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VEGF-A Regulates Angiogenesis during Osseointegration of Ti Implants via Paracrine/Autocrine Regulation of Osteoblast Response to Hierarchical Microstructure of the Surface

Andrew L. Raines¹, Michael B. Berger², Nehal Patel¹, Sharon L. Hyzy^{1,2}, Barbara D. Boyan^{1,2}, Zvi Schwartz^{2,3}

¹Wallace H. Coulter Department of Biomedical Engineering at Georgia Tech and Emory University, Georgia Institute of Technology, Atlanta, GA, USA;

²Department of Biomedical Engineering, Virginia Commonwealth University, Richmond, VA, USA;

³Department of Periodontology, University of Texas Health Science Center at San Antonio, San Antonio, TX, USA

Abstract

Establishment of a patent vasculature at the bone-implant interface plays a significant role in determining overall success of orthopaedic and dental implants. Osteoblasts produce vascular endothelial growth factor-A (VEGF-A), an important regulator of angiogenesis during bone formation and healing, and the amount secreted is sensitive to titanium (Ti) surface microtopography and surface energy. The purpose of this study was to determine if surface properties modulate cellular response to VEGF-A. MG63 osteoblast-like cells were transfected with shRNA targeting VEGF-A at >80% knockdown. Cells stably silenced for VEGF-A secreted reduced levels of osteocalcin, osteoprotegerin, FGF-2, and angiopoietin-1 when cultured on grit-blasted/acid-etched (SLA) and hydrophilic SLA (modSLA) Ti surfaces and conditioned media from these cultures caused reduced angiogenesis in an endothelial tubule formation assay. Treatment of MG63 cells with 20 ng/mL rhVEGF-A₁₆₅ rescued production in silenced cells and increased production of osteocalcin, osteoprotegerin, FGF-2, and angiopoietin-1, with greatest effects on control cells cultured on modSLA. Addition of a neutralization antibody against VEGF receptor 2 (VEGFR2; Flk-1) resulted in a significant increase in VEGF-A production. Overall, this study indicates that VEGF-A has two roles in osseointegration: enhanced angiogenesis and an autocrine/paracrine role in maturation of osteoblast-like cells in response to Ti surface properties.

Keywords

Titanium; VEGF; Osteoblast; Osseointegration; Angiogenesis

INTRODUCTION

The surface properties of biomaterials directly influence attachment, proliferation, and phenotypic expression of cells both *in vitro* and *in vivo*.^{1–3} The first events after implantation of material into the body involve adsorption of proteins onto the surface from surrounding blood and tissue fluid. The topography, chemistry, and energy of a biomaterial surface are all known to affect this process and the subsequent cellular response.

In orthopaedics and dentistry, titanium (Ti) is a commonly used material for implants in bone due to its biocompatibility, good mechanical properties and resistance to wear. The biocompatibility and wear resistance of Ti is due to the thin oxide layer (TiO₂) that forms on its surface. In vitro studies show that Ti substrates presenting a rough micron-scale topography affect the attachment and differentiation of osteoblast lineage cells.⁴ Mesenchymal stem cells (MSCs) and osteoblasts cultured on these substrates display a more differentiated osteoblast phenotype with a reduction in proliferation and an increase in the production of factors that generate an osteogenic microenvironment, including osteocalcin, prostaglandin E2 (PGE₂), transforming growth factor beta-1 (TGFβ₁), and bone morphogenetic proteins (BMP2, BMP4, and BMP7), as well as factors that reduce osteoclastic resorption like osteoprotegerin.^{5,6} Combining a rough surface microtopography with high surface energy further enhances the differentiation of osteoblasts and generation of an osteogenic environment.¹⁰ These in vitro results provide mechanistic support for the observation that micro-rough Ti implants exhibit greater bone to implant contact and have higher torque removal strengths *in vivo* than Ti implants with smooth surfaces.^{11,12}

The establishment of a vascular supply is of critical importance in the osseointegration of implants, both for delivery of nutrients and removal of wastes, as well as for the migration of osteoprogenitor cells to the site.^{13–15} We have shown that development of the neovasculature during osteogenesis around Ti implants placed in the medullary canal of aged rats is influenced by the surface properties of the implants.¹⁶ This suggests that factors generated by cells on the implant surface are angiogenic, stimulating the growth of small blood vessels from the existing vasculature. Osteoblast and osteoprogenitor cells have been demonstrated to produce and secrete several pro-angiogenic growth factors, including vascular endothelial growth factor-A (VEGF-A), basic fibroblast growth factor (FGF-2), and angiopoietin-1 (Ang-1).¹⁷ Expression of these growth factors depends on the state of maturation of the cell in the osteoblast lineage^{18,19} and on the surface properties of their substrate.^{17,20,21}

