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Hyperglycemia Induced and Intrinsic Alterations in Type 2 Diabetes-Derived Osteoclast Function

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Abstract

Periodontal disease-associated alveolar bone loss is a co-morbidity of type-2-diabetes, where the roles of osteoclasts are poorly understood.

Objective—To evaluate osteoclast differentiation and function in the context of type-2-diabetes.

Materials/Methods—Bone marrow-derived osteoclasts from db/db mice, a model of type-2-diabetes, as well as human osteoclasts derived from peripheral blood of individuals with type-2-diabetes were evaluated for differentiation, resorption, and soluble mediator expression.

Results—While db/db mice were hyperglycemic at time of cell harvest, human participants were glycemically controlled. Although db/db cultures resulted in a higher number of larger osteoclasts, individual cell RANKL-mediated bone resorption was similar to that observed in diabetes-free OCs. Osteoclasts derived from individuals with type-2-diabetes differentiated similarly to controls with again no difference in bone resorbing capacity. Murine and human type-2-diabetes cultures both displayed inhibition of LPS-induced deactivation and increased pro-osteoclastogenic mediator expression.

Conclusions—Hyperglycemia plays a role in aberrant osteoclast differentiation leading to an increased capacity for bone resorption. Osteoclasts derived from murine models of and individuals with type-2-diabetes are unable to be inhibited by LPS, again leading to increased capacity for bone resorption. Here environmental and intrinsic mechanisms associated with the increased alveolar bone loss observed in periodontal patients with type-2-diabetes are described.

Keywords

Osteoclasts; Type 2 Diabetes; Bone Resorption; Inflammatory Mediators; LPS

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Conflicts of Interest

All authors have no conflicts of interest.

Introduction

Periodontitis is a cumulative inflammatory condition, initiated by bacteria but perpetuated by the host's immune system which leads to the destruction of the soft and hard tissues of the periodontium and, if left untreated, can lead to tooth loss (Kinane, 2001). Susceptibility to periodontitis is enhanced by the interaction between acquired, environmental and genetic factors which modify the host response toward the subgingival biofilm (Page & Kornman, 1997). Many systemic diseases including type-2-diabetes [T2D] modify the progression of periodontal disease contributing to increased prevalence, incidence and/or severity of disease (Bascones-Martinez et al., 2011, Hallmon & Mealey, 1992, Mealey & Rose, 2008, Ryan et al., 2003, Nanci & Bosshardt, 2006). T2D, previously referred to as non-insulin-dependent diabetes or adult-onset diabetes, accounts for 90–95% of diabetes cases, where the development of insulin resistance in target tissues leads to hyperglycemia and if left unchecked can result in the loss of insulin secretion by the islets of Langerhans in the pancreas (Fernandez-Real & Pickup, 2008). Just as T2D is a major risk factor for periodontitis, periodontitis is now classified as the sixth complication of diabetes where the level of glycemic control is an important determinant in the relationship (Papapanou, 1996, Lalla et al., 2006, Loe, 1993).

The periodontium is comprised of gingiva, periodontal ligament, and alveolar bone which work together to anchor the teeth where the alveolar bone lines the tooth socket and serves as an attachment site of the periodontal ligament fibers (Nanci & Bosshardt, 2006). Increased alveolar bone loss is observed in periodontitis patients with T2D when compared to diabetes-free individuals (Nelson, Shlossman 1990). This progressive periodontal bone loss in T2D is related to changes that alter the balance between resorption and deposition phases of bone metabolism (Taubman et al., 2005, Graves, 2008). Traditional bone cells including osteoclasts [OC] and osteoblasts [OB] are responsible for bone resorption and formation, respectively, although immune cells have also been implicated in their regulation (Raggatt & Partridge, 2010). While changes in impaired osseous healing, OB proliferation and function as well as collagen function and deposition have been described in T2D-associated periodontitis, the effect of T2D on OC differentiation and function has not been reported (Mealey & Oates, 2006).

OC differentiation is regulated by macrophage colony-stimulating factor [M-CSF] and the receptor activator of nuclear factor kappa B ligand [RANK-L]. Resorption of bone is initiated by binding of the OCs to the mineralized bone surface via α v β 3 integrins forming a sealing zone membrane and a resorption lacuna (Del Fattore et al., 2008). Vesicles containing osteoclastic enzymes such as tartrate-resistant acid phosphatase [TRAP], the serine protease cathepsin K, and matrix metalloproteinase-9 [MMP-9] induce collagen degradation after bone demineralization through acidification of the lacunae by the vacuolar H⁺-ATPase (Hayman, 2008).

