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ORIGINAL ARTICLE

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Hypoxia triggers a HIF-mediated differentiation of peripheral blood mononuclear cells into osteoclasts

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Structured Abstract

Background – The source and mechanisms leading to osteoclast (OC) generation during tooth movement are not clearly understood. We hypothesized that during tooth movement, OC differentiate from peripheral blood mononuclear cells (PBMNC) downstream of the global hypoxia-inducible transcription factor hypoxia-inducible factor (HIF)-1α.

Objective – The objective of this study was to demonstrate up-regulation of OC growth factors from osteoblasts (OB) and subsequent conversion of PBMNC into functional OC under hypoxic stress.

Material and Methods – Human primary PBMNC were cocultured with/without OB and subjected to either hypoxia (2.5% O_2) or normoxia (21% O_2) over 14 days. Levels of HIF, vascular endothelial growth factor (VEGF) and receptor activator for nuclear factor kappa-β ligand (RANKL) were measured. Conversion of PBMNC into OC was measured using resorption and TRAP assays.

Results – Functional OC were only observed in response to hypoxia during coculture of PBMNC and OB and only after up-regulation of HIF, VEGF and RANKL in the hypoxic conditions. YC-1, a HIF inhibitor, reduced OC formation in response to hypoxia.

Conclusion – Hypoxia triggers the differentiation of PBMNC into functional OC in the presence of OB in a HIF-dependent manner as would occur during orthodontic loading of the periodontal ligament space.

Key words: hypoxia; monocyte; osteoclast; RANKL; vascular endothelial growth factor

Introduction

Orthodontic tooth movement involves a remodeling process wherein resorption of bone of the alveolar wall occurs in the direction of the displacement and deposition on the contralateral (tension) side (Fig. 1). Two forms of orthodontic-induced bone

resorption have been described: direct (frontal) and undermining resorption. Direct resorption occurs from the side of the periodontal ligament (PDL), whereas undermining resorption, which occurs after occlusion of the PDL, is from the marrow space (1, 2).

Osteoblasts (OB) are important in the bone remodeling process as they are responsible for the recruitment and maturation of osteoclasts (OC) through receptor activator for nuclear factor kappa-β ligand (RANKL) and macrophage colony-stimulating factor (MCSF) (3, 4). OB also release angiogenic factors that include vascular endothelial growth factor (VEGF) (5–7), a downstream target of hypoxia-inducible factor (HIF) (8). VEGF has been reported to expedite tooth movement (9, 10). However, the molecular mechanisms underlying that process have not been elucidated.

While it has been indicated that OC involved in tooth movement originate from the bone marrow (11), several investigators have cultured OC *in vitro* from peripheral blood mononuclear cells (PBMNC) (12–16), demonstrating that OC do not originate from the bone marrow alone.

Application of orthodontic forces leads to compression of the PDL (Fig. 1), thereby occluding the vasculature (17) resulting in localized areas of ischemia within the PDL. High levels of RANK/RANKL have also been detected in resorptive areas induced by orthodontic loading (18). In addition, osteoblasts up-regulate HIF in response to hypoxia with concomitant production of VEGF (6, 19). We hypothesize that this hypoxia triggers the production of factors involved in vascular regeneration as well as OC differentiation. Such factors include HIF, VEGF and RANKL. The purpose of this project, therefore, was to demonstrate up-regulation of these cytokines in OB in response to hypoxic conditions as well as to show differentiation of PBMNC to OC when cocultured with OB.

Material and methods

Isolation of PBMNC and OB

Upon approval from the Institutional Review Board, blood obtained with consent from healthy donors was purchased from the Oklahoma Blood Institute (http://www.obi.org). PBMNC were then isolated using standard Ficoll-Paque procedure (Stem Cell Technologies, Vancouver, BC, Canada). The cells were either used immediately or frozen and stored in liquid nitrogen for future use. Previously characterized primary human calvarial osteoblasts were generously donated by Dr Michael Cunningham (University of Washington, Seattle).

