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Regulation of Osteoclasts by Osteoblast Lineage Cells Depends on Titanium Implant Surface Properties

Ethan M. Lotz^a, Michael B. Berger^a, Zvi Schwartz^{a,b}, and Barbara D. Boyan^{a,c}

^aDepartment of Biomedical Engineering, School of Engineering, Virginia Commonwealth University, Richmond VA 23284, USA

^bDepartment of Periodontics, University of Texas Health Science Center at San Antonio, San Antonio, Texas 78229, USA

^cWallace H. Coulter Department of Biomedical Engineering, Georgia Institute of Technology, Atlanta, GA 30332, USA

Abstract

Acritical stage during osseointegration of a titanium (Ti) implant is primary bone remodeling, which involves cross talk among osteoclast precursors, osteoclasts, mesenchymal stem cells (MSCs), and osteoblasts. This phase couples the processes of bone formation and resorption. During remodeling, osteoclasts produce factors capable of regulating MSC migration and osteogenesis. Furthermore, they degrade primary bone, creating a foundation with a specific chemistry, stiffness, and morphology for osteoblasts to synthesize and calcify their matrix. MSCs and osteoblasts receiving cues from the implant surface produce factors capable of regulating osteoclasts in order to promote net new bone formation. The purpose of this study was to determine the effects Ti implant surfaces have on bone remodeling. Human MSCs and normal human osteoblasts (NHOs) were cultured separately on 15mm grade 2 smooth PT, hydrophobic-microrough SLA, hydrophilic-microrough Ti (mSLA) (Institut Straumann AG, Basel, Switzerland), or tissue culture polystyrene (TCPS). After 7d, conditioned media from surface cultures were used to treat human osteoclasts for 2d. Activity was measured by fluorescence of released collagen followed by mRNA quantification. This study demonstrates that MSC and NHO cultures are able to suppress osteoclast activity in a surface dependent manner and osteoclast mRNA levels are selectively regulated by surface treatments. The substrate-dependent regulatory effect was mitigated when MSCs were silenced for integrin subunits and when conditioned media were denatured. These results indicate that MSCs and NHOs regulate at least two aspects of remodeling: reduced fusion of new osteoclasts and reduced activity of existing osteoclasts.

Address for Correspondence: Barbara D. Boyan, Ph.D., School of Engineering, Virginia Commonwealth University, 601 West Main Street, Richmond, VA 23284-3068, Phone: 804-828-0190, FAX: 804-828-9866, bboyan@vcu.edu.

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Graphical abstract

Keywords

Titanium; Bone Remodeling; Osteoclast; Osteoblast; Mesenchymal Stem Cells

Introduction

Osseointegration is a complex cascade of biological events responsible for providing a direct structural and functional connection between the surface of a load-bearing implant and living bone. Greater bone apposition to implant materials results in functional stability, reducing the risk of failure while maintaining implant longevity. In addition to various intrinsic factors that enhance or inhibit osseointegration, the implant surface directly contacts the biological tissues and influences the outcome.

Titanium (Ti) and its alloys are choice materials for both dental and orthopaedic implants because of their ability to form a passive oxide layer bestowing high biocompatibility and corrosion resistance [1]. Because of its stability in biological systems, Ti has been used extensively to investigate the process of osseous wound healing and osseointegration. Our previous studies have identified a number of important surface characteristics including microroughness, hydrophilicity, and chemistry that stimulate osteogenesis, resulting in enhanced osseointegration. When grown on Ti surfaces that are microstructured and hydrophilic, mesenchymal stem cells (MSCs) and osteoblasts (OBs) produce factors that create an osteogenic environment by stimulating osteoblastic differentiation in progenitor cells distal to the implant; promoting vasculogenesis; reducing inflammation; and regulating osteoclastic resorption to achieve net new bone formation [2–7].

Much of the research regarding osseointegration revolves around bone-forming OBs and MSCs due in part to the necessity to achieve net new bone formation. Materials that do not promote osteogenesis, such as polymers like polyether-ether-ketone, result in a fibrous connective tissue interface [8]. Although it is clear that implant materials should actively promote bone formation, long-term stability requires solutions that not only result in net new primary bone formation but also provide continued osteogenic signals to maintain healthy bone remodeling. Bone-resorbing osteoclasts (OCs) are present during peri-implant healing, significantly influencing implant outcomes by playing an important role during the initial and late phases of osseointegration [9]. Bone is continuously remodeled through a finely balanced equilibrium of cell mediated extracellular matrix (ECM) degradation and formation. Bone remodeling is a versatile and ubiquitous process providing the mechanism for adaptation to mechanical stress, repair of micro-damage, and replacement of primary bone during osseointegration of implanted materials [10]. Because it can occur in separate areas asynchronously, locally generated regulatory factors and environmental cues ensure

the appropriate balance among OCs, OBs, and their precursors. Although it seems counterintuitive, implant materials for dental and orthopaedic applications should not inhibit OC-mediated bone resorption to promote net new bone formation, but instead promote a healthy communication among these cells to achieve osseointegration and maintain its longevity.