VEGF-A is a member of the VEGF family of proteins, which includes VEGF-A, VEGF-B, VEGF-C, and VEGF-D, as well as placental growth factor 1 and 2 (PLGF-1 and -2),²² all of which have the ability to stimulate endothelial cell proliferation and differentiation.^{14,23} It exerts its effects through two tyrosine kinase receptors, VEGFR1/Flt-1 and VEGFR2/KDR/Flk1 and both VEGF receptors are expressed by osteoblasts during their differentiation.^{18,24}

Binding to Flk1 receptor has been shown to mediate angiogenesis.²⁵ VEGF-A has been identified as a particularly important growth factor during bone formation and remodeling.^{26,27} In human primary osteoblasts, this mechanism of VEGF-A signaling has been shown to signal through the Flt-1 receptor.²⁸ In addition to being expressed by osteoblasts, VEGF-A is

expressed by hypertrophic chondrocytes and may be involved directly in osteoblast differentiation.^{29,30} While it is known that osteoblasts express VEGF-A and the receptors for VEGF-A³¹ and that disruption of VEGF-A signaling in osteoblasts inhibits bone formation during endochondral ossification,²⁶ it is not known whether VEGF-A production by osteoblasts in response to Ti surface microtopography and energy has an effect on the differentiation of these cells in parallel to its effects on angiogenesis.

To address this question, we selected MG63 osteoblast-like cells as the model system. They are well-established model cell line for osteoblast progenitor cells and can be stably silenced.⁷⁻⁹ Accordingly, we stably silenced VEGF-A in MG63 osteoblast-like cells using shRNA targeting VEGF-A and compared the production of osteogenic and angiogenic factors produced by these cells to production by wildtype MG63 cells cultured on Ti surfaces presenting different surface roughness and energy. To determine if VEGF-A produced by MG63 cells has a paracrine effect on endothelial cells, we used conditioned media from wild-type, and VEGF-A silenced MG63 cell cultures in an *in vitro* fibrin gel assay to assess endothelial tubule formation. To see if endogenous VEGF-A produced by MG63 cells in response to Ti surface roughness and energy has an autocrine effect on MG63 cell differentiation through interaction with VEGFR2/Flk-1, we used a monoclonal antibody against human VEGFR2/Flk-1 in wild-type MG63 cell cultures on Ti substrates. We also treated MG63 cells on Ti surfaces with rhVEGF-A or rhFGF-2 to determine if osteoblastic differentiation is enhanced by treatment of either of these growth factors. Finally, to see if the production of osteogenic and angiogenic factors in VEGF-A silenced MG63 cells could be restored to wild-type levels, we treated VEGF-A silenced MG63 cells with exogenous rhVEGF-A or rhFGF-2.

MATERIALS AND METHODS

Preparation of Ti substrates

Ti disks were prepared from 1 mm thick sheets of grade 2 unalloyed commercially pure Ti punched into 15mm diameter disks and supplied by Institut Straumann AG (Basel, Switzerland). The production and characterization of smooth pretreatment (PT), grit-blasted and acid-etched (SLA), and hydrophilic SLA (modified SLA; modSLA) surfaces have been described previously.³² The PT surface has an overall average roughness (Ra) of less than 0.7 μm . SLA and modSLA surfaces have a complex microtopography with craters varying from 30 to 100 μm in diameter overlaid with pits from 1 to 3 μm in diameter.³³ The acid-etch creates sharp edges approximately 700 nm in height, resulting in an overall Ra of approximately 4 μm . PT, SLA and modSLA Ti disks all have a TiO_2 surface layer, with the PT and SLA surfaces being hydrophobic due to the adsorption of atmospheric hydrocarbons while the modSLA surface is hydrophilic. Advancing contact angles were used to calculate the hydrophilicity of the surfaces as PT (95.8°), SLA (139.80°), and modSLA (~0°).^{32,33}

General Cell Culture

MG63 osteoblast-like cells were maintained in culture using Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin (P/S). MG63 cells stably silenced for VEGF-A were cultured in DMEM

containing 10% FCS, 1% P/S and 0.5 µg/mL puromycin for selection of successfully transduced cells. For experiments, both MG63 cells and VEGF-A silenced MG63 cells were plated at an initial seeding density of 10,000 cells/cm² in 24-well standard tissue culture plates. Media were changed 24 hours after plating and every 48 hours thereafter until the cells were 100% confluent. When cells reached 100% confluent on TCPS, media from all cultures were collected and examined for secreted levels of VEGF-A, FGF-2, Ang-1, osteoprotegerin (OPG), and osteocalcin where appropriate.