Culture conditions of the PBMNC and OB under normoxic (21% oxygen) and hypoxic (2.5 and 5% oxygen) conditions

Peripheral blood mononuclear cells were plated in 24-well plates at a density of 4.5×10^5 cells per well in 0.5 ml of Dulbecco's modified Eagle's medium (D-MEM/F-12) (Invitrogen, Grand Island, NY, USA) supplemented with 10% cosmic

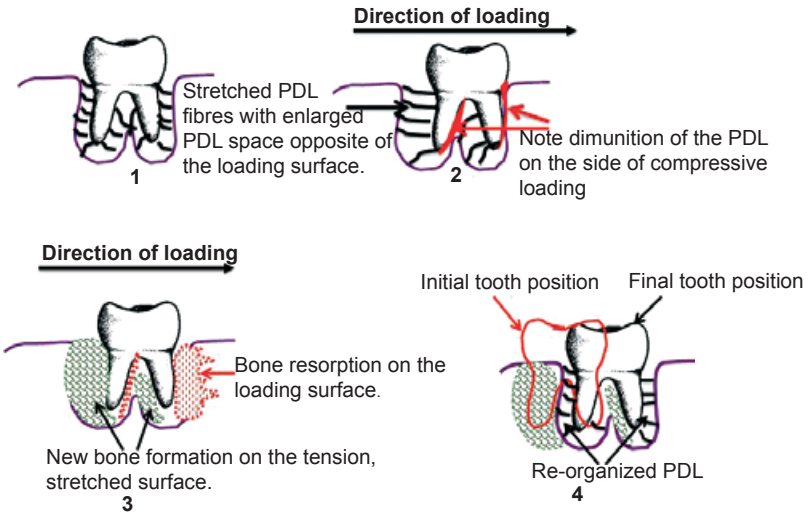


Fig. 1. Schematics of tooth movement showing resorptive and depository surfaces. (1) Initial tooth position before application of orthodontic loading. (2) Loading force applied. Note direction of tooth movement and the tissue response on the different surfaces. Red is indicative of the ischemia experienced on the compressive region. (3) As the tooth translates in the respective direction, new bone (green) is formed on the tension side, whereas resorption is imminent on the ischemic, compressive loading surface. (4) Initial and final tooth position after completion of tooth movement. The periodontal ligament (PDL) is now fully remodeled with symmetrical distribution of the spaces around the tooth surfaces.

calf serum (Hyclone, Logan, UT, USA), 1% sodium pyruvate, 1% L-glutamine and 2% penicillin–streptomycin. All media were changed every 3.5 days by semi-depletion. The OB were either plated onto 12-mm inserts with 0.1 ml of media or directly onto the 24-well plate with 0.5 ml of media. The 12-mm inserts containing OB were placed into the 24-well plate with PBMNC at the bottom of the well plate. Both OB and PBMNC were cultured either under normoxia (21% oxygen) or hypoxia (2.5 and 5% oxygen) for a total of 14 days with media change as described. YC-1 (Sigma-Aldrich, St Louis, MO, USA), a HIF inhibitor, was added to the respective wells at 5 μM, a concentration found to significantly reduce HIF levels without killing the cells (20).

CFDA-AM cell proliferation assay

After treatment, 5 μM 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM; Molecular Probes, Eugene, OR, USA), a substrate cleaved multiple times by non-specific cellular esterases to form fluorescein, was added for 2 h at 37°C. The fluorescence was read on a plate reader (BMG Labtech, Durham, NC, USA) using the fluorescein channel as a marker of cell proliferation (21). Data were then graphed as a percent of the fluorescence of untreated or normoxic cells. For the CFDA-AM assay, the cells were exposed to either 21% O_2 (normoxia) for 72 h, either 2.5 or 5% O_2 for 24 h or 2.5 and 5% O_2 for the first 24 h and then moved to normoxia for the next 48 h for a total of 72 h of exposure.