Achieving implant stability is critical for successful osseointegration; however, insertion of a dental or orthopaedic implant changes the nature of bone healing [11]. Immediately after insertion, implant stability depends on the initial mechanical interlock with the surrounding bone. Unless primary fixation is achieved via new bone formation between the implant surface and the existing bone bed, micromotion of the implant and localized inflammation may occur, frequently ending with osteolysis and implant failure. In addition, the trauma of implant placement ruptures blood vessels and releases growth factors and proteins [12, 13], which provide the local environment with a provisional ECM important for blood clot formation [14]. The adsorption of these early proteins is dependent upon the surface topography with superior anchorage occurring on micro-roughened implant surfaces compared to smooth surfaces [15, 16]. Moreover, this protein layer serves as a scaffold for the migration of MSCs to the implant surface [17, 18].

Surface design that facilitates this contact osteogenesis has been shown to control the fundamental processes that drive the osteoblastic differentiation of MSCs [19, 20]. A cell-rich immature bone (woven bone) is seen in the provisional connective tissue that surrounds newly formed blood vessels [21]. Woven bone forms in direct contact with the surface of the Ti implant and continues to form over 2 weeks [22]. Although OCs are present in the peri-implant tissues as early as 1 week following implantation, it is not until after formation of the woven bone that OC activity in relation to osseointegration begins [9]. This marks the beginning of the progressive removal of woven bone, creating space for new lamellar bone formation and replacing passive primary stability with active secondary fixation through biological bonding [23].

Modifications to the implant surface topography, chemistry, and hydrophilicity are employed as a means of controlling bone–implant interactions and shortening the time of bone fixation. Furthermore, it is known that the communication among OCs, MSCs, and OBs is coupled to provide control of bone mass and healing. Commitment of OC precursors to mature OCs is dependent on production of RANKL (receptor activator of the NF κ B ligand) [24] and its physiologic inhibitor OPG (osteoprotegerin) [25] by OBs and MSCs. Implant surface microstructure and hydrophilicity facilitate increased production of OPG by MSCs and OBs, leading to increased bone formation. In this regard, much attention has been given to the anabolic aspects of osseointegration. In contrast, there is little information regarding the OC-mediated catabolic processes associated with bone remodeling. It remains unclear as to whether implant surface topography can affect the process of osteoclastogenesis or OC function. A thorough understanding of healing, repair, modeling, and remodeling of bone is critical for the establishment and maintenance of osseointegration of dental and orthopaedic implants. Given that the process of bone adaptation and maturation are critical features for the long-term success of osseointegration, continued attention to the molecular processes underlying these events may create new biological design criteria for implant surface design.

This insight is critical to optimize an implant surface capable of simultaneously enhancing peri-implant osteogenesis while also positively influencing the later stages of osseointegration. Therefore, the objective of this study was to determine the effects microrough and hydrophilic Ti surfaces have on a novel *in vitro* model of bone remodeling by studying their distal influence on OC formation and activity and the mechanisms responsible for the influence.

2. Materials and Methods

2.1 Ti disk preparation

Grade 2 Ti disks, 15mm in diameter and 1mm in thickness, were provided by Institut Straumann AG (Basel, Switzerland). Disks were degreased in acetone and processed for 30s in a 55°C 2% ammonium fluoride/2% hydrofluoric acid/10% nitric acid solution to produce pretreatment Ti disks (PT). SLA substrates were prepared by subjecting PT substrates to sand blasting (250 – 500µm corundum) and acid etching (HCl/H₂SO₄). Disks were cleaned in HNO₃, rinsed in deionized water, air dried, and packed in aluminum foil. mSLA substrates were produced using the same sandblasting and acid etching procedure as used for SLA, but subsequent steps took place under nitrogen gas to prevent exposure to air. mSLA disks were rinsed and stored in 0.9% NaCl solution. The methods used to characterize the surface microroughness, contact angle, and carbon contamination of the surfaces (PT [$S_A=0.59\mu\text{m}$; $\theta_{CA}=93.6^\circ$; %C=31], SLA [$S_A=3.58\mu\text{m}$; $\theta_{CA}=120.9^\circ$; %C=35], mSLA [$S_A=3.64\mu\text{m}$; $\theta_{CA}=0^\circ$; %C=17]) have been reported previously [26]. All disks were γ -irradiated prior to use.

2.2 OC Cell Culture

Human OC precursors (OCPs; Lonza Biosciences, Walkersville, MD) were cultured on a human type I collagen coated, 96-well OsteoLyse™ Assay Kit (Lonza Biosciences). OCPs were differentiated into mature OCs using osteoclast precursor growth medium (OCGM, Lonza Biosciences) supplemented with 33ng/ml macrophage colony stimulating factor (M-CSF, Lonza Biosciences) and 66ng/ml receptor activator of nuclear factor kappa-B ligand (RANKL, Lonza Biosciences). OCPs were also cultured in OCGM supplemented with 33ng/ml M-CSF without RANKL for use as a negative control.

2.3 Establishment of Osteoclast Phenotype and Treatment Period

In order to establish the OC phenotype and treatment period, OCPs were harvested at 3d, 7d, 9d, and 14d and subjected to the following assays.

2.3.1 OC Activity—OC activity was measured by the release of the europium conjugated human type I collagen coated on the bottom of the OsteoLyse™ Assay Kit at each time point. 200µl of a fluorophore releasing reagent (Lonza Biosciences) was placed in each well of a 96-well black, clear-bottom assay plate (Corning Inc., Corning, NY). 10µl of cell culture supernatant was transferred to each well of the assay plate containing the fluorophore releasing reagent. Fluorescence of each well of the assay plate was measured with an excitation wavelength of 340nm and an emission wavelength of 615nm over a 400µs period after an initial delay of 400µs.

