bone Resorption Assay

Osteoclast precursors were cultured on calcium phosphate-coated 16-well plates^{††} in α -MEM alone or osteoclastogenic media. After 10 days, cells were removed, and total resorption pit area was visualized by von Kossa stain. Using a light microscope camera, an image of each well was captured at $\times 2$ magnification. The total resorbed area of each well was measured using imaging software.^{‡‡}

RNA Isolation and Real-Time Quantitative Polymerase Chain Reaction

Total RNA from BMSC culture was collected at days 7 and 14, peak osteogenic potential time points previously described,⁹ using reagent,^{§§} and used for quantitative polymerase chain reaction (PCR), performed in triplicate for at least three independent experiments, with PCR mix^{|||} and rat gene-specific primers, including: RANKL (NM_057149) 5'-GGA-GAGCGAAGACACAGAAGCACTAC-3' (forward), 5'-CGAGCCACGAACCTTCCAT-CATAGC-3' (reverse); OPG (NM_012870) 5'-TGTCCCTTGCCCTGACTAC-TCTTATAC-3' (forward), 5'-CCTTCCTCACATTCGC-ACACTCG-3' (reverse); and GAPDH (NM_017008) 5'-TTCAACGGCAGTCAAGG-3' (forward), 5'-AT-ACTCAGCACCAGCATCAC-3' (reverse). The maximum gene expression normalized to GAPDH was set

§ Charles River, Wilmington, MA.

|| BioLegend, San Diego, CA.

¶ Mediatech, Herndon, VA.

PeproTech, Rocky Hill, NJ.

** Sigma-Aldrich, St. Louis, MO.

†† BioCoat Osteologic MultiTest Slides, BD Biosciences, Bedford, MA.

‡‡ CellSens, Olympus, Center Valley, PA.

§§ Trizol, Invitrogen, Carlsbad, CA.

||| iQ SYBR Green supermix, Bio-Rad, Hercules, CA.

at 100%, and the remaining expression levels were calculated as percentage of maximum induction. The authors elected to express gene levels as percentage of maximum instead of fold change to avoid large and artificial fluctuations of gene induction in cases where control levels were low.

In Vivo Assessment of Osteoclastogenesis

Eight male 3-month-old Sprague-Dawley rats were used. Subcutaneously implanted mini-osmotic pumps^{¶¶} continuously infused vehicle or 40 µg/kg/day human PTH (1-34)^{##} and 2 µg/kg/day 1,25D₃^{***} for 3 days. This timing of PTH and 1,25D₃ treatment has shown peak osteoclastic induction in vivo.^{22,23} Animals were sacrificed at day 4, and MB and tibiae were fixed in 4% formaldehyde solution for 48 hours and stored in 70% ethanol.

Bones were decalcified in 14.5% EDTA (pH 7.2) for 4 weeks. Paraffin-embedded 4-µm-thick coronal sections at the interproximal area between the first and second mandibular molars and cross sections of proximal tibiae were stained with hematoxylin and eosin (H&E) and digitally scanned using an automated slide scanner^{†††} and software.^{†††} Osteoclasts (≥2 nuclei represent multinucleation, distinguishing from mononucleated preosteoclasts²⁴) in contact with the bone surface and bone area were measured (software annotation tool) within the alveolar bone region of the mandible and the trabecular bone region of the proximal tibia.

Statistical Analyses

Data were expressed as mean ± SEM from at least three independent experiments. Data among groups were compared with one-way analysis of variance, and statistical differences among groups were identified using Student *t* test. *P* < 0.05 was considered significant.

RESULTS

Higher Number of TRAP⁺ Multinucleated Cells and Increased Resorption of MB Versus LB Marrow

To study the differences between the osteoclastogenic potential of rat MB versus LB marrow, non-adherent marrow cells were differentiated into mature OCs by M-CSF and RANKL. At day 6 of culture, TRAP⁺ multinucleated cells (MNCs) were observed in both MB and LB cultures (white arrows, Figs. 1A and 1B). However, significantly more TRAP⁺ MNCs were seen in MB cultures (Fig. 1C). To verify that the observed TRAP⁺ MNCs were functional osteoclasts, osteoclast precursors were differentiated on calcium phosphate substrates. MB cultures showed increased resorptive pit formation (Figs. 1D and 1E) with significantly higher total resorbed area (Fig. 1F) than the LB cultures.