Functional assay for OC formation

Twelve-millimeter osteologic disks (BD Biosciences, Bedford, MA, USA) were placed at the bottom of the 24-well plates into which either PBMNC alone, PBMNC cocultured with OB or OB alone were added at day one of the experiment. Another set of osteologic disks was placed into three wells of a 24-well plate into which neither PBMNC nor OB were placed as a control. After 14 days, the osteologic disks were washed with distilled water, bleached with NaOCl (6%) to remove the adherent cells and rinsed with running distilled water, then viewed under light microscope to assess the presence/absence of resorption pits (22, 23) as per manufacturer's instructions.

TRAP assay

Another assay to assess for osteoclastic formation was conducted by way of the tartrate-resistant acid phosphatase procedure (TRAP) (24), which measures acid phosphatase activity, using a commercially available tartrate-resistant acid phosphatase antibody (Abcam, Cambridge, MA, USA). Briefly, at the end of 14 days, media were removed and the cells were washed with PBS then fixed in 4% formaldehyde for 10 min at room temperature, washed twice with PBS, incubated with 0.5% Triton at room temperature for 10 min, washed three times with PBS and blocked in 10% goat serum in PBS for 30 min. The cells were incubated in primary antibody made up in 10% goat serum in PBS for 1 h at room temperature, washed three times with PBS and incubated with secondary antibody (Alexa-488 Mouse; Invitrogen, Carlsbad, CA, USA) in 10% goat serum in PBS. The cells were then washed three times with PBS, rinsed with DAPI (5 mg/ml stock diluted 1:2000) for 1 min, washed again three times with PBS and mounted with a coverslip with SlowFade Gold reagent (Invitrogen). Captured Z-stack images were averaged together and assessed for the presence of TRAP antibody.

Western blot analysis of RANKL and HIF-1α

Whole cell lysates were made using M-PER lysis buffer (Pierce Chemical, Rockford, IL, USA) with 300 mM sodium chloride and containing protease inhibitor cocktail (EMD Bioscience, San Diego, CA, USA) and phosphatase inhibitors (Sigma Chemical, St Louis, MO, USA). For HIF-1α, either 30 or 50 μg protein lysates was separated by 10% SDS-PAGE. For RANKL, 30 μl of cellular supernatants was run in 10% gels. Gels were then transferred to 0.2-μm nitrocellulose membranes, blocked with Superblock (Pierce Chemical), and incubated with primary antibodies overnight at 4°C. Membranes were then washed in Tris-buffered saline (TBS), appropriate secondary added,

and then washed again with TBS. The substrate, Supersignal Dura chemiluminescence (Pierce Chemical), was added and membranes developed using a digital imaging system with a charge-coupled device camera (Image-Station 4000; Kodak, New Haven, CT, USA). To assess loading, before blocking membranes were stained with the reversible memcode dye (Pierce Chemical) by first converting images to grayscale, setting calibration to uncalibrated optical density and capturing the mean integrated density of regions of interest around particular bands. Ratios of band intensity as compared to normoxic (21% O_2) control from each blot were used to generate percent control values and these percent control values combined from each replicate and from each experiment to generate data for graphs. Densitometric analysis of bands was performed using NIH ImageJ 1.41 software. HIF-1α (Chemicon, Millipore, Bellerica, MA, USA) and RANKL antibodies (R&D Systems, Minneapolis, MN, USA) were used at a concentration of 2 μg/ml.

VEGF ELISA

Supernatants and standard curves derived from recombinant VEGF were loaded in a high-binding ELISA plate (Corning/COSTAR, Corning, NY, USA) and incubated at 4°C overnight. Wells were then washed with PBS, polycolonal anti-VEGF antibody (Ab2992; 1:1000) (Abcam) added and incubated for 2 h at room temperature. Wells were washed with PBS, and secondary antibody was added for 45 min at room temperature. The wells were washed again and ECL ELISA (Pierce Chemical) substrate was added. Luminescence was measured after 5 min using a FlUOstar Optima plate reader (BMG Labtech, Cary, NC, USA).