Generation of VEGF-A silenced MG63 Cells

To generate stably silenced MG63 cells for VEGF-A, we used MISSION® shRNA lentiviral transduction particles targeting the human VEGF-A gene (VEGFA) (Sigma-Aldrich, St. Louis, MO). Five candidate shRNA clones were designed using MISSION pLOK.1-puromycin vectors, and packaged into self-inactivating replication incompetent lentiviral particles for transduction. shRNA clone providing the highest degree of gene expression knockdown was used for experiments. The sequences for the 5 clones are described in Supplemental Table 1. In addition, an empty shRNA vector that did not contain a hairpin insert (PLK0.1) and a shRNA sequence not targeting any gene in the human genome (NT) were used as controls.

RT-qPCR for human VEGFA and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was run to test for silencing efficiency of VEGFA in transduced MG63 cell cultures using the primers outlined in Supplemental Table 2. Quantification of VEGFA and GAPDH gene expression was done using real-time qPCR using SYBR Green as a fluorescent marker. Fluorescence values were quantified as starting quantities using known dilutions of MG63 cell RNA. VEGFA mRNA levels were normalized to expression of GAPDH for all cell types.

Endothelial Cell Differentiation

We performed an initial screen of the conditioned media from the wildtype and VEGFA-silenced cells to determine if the silencing of VEGFA resulted in a loss in angiogenic activity. To do this, we used a Matrigel™ endothelial cell tubule formation assay kit (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. Based on the results of this initial study, we selected clone H4 for all subsequent experiments.

To determine if the effects of surface topography and hydrophilicity on angiogenesis were dependent on VEGF-A, we examined the ability of conditioned media from cultures of wildtype and silenced MG63 cells to support endothelial tubule formation using a fibrin gel assay kit (Millipore, St. Charles, MO). Briefly, each well of a 96-well tissue culture plate was coated with 30 µL of a fibrinogen solution and 20 µL of a thrombin solution and the mixture was allowed to polymerize for at least 1 hour at 37°C. Human aortic endothelial cells (HAECs) were plated on fibrin gel-coated 96-well plates in 100 µL of endothelial growth medium containing 2% FBS and VEGF (EGM-2, Lonza, Walkersville, MD) at a density of 5×10^3 cells/well and cultured at 37°C for 24h. At 24h, media were removed and a second layer of fibrin was added on top of cells by again mixing 30 µL of fibrinogen and

20 μ L of thrombin. The mixture was allowed to polymerize for 5 minutes before 100 μ L of conditioned media from either MG63 cell cultures or VEGFA silenced MG63 cell cultures (clone H4) on TCPS, PT, SLA, or modSLA substrates were added. At 12, 24, and 36 after the addition of conditioned media, images were taken for morphometric analysis to determine total endothelial tube length.

Mechanism of VEGF-A Action

Flk-1/IgG Antibody Treatment: MG63 cells plated on tissue culture polystyrene (TCPS, control for all studies), PT, SLA, and modSLA Ti substrates were treated with 100 ng/mL Flk-1 mouse anti-human monoclonal neutralization antibody (R&D Systems, Minneapolis, MN) or 100 ng/mL rabbit anti-human nonspecific IgG polyclonal antibody (NeoMarkers, Fremont, CA) for the duration of cell culture.

rhVEGF-A & rhFGF-2 Treatment: For studies examining the effect of exogenous VEGF-A or rhFGF-2, cell culture media were supplemented with 20 ng/mL rhVEGF-A₁₆₅ (R&D Systems, Minneapolis, MN) or 10 ng/mL rhFGF-2 (R&D Systems, Minneapolis, MN). MG63 cells and VEGF-A silenced MG63 cells were treated with supplemented media for the duration of cell culture.

Analysis of Cell Response

Cell Number: Total cell number was determined at the time of harvest. Cells were released from TCPS and Ti surfaces using two sequential incubations with 0.25% trypsin for 10 minutes at 37°C to ensure that no cells remained on the rough Ti surfaces and counted using an automated cell counter (Z1 Particle counter, Beckman Coulter, Fullerton, CA).

Alkaline Phosphatase Specific Activity: Alkaline phosphatase specific activity (orthophosphoric monoester phosphohydrolase, alkaline; E.C. 3.1.3.1) was measured in cell lysates as a marker of osteoblastic differentiation. Enzyme activity was determined using a colorimetric assay measuring the release of *p*-nitrophenol from *p*-nitrophenyl phosphate at 37°C. Samples were read on a plate reader at 415nm.

Osteocalcin: Osteocalcin in the conditioned media was determined as a marker of osteoblast maturation using a commercially available radioimmunoassay (Biomedical Technologies, Inc., Stoughton, MA) following the manufacturer's protocol.

