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Role of integrin subunits in mesenchymal stem cell differentiation and osteoblast maturation on graphitic carbon-coated microstructured surfaces

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Surface roughness, topography, chemistry, and energy promote osteoblast differentiation and increase osteogenic local factor production *in vitro* and bone-to-implant contact *in vivo*, but the mechanisms involved are not well understood. Knockdown of integrin heterodimer alpha2beta1 (α2β1) blocks the osteogenic effects of the surface, suggesting signaling by this integrin homodimer is required. The purpose of the present study was to separate effects of surface chemistry and surface structure on integrin expression by coating smooth or rough titanium (Ti) substrates with graphitic carbon, retaining surface morphology but altering surface chemistry. Ti surfaces (smooth [R_a < 0.4 μm], rough [$R_a \geq$ 3.4 μm]) were sputter-coated using a magnetron sputtering system with an ultrapure graphite target, producing a graphitic carbon thin film. Human mesenchymal stem cells and MG63 osteoblast-like cells had higher mRNA for integrin subunits α1, α2, αv, and β1 on rough surfaces in comparison to smooth, and integrin αv on graphitic-carbon-coated rough surfaces in comparison to Ti. Osteogenic differentiation was greater on rough surfaces in comparison to smooth, regardless of chemistry. Silencing integrins β1, α1, or α2 decreased osteoblast maturation on rough surfaces independent of surface chemistry. Silencing integrin αv decreased maturation only on graphitic carbon-coated surfaces, not on Ti. These results suggest a major role of the integrin β1 subunit in roughness recognition, and that integrin alpha subunits play a major role in surface chemistry recognition.

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1. Introduction

Surface characteristics determine which biological molecules adsorb to an implant surface and how these molecules interact with cells in the host tissue [1]. Cells interact with proteins adsorbed on the implant surface via integrins [2], which are transmembrane heterodimeric receptors consisting of non-covalently associated alpha (α) and beta (β) subunits [3]. Integrins function as interface between the extra- and intra-cellular compartments and, depending on the α/β heterodimer, trigger specific signaling cascades. The composition of the α/β heterodimer results in multiple specific receptors that can recognize extracellular matrix components like collagens, laminins, fibronectin, vitronectin, thrombospondin, osteopontin, and tenascin. Thus, external ligands can modulate cell attachment, adhesion, spreading, migration, and differentiation via integrin signaling in very specific ways [3].

Integrin signaling also plays a role in mediating cell attachment, spreading, proliferation, and differentiation on several biomaterials used in dental and orthopedic implants [4–6]. Previously we showed that osteoblastic cells grown on microstructured Ti or Ti alloys regulated integrin expression depending on surface roughness [7–9]. Cells on rougher surfaces expressed a different integrin profile than cells on smoother substrates, notably exhibiting increased levels of α1, α2, αv, and β1. Additionally, knockdown of integrin α2 and integrin β1 abolished terminal differentiation and

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the production of an osteogenic environment on the rough surface, indicating that signaling via α2β1 was required [7].

Other studies have shown that surface chemistry is an important variable in determining cell response to surface microarchitecture and surface chemistry [10,11]. A number of approaches have been used to modify surface chemistry, including thin films [12,13], self-assembled monolayers [14–16], and various kinds of attachment ligands [17–20]. While these studies have provided information on cell response to the functionalized surface, they have not clarified the respective roles of surface microarchitecture and surface chemistry. To address this question more accurately, we created a nanometric surface modification that does not affect surface microstructure but completely alters surface chemistry to elucidate the role of surface chemistry on integrin-mediated osteoblast terminal differentiation.

2. Materials and methods

2.1. Disk preparation

Ti disks 15 mm in diameter were punched from 1 mm thick sheets of grade 2 unalloyed Ti (Institut Straumann AG, Basel, Switzerland) to fit snugly in a 24-well plate. Disks were prepared as described previously and sterilized overnight with 25-kGy gamma irradiation [21]. Briefly, disks were degreased in acetone and processed for 30 s in a 2% ammonium fluoride/2% hydrofluoric acid/10% nitric acid solution at 55 °C to produce pretreatment Ti disks (PT). SLA substrates were produced by grit blasting with 0.25–0.50 mm corundum grit at 5 bar followed by acid etching in HCl/H_2SO_4.

2.2. Graphitic carbon coatings

Amorphous graphitic carbon films were produced by a DC-magnetron sputtering system attached to a high vacuum chamber (base pressure 1.3×10^{-4} Pa) using a 4-inch diameter high purity graphite cathode. The deposition was carried out at 4 Pa of pressure using argon as the sputtering gas (20 standard cubic centimeters per minute) and 0.4 A of DC current for 5 min.

2.3. Surface characterization

Surface morphology of PT and SLA surfaces with carbon films was examined by scanning electron microscopy (SEM, Carl Zeiss SMT Ltd., Cambridge, UK). Surface roughness was measured by confocal laser microscopy (CLM, Olympus America Inc., PA), using LEXT OLS4000 software. Surface chemistry was measured by Thermo K-Alpha X-ray photoelectron spectroscopy (XPS, Thermo Fisher Scientific Inc., MA), equipped with the Thermo Advantage 4.43 software package. Contact angle measurement (Ramé-Hart goniometer, 250-F1, NJ, USA) with DROPimage software analysis package was used to measure surface energy.

2.4. Cell culture methods

Human osteoblast-like MG63 cells (American Type Culture Collection, Manassas, VA) were seeded at an initial density of 10,000 cells/cm^2 and cultured in Dulbecco's modification of Eagle's medium (DMEM, Mediatech, Inc., Manassas, VA) supplemented with 10% fetal bovine serum (Life Technologies, Carlsbad, CA) and 1% penicillin-streptomycin (Life Technologies). Human bone marrow mesenchymal stem cells (MSCs, Lonza, Walkersville, MD) were plated at 5000 cells/cm^2 and cultured in Mesenchymal