2.3.2 Gene Expression—To quantify mRNA, cells were plated as described above (Section 2.2). At each time point, mRNA was isolated from cells using a TRIzol[®] (Invitrogen, Carlsbad, California) extraction method. RNA was quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and then reverse transcribed into 250 ng/ μ L cDNA. Real-time PCR was performed using a fluorescent dye (Power SYBR Green, Applied Biosystems, Foster City, CA) to quantify starting mRNA levels using gene specific primers (Table 1). Levels of mRNA are normalized to GAPDH.

2.3.3 Scanning Electron Microscopy (SEM) Imaging—OCs were qualitatively evaluated using SEM (Hitachi FE-SEM Su-70, Tokyo, Japan) at each time point. In addition, the collagen coating of the OsteoLyse[™] Assay Kit was assessed before and after OC treatment. For assessment of the collagen coating, OCs were trypsinized for 5min. All samples were then fixed in 4% paraformaldehyde for 15min after three rinses in 1x PBS. Samples were then dehydrated stepwise in increasing ethanol concentrations of 15%, 30%, and 45% for two hours, followed by 60%, 75%, 90% and 100% (x3) for at least 1 hour. The samples were then critically dried using a 1:1 exchange of 100% ethanol and hexamethyldisilazane (HMDS, Sigma-Aldrich, St. Louis, MO) for 30min in a chemical safety hood, then twice with 100% HMDS for 30min. Sample wells were trimmed to allow even sputter coating, then dried in a desiccator for 24hrs prior to platinum sputter coating. Samples were imaged with 56 μ A ion current, 5kV accelerating voltage and 8mm working distance. Representative images were selected from 18 images per well and n=4 per group.

2.3.4 Tartrate-Resistant Acid Phosphatase (TRAP) Staining—OCs were stained for tartrate-resistant acid phosphatase (TRAP) and were analyzed using light microscopy. OC groups (n=4) were prepared based on a modified manufacture's protocol (Sigma-Aldrich). Briefly, OCs were rinsed three times with 1x PBS, fixed in a fixative solution comprising of 25mL citrate solution, 65mL acetone, and 8mL of 37% formaldehyde for 15min. Followed by 3 rinses in deionized water. Staining solution consisting of 45mL of deionized water at 37°C, 1mL diazotized Fast Garnet GBC solution, 0.5mL Naphthol AS-BI Phosphate Solution, 2mL Acetate solution, and 1mL Tartrate solution was prepared and 200 μ L of staining solution was added to each well and allowed to incubate for 1hr at 37°C. Samples were counterstained with 200 μ L hematoxylin solution for 2min and rinsed in deionized water prior to imaging with a Carl Zeiss Primo Vert[™] Inverted Microscope (Zeiss, Oberkochen, Germany). Representative images were selected from 18 images per well and n=4 per group.

2.4 MSC and NHOst Cell Culture

Human MSCs (Lonza Biosciences) were cultured in MSC growth medium (MSCGM; Lonza Biosciences). Normal human osteoblasts (NHOst, Lonza Biosciences) were cultured using Dulbecco's modified Eagle medium (DMEM, CellGro[®]; Mediatech, Inc., VA) supplemented with 10% fetal bovine serum (FBS, Life Technologies, Carlsbad, CA) and 1% penicillin-streptomycin (Life Technologies). MSCs and NHOst were separately cultured on tissue culture polystyrene (TCPS), PT, SLA, or mSLA at a density of 10,000 cells/cm² at 37°C in 5% CO₂ and 100% humidity. Media were changed 24h after plating and every 48h thereafter for 7d. At 7d, cells were incubated with fresh media for 24h, which was used for subsequent

experiments. An illustrative schematic detailing the different sub-studies together with a time line is provided in Supplementary Fig.1.

2.5 Indirect Effects of MSC/NHost Conditioned Media on OCs

MSCs and NHosts were separately cultured as described in section 2.4. OCPS were also cultured using the OsteoLyse™ Assay Kit as described in section 2.2. After culture for each cell type, 200µl media from MSCs or NHosts were transferred to wells of the OsteoLyse™ Assay Kit containing differentiated OCs. In order to control for the initial concentrations of M-CSF and RANKL used to differentiate the OCs, an additional group of differentiated OCs was incubated with 200µl of MSC or NHost media supplemented with 33ng/ml M-CSF and 66ng/ml RANKL. For each experiment, positive (differentiated OCs receiving OCGM supplemented with 33ng/ml M-CSF and 66ng/ml RANKL) and negative (undifferentiated OCPs receiving OCGM supplemented with 33ng/ml M-CSF) controls were used. Differentiated OCs were treated for 2d, followed by quantification of activity (Section 2.3.1) and gene expression (Section 2.3.2).

2.6 Cell Presence and Conditioned Media Effects on OCs

In order to determine whether the observed indirect effects of conditioned media were dependent on the presence of cells, MSCs were cultured on TCPS or mSLA and compared to conditioned media collected from TCPS and mSLA without seeded cells. Both the cellular and acellular TCPS and mSLA were incubated and received media changes in the exact same manner for 7d followed by treatment with MSCGM for 24hr (Section 2.3). After treatment, conditioned media were supplemented with 33ng/mL M-CSF and 66ng/mL RANKL and used to treat differentiated OCS for 2d. Positive and negative controls (Section 2.4) were also used.