MB Versus LB BMSCs Possess a Lower Osteoclastogenic Potential Under Hormonal Stimulation

BMSCs support osteoclastogenesis through modulation of the RANKL-OPG system. Hormones that regulate bone homeostasis, such as PTH and 1,25D₃, regulate production of osteoclast regulatory cytokines.^{21,25} The authors thus explored RANKL and OPG expression in basal and hormone-stimulated conditions at 7 and 14 days of rat MB versus LB BMSC cultures. Basal RANKL and OPG mRNA expression was similar in MB versus LB BMSCs (Figs. 2A and 2B). However, in the presence of 1,25D₃ alone or in combination with PTH, RANKL expression was higher in LB versus MB cultures at both 7 (*P* < 0.05) and 14 (*P* < 0.01) days (Fig. 2A). Under the same treatments, the inhibition of OPG expression was greater in LB versus MB BMSCs with PTH+1,25D₃ (*P* < 0.01) and 1,25D₃ alone (*P* < 0.05) at day 14 (Fig. 2B). Importantly, the RANKL:OPG ratio was substantially enhanced in LB versus MB BMSCs under 1,25D₃ alone or in combination with PTH at 7 and 14 days of culture (Fig. 2C).

LB Versus MB Whole Marrow Generates More TRAP⁺ MNCs Under Hormonal Induction

These data showed that although rat MB marrow has a higher osteoclastogenic potential in the presence of M-CSF and RANKL, hormonally stimulated LB BMSCs express higher levels of RANKL and RANKL:OPG ratio that would favor increased osteoclastogenesis. To evaluate the osteoclastogenic ability of rat MB versus LB marrow, the authors cultured whole marrow under basal and PTH+1,25D₃ treatment. TRAP staining revealed TRAP⁺ MNCs in both LB and MB culture under PTH+1,25D₃ stimulation but not at baseline (Fig. 3A). Quantitatively, LB marrow cultures contained significantly more TRAP⁺ MNCs compared with MB culture (Fig. 3B).

Higher Basal and PTH+1,25D₃-Stimulated MNC Number in Tibia Versus Mandible in Rats In Vivo

To evaluate basal and hormonal stimulation of osteoclast formation in vivo, mini-osmotic pumps containing either vehicle or PTH+1,25D₃ were subcutaneously implanted for 3 days in adult rats. Then MB and tibiae were decalcified, and H&E staining was performed on sections from the trabecular bone of the proximal tibia metaphyseal area (Figs. 4A through 4C) and the mandibular alveolar bone in the interproximal area between the first and second molars (Figs. 4D through 4F). MNCs abutting the

¶¶ Alzet model 1003D, Alza, Palo Alto, CA.

Bachem, Torrance, CA.

*** Sigma-Aldrich.

††† Aperio XT, Aperio Technologies, Vista, CA.

†††† Aperio Imagescope v.11, Aperio Technologies.

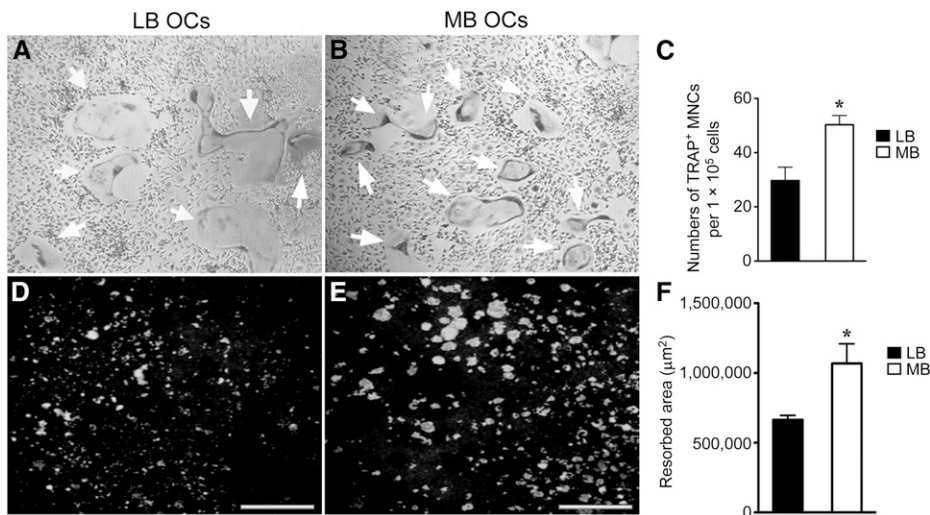


Figure 1.