Activation of OC-mediated bone resorption can be augmented by infection and inflammation, as well as hormonal alterations (Takayanagi, 2007). Lipopolysaccharide [LPS], a cell wall component of gram negative bacteria, has been found to be highly immunogenic and induces the production of pro-inflammatory cytokines by various immune cells. OCs and their precursors, which share the same lineage as macrophages and dendritic cells, express many innate immune receptors including toll-like receptors [TLRs] and thus can respond to bacterial components (Dumitrescu et al., 2004, Jiang et al., 2003, Matsuo & Irie, 2008). In a co-culture of osteoclasts and osteoblasts, LPS, the ligand for TLR4, augments bone resorption (Jiang et al., 2003). However, when supporting cells such as osteoblasts or other immune cells are absent, LPS inhibits bone resorption in OC pure cultures, although the exact mechanism(s) is not known (Liu et al., 2009). In addition, pro-

inflammatory cytokines including TNF- α , IL-1 β , and IL-6 stimulate differentiation and activation of OCs with IL-1 β and TNF- α being directly involved in activating resorption (D et al., 2004, Grey et al., 1999). Importantly, inflammatory cytokines and bacterial components are found in abundance within the periodontal lesion where they can directly affect OC differentiation and function thereby shaping the bone remodeling process.

T2D leads to alterations of the function of innate immune cells such as monocytes and macrophages resulting in the production of increased levels of pro-inflammatory cytokines, namely IL-1 β and TNF- α , which are also prolonged in duration (Mealey & Rose, 2008, Salvi et al., 1997a, Salvi et al., 1997b, Naguib et al., 2004, Mealey, 1999b, Mealey, 1999a). In addition, T2D-associated neutrophil defects lead to decreased clearance of microbes, further amplifying the inflammatory process (McMullen et al., 1981, Ayilavarapu et al., 2010) (Karima et al., 2005).

Thus, since a similar heightened inflammatory response is evident in the periodontium of T2D individuals which is exacerbated in poorly-controlled diabetics (Engelbrecht et al., 2004), it is entirely possible that these individuals would be more susceptible to severe alveolar bone loss than T2D-free individuals given the effects of these soluble mediators on OC activation and function. Similarly, given the phylogenetic relationship of OCs to macrophages and dendritic cells and the aberrant TLR-responsiveness of these cell types in T2D, we postulate that T2D-derived OCs have heightened sensitivity to stimulation resulting in augmented differentiation and activation. Therefore in the present study, we characterized T2D-associated OC-specific differentiation, activation and function in the presence and absence of inflammatory stimuli utilizing both a murine model of T2D, namely the db/db mouse model, and human primary osteoclasts derived from T2D individuals.

Materials and Methods

Participant Population

The Institutional Review Board [IRB] for protection of human subjects at the University of Florida approved this protocol in accordance with the World Medical Association Declaration of Helsinki. All data and samples were obtained under informed consent. Participants were recruited from the University of Florida, College of Dentistry from January 2009 until January 2012. Inclusion criteria: aged 13–75 years old, diagnosed with type 2 diabetes [T2D] without diabetic ketoacidosis, currently under the care of a physician or diabetes-free. Exclusion criteria: diabetic complications that would affect the safety or compliance or that could influence the course of either periodontal disease or diabetes care and glucose control; received immunosuppressive, antibiotic, glucocorticoid, or bisphosphonate therapy over the last 6 months; and current tobacco use. A single venous blood sample of 30mL was collected from all participants, where non-fasting blood glucose levels and glycated hemoglobin [HbA1c] were measured with the Ascensia Contour blood glucose meter (Bayer, Tarry Town, NY) and A1CNow meter (Bayer), respectively.

Peripheral Mononuclear Cell [PBMC] Isolation, Osteoclast [OC] Differentiation and Activation

Whole blood samples were collected from T2D (n=20) and diabetes-free participants (n=15) divided equally and diluted 1:1 with balanced working salt solution [1 volume salt solution A:9 volumes salt solution B]. Salt solution A: 1g/L anhydrous D-glucose, 0.0074g/L CaCl₂, 0.1992g/L MgCl₂, 0.4026g/L KCl and 17.565g/L Tris in dH₂O. Salt solution B: 8.19g/L NaCl in dH₂O. Diluted blood was overlaid onto 15mL of Ficoll-Paque (GE Healthcare Life Sciences, Waukesha, WI) and centrifuged for 40min at 1200rpm. The interface layer containing PBMCs was removed and washed 3 times with 6mL of working salt solution.