2.7 Proteins and Conditioned Media Effects on OCs

Whether proteins were the primary factor for the observed effects of conditioned media on OCs was determined by two methods of denaturation: 1) proteinase K or 2) heat. For proteinase K denaturation, MSCs were cultured on either TCPS or mSLA as described previously (Section 2.3). After treatment with MSCGM, conditioned media were collected and treated with 200µg/ml proteinase K (Sigma-Aldrich) for 1h at 37°C. Proteinase K activity was then neutralized with 5mM Pefabloc® SC (Sigma-Aldrich) for 2h at 37°C. For heat treatment, conditioned media were placed in an 80°C water bath for 2h. Heat treated media were then cooled to room temperature by placing samples on ice for 10 min. Denatured conditioned media were then supplemented with 33ng/mL M-CSF and 66ng/mL RANKL prior to treating differentiated OCs for 2d using positive and negative controls (Section 2.4).

2.8 ITGA2 and ITGB1 Silencing

MISSION® shRNA lentiviral transduction particles (Sigma-Aldrich) were used to silence human MSCs for either ITGA2 (SHCLNV-NM_002203) or ITGB1 (SHCLNV-NM_002211). MSCs were plated at 20,000 cells/cm² and cultured overnight at 37°C in 5% CO₂ and 100% humidity. Particles were added to the cells at a multiplicity of infection of 2

in culture media supplemented with 8µg/ml hexadimethrine bromide for 18h. Transduced cells were selected using MSCGM supplemented with 0.5 µg/ml of puromycin. Silencing of ITGB1 and ITGA2 were confirmed using real-time qPCR and maintained over the period of culture (Supplementary Fig.2). A 70% reduction in mRNA levels compared to the wild-type control was considered the minimum acceptable decrease.

2.9 Effects of ITGA2 and ITGB1 Silencing on Indirect Effects of MSC Conditioned Media

To determine if signaling via the $\alpha 2$ or $\beta 1$ integrin subunit was required for the indirect effects of conditioned media on OCs, wild type (WT) and silenced MSCs were cultured on TCPS or mSLA for 7d in MSCGM followed by a 24hr treatment with MSCGM. Conditioned media were supplemented with 33ng/ml M-CSF and 66ng/mL RANKL and used treat differentiated OCS for 2d. Positive and negative controls (Section 2.4) were also used.

2.10 Paracrine Effects of OPG and TGF β 1 in Conditioned Media

The contribution of osteoprotegerin (OPG) and transforming growth factor β 1 (TGF β 1), two known regulators of OC activity and function, to the indirect effects of conditioned media on OCs were also evaluated. Conditioned media were collected from cultures of MSCs on TCPS or mSLA as previously described (Section 2.3). Conditioned media were supplemented with either 2µg/ml or 0.5µg/ml anti-TGF β 1 type II receptor antibody (R&D Systems, Minneapolis, MN); 1µg/ml or 0.1µg/ml soluble type II receptor (R&D Systems); or 2µg/ml or 0.5µg/ml anti-OPG antibody (R&D Systems, Minneapolis, MN). All conditioned media were then supplemented with 33ng/ml M-CSF and 66ng/mL RANKL and used treat differentiated OCs for 2d. Positive controls (Section 2.4) supplemented with each treatment were also used.

2.11 Statistical Analysis

Data from surface characterization experiments and *in vitro* assays are presented as the mean $\pm$ standard error (SE) per variable (n=6). A one-way analysis of variance was performed with a two-tailed Tukey correction and $\alpha = 0.05$. All statistical analyses were performed using JMP statistical software.

Results

Initial studies established the OC phenotype and treatment period (Figure 1). Activity of OCs is shown as differentiated OCs (OCPs receiving M-CSF and RANKL) per undifferentiated OCs (OCPs receiving M-CSF) within the same time point. A significant increase in OC activity was observed at each time point (Fig.1A). Gene expression data are shown by indicating the source of the media (OCGM) and the presence (+) or absence (–) of RANKL. No difference in OCP CA2 mRNA was detected at 3d, 7d, and 9d (Fig.1B). At 14d, CA2 mRNA increased to levels comparable to 3d OC cultures. CA2 mRNA further increased in OCs 7d post-culture then decreased at 14d. Similar CTSK expression was observed among OCPs and 3d OC cultures (Fig.1C). CTSK expression in 7d OCs was highest and subsequently decreased at 9d and 14d. OCP expression of CSTB (Fig.1D), ITGAV (Fig.1E), ITGB3 (Fig.1F), and OCSTAMP (Fig.1G) was lower compared to OCs.

CSTB and ITGAV expression increased to similar levels on 7d, 9d, and 14d OCP cultures compared to 3d. No differences were detected in OCP ITGB3 and OCSTAMP expression. For OCs, similar levels of CSTB, ITGAV, and ITGB3 mRNA were detected at 3d and 14d cultures; however, CSTB was highest in 9d OC cultures while ITGAV and ITGB3 were highest in 7d OC cultures. Peak OCSTAMP expression was observed in 3d OC cultures. OCSTAMP expression declined at 7d and further decreased to similar levels at 9d and 14d.