Statistical comparisons were conducted using anova followed by the Bonferroni test.

Results

OB and PBMNC stably survive hypoxia

To assess whether both OB and PBMNC could survive long-term hypoxic conditions, CFDA-AM, a fluorescent proliferation assay was employed. There was no significant difference in proliferative index between hypoxic (2.5 and 5% oxygen) and normoxic (21% oxygen) conditions over 72 h (Fig. 2).

RANKL is up-regulated by hypoxia

Densitometric comparison of the levels of RANKL in OB subjected to hypoxia and normoxic conditions showed substantial up-regulation of RANKL in the hypoxic conditions (Fig. 3A, B). Western blot showed steady increase in the levels of RANKL in OB subjected to hypoxia (Fig. 3B). Such up-regulation reached its maximum after 48 h with no further increase observed after that period. There was no change in the RANKL levels in the normoxic conditions during the entire period.

VEGF and HIF-1 are up-regulated in OB during hypoxia

Vascular endothelial growth factor protein levels were significantly up-regulated starting at 24 h after exposure to hypoxia and remained induced out to 72 h (Fig. 3C). The transcription factor HIF-1α known to regulate VEGF was likewise significantly increased to similar levels (i.e., 2.5-fold that of normoxic levels) in response to 4- and 72-h

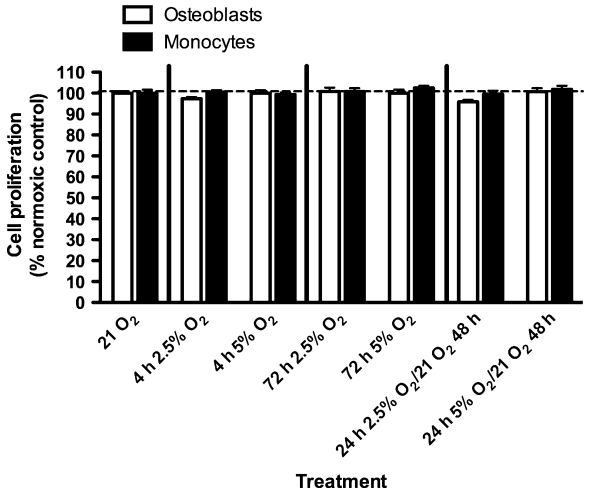
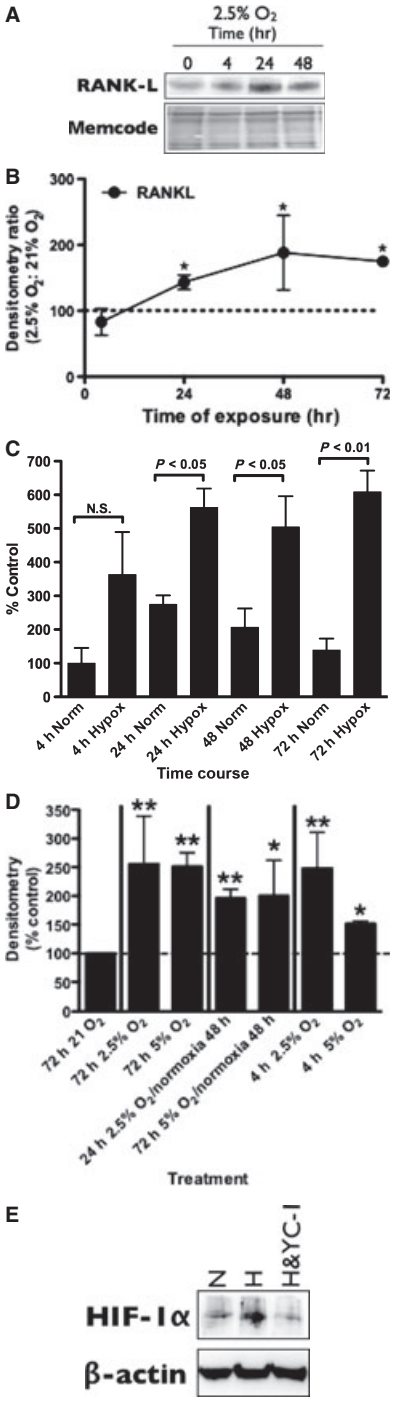