VEGF-A, FGF-2, Ang-1, OPG: Secreted levels of VEGF-A, FGF-2, Ang-1, and OPG by MG63 cells and VEGF-A silenced MG63 cells were determined in the conditioned media using commercially available ELISA assays (DuoSet ELISA Development Systems, R&D Systems, Minneapolis, MN) following the manufacturer's protocols.

Statistical Analysis: The data presented here are from one of at least two separate sets of experiments. Both sets of experiments yielded comparable observations. For any given experiment, each data point represents the mean $\pm$ standard error of six individual cultures. Data were analyzed by ANOVA, and when statistical differences were detected, Student's *t*-

test for multiple comparisons using Bonferroni's modification was used. p-values < 0.05 were considered significant.

RESULTS

VEGF-A Silencing

To verify successful silencing of VEGF-A in MG63 cells, we examined both the total secreted protein levels in the conditioned media of cultures transduced with one of the five candidate shRNA sequences for human VEGF-A as well as a scrambled shRNA sequence (NT) and an empty vector (PLK0.1). VEGF-A protein levels in conditioned media from MG63 cells, NT, and PLK0.1 cell lines were comparable to each other. VEGF-A shRNA clones H1–H5 secreted varying levels of VEGF-A into the conditioned media (Figure 1A). Notably, clone H4 resulted in a 94.1% reduction in VEGF-A compared to MG63 cells. RT-qPCR showed that VEGF-A expression levels for MG63 cells, NT, and PLK0.1 vectors were similar, while the VEGF-A shRNA clones H1–H5 had varying levels of expression compared to MG63 cells (Figure 1B). We selected clone H4 for further experiments due to clone H4's maximal silencing for both gene and protein expression.

Conditioned media from wildtype MG63 cells caused a time-dependent increase in endothelial cell length in the Matrigel assay. Silencing VEGF-A resulted in reduced endothelial tubule length in HAEC cultures incubated with H4-cell conditioned media at each time point using the fibrin gel assay (Figure 1C).

Requirement for VEGF-A in Osteoblast Response to Surface Properties

VEGF-A was increased in conditioned media of wildtype MG63 cells cultured on the micro-rough SLA and modSLA surfaces compared to TCPS and smooth PT (Figure 2A). Silencing VEGF-A resulted in more than 80% reduction in VEGF-A on all surfaces, but the surface dependent increase seen in wildtype cultures on modSLA was also evident in the silenced MG63 cells.

FGF-2 also exhibited a surface dependent increase in wildtype cultures grown on SLA and modSLA. Silencing of VEGF-A did not alter FGF-2 production on smooth TCPS and PT surfaces, but it reduced the stimulatory effect of SLA and modSLA (Figure 2B). Ang-1 production by wildtype MG63 cells was unaffected by surface microtopography or surface energy (Figure 2C). Silencing VEGF-A reduced Ang-1 by 50% on all surfaces.

Mechanism Mediating Surface-Dependent VEGF-A Action

Treatment of wild-type or shVEGF-A cells with rhVEGF-A₁₆₅ had no effect on cell number (Figure 3A). Silencing VEGF-A caused greater than 80% reduction in alkaline phosphatase specific activity on all surfaces (Figure 3B). Whereas exogenous rhVEGF-A did not alter enzyme activity in wildtype cultures or in shVEGF-A cultures on smooth substrates, it stimulated activity in shVEGF-A cells on SLA. Total cell number was reduced in response to surface topography and energy for all groups. Silencing VEGF-A reduced osteocalcin production by 50% on all surfaces (Figure 3C). Treatment with rhVEGF-A₁₆₅ restored osteocalcin production by the silenced cells to levels seen in wildtype cells on TCPS, PT,

and SLA. Moreover, it stimulated activity in wild-type cells on modSLA, and it restored activity in silenced levels to levels observed in wild-type cells. Silencing VEGF-A also reduced osteoprotegerin production on all surfaces. Treatment with rhVEGF-A did not affect osteoprotegerin levels on TCPS, PT or SLA, but it caused a 100% increase in wild-type cells on modSLA, and it restored production to wildtype levels in VEGF-A silenced cells. Silencing VEGF-A reduced FGF-2 production only on modSLA and treatment with rhVEGF-A restored FGF-2 to wildtype levels on this surface (Figure 3E). Silencing VEGF-A reduced Ang-1 production on all surfaces (Figure 3F). rhVEGF-A caused a marked increase in Ang-1 in wild-type cells only on modSLA, and restored Ang-1 in silenced cells to levels seen in wildtype cultures.