The resulting PBMC cell pellet was resuspended in α -MEM complete media. and seeded in T25 flasks containing 10mL α -MEM complete media (Sigma-Aldrich, St. Louis, MO) [α -MEM with 10% fetal bovine serum (Mediatech Inc., Manassas, VA), 1% L-glutamine (Thermo Fisher Scientific Inc., Waltham, MA), and 1% penicillin/streptomycin/amphotericin B (Thermo Fisher Scientific Inc.)] supplemented with 10ng/mL recombinant human M-CSF [rhM-CSF] (Peprotech, Rocky Hill, NJ) at 37C and 5% CO₂ for 14d with media refreshed every 3d. Non-adherent cells were removed and 5.9×10^5 cells/mL of adherent cells were seeded in 24-well plates on either glass coverslips (Thermo Fisher Scientific Inc.) or 1 cm² bovine bone slices cut with an Isomet Low Speed Saw (Buehler, Lake Bluff, IL). All cultures were supplemented with 10ng/mL rhM-CSF and 50ng/mL recombinant human soluble RANK-L [rhRANK-L] (Peprotech) and allowed to culture for 12d with complete media refreshed every 3d. After 12d of differentiation, media was refreshed with α -MEM complete media supplemented with 10ng/mL rhM-CSF and 50ng/mL rhRANK-L. Cells were allowed to resorb bone for 72hrs in the presence or absence of 1 μ g/mL *Escherichia coli* LPS [LPS] (Sigma-Aldrich). Cultures were permeablized with 1% Triton X-100 for 10mins. Supernatants were stored at -80°C until cathepsin K ELISA, collagen type I telopeptide ELISA, and cyto/chemokine multi-plex analysis were performed. All outcome measures were normalized to total number of TRAP+ cells.

Mouse Models

All experimental procedures were conducted in accordance with the guidelines of the University of Florida Institutional Animal Care and Use Committee [IACUC] where measures were taken to minimize pain or discomfort. B6.BKS(D)-Lepr^{db}/J [db/db] and C57Bl/6J mice were maintained in a specific pathogen-free environment at the breeding facilities of the University of Florida. Non-fasting blood glucose levels and glycated hemoglobin [HbA1c] were measured with the Ascensia Contour blood glucose meter and A1CNow meter, respectively, at the time of sacrifice.

Bone Marrow Osteoclast [BMOC] Differentiation and Activation

Bone marrow was harvested from 10–12 wk old db/db (n=8) and C57Bl/6 (n=11) mice. Femora and tibiae were surgically isolated, and marrow expelled from bones with α -MEM complete media (Sigma-Aldrich) [α -MEM with 10% fetal bovine serum (Mediatech Inc., 1% L-glutamine (Thermo Fisher Scientific Inc.), and 1% penicillin/streptomycin/amphotericin B (Thermo Fisher Scientific Inc.)]. Cells were seeded in T75 flasks at a concentration of 1.5×10^6 cells/mL supplemented with 5ng/mL recombinant murine M-CSF [rmM-CSF] (Peprotech) and allowed to culture for 24hrs at 37C and 5% CO₂. Non-adherent cells were removed and 5.9×10^5 cells/mL of adherent cells were seeded in 24-well plates on either glass coverslips (Thermo Fisher Scientific) or 1 cm² bovine bone slices cut with an Isomet Low Speed Saw (Buehler). All cultures were supplemented with 10ng/mL rmM-CSF and 50ng/mL recombinant murine soluble RANK-L [rmsRANK-L] (Peprotech) and allowed to culture for 6d with complete media refreshed every 3d. After 6d of differentiation, media was refreshed with α -MEM complete media supplemented with 10ng/mL rmM-CSF and 50ng/mL rmsRANK-L. Cells were allowed to resorb bone for 72hrs in the presence or absence of 1 μ g/mL *Escherichia coli* LPS (Sigma-Aldrich). Cultures were permeablized with 1% Triton X-100 for 10mins. Supernatants were stored at -80°C until cathepsin K ELISA, collagen type I telopeptide ELISA, and cyto/chemokine multi-plex analysis were performed. All outcome measures were normalized to total number of TRAP+ cells.