Qualitative assessment of the OC phenotype at each time point is shown in Figure 2. As early as three days, RANKL supplemented OCPs began to aggregate and fuse into multinucleated OCs as indicated by TRAP stain. At 7d, increased TRAP stain multi-nucleation as well as ruffled border formation in SEM images was seen. This phenotype continued to develop through 9d. The 9d RANKL negative cultures had larger cells attributed to the actions of M-CSF; however, they lacked the magnitude of surface area, TRAP stain intensity, and multi-nucleation compared to OCs. Interestingly, TRAP stain intensity and multi-nucleated cell density decreased in the 14d RANKL positive cells while both increased in the 14d RANKL negative cells suggesting that the phenotypic integrity of OCPs and OCs was lost by 14d. SEM images of the collagen coating before and after OC culture are shown in Supplementary Fig. 3. Although the collagen coating is slightly altered by trypsin, differentiated OCs degrade much of the collagen coating by 9d of culture and only a few fragments of protein are left by 14d. Figure 3 shows the indirect effects microstructured Ti substrates have on OC activity via MSCs (Fig.3A,B) and NHOs (Fig. 3C,D) after treatment for 2d. The sources of the treatment media (OCGM or MSCs/NHOs cultured on TCPS or PT, SLA, or mSLA disks) are shown below each group. Furthermore, indications of whether treatment media were supplemented with (+) or without (–) RANKL are shown below each group. For each cell type, the OC activity was highest in the positive control and lowest in the negative control. Compared to the positive control, OC activity was reduced following treatment with media collected from cultures of MSCs (Fig.3A) and NHOs (Fig.3C) grown on TCPS and microstructured Ti. Similar effects were seen when media collected from MSCs and NHOs were supplemented with RANKL and M-CSF (Fig.3B,D respectively). OC activity was reduced to comparable levels in groups treated with media collected from MSCs and NHOs cultured on TCPS and PT with or without RANKL and M-CSF supplementation. A further reduction in OC activity was observed after treatment with media from SLA cultured MSCs followed by mSLA cultured MSCs. Supplementing MSC treatment media with RANKL and M-CSF abrogated this effect. OCs treated with media collected from SLA and mSLA cultured NHOs were not sensitive to the effects of RANKL and M-CSF supplementation observed with MSC treatment.

Data regarding gene expression following treatment with MSC and NHO conditioned media (CM) supplemented with M-CSF and RANKL are shown in Figure 4. For all genes, expression was higher in the positive control compared to the negative control. MSC-CM treatment reduced OC CA2 (Fig.4A) and CSTB (Fig.4B) expression compared to the positive control. CA2 expression further decreased when OCs were treated with SLA and mSLA MSC-CM. CSTB expression decreased after MSC-CM treatment and continued to decrease in a surface dependent manner with no differences detected between SLA and mSLA MSC-CM. OCSTAMP (Fig.4C) was reduced to similar levels for all MSC-CM treatments. CTSK (Fig.4D) mRNA levels decreased when treated with mSLA MSC-CM.

Although not different from the positive control, OC CTSK expression decreased when treated with SLA MSC-CM compared to TCPS and PT MSC-CM. No differences in OC ITGAV (Fig.4E) expression were detected between the positive control and OCs treated with SLA and mSLA MSC-CM; however, OCs treated with TCPS and PT MSC-CM had significantly higher ITGAV expression compared to the positive control and mSLA MSC-CM. OC ITGB3 expression (Fig.4F) increased compared to the positive control following treatment.

Treatment with NHOst-CM reduced CA2 (Fig.4G) expression to similar levels. OC CSTB (Fig.4H) mRNA levels decreased when treated with TCPS and PT NHOst-CM and further decreased when treated with SLA and mSLA NHOst-CM. OCSTAMP (Fig.4I) mRNA levels were reduced in OCs to similar levels; however, OCs treated with TCPS NHOst-CM had significantly higher OCSTAMP mRNA levels compared to those treated with mSLA NHOst-CM. CTSK (Fig.4J) expression was reduced in OCs treated with TCPS, PT, and SLA NHOst-CM and further reduced in OCs treated with mSLA NHOst-CM. No differences in ITGAV (Fig.4K) expression were detected in OCs treated with NHOst-CM compared to the positive control. However, mSLA NHOst-CM reduced ITGAV expression compared to other treatments. ITGB3 (Fig.4L) mRNA levels were highest in OCs treated with TCPS and PT NHOst-CM. ITGB3 mRNA remained unchanged compared to the positive control in OCs treated with SLA and mSLA NHOst-CM. Similar effects were observed when CM was not supplemented with RANKL and M-CSF prior to treatment (Supplementary Fig.4).

For experiments to determine the mechanisms responsible for the indirect regulation of OCs, a simplified model was used that included only MSCs cultured on either TCPS or mSLA. Figure 5A demonstrates that the observed inhibitory effect is dependent on the presence of cells conditioning the media prior to treatment, although there is an equal inhibitory effect on OCs due to treatment with acellular MSCGM. ITGA2 silenced MSCs (Fig.5B) and ITGB1 silenced MSCs (Fig.5C) decreased OC activity compared to the positive control. shITGA2 MSCs cultured on TCPS decreased OC activity compared to WT. The decrease in OC activity by mSLA MSC-CM was attenuated by silencing ITGA2. Similar results were observed for shITGB1 MSCs; however, no difference in OC activity was detected between TCPS WT MSC-CM and TCPS shITB1 MSC-CM. In order to determine whether the inhibitory effect was due to proteins, two methods of protein denaturation were employed. Treatments subjected to proteinase K digestion (Fig.5D) decreased OC activity to levels comparable to the negative control regardless of the initial surface. Interestingly, heat denaturation of proteins (Fig.5E) increased OC activity compared to the intact protein treatments, although the activity was significantly lower than the positive control.