TRAP staining of cultures from LB (A) and MB (B) after 6 days of differentiation with M-CSF and RANKL. TRAP⁺ MNCs (indicated by white arrows) are shown at $\times 4$ magnification. (C) Quantification of total TRAP⁺ MNCs (>3 nuclei) formed by LB and MB marrow after 6 days of differentiation (mean \pm SEM). Von Kossa stain reveals resorptive pits formed by LB (D) and MB (E) marrow cultures after 10 days of differentiation in the presence of M-CSF and RANKL on calcium phosphate substrate. (F) Quantification of total resorbed area formed in LB and MB cultures (mean \pm SEM). * $P < 0.05$.

bone, presumably representing osteoclasts, were observed at baseline and under PTH+1,25D₃ stimulation in both tibia and MB (Figs. 4B, 4C, 4E, and 4F, green arrows). At baseline, more osteoclasts were observed in tibia versus MB. PTH+1,25D₃ significantly increased osteoclast numbers in both tibia and MB. Importantly, greater numbers of MNCs were observed under hormonal stimulation in tibia compared with MB (Fig. 4G).

DISCUSSION

Mandible, a bone of the orofacial complex, possesses unique metabolic and functional properties and demonstrates discrete responses to homeostatic, mechanical, and developmental stimuli.²⁶ Bone diseases such as cherubism,⁶ hyperparathyroid jaw tumor syndrome,⁷ and ONJ^{8,27,28} affect the jaws while sparing the remaining skeleton. Differences in the osteogenic potential of LB versus MB BMSCs suggest a skeletal site-specific BMSC response to various stimuli during bone remodeling and healing.^{10,29} This study examines potential differences in the osteoclastogenic ability between MB and LB under basal and stimulated conditions. Exploring mandibular osteoclastogenic potential under basal and hormonal regulation is significant, since it would provide valuable understanding of jawbone homeostasis and might lead to better target approaches for bone conditions that selectively affect the face.

The authors first investigated the *in vitro* potential of rat MB versus LB marrow cells to form osteoclasts

in the presence of M-CSF and RANKL. MB marrow generated significantly more TRAP⁺ MNCs. The authors tested marrow osteoclastogenesis at 6 days of culture.³⁰ To test whether differences in TRAP⁺ MNC numbers result in increased resorption, the authors used a calcium phosphate substrate as a neutral culturing surface. Increased osteoclast formation was mirrored by larger total resorbed area generated by MB osteoclasts in comparison to LB cultures. The findings suggested an increased osteoclastic potential of MB marrow.

Previous reports utilizing mouse-derived MB and LB marrow found higher osteoclast numbers in LB marrow cultures at earlier time points, although at later time points no difference was observed. A difference in the MB versus LB marrow cellular composition, with monocytes and myeloid blasts as principal cells in the jaw and LB, respectively, could have accounted for the observed difference at the earlier time points.¹⁸ Interestingly, differences in osteoclast number between MB and LB appear to depend on the substrate the cells are cultured on. More osteoclasts were observed in LB marrow when cells were cultured in plastic or bone slices, while the contrary was true when cells were cultured on dentin slices.¹⁷ Utilization of rats versus mice as well as experimental differences in preparing and culturing marrow cells could account for the disparate findings in this study, where the authors observed differences in osteoclastogenic potential between the two skeletal sites, and other studies, where no differences were observed.¹⁸

Because the authors observed a higher osteoclastic potential of rat MB marrow when M-CSF and RANKL was added to the culture, they sought to investigate whether there is a difference in the ability of MB versus LB BMSCs to support osteoclast formation. Such differences should not be surprising, since diverse function of BMSCs from various skeletal sites, including the jaws, have been reported for humans and rodents.^{9,10,31} BMSCs can support osteoclastogenesis through the production of RANKL and OPG. Furthermore, systemic hormones such as PTH and 1,25D₃ induce osteoclast formation by, among others, altering RANKL and OPG expression and thus regulating the RANKL:OPG ratio.^{20,21,32}