Fig. 2. CFDA-AM cell proliferation assay for osteoblast (OB) and PBMNC. The cells were exposed to either 21% O_2 (normoxia) for 72 h, which was used as the baseline for comparison, 2.5 or 5% O_2 for 24 h, 2.5 and 5% O_2 for the first 24 h and then moved to normoxia for the next 48 h for a total of 72 h of exposure. Cells were counted by hand in 24-well plates at the defined times. Data are taken from three separate runs and 2–3 samples per run. Error bars indicate standard deviation.

hypoxia (Fig. 3D). These levels were higher in response to 4 h, 2.5% oxygen compared with 5% oxygen with no difference at 72 h. Based on the higher levels of HIF1α in the 2.5% oxygen group at 4 h, the rest of the experiments were conducted at the 2.5% level. Addition of the HIF inhibitor YC-1 to OB reduced HIF-1α protein levels (Fig. 3E).



PBMNC can differentiate to OC under hypoxic conditions

When PBMNC were cocultured with OB and the coculture exposed to either normoxia or hypoxia, functional OC formed in the hypoxic coculture system only. Presence of OC was shown using TRAP immunofluorescence and confirmed by resorption pits (22–24) on osteologic material (Fig. 4). The addition of the HIF inhibitor YC-1 to OB reduced OC formation in the coculture wells as measured by reduction in resorption pits (Fig. 4, column D), which coincided with reduction in HIF-1α protein levels (Fig. 3D). Functional resorptive assays were preferred over TRAP because a positive TRAP test is not highly specific for OC only but other conditions such as hairy cell leukemia (25, 26), for which the test was initially developed.

Discussion

Considerable debate has gone on over the years as to the origin of OC involved in tooth movement. Some investigators have concluded that OC can only originate from the bone marrow (11). However, these OC could also have derived from PBMNC because numerous investigators have successfully converted, *in vitro*, PBMNC into functional OC (14–16). The PBMNC used in the *in vitro* studies were, in general, from a tissue bank. In the present study, primary PBMNC differentiated into OC under hypoxic stress in the presence of OB as would occur during orthodontically induced hypoxic stress.

During orthodontically induced bone remodeling, the PDL is compressed in the direction of orthodontic tooth movement, which can create an

Fig. 3. Effect of hypoxia on RANK, hypoxia-inducible factor (HIF)-1α and vascular endothelial growth factor (VEGF) protein expression. (A) Western blot against RANKL. (B) Densitometry of Western blot for RANKL. (C) VEGF ELISA. VEGF was significantly up-regulated in the hypoxic compared with the normoxic conditions. (D) Densitometric comparison of Western blot for HIF-1α. At the 4-h exposure, higher levels of HIF were observed in 2.5% compared with 5% O2. (E) Western blot loading for HIF-1α protein. Addition of YC-1 reduced HIF-1α protein levels. $^{*}p < 0.05$, $^{**}p < 0.01$ from three runs with three samples per run. Error bars indicate standard deviation.