TRAP Staining

After differentiation, human and murine OCs [hOCs and mOCs, respectively] plated on glass coverslips were fixed with 2% paraformaldehyde/PBS (Thermo Fisher Scientific Inc.). Cells were washed with PBS and permeablized in 0.5% Triton X-100/PBS (Thermo Fisher

Scientific Inc.). Cells were washed and probed for leukocyte acid phosphatase [TRAP [1:1:1:2:4 Fast Garnet GBC Base Solution:Sodium Nitrite Solution:Naphthol AS-BI Phosphate Solution:Tartrate Solution:Acetate Solution] (Sigma-Aldrich) after which cells were washed and mounted on glass slides with MOWIOL 4–88 solution (Calbiochem, San Diego, CA). TRAP positive cells [purple in color] were counted according to number of nuclei present: mononuclear cells [1 nucleus], multinucleated osteoclasts [2–10 nuclei], and giant osteoclasts [11+ nuclei] using light microscopy at 40× magnification. Two whole coverslips per mouse or participant were counted for each cell type.

Collagen Teloepitope ELISA

Collagen carboxy-terminal telopeptides were detected using an ELISA according to manufacturer instructions (Immunodiagnostic Systems, Scottsdale, AZ). Supernatants were pre-incubated with biotin conjugated anti-teloepitope and horseradish-peroxidase [HRP] conjugated anti-teloepitope and added to an ELISA plate coated with streptavidin [SAV]. Following five washes, tetramethylbenzidine [TMB] substrate was used to develop reactivity followed by quenching with H₂SO₄. Colorimetric reactions were detected using an Epoch microplate spectrophotometer (Biotek, Broadview, IL) set at a dual wavelength reading of 450nm with a reference of 655nm. Gen5 Software (Biotek) and a standard curve were used to determine nM concentrations. Assay was performed in duplicate per mouse or participant.

Cathepsin K ELISA

Active cathepsin K was detected using an ELISA according to manufacturer instructions (Alpco Diagnostics, Salem, NH). Supernatants pre-incubated with HRP-conjugated anti-cathepsin K were added to an ELISA plate pre-coated with polyclonal sheep anti-cathepsin K. Following five washes, TMB substrate was used to develop reactivity followed by quenching with STOP solution. Colorimetric reactions were detected using an Epoch microplate spectrophotometer (Biotek) set at a dual wavelength reading of 450nm with a reference of 655nm. Gen5 Software (Biotek) and a standard curve were used to determine pM/L concentrations of active cathepsin K. Assay was performed in duplicate per mouse or participant.

Soluble Mediator Analysis

Cytokines and chemokines from resorption supernatants were detected and quantified using a mouse 22-cyto/chemokine multiplex or a human 14-cyto/chemokine multiplex (Millipore, Billerica, MA) according to the manufacturer's instructions. Supernatant and antibody-coated beads were allowed to incubate overnight at 4C in a 96-well primed plate. Following two washes, reactivity was probed with biotinylated detection antibodies and SAV-phycoerythrin [PE]. All incubations occurred while gently shaking in the dark. Following two washes, beads were resuspended in sheath fluid and reactivity acquired using a Luminex 200 IS system with Xponent software (Millipore). Milliplex analyst software (Viagene, Beverly Hills, CA), 5-parameter logistics and a standard curve were used to determine pg/ml concentrations. Assay was performed in duplicate per mouse or participant.

Statistical Analysis

One-way ANOVA with Bonferroni's multiple comparisons were used to analyze and determine statistical significance (p<0.05).

Results

Hyperglycemia enhances differentiation of T2D-derived OCs

Db/db mice on the C57Bl/6 genetic background provide an animal model of obese, insulin-resistant type 2 diabetes (Coleman, 1982, Leibel et al., 1997) whereby plasma insulin begins to rise around 10 to 14 days of age resulting in obvious obesity around 3 to 4 weeks after which hyperglycemia sets in around four to eight weeks of age. Osteoclasts [mOCs] were derived from the bone marrow of 10–12 week old db/db and C57Bl/6 mice and the differentiation potential evaluated. As expected, at the time of bone marrow harvest, db/db mice had significantly higher non-fasting glucose and %HbA1c compared to C57Bl/6 control mice (Fig. 1A, B). TRAP staining was used to determine the number of multinucleated osteoclasts defined as having 2–10 nuclei and giant cells containing 11 nuclei (Fig. 1C). Here following long term and severe hyperglycemia, a significantly higher number of multi-nucleated and giant mOCs in db/db-derived cultures were observed compared to C57Bl/6 controls (Fig. 1D, E).