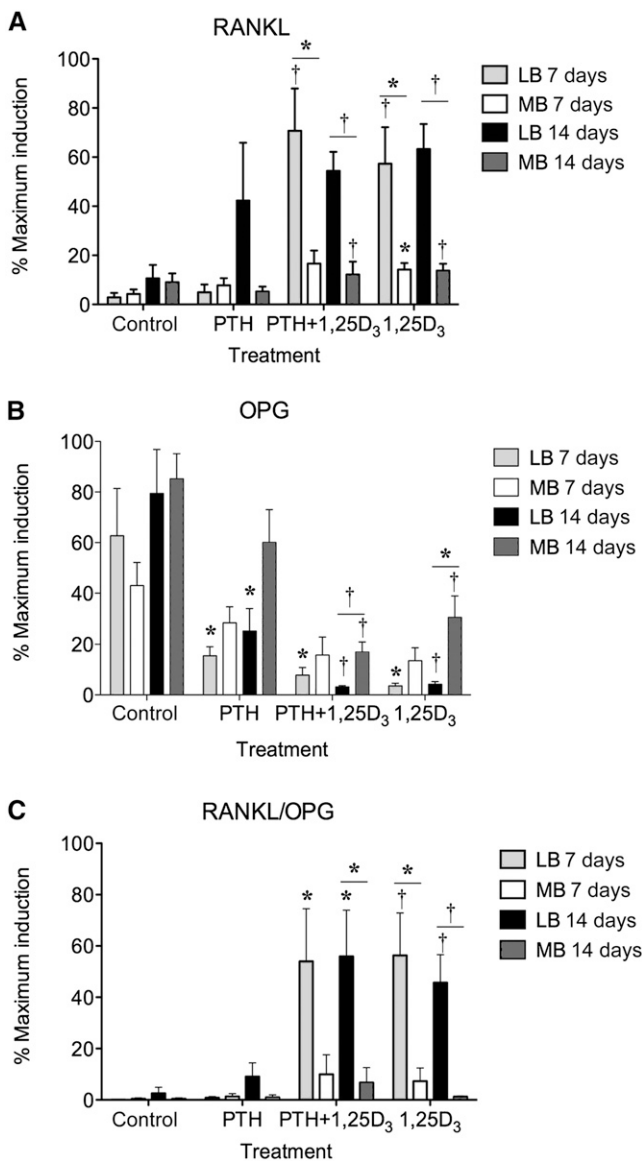


Figure 2. Effect of PTH (10 nM) and 1,25D₃ (10 nM), alone or in combination, on the expression of RANKL (A), OPG (B), and RANKL:OPG ratio (C) by LB versus MB BMSCs cultured in osteogenic media for 7 or 14 days. mRNA levels were normalized to GAPDH and expressed as percentage of maximum expression (mean ± SEM). For control, vehicle-treated BMSCs were cultured in osteogenic media for 7 or 14 days. *P < 0.05; †P < 0.01.

1,25D₃, alone or in combination with PTH, induced RANKL expression in both MB and LB BMSCs. However, the RANKL induction in LB cells was significantly greater than that in MB cells. PTH, 1,25D₃, and PTH+1,25D₃ significantly attenuated OPG expression in LB BMSCs. Only PTH+1,25D₃ inhibited OPG expression in MB BMSCs at 14 days of culture. Importantly, the inhibition of OPG expression in LB BMSCs was more pronounced. This differential RANKL induction and OPG inhibition resulted in an elevated RANKL:OPG ratio of LB BMSCs under stim-

ulated conditions, suggesting a disparate response of LB versus MB BMSCs to hormonal stimulation.

The ability of cells along the osteoblastic lineage to support osteoclastogenesis has been explored.³³ Although immature versus differentiated osteoblasts exhibit a strong potential to support osteoclast formation and differentiation in vitro,³⁴ the vital role of osteocytes in regulation of osteoclast formation in vivo at baseline, as well as during unloading, is well established.^{35,36} In these experiments, the authors studied RANKL and OPG expression at two culture points, representing different osteoblastic potential of marrow stromal cells.⁹ Basal RANKL levels were higher at later time points of culture, suggesting increased ability for support of osteoclastogenesis of more differentiated stromal cells. However, PTH and 1,25D₃ induced RANKL at a similar level for all time points.