Treatment of MG63 cells and VEGF-A silenced MG63 cells with rhVEGF-A had an effect on the production of angiogenic growth factors in response to surface topography and energy. Secreted levels of FGF-2 were similar amongst all groups on TCPS, PT, and SLA surfaces. The combination of a rough surface microtopography and high surface free energy observed on modSLA surfaces increased FGF-2 production in MG63 cells. Treatment of MG63 cells with rhVEGF-A displayed a further, modest increase in FGF-2 production compared to untreated cells, though this increase was not statistically significant (Figure 3E). In VEGF-A silenced MG63 cells, FGF-2 production was significantly reduced compared to MG63 cells on modSLA only and treatment with rhVEGF-A165 increased FGF-2 production to levels comparable to wild-type MG63 cell cultures (Figure 3E). Ang-1 levels were unchanged in response to surface microtopography and surface energy in both MG63 cells and VEGF-A silenced MG63 cells, though levels in VEGF-A silenced MG63 cells were significantly reduced. Treatment with rhVEGF-A165 had no effect on Ang-1 production in response to surface topography. However, on modSLA surfaces, levels of Ang-1 in both MG63 cells and VEGF-A silenced MG63 cells were significantly increased compared to control TCPS cultures. In addition, levels of Ang-1 in rhVEGF-A165 treated VEGF-A silenced MG63 cells were comparable to those observed in wild-typeMG63 cells (Figure 3F).

In order to determine if VEGF-A produced by MG63 cells has an autocrine effect mediated by VEGF-A receptor, we added an Flk-1 neutralization antibody to MG63 cell cultures on TCPS and Ti substrates. Consistent with previous results, total cell number in MG63 cell cultures were reduced on micro-rough SLA substrates, and micro-rough, hydrophilic modSLA surfaces compared to TCPS. Addition of either Flk-1 neutralization antibody or nonspecific IgG antibody had no effect on cell number (Figure 4A). Alkaline phosphatase specific activity in MG63 cells were unchanged amongst any of the surfaces examined and the addition of either Flk-1 neutralization antibody or IgG control antibody had no effect on alkaline phosphatase specific activity (Figure 4B). Secreted levels of osteocalcin, a late marker of osteoblast differentiation, were significantly increased ($p<0.05$) in MG63 cells cultured on SLA and modSLA surfaces compared to TCPS substrates while the addition of either antibody had no effect on osteocalcin levels compared to MG63 cells (Figure 4C). Osteoprotegerin (OPG) secretion by MG63 cells were similar on TCPS and smooth PT Ti substrates. Addition of a rough surface microarchitecture observed on SLA substrates had no effect on secreted levels of OPG by MG63 cells. However, on rough, hydrophilic, modSLA substrates, OPG levels were significantly increased. Addition of either an Flk-1

neutralization antibody or nonspecific IgG antibody had no effect on OPG levels compared to MG63 cells (Figure 4D).

Consistent with what we have found previously, production of VEGF-A by MG63 cells was significantly increased on both SLA and modSLA substrates compared to TCPS and smooth PT surfaces. The effect was more robust on the hydrophilic modSLA but not different from SLA. Addition of a Flk-1 neutralization antibody to MG63 cell cultures significantly increased production of VEGF-A on all surfaces compared to MG63 cells with no antibody, with the largest increase occurring on modSLA substrates. A nonspecific IgG antibody had no effect on VEGF-A production versus MG63 cell cultures (Figure 4E). FGF-2 production by MG63 cells was not changed in response to treatment with either a Flk-1 neutralization antibody or a nonspecific IgG antibody. However, surface roughness and energy dependent increases in FGF-2 production were observed in all three treatment groups (Figure 4F). Angiopoietin-1 levels were unaffected by treatment with either antibody or by any surface type tested (data not shown).

rhFGF-2 Treatment

Since endogenous knockdown of VEGF-A in MG63 cells resulted in decreased production of FGF-2 in response to Ti surface microtopography and energy, we treated VEGF-A silenced MG63 cells with exogenous rhFGF-2 to see if FGF-2 was mediating MG63 cell response to Ti surface properties. In MG63 cells, treatment with 10 ng/mL rhFGF-2 resulted in a significant increase in total cell number at the time of harvest against untreated MG63 cells on all surfaces examined (Figure 5A). On control TCPS surfaces, VEGF-A silenced MG63 cells treated with rhFGF-2 displayed increased cell number compared to untreated VEGF-A silenced MG63 cells. However, treatment with rhFGF-2 did not have any effect on cell number in response to Ti surface topography or energy (Figure 5A). In both MG63 cells and VEGF-A silenced MG63 cells, alkaline phosphatase specific activity was increased compared to untreated control cells on control TCPS, smooth PT, and SLA Ti surfaces. Further, an increase in alkaline phosphatase specific activity in response to rhFGF-2 treatment was seen on micro-rough, hydrophilic modSLA Ti surfaces in MG63 cell cultures but not in VEGF-A silenced MG63 cell cultures. In all cell types examined, surface topography and energy had no effect on alkaline phosphatase specific activity (Figure 5B).