In order to determine if similar phenomenon occurred in human disease, osteoclasts [hOCs] were derived from peripheral blood monocytes of individuals with and without T2D. Although the T2D cohort presented with a statistically higher non-fasting blood glucose and %HbA1c, when compared to the diabetes-free cohort (Fig. 2A, B), the levels of non-fasting glucose and %HbA1c present within the T2D cohort indicate that the cohort was under glycemic control as described by the American Diabetes Association (Aguilar, 2011, Defronzo, 2009). Again, TRAP staining was used to determine the number of multinucleated osteoclasts (Fig. 2C). Here in the absence of long term hyperglycemia, similar numbers of multi-nucleated and giant hOCs were observed in T2D-derived cultures compared to those derived from diabetes-free individuals (Fig 2D, E).

Enhanced Differentiation Results in Exacerbated Bone Resorption

While the differentiation data suggests that hyperglycemia enhances OC differentiation resulting in a greater number of large OC, these assays do not address the bone resorbing function of these cells. In order to determine the bone resorbing capabilities of T2D-derived osteoclasts, mOCs and hOCs were seeded onto bovine bone slices and stimulated with RANK-L to promote bone resorption. During bone resorption, after demineralization by the H⁺-ATPase, serine proteases such as cathepsin K in the resorption lacunae degrade the organic portion of bone containing type I collagen, where vesicles containing collagen telopeptides are transcytosed out of the osteoclast and released into the extracellular milieu (Bar-Shavit, 2007). Therefore, in order to quantify bone resorption, levels of intra- and extra-cellular cathepsin K and collagen telopeptides were evaluated (Fig. 3). mOCs derived from db/db mice produced significantly more cathepsin K (Fig. 3A; black bars) and resorbed significantly more bone (Fig. 3B, black bars) than control mice (Fig. 3A, B; white bars). Due to the increased numbers of multi-nucleated and giant mOCs in db/db cultures, it would be expected for these cultures to have both increased cathepsin K and bone resorption capabilities. Thus cathepsin K expression and collagen degradation was normalized on a per cell basis where the bone resorption was now similar between db/db and C57Bl/6 mOCs (Fig. 3C, D), suggesting that the increased resorption observed in mOC cultures was simply due to increased numbers of multi-nucleated and giant mOCs in the culture (Fig. 2), rather than enhanced function due to the metabolic disease. Similarly, no significant differences in cathepsin K expression (Fig. 3E; black bars) nor bone resorption (Fig. 3F; black bars) was observed in hOC derived cultures compared to hOC diabetes free controls (Fig. 3E, F; white bars).

T2D-derived Osteoclasts Respond Aberrantly to LPS

The inflammatory pathology of periodontal diseases includes a large bacterial burden, where activation of bone resorption can be induced by bacterial components. Bacterial components such as LPS act on osteoblasts and activated T-cells to produce more RANK-L to stimulate osteoclasts to differentiate and resorb bone (Takahashi et al., 1994, Teng, 2006). However, LPS can also act on osteoclasts directly by inhibiting resorption by mature osteoclasts (Liu et al., 2009, Takami et al., 2002). Thus the affect of LPS on T2D-derived osteoclast bone resorbing capacity was evaluated, whereby mOCs and hOCs were seeded onto bovine bone slices and stimulated to resorb bone with RANK-L in the presence of LPS. LPS-induced deactivation of OC function was observed in C57Bl/6 mOCs and diabetes-free hOC-derived cultures indicated by a decrease in the cathepsin K secretion (Fig. 3C, E; white bars) and collagen degradation (Fig. 3D, F; white bars). However, this LPS-induced deactivation did not occur in the db/db-derived mOCs cultures (Fig. 3C, D; black bars) nor the T2D-derived hOC cultures (Fig. 3E, F; black bars). Interestingly, while db/db-derived mOCs also displayed an increase in collagen degradation secretion in the presence of LPS compared to RANK-L stimulation alone (Fig. 3D; black bars), T2D-derived hOCs did not (Fig. 3F; black bars).

Elevated Pro-Osteoclastic Milieu in T2D-derived OC Cultures is Augmented by LPS

Because osteoclast function is regulated by many soluble immune mediators, the cytokine and chemokine profile within mOC and hOC cultures were evaluated. Following RANK-L stimulation, db/db mOC-derived cultures had elevated levels of the chemokines IL-6, IP-10, MIP-1 and TNF- as well as the anti-osteoclastic mediator IL-10 when compared to C57Bl/6 mOC-derived cultures (Fig. 4). Similarly, T2D hOC-derived cultures had elevated levels of IL-6, MIP-1 and TNF- , but not IP-10 nor the anti-osteoclastic mediator IL-10 when compared to those derived from the diabetes-free cohort (Fig. 5).

LPS stimulation of OC cultures resulted in an upregulation of all cyto/chemokines measured in all mOC and hOC cultures. Significantly higher levels of MCP-1, IP-10 and MIP-1 were observed in T2D-derived mOC and hOC cultures (Fig. 4, 5), while hOC cultures also had significantly higher levels of IL-6 and TNF- (Fig. 5). Interestingly, both T2D-derived mOC and hOC cultures presented with a significantly lower amount of the anti-osteoclastic cytokine IL-10 (Fig. 4C, 5C).

Discussion

Individuals with T2D have a greater risk of developing periodontal disease, where the degree of hyperglycemia is a confounding factor (Papapanou, 1996, Lalla et al., 2006, Loe, 1993). Specifically, gingival inflammation as well as soft and hard periodontal tissue destruction are more severe in individuals with T2D, where the risk of progressive alveolar bone loss increases over time although the exact mechanism is unclear (Bascones-Martinez et al., 2011, Mealey & Rose, 2008, Tsai et al., 2002, Sandberg et al., 2000, Ryan et al., 2003, Pacios et al., 2011). Importantly, other bone metabolism pathologies are prevalent in individuals with T2D, including osteopenia and delayed fracture healing (Blakytyn et al., 2011).

Previous studies have demonstrated both defective bone formation and osteoblast maturation under hyperglycemic conditions in murine and rat models of T2D. Specifically, Graves and colleagues have demonstrated increased osteoblast apoptosis and less osteoid formation in the Goto-Kakizaki rat model of periodontal disease in T2D (Pacios et al., 2011). This result was also observed by Liu and colleagues in the Zucker diabetic fatty rat, where by bone formation did not occur after resolution of inflammation as was observed in T2D-free

controls (Liu et al., 2006). Hyperglycemia also affects osteoblast differentiation and mineralization capabilities whereby the production of osteocalcin and calcium deposition are known to be decreased in the presence of high blood glucose levels (Blakytyn et al., 2011), thus shutting mesenchymal stem cells (MSCs), which serve as precursors for osteoblasts, towards the adipocyte lineage leading to adipose tissue formation in bone marrow (Botolin & McCabe, 2007).

Conversely, the role of osteoclasts in T2D-associated bone loss are less defined, although the literature describing the direct effect of hyperglycemia on osteoclast differentiation and function vary with some models suggesting high glucose leads to increased function (Larsen et al., 2005, Larsen et al., 2002, Williams et al., 1997) while others suggest decreased resorptive capabilities under hyperglycemic conditions (Wittrant et al., 2008, Liu et al., 2006, Suzuki et al., 2005, Mabileau et al., 2008, Tamasi et al., 2003, Gough et al., 1997, Baumhauer et al., 2006). For instance, bone mineral density was found to be decreased in individuals with uncontrolled T2D compared to T2D-free controls, whereby urinary levels of collagen degradation products from bone resorption such as carboxy-terminal collagen crosslinks (CTx), deoxypyridinoline (DPD), and type 1 cross-linked N-telopeptide (NTx) as well as serum TRAP levels were found to be significantly elevated (Suzuki et al., 2005). In addition, augmented osteoclastogenesis and resorptive capabilities from peripheral blood mononuclear cells were found in a subset of individuals with diabetes suffering from Charcot's osteoarthopathy, a rare osteolytic complication characterized by neuropathy and bone degradation in the foot (Mabileau et al., 2008).