Osteoclast formation depends on the availability of osteoclastic precursors, as well as on appropriate signals that will promote their differentiation and fusion into mature multinucleated osteoclasts. To compare the ability of rat LB versus MB marrow to form MNC TRAP⁺ cells, whole marrow in the absence of added RANKL and M-CSF was cultured in the presence of hormonal stimulation. LB marrow generated significantly greater TRAP⁺ MNCs, reinforcing the higher osteoclastogenic ability of LB marrow under PTH+1,25D₃ treatment. Ultimately the authors evaluated basal and hormonal stimulation of osteoclast formation in vivo. Interestingly, higher MNC numbers were identified in tibiae versus MB in control animals, indicating that baseline bone homeostasis favors increased osteoclast presence that might reflect higher basal bone remodeling in long bones. Hormonal treatment significantly induced osteoclast formation in both skeletal sites with a higher overall osteoclast number in tibiae, similar to the in vitro findings of PTH+1,25D₃ increase in whole marrow MNCs.

Overall, the authors detected distinct osteoclastogenic ability of rat MB versus LB marrow. MB marrow appeared to possess more basal osteoclast precursors. Alternatively MB pre-osteoclasts could demonstrate increased proliferation under M-CSF and RANKL stimulation. However, LB marrow cells responded to hormonal treatment with enhanced RANKL gene expression and RANKL:OPG ratio, and with increased osteoclast numbers. These observations point to discrete and complex regulation of jaw versus appendicular bone homeostasis. These findings have potential implications for pharmacologic treatment of periodontal bone loss. Osteoclast activation is central in the pathogenesis of periodontitis.³⁷⁻³⁹ Pharmacologic inhibitors of osteoclast function or differentiation attenuate bone loss in animal models of periodontitis and have been entertained as potential interventions for periodontal

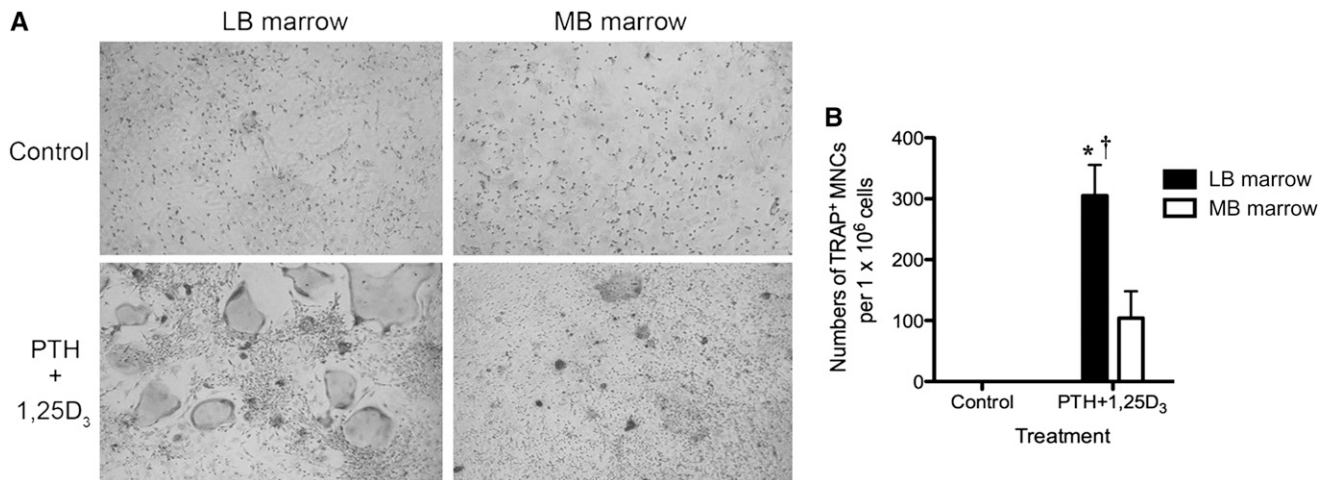


Figure 3.

A) TRAP staining of LB versus MB whole marrow under control or PTH+1,25D₃ treatment after 7 days. (Original magnification ×4X.) **B)** Quantification of TRAP⁺ MNC numbers in LB or MB marrow cultures (mean ± SEM). *P < 0.05 compared with vehicle-treated control; †P < 0.05 compared with PTH+1,25D₃-treated group.