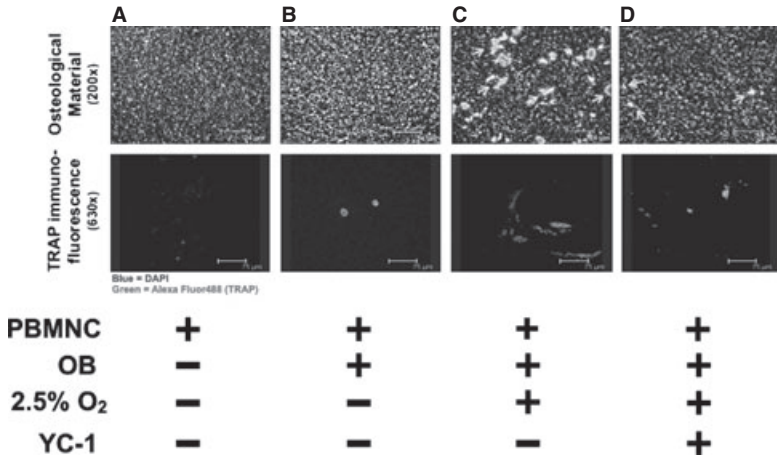


Fig. 4. Resorption and TRAP immunofluorescence assays. (A) Normoxia with PBMNC only without osteoblast (OB). No resorption pits are present and the immunofluorescence is negative. (B) PBMNC/OB coculture at normoxic levels. Both resorption and immunofluorescence were negative. (C) Coculture at 2.5% hypoxia. Note the resorption pits (arrows) and green fluorescence, positive for TRAP. (D) YC-1, the hypoxia-inducible factor (HIF) inhibitor, was added and abrogated the effects of HIF seen in (C) (see Fig 3E).

ischemic (hypoxic) environment. Hypoxia is known to induce production of VEGF in OB (5, 6), a downstream target of HIF, the master regulator of hypoxia (27). We have shown here that both HIF-1α and VEGF are up-regulated in OB when they are subjected to hypoxic stress.

In the present study, RANKL levels were shown to be directly correlated to those of HIF-α and VEGF. RANKL, a soluble growth factor released by OB (3, 4), is required for activation and maturation of OC. OC are dysfunctional or nonfunctional in the absence of RANKL/MCSF (13). The permeable barrier allowed migration of RANKL into the bottom of the well that contained the PBMNC. While it is possible that the RANKL could have been produced endogenously by the PBMNC, RANKL is known to be OB in origin and wells that did not contain OB did not produce OC. Furthermore, few OC were produced when YC-1, a HIF inhibitor (20), was added to the wells.

While orthodontists have relied on compressive forces to move teeth in a desired direction, little is known about the role of hypoxia during tooth movement. It is known that the level of local compression may result in either direct resorption of the alveolar wall or indirect resorption starting from the marrow space, often combined with root resorption. It is demonstrated in this investigation that there is up-regulation of HIF-1α when OB are subjected to hypoxia, which is followed by up-regulation of VEGF, a finding which is consistent with that of other investigators (5–7).

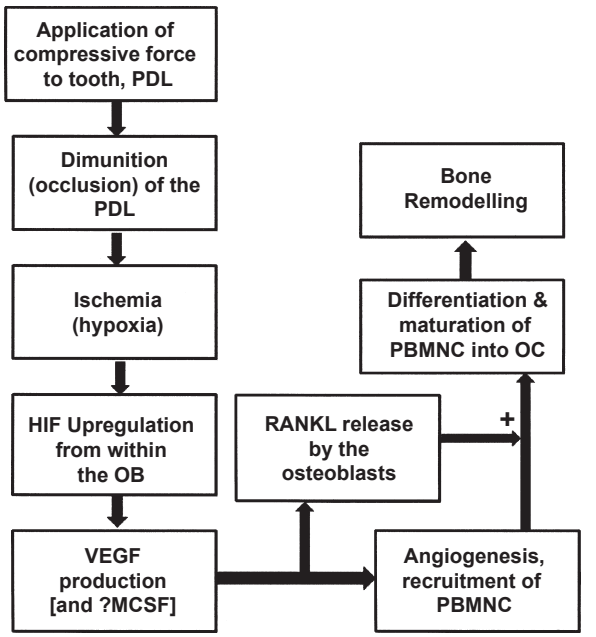


Fig. 5. Hypothetical schema describing the cascade of events during bone remodeling.