Type 2 diabetes is accompanied by an increased inflammatory state which can have direct and indirect effects on osteoclast survival and function (Motyl & McCabe, 2009). Specifically, elevated levels of pro-inflammatory mediators such as TNF α , are pro-osteoclastic whereby osteoclast survival is promoted (Hotamisligil et al., 1993). In addition, elevated levels of the inflammatory cytokines TNF α , IL6 and IL1 result in insulin resistance ultimately leading to hyperglycemia (Roszer), where experimentally, lack of insulin signaling *in vivo* results in reduced bone formation along with reduced bone resorption (Roszer). Although this observed decrease in osteoclast activity is most likely attributable to changes in osteoblast function whereby increased osteoprotegerin (OPG) secretion results in decreased osteoclast expression of *Cathepsin K (Ctsk)* and *Tcirl1*, which are both required for bone resorption (Ng). While osteoclasts do express an insulin receptor the effect of insulin resistance on their phenotype and function were not addressed in this study. The role of insulin signaling on both osteoclast and osteoblast function are currently being investigated in our laboratory.

Hyperglycemia can have both immediate and long term effects on osteoclast biology. For instance, glucose is the primary energy source of osteoclasts which can increase the expression of the V-ATPase responsible for acidification of the resorptive lacunae (Larsen et al., 2002). On the other hand, long term hyperglycemia results in the formation of advanced glycated end products (AGES) which not only aggravate inflammation, but also increase osteoclast activity again by augmenting RANK signaling, although the mechanism of action is still unknown (Braun & Schett, 2012). It is important to note that the consequences of chronic inflammation and hyperglycemia, including insulin resistance and AGE production, remain long after glycemic control is achieved making the uncoupling of their effects on osteoclast biology difficult.

Here we report that in a murine model of T2D, chronic inflammation and long term hyperglycemia enhances the differentiation/fusion of osteoclast precursors resulting in more resorptive cultures. On the other hand, human osteoclasts from participants with chronic inflammation yet well-controlled T2D, differentiated similarly to those derived from

diabetes-free participants. Cell surface receptors such as DC-STAMP are critical for fusion from mononuclear precursors into multinucleated osteoclasts. Thus, it is plausible that the expression of these fusion receptors is altered by hyperglycemia allowing for more efficient fusion (Oursler, 2010). Mechanisms associated with hyperglycemia-induced augmented differentiation are currently being investigated in our laboratory.

While initially, our data suggested a hyper-responsiveness of db/db-derived osteoclasts to RANK-L, when resorption data was normalized to number of multi-nucleated cells, the observed increased resorption could only be attributed to a significant increase in number of larger osteoclasts. In addition, no significant differences in bone resorption was observed in hOC derived cultures when compared to diabetes-free controls. Together these data suggest that the level of chronic inflammation present in these models have little effect on the RANKL responsiveness of T2D-derived OCs.

With that said, both human and murine osteoclasts derived from T2D sources did display altered responses to LPS where deactivation did not occur as was observed in T2D-free cultures. This alteration in function appears to be independent of hyperglycemia considering that hOCs derived from well-controlled hyperglycemic environments also displayed this phenotype. It is important to note that the long term consequences of hyperglycemia such as the formation of AGEs was not addressed in this study and thus cannot be discounted. Aberrant LPS responsiveness in innate immune cells such as monocytes and macrophages, relatives of osteoclasts, have already been described in both type 1 and type 2 diabetes independent of hyperglycemia, resulting in augmented pro-inflammatory cytokine secretion (Nikolajczyk et al., 2011). Together, these data suggest that the level of chronic inflammation present in these models may be sufficient to over-ride LPS induced deactivation of RANKL mediated OC resorption.

Indeed, in conjunction with a lack of inactivation, osteoclasts derived from T2D individuals and db/db mice displayed LPS-induced augmented pro-inflammatory cytokine and chemokine secretion compared to T2D-free controls. Here chemokines such as MCP-1, IP-10, and MIP-1 which recruit osteoclast precursors were elevated (Gillespie, 2007, Knowles & Athanasou, 2009, Takayanagi, 2009). Similarly, the cytokines IL-6 and TNF- α which are innate and adaptive immune cell activators and can aid in osteoclastogenesis were also elevated (Gillespie, 2007, Knowles & Athanasou, 2009, Takayanagi, 2009). On the other hand, the anti-osteoclastic cytokine IL-10 was significantly lower in T2D-derived cultures, suggesting a lack of regulatory function in T2D-derived osteoclasts (Gillespie, 2007, Knowles & Athanasou, 2009, Takayanagi, 2009). Indeed, others have demonstrated that peripheral blood-derived osteoclasts from T2D individuals are less sensitive to the soluble decoy receptor for RANK-L, osteoprotegerin [OPG] than those derived from diabetes-free controls again resulting in increased bone resorption (Mabilleau et al., 2008, Poubelle et al., 2007).