We next examined whether treatment with rhFGF-2 had an effect on osteoblast phenotype in response to Ti surface topography and energy. Similar to our earlier results, both MG63 cells and VEGF-A silenced MG63 cells displayed a surface roughness and energy dependent increase in osteocalcin and osteoprotegerin production, though production of these growth factors was reduced by at least 50% in VEGF-A silenced MG63 cell cultures on all surfaces (Figure 5C, D). Treatment with rhFGF-2 had no effect on the production of either osteocalcin or osteoprotegerin in either cell type on any surface examined (Figure 5C, D).

VEGF-A levels in MG63 cells were increased in an energy-dependent manner and treatment with 10 ng/mL rhFGF-2 did not have any effect on the production of VEGF-A in these cells. In VEGF-A silenced cells, secreted levels of VEGF-A were reduced by at least 60% compared to MG63 wild-type cells on all surfaces and rhFGF-2 treatment did not affect secretion of this growth factor (Figure 5E). For both cell types, Ang-1 production was

unaffected by either surface roughness or energy, though levels in VEGF-A silenced MG63 cells were significantly reduced compared to MG63 cells, and rhFGF-2 treatment also had no effect on secretion of this growth factor (Figure 5F).

DISCUSSION

Osseointegration of orthopaedic and dental implants is dependent not only on direct apposition of bone to the implant surface, but also on the formation of a patent vasculature in the peri-implant space to allow delivery of oxygen, nutrients, and cells. Osteoblasts are known to secrete VEGF-A, a potent endothelial cell mitogen, in response to a number of different stimuli, including several growth factors and hormones.^{34–36} VEGF-A has also been found to have a direct role in osteoblast differentiation and maturation.^{30,37,38} In the present study we show that osteoblasts grown on the Ti substrates produce factors that induce angiogenesis and this is dependent on the surface roughness and energy. In addition, VEGF-A was shown to have a direct effect on osteoblast differentiation in response to Ti surface roughness and energy. This suggests that properties of the implant surface can have direct effects on osteoprogenitor cells, which can affect cells that are distal to the implant by promoting maturation of vessels via the production of angiogenic factors.

Moreover, our results indicate that VEGF-A and FGF-2 regulated the response of MG63 cells to surface properties, suggesting surface-dependent autocrine/paracrine feed-back regulation of osteogenesis. Secreted levels of osteocalcin and osteoprotegerin, later markers of osteoblast differentiation, as well as levels of VEGF-A and FGF-2, two pro-angiogenic growth factors were increased by MG63 cells on micro-rough SLA and micro-rough, high surface energy modSLA Ti substrates compared to TCPS and smooth PT Ti substrates, while levels of Ang-1 were unaffected by either Ti substrate roughness or energy, consistent with earlier findings.^{17,21,39} This suggests that the surface effects were limited to induction of angiogenesis rather than growth of mature vessels, as Ang-1 was not affected. However, in VEGF-A silenced MG63 cell cultures, secretion of these growth factors was reduced compared to wild-type cells. These data suggest that VEGF-A has an autocrine role in mediating osteoblast-like cell differentiation on Ti substrates *in vitro*.

Addition of VEGF to fetal bovine osteoblasts increased migration and alkaline phosphatase expression, but had no effect on proliferation,⁴⁰ while exogenous VEGF-A₁₆₅ increased alkaline phosphatase activity and mineralized nodule formation in cultures of primary human osteoblasts³⁰. Our results here show that VEGF-A can enhance osteoblast differentiation in response to Ti surface microtopography and energy. Addition of 20 ng/mL rhVEGF-A₁₆₅ to MG63 cells increased levels of osteocalcin and osteoprotegerin as well as levels of Ang-1 and FGF-2 on micro-rough, high surface energy modSLA surfaces, but had no effect on either cell number or alkaline phosphatase specific activity. Further, in MG63 cells that have been permanently silenced for expression of VEGF-A, addition of exogenous VEGF-A increased secreted levels of osteocalcin, osteoprotegerin, FGF-2, and Ang-1 to those observed in wild-type MG63 cell cultures on modSLA Ti surfaces, further supporting the hypothesis that interaction of VEGF-A with its receptors is necessary for osteoblast maturation.