The addition of an antibody against TGF β type 2 receptor (Fig.6A) or soluble TGF β type 2 receptor (Fig.6B) decreased OC activity when added to TCPS MSC-CM to levels similar to mSLA MSC-CM. OC activity was unchanged when these factors were added to mSLA MSC-CM. Furthermore, no effects on OC activity were detected when an antibody against osteoprotegerin was added to either TCPS or mSLA MSC-CM, although the activity was significantly lower in mSLA MSC-CM compared to TCPS MSC-CM (Fig.6C). In all instances, addition of either antibody or soluble protein had no effect on the positive control.

Discussion

Implant design that promotes both early bone formation while maintaining healthy cellular signaling throughout the remaining stages of osseointegration will achieve long-term implant stability. It is now understood that surface properties modulate immune cell function and factors produced by macrophages influence osteogenesis [26, 27]. Others have shown that osteocytes make direct contact with the implant surface, suggesting their importance in maintaining healthy bone remodeling as well as the longevity of implant osseointegration [28, 29]. However, no studies have sought to investigate how implant surface properties modulate MSC and NHOst communication with OCs, an integral part of the bone remodeling phase of osseointegration. The results of this study demonstrate that implant surface properties can impact downstream events during the process of osseointegration. A novel *in vitro* model of bone remodeling was developed and used to assess the influence commercially available Ti implant surfaces have on osteoclastogenesis and OC activity. CM collected from MSCs and NHOsts cultured on smooth, microrough, and/or hydrophilic Ti surfaces decreased OC activity with NHOst cultures having a more robust effect. Gene expression was selectively regulated rather than completely shut down suggesting that the OCs have the capacity to return once the regulatory stimulus favors bone resorption.

The OsteoLyse™ assay kit is a cell culture plate coated with europium-conjugated human type I collagen used in experiments of OC differentiation and activity [30–32]. OC resorptive activity is directly proportional to the release of Eu-labeled collagen fragments. Although type I collagen is the most abundant protein in bone, the assay lacks the complexity of native bone, including the presence of bone mineral. Furthermore, ITGAV and ITGB3 complex binding can occur on multiple bone matrix proteins not present in the assay substrate. Shifting to bone or dentine may alter the expression patterns observed in the present study and may be necessary to confirm our results. Lastly, the assay does not allow cell culture beyond 14d as indicated by the phenotypic changes in both the OCPs and OCs. This could be due to the complete resorption of the collagen coating leading to cell death or foreign body giant cell (FBGC) fusion. Because OCs and OCPs are from the hematopoietic lineage [33], they are sensitive to many chemokines including interleukin 4 (IL-4). IL-4 is the driving factor for FBGC fusion and is produced by OCs and OCPs [34, 35]. As a result, the chosen period of our model permits only 9d of total OC culture.

Despite these limitations, our model proved to be a practical *in vitro* tool for studying bone remodeling. Our results confirm countless observations that MSCs and NHOsts release factors capable of limiting the degree and extent to which OCs resorb bone. However, for the first time we have shown that the attenuating effect is enhanced when cultured on microstructured and/or hydrophilic Ti surfaces. CM were supplemented with RANKL and M-CSF to ensure that their abrupt removal was not the cause of the decreased OC activity. The observed effect went largely unchanged, although differences were detected between SLA and mSLA MSC-CM without supplementation. Despite this observation, our results suggest that surface microroughness may play a larger role in mitigating OCs than hydrophilicity. Furthermore, these observed effects are not due to the influence Ti surfaces have on cell attachment, proliferation, or death. Previously we have shown that cell proliferation is reduced on Ti surfaces compared to TCPS but osteogenic differentiation is

increased [3, 4, 36]. Although, SLA and mSLA have the fewest number of cells attached, CM from these surface cultures resulted in the greatest decrease in OC activity.

Differential gene expression in osteoclasts following treatment with CM suggests that pathways in OCs are selectively targeted. The fusion marker, OCSTAMP, was markedly reduced in OCs following treatment with CM from both cell types suggesting that a portion of the regulatory influence acts to prevent OC fusion. In order to initiate resorption, mature OCs attach to the mineralized bone matrix, forming a sealing zone. Their initial attachment and subsequent activation is mediated by integrins, specifically, the vitronectin receptor, $\alpha V\beta 3$ [37–39]. Attachment of $\alpha V\beta 3$ induces signaling mechanisms that control cytoskeletal reorganization, which is important for the spreading, motility, and sealing zone organization [40–42]. Interestingly, expression of either subunit did not decrease, suggesting that this signaling complex maintains its activity after treatment with MSC or NHOst CM. Following formation of the sealing zone by $\alpha V\beta 3$ signaling, the resorption lacuna beneath the OC forms where its membrane is folded forming the ruffled border. The OC is able to release hydrogen ions through the ruffled border via activity of carbonic anhydrase II (CA2), thereby reducing the pH and dissolving the mineralized portion of the ECM. In humans, CA2 deficiency causes non-functional osteoclasts and osteopetrosis [43]. Its expression is induced through RANKL dependent pathways so its reduction would reduce the ability of the osteoclast to maintain optimal pH gradients, thus decreasing activity [44].