Data presented here describe environmental and intrinsic mechanisms associated with the increased alveolar bone loss observed in periodontal patients with T2D. Specifically, hyperglycemia augmented osteoclast differentiation/fusion resulting in a more resorptive environment. In addition, independent of hyperglycemia, T2D-derived osteoclasts were less responsive to regulation whereby they were refractive to LPS-induced deactivation and secreted lower anti-osteoclastic soluble mediators. Finally, T2D-derived osteoclast cultures were more osteoclastic in response to LPS perpetuating the uncontrolled bone resorption. Thus, one can imagine that the presence LPS during infections such as periodontal disease in a chronically inflamed host would lead to a vicious cycle of uncontrolled osteoclast differentiation and activation that would lead to excessive alveolar bone loss in periodontal patients with T2D.

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Figure 1. Glycemic Indices Associated with Differentiation of mOCs

(A) Blood glucose and (B) HbA1c were measured at time of bone marrow harvest. Red line is indicative of the high limit of normal indices. Non-fasting blood glucose = 250mg/dL; glycated hemoglobin (HbA1c) = 7.5%. (C–E) 6d post-differentiation, bone marrow-derived mOCs were imaged with light microscopy at 10× magnification. (C) Representative images of TRAP positive [purple in color] (i) mononuclear osteoclasts, (ii) multinucleated osteoclasts, and (iii) giant osteoclasts. Number of (D) multinucleated and (E) giant mOCs per coverslip were enumerated using 40× magnification. **p* value < 0.05. Unpaired t-test with Welch's correction.

Figure 2. Glycemic Indices Associated with Differentiation of hOCs

(A) Blood glucose and (B) HbA1c were measured at time of peripheral blood collection. Red line is indicative of the high limit of normal indices. Non-fasting blood glucose = 250mg/dL; glycated hemoglobin (HbA1c) = 7.5%. (C–E) 12d post differentiation, peripheral blood-derived hOCs were imaged with light microscopy at (C) (i) 20× and (ii) 40× magnification. TRAP positive [purple in color] (D) multinucleated and (E) giant hOCs were enumerated using 40× magnification. **p* value < 0.05. Unpaired t-test with Welch's correction. ND = diabetes-free participants, T2D = participants with type 2 diabetes.

Figure 3. Type 2 diabetes-derived osteoclasts are less responsive to LPS-induced deactivation (A–D) mOCs and (E–F) hOCs were stimulated with RANK-L in the (A–F) absence and (C–F) presence of *E. coli* LPS for 72hrs on bovine bone slices. Supernatants collected after 1% Triton X-100 solubilization were evaluated for (A, C, E) cathepsin K and (B, D, F) collagen I telopeptide by ELISA. Data shown as pM and nM respectively. (C, D) mOC collagen and cathesin K expression normalized to number of multinucleated cells. Normalized outcome measure = [raw value × cell number corrective ratio]. Cell number corrective ratio = [lowest average cell number within sample set] / [average cell number for group of interest]. *p

value < 0.05. One-way ANOVA with Bonferroni's multiple comparison correction. ND = diabetes-free participants, T2D = participants with type 2 diabetes.

Figure 4. LPS-induced elevation of pro-inflammatory and pro-osteoclastic soluble mediators in db/db osteoclast cultures

mOCs were stimulated with RANK-L in the presence or absence of *E. coli* LPS for 72hrs on bovine bone slices. (A) IL-6, (B) MCP-1, (C) IL-10, (D) IP-10, (E) MIP-1 α , and (F) TNF- α levels were evaluated in the supernatants and using Milliplex technology. Data shown as pg/mL. *p value < 0.05. One-way ANOVA with Bonferroni's multiple comparison correction.

Figure 5. LPS-induced elevation of pro-inflammatory and pro-osteoclastic soluble mediators in human type 2 diabetes osteoclast cultures

hOCs were stimulated with RANK-L in the presence or absence of *E. coli* LPS for 72hrs on bovine bone slices. (A) IL-6, (B) MCP-1, (C) IL-10, (D) IP-10, (E) MIP-1 α , and (F) TNF- α , levels were evaluated in the supernatants and using Milliplex technology. Data shown as pg/mL. *p value < 0.05. One-way ANOVA with Bonferroni's multiple comparison correction.