Differences in response between modSLA and the other surfaces can be attributed to differences in surface energy. modSLA surfaces are super hydrophilic and have been shown previously to enhance osteoblast maturation compared to microroughened SLA and smooth PT titanium surfaces.^{5,17} This can explain the robust increases in production or alterations in response when treated with exogenous VEGF-A or Flk-1 antibodies.

Our results *in vitro* support *in vivo* genomic and proteomic analyses of osseointegration over 14 days using Ingenuity Pathway Analysis (IPA).⁴¹ These studies showed that modSLA increased VEGF-A *in vivo* at 7 days compared to SLA with maximal pathway signaling at 14 days. This increase was closely correlated to increased osteogenesis at 7 days post implantation on modSLA surfaces.

VEGF-A exerts its effects through binding to two receptor tyrosine kinases, VEGFR1 (Flt1) and VEGFR2 (Flk1/KDR), which are expressed by not only endothelial cells, but also chondrocytes, osteoblasts, and osteoclasts.^{27,37} In endothelial cells, VEGFR2 is recognized as the primary mediator of the mitogenic effects of VEGF-A during neo-vascularization.²⁵ Upon binding, VEGFR2 dimerizes and undergoes strong autophosphorylation, inducing the downstream phosphorylation of multiple proteins in endothelial cells, including phospholipase C- γ , PI-3 kinase, Ras, and members of the Src family.^{42,43} However, a possible role for both VEGFR1 and VEGFR2 has been implicated in bone growth and development. MG63s exhibit both VEGFRs and primary human osteoblasts have been shown to mature through the activation of VEGFR1.^{28,44}

In Flt1 (VEGFR1) null mice, trabecular bone volume, and bone formation rate were reduced *in vivo*, and bone marrow stromal cell cultures from Flt1 null mice exhibited decreased mineralization *in vitro* compared to wild-type animals.⁴⁵ Interaction of VEGF-A with Flk1 has been demonstrated in primary human osteoblasts, and prostanoid mediated upregulation of VEGF-A by these cells is inhibited by treatment with a VEGFR tyrosine kinase inhibitor.²⁴ It has also been found that VEGF-A increases bone mass through a Flk1 and PI-3 kinase-mediated pathway.^{29,41}

In our study, we found that addition of a neutralizing antibody specific for Flk1 did not have any effect on the production of osteocalcin, osteoprotegerin, FGF-2 or Ang-1 in MG63 cells in response to Ti substrate features. However, VEGF-A production in response to treatment with a Flk1 antibody was significantly increased. It may be possible that VEGF-A interaction with Flk1 in MG63 cells is necessary for maturation of these cells and to overcome the effect of the neutralization antibody, production of VEGF-A was increased. In addition, these data suggest that the full effect of VEGF-A on osteoblasts is also mediated by the receptor Flt1.

FGF-2 has been reported to be a modulator of bone formation *in vitro* and *in vivo*.^{46,47} In our study, we found that treatment of MG63 cells with 10 ng/mL rhFGF-2 increased cell number and alkaline phosphatase specific activity but had no effect on the production of either osteogenic or angiogenic markers. FGF-2 has also been found to induce proliferation of human mesenchymal progenitor cells and MC3T3-E1 cells.^{48,49} Moreover, treatment of mineralized MC3T3-E1 cultures with FGF-2 reduced the expression of collagen I, BMP-2,

and osteocalcin.⁴⁹ In VEGF-A silenced cells, the addition of rhFGF-2 did not increase cell number to the same extent as seen in MG63 wild-type cell cultures but did increase alkaline phosphatase specific activity compared to untreated VEGF-A silenced cells. However, no increase in the production of osteogenic or angiogenic markers by VEGF-A silenced cells were observed in response to FGF-2 treatment. These results indicate that VEGF-A, but not FGF-2 is necessary for osteoblast differentiation. Furthermore, IPA analysis of angiogenesis during osseointegration further supports these results and suggests that hypoxia-inducible factor 1 (HIF1) may work together with VEGF to increase angiogenesis during osseointegration on modSLA implant surfaces.⁴¹

Alkaline phosphatase activity in osteoblast-like cells is highly dependent on the state of maturation of the cells at the time of assay. In early osteoblast differentiation, alkaline phosphatase activity increases until the onset of mineralization, at which time its activity decreases, and osteocalcin production is increased.⁵⁰ This biphasic curve for alkaline phosphatase activity during osteoblast maturation may explain observed differences in alkaline phosphatase specific activity in response to Ti substrate features seen in this study compared to other studies.