In addition to regulating pH in the resorption lacunae, osteoclasts secrete enzymes like lysosomal cysteine proteinases and matrix metalloproteinases are then secreted to degrade the organic matrix [45, 46]. Cathepsin K (CTSK) is one of the main cysteine proteinases secreted into the resorption lacuna [45, 47]. CTSK has been shown to degrade insoluble type I collagen, and inhibition of its enzymatic activity *in vitro* and *in vivo* models prevents matrix degradation [48, 49]. Osteoclasts are also rich in cystatin B (CSTB), a natural inhibitor of cysteine proteinases. A balance between CTSK and CSTB is important for regulating cell survival as addition of CSTB to osteoclasts protects them from experimentally induced apoptosis [50]. The decreased CTSK and CSTB accompanying CM treatment suggests that organic matrix degradation is subsiding and OCs may be attempting to regulate their survival. Interestingly, NHOsts had a more robust effect on CTSK expression compared to MSCs. In general, NHOsts produce higher quantities of regulatory factors compared to MSCs, suggesting that regulation may be dependent on the cell type.

The potential regulatory difference between cell types was the impetus for choosing MSCs to study mechanisms mediating OCs. In addition, we focused on the modulating effects of mSLA. Microroughness was the more important surface feature in terms of reducing OC activity, but our previous studies indicated that mSLA is superior at facilitating MSC differentiation [2, 4, 20, 36, 51, 52]. MSCs are attachment dependent and interact with their underlying substrate via integrins such as $\alpha 2\beta 1$. Silencing of both the ITGA2 and ITGB1 subunits in MSCs attenuated the inhibitory effect on OCs. Interestingly, TCPS shITGA2 MSC-CM decreased OC activity suggesting a unique signaling through the $\alpha 2$ integrin subunit.

Proteins produced by MSCs cultured on Ti surfaces are crucial to the observed phenomena in the present study. Although there is an inhibitory effect on OCs due to factors present in the basal growth media, MSC-CM decreased OC activity, which further declined with mSLA MSC-CM treatment. Heat denaturation of the CM mitigated the inhibition of OC activity. Interestingly, proteinase K digestion inhibited OC activity more. Proteinase K cleaves peptide bonds adjacent to the carboxylic group of aliphatic and aromatic amino acids [53]. If proteins have complicated secondary, tertiary, and/or quaternary structures, proteinase K may be unable to access certain amino acids preventing their complete degradation. This could lead to selective degradation and exacerbate the effect if antagonistic proteins are denatured over agonistic proteins. Moreover, susceptible proteins could be digested in a way that leaves the residual fragments active. Alternatively, addition of proteinase K and its neutralizer may have diluted the CM preventing OCs from receiving sufficient nutrients to stay healthy.

There is a myriad of signals capable of regulating OCs. One mechanism by which MSCs control OCs is mediated by TGF β 1-dependent regulation of OPG production [7]. TGF β 1 enhances the proliferation of MSCs and osteoblasts and stimulates ECM protein production like type I collagen. Latent TGF β 1 and latent TGF β binding protein are synthesized and stored in the ECM. Most of the TGF β 1 produced by OBs cultured on Ti is in latent form [54], and the amount incorporated into the matrix is increased on rougher surfaces. Once the bone remodeling phase of osseointegration begins, OCs degrade the newly synthesized matrix releasing the stored, latent TGF β 1 and convert it into active TGF β 1. Once activated, TGF β 1 regulates OCs, in part, by regulating production of OPG [7]. OPG serves as a decoy receptor for RANKL, which prevents RANKL-RANK binding on the OCP surface inhibiting OC differentiation [55]. A feedback mechanism is in place that allows osteoblasts to produce soluble RANKL in order to deplete excess OPG if the regulatory stimulus favors new osteoclast formation. Microrough Ti substrates facilitate increased production of OPG by osteoblasts; however, levels of RANKL do not change. Thus, the net effect is bone formation without bone resorption. Soluble TGF β type 2 receptor and anti-TGF β type 2 receptor antibody will compete with endogenous TGF β . Addition of these factors to TCPS MSC-CM but not mSLA MSC-CM decreased OC activity, suggesting that TGF β signaling is an important regulatory protein produced by MSCs cultured on TCPS. However, TGF β was not a contributing factor to the regulation of OCs by surfaces. This could be due to the overwhelming presence of other factors. Furthermore, the addition of anti-OPG antibody to both the TCPS and mSLA MSC-CM did not mitigate the decreased OC activity confirming the importance of OPG as a regulator of OC fusion rather than a regulator of OC activity.

Conclusions

We have shown that microstructured Ti surfaces indirectly regulate OC activity and gene expression. After surface recognition by integrins, MSC and NHOst protein production is modulated and responsible for the decreased activity of existing OCs and reduced fusion of new OCs. In addition, OC gene expression is selectively regulate suggesting more than one pathway of control may be involved. Of the proteins responsible, TGF β and OPG do not contribute to the surface dependent regulation *in vitro*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Statement of Significance

In this study, we developed a novel *in vitro* model to study how microstructured and hydrophilic titanium implants impact bone remodeling for dental and orthopaedic applications. Our approach intersects biomaterials and systems physiology, revealing for the first time that implant surface properties are capable of regulating the communication among the cells involved in remodeling of primary bone during osseointegration. We believe that the basic research presented in our manuscript will provide important knowledge in our understanding of factors that impact implant success. Furthermore, it provides a solid foundation for the development of materials that enable rapid osseointegration and earlier loading times for implants in bone that has been compromised by trauma or disease.