Vascular endothelial growth factor seems important in bone remodeling and can expedite tooth movement (9, 10). This experiment shows that HIF is upstream of RANKL in the OC activation/maturation cascade. When PBMNC were cocultured with OB under hypoxic conditions, high levels of VEGF were observed in culture. After 14 days, functionally active OC were detected only in the culture system subjected to hypoxia. The baseline levels of VEGF in the normoxic chambers were maintained and no OC formed in these chambers. Furthermore, YC-1, a HIF inhibitor (20) abrogated OC formation when it was added to the coculture system. The OC that were

detected in this culture system could only have been derived from the PBMNC and been induced by the hypoxia. Two important aspects of osteoclastic development are shown: (1) OC do not exclusively originate from the bone marrow but can differentiate from circulating PMBNC and (2) OC development can be initiated from PBMNC downstream of HIF. While other investigators have shown that VEGF can substitute for M-CSF to support OC development and that the HIF-VEGF system can be generated *in vivo* (28–30), this is the first experiment to show that the system can be self-sustaining without addition of exogenous growth factors.

Markers of OC can be detected as early as 1 week in a culture of PBMNC supplemented with the appropriate growth factors (16). Like arthritic joints, regions subjected to orthodontic tooth movement are heavily infested with PBMNC (31). It is possible that these are the same PBMNC that convert into OC. So far, there is no conclusive evidence to suggest that OC are formed from a coalescence of PBMNC but this cannot be ruled out. Fusion of PBMNC to form OC has been demonstrated *in vitro* (31, 32). It is possible that PBMNC undergo nuclear division but not cytokinesis, thereby giving rise to the OC. Alveolar macrophages have been shown to express fusion regulatory proteins (31), and PBMNC show heavy attachment to the walls of newly formed blood vessels (33). Formation of new blood vessels during tooth movement has been demonstrated (17). Brief compressions (that create hypoxia) can produce tooth movement comparable to that of compression over longer periods of time (34). Once the hypoxia transcription factor HIF-1α is activated, a cascade of events is initiated that results in recruitment and activation of OC. PBMNC are recruited to the region of inflammatory compression, which as well is hypoxic, making the hypoxia-HIF-RANKL system an attractive target to enhance tooth movement.

Conclusion and future studies

This study showed that OB under hypoxic stress can signal conversion of PBMNC to OC and that the conversion of PBMNC into OC was HIF dependant. OC formation was correlated to up-regulation of RANKL downstream of HIF-1α and VEGF. From this, we derived the schematic of events shown in Fig. 5 as what possibly occurs after orthodontic loading of the PDL. We are exploring this schematic to enhance bone remodeling via HIF and VEGF agonists. Future experiments will include chemical and genetic inhibitors of the HIF and NF-κB pathways in our coculture model to functionally link these pathways with VEGF, RANKL, M-CSF and PBMNC differentiation. Stable transfections of OB with the HIF DNA–binding element (hypoxia response element) and canonical NF-κB DNA–binding site linked to a reporter gene will be exposed to different levels of HIF and NF-κB agonists/antagonists to further assess the role of HIF and NF-κB signaling in osteoclastic generation.

Clinical relevance

We hypothesized that OC are formed from PBMNC upon activation of OC growth factors from OB subjected to hypoxia. The relationship between osteoclastic bone remodeling and ischemia is demonstrated. Ischemia, herein hypoxia, initiates a cascade of events that result in recruitment of osteoclasts not from the marrow space, but circulating PBMNC that convert to osteoclasts. OC growth factors that included RANKL and VEGF were produced downstream of the hypoxia master regulator HIF. Manipulation of the relationship between OB and PBMNC can improve our understanding of tooth movement.

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