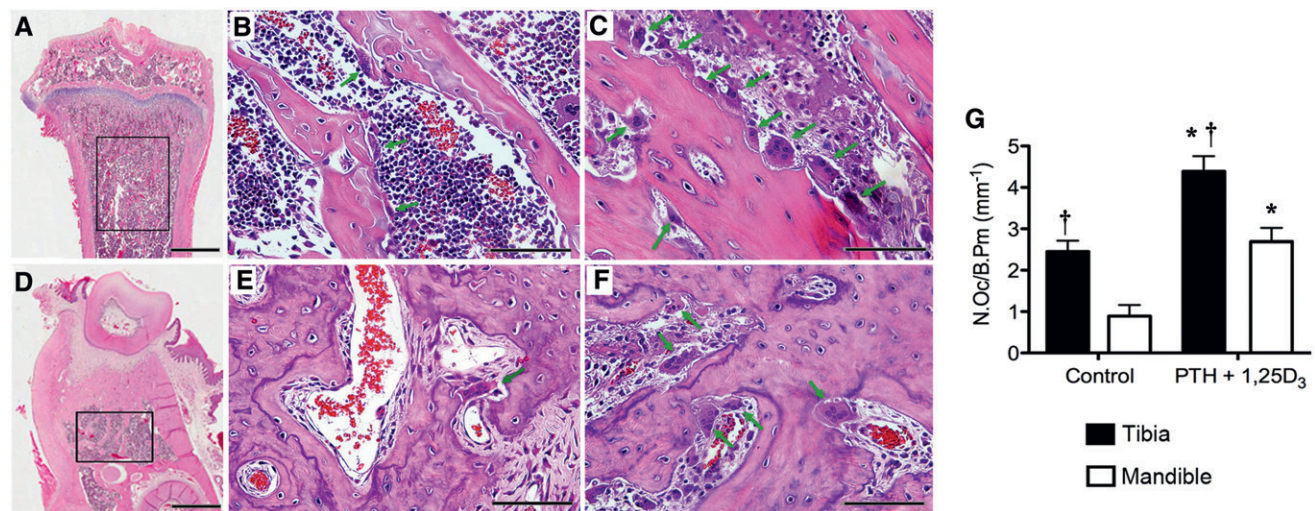


Figure 4.

H&E-stained sections of tibia (**A through C**) and MB (**D through F**) at ×2 (**A, D**) and ×40 (**B, C, E, F**) magnification. Sprague-Dawley rats were continuously infused with vehicle (**B and E**) or PTH + 1,25D₃ (**C and F**) for 3 days. **G)** MNCs abutting bone surface (indicated by green arrows in **B, C, E, and F**) were measured within the trabecular bone of tibia and MB (black rectangular outline, **A and D**). N.Oc/B.Pm = number of osteoclasts per bone perimeter. *P < 0.05 compared with vehicle-treated control; †P < 0.05 compared with MB.

disease.⁴⁰⁻⁴⁵ Systemic hormones and inflammatory cytokines play key roles in osteoclast formation and activation.^{38,43,46,47} Indeed, association of systemic diseases that show increased osteoclast activity with periodontal bone loss has been explored.⁴⁸⁻⁵¹ These results suggest a bone site-specific regulation of osteoclast activation. Combined with other published data that show bone site-specific differences in osteoclastic function,^{13,14,17} potential specific inhibitors of alveolar bone osteoclast activation and/or function could be developed. Such pharmacologic

interventions would be important in regulating periodontal bone loss, while sparing bone homeostasis in the remainder of the skeleton. Alternatively, osteoclastic inhibitors for the rest of the skeleton but not the jaws would be valuable for management of bone metabolic diseases, such as osteoporosis or bone cancer, while reducing the risk for ONJ.

CONCLUSIONS

In summary, these data demonstrate a diverse osteoclastogenic capacity of rat MB versus LB marrow.

Although MB marrow appeared to possess intrinsically more osteoclast precursors, LB BMSCs showed enhanced response to hormonal increase of RANKL expression and RANKL:OPG ratio. Furthermore, PTH and 1,25D₃ induced higher TRAP⁺ MNC numbers in whole marrow of LB versus MB. Paralleling these findings, tibiae showed higher osteoclasts at basal and PTH+1,25D₃-stimulated conditions. The diverse osteoclastic potential of the MB versus other skeletal sites could explain, in part, the differential response of the jaws to mechanical, hormonal, and nutritional signals, as well as to antiresorptive therapies.

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