Figure 1. Establishment of OC phenotype and treatment period

OCPs were differentiated with M-CSF (negative control) or M-CSF and RANKL (positive control) on an OsteoLyse Kit. After 3d, 7d, 9d, and 14d OC activity was measured by absorbance following the addition of 10µl of media to 200µl of a fluorophore releasing agent. Afterwards, mRNA was isolated from OCs and CA2, CTSK, CSTB, ITGAV, ITGB3, and OCSTAMP were measured. Data shown are the mean $\pm$ standard error (SE) of six independent samples. Groups not sharing a letter are statistically significant at an $\alpha=0.05$.

Figure 2. Qualitative assessment of OCs
 TRAP staining (Left) and SEM imaging (Right) of OCs at each harvest point. OCPs cultured with M-CSF and without RANKL serve as negative controls while OCPs cultured with M-CSF and RANKL serve as positive controls.

Figure 3. The indirect effects of Ti surface cultured osteoblast lineage cell conditioned media on osteoclast activity
 OCPs were differentiated on an OsteoLyse Kit with M-CSF and RANKL. MSCs and NHOst were separately cultured on TCPS, PT, SLA, or mSLA. After 7d, MSC and NHOst media were collected, supplemented with or without M-CSF and RANKL, and used to treat OCs, leaving a negative (OCPs + M-CSF) and positive (OCPs + M-CSF + RANKL) control. After treatment for 2d, 10µl of media was placed into 200µl of a fluorophore releasing agent and absorbance measured. Data shown are the mean ± standard error (SE) of six independent samples. Groups not sharing a letter are statistically significant at an $\alpha=0.05$.

Figure 4. The indirect effects of Ti surface cultured osteoblast lineage cell conditioned media on osteoclast gene expression

OCPs were differentiated on an OsteoLyse Kit with M-CSF and RANKL. MSCs and NHOst were cultured on TCPS, PT, SLA, or mSLA. After 7d, MSC and NHOst media were collected, supplemented with M-CSF and RANKL, and used to treat OCs, leaving a negative (OCPs + M-CSF) and positive (OCPs + M-CSF + RANKL) control. After treatment for 2d, OCs were harvested and mRNA levels of CA2, CSTB, OCSTAMP, CTSK, ITGAV, and ITGB3 quantified. Data shown are the mean $\pm$ standard error (SE) of six independent samples. Groups not sharing a letter are statistically significant at $\alpha=0.05$.

Figure 5. The indirect effects of conditioned media are mediated through integrin signaling and dependent on cells and proteins

(A) MSCs were cultured on TCPS or mSLA and compared to their acellular counterparts. TCPS and mSLA containing no cells were incubated with MSCGM and received media changes identical to MSC cultured TCPS or mSLA. MSCs silenced for either ITGA2 (B) or ITGB1 (C) were cultured on TCPS or mSLA and compared to their WT controls. Conditioned media were subjected to protein denaturation via proteinase K (D) or heat (E) and compared to non-denatured controls. After 7d, conditioned media were collected, supplemented with M-CSF and RANKL, and used to treat OCs, leaving a negative (OCPs +

M-CSF) and positive (OCPs + M-CSF + RANKL) control. After treatment for 2d, 10µl of media was placed into 200µl of a fluorophore releasing agent and absorbance measured. Data shown are the mean $\pm$ standard error (SE) of six independent samples. Groups not sharing a letter are statistically significant at $\alpha=0.05$.

Figure 6. The influence of TGF- β 1 and Osteoprotegerin on the indirect effects of conditioned media

MSCs were cultured on TCPS or mSLA for 7d. Conditioned media was collected and supplemented with M-CSF and RANKL as well as either (A) anti-TGF- β type 2 receptor antibody, (B) soluble TGF- β type 2 receptor, or (C) anti-osteoprotegerin antibody. Conditioned media was then used to treat OCs, leaving a negative (OCPs + M-CSF) and positive (OCPs + M-CSF + RANKL) control. After treatment for 2d, 10 μ l of media was placed into 200 μ l of a fluorophore releasing agent and absorbance measured. Data shown are the mean $\pm$ standard error (SE) of six independent samples. Groups not sharing a letter are statistically significant at $\alpha=0.05$.

Table 1

Human Primers Used in Real-Time PCR Analysis

Name	Gene		Primer Sequence
Carbonic Anhydrase II	CA2	F	CACTCGTAAATGCCAAGAAAAG
		R	TAGAACCCAACACAAAGATGC
Cystatin B	CSTB	F	ACTGTTCAAGGAGGAGAC
		R	GGTCAAAGGCTTGTTTAGG
Cathepsin K	CTSK	F	ATCTGTGTGCCTGACCTG
		R	AAGTTCCTGGGTGTCTG
Osteoclast Stimulatory Transmembrane Protein	OCSTAMP	F	ATTACTCAGATCCAACCAC
		R	TCCTCCTCATTTTCATTCATC
Integrin $\beta 3$	ITG $\beta 3$	F	ACGGTGGGAACTTTTGATGTG
		R	CGAGTCTGATGGAGGTGAGTC
Integrin αV	ITG αV	F	AGCAGCAGCGACCAGAGG
		R	ACAGTAGTAGGCGGCGTAGC
Glyceraldehyde 3-phosphate dehydrogenase	GAPDH	F	GCTCTCCAGAACATCATCC
		R	TGCTTCACCACCTTCTTG