In conclusion, we show here that osteoblasts grown on modified Ti substrates produce the angiogenic factor VEGF-A, which has an autocrine/paracrine effect on the production of osteogenic and angiogenic markers by these cells. shRNA targeted knockdown of VEGF-A resulted in a reduction in secreted levels of osteocalcin, osteoprotegerin, FGF-2, and angiopoietin-1. However, treatment with exogenous VEGF-A increased secreted levels of these markers and was able to recover the effect of silencing VEGF-A. FGF-2 increased cell number and alkaline phosphatase, but had no effect on the production of osteogenic or angiogenic markers and did not recover the effect of silencing VEGF-A. Our results also indicate that the ability to recover the effect of silencing VEGF-A is not mediated by FGF-2.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Verification of VEGF-A silencing in an MG63 cell line using lentiviral-mediated transduction of shRNA specific for human VEGF-A. (A) VEGF-A protein levels and (B) VEGF-A mRNA levels were determined in 5 separate MG63 cell lines established using shRNA sequences specific for VEGF-A as well as a scrambled shRNA sequence (NT) and empty vector control (PLK0.1). * $p < 0.05$ vs. MG63. (C) Total endothelial tube length in the presence of conditioned media from MG63 cells and VEGF-A silenced clone H4 cells. * $p < 0.05$ vs. 4h; # $p < 0.05$ vs. MG63.

Figure 2.

Paracrine effect of angiogenic growth factor production by MG63 and VEGF-A silenced MG63 cells. (A) VEGF-A, (B) FGF-2, and (C) Angiopoietin-1 levels in the conditioned media of MG63 and VEGF-A silenced MG63 cells were determined. Cells were cultured on control (TCPS), PT, SLA, and modSLA Ti surfaces. The ability of angiogenic growth factors to promote endothelial cell tubule formation was assessed using a fibrin gel assay. Total endothelial tube length was measured at (D) 12, (E) 24, and (F) 36 hours after the addition of conditioned media. Representative images using conditioned media from (G) siVEGF-A and (H) MG63 cell cultures. Values presented are mean $\pm$ SEM of six independent cultures. The data presented are from one of two separate experiments with comparable results. Data were analyzed using ANOVA, and statistical significance between groups was determined using Bonferroni's modification of Student's t-test. * $p < 0.05$ vs. TCPS; # $p < 0.05$ vs. MG63.

Figure 3.

MG63 and VEGF-A silenced cell response to treatment with exogenous rhVEGF-A₁₆₅. (A) Cell number, (B) alkaline phosphatase specific activity in the cell lysate, (C) osteocalcin, (D) osteoprotegerin, (E) FGF-2, and (F) Angiopoietin-1 levels were determined for both MG63, and VEGF-A silenced MG63 cells as well as MG63 and VEGF-A silenced MG63 cells treated with 20 ng/mL of rhVEGF-A₁₆₅. Values presented are mean $\pm$ SEM of six independent cultures. Data were analyzed using ANOVA, and statistical significance between groups was determined using Bonferroni's modification of Student's t-test. * $p < 0.05$ vs. TCPS; ^ $p < 0.05$ vs. MG63; # $p < 0.05$ vs. no rhVEGF-A treatment.

Figure 4.

The response of MG63 cells cultured on TCPS and Ti surfaces in the presence of an Flk-1 neutralizing antibody. (A) Cell Number, (B) Alkaline phosphatase specific activity in the cell lysate, (C) osteocalcin, (D) osteoprotegerin, (E) VEGF-A, (F) FGF-2, and (G) Angiopoietin-1 levels in the conditioned media of MG63 cells cultured in the presence of 100 ng/mL of an Flk-1 or control IgG antibody were determined. Cells were cultured on control (TCPS), PT, SLA, and modSLA Ti surfaces. Values presented are mean $\pm$ SEM of six independent cultures. The data presented are from one of two separate experiments with comparable results. Data were analyzed using ANOVA, and statistical significance between groups was determined using Bonferroni's modification of Student's t-test. * $p < 0.05$ vs. TCPS.

Figure 5.

MG63 and VEGF-A silenced cell response to treatment with exogenous rhFGF-2. (A) Cell number, (B) alkaline phosphatase specific activity in the cell lysate, (C) osteocalcin, (D) osteoprotegerin, (E) VEGF-A, and (F) Angiopoietin-1 levels were determined for both MG63, and VEGF-A silenced MG63 cells as well as MG63 and VEGF-A silenced MG63 cells treated with 10 ng/mL of rhFGF-2. Values presented are mean $\pm$ SEM of six independent cultures. Data were analyzed using ANOVA, and statistical significance between groups was determined using Bonferroni's modification of Student's t-test. * $p < 0.05$ vs. TCPS; $\Delta p < 0.05$ vs. MG63; # $p < 0.05$ vs. no rhFGF-2 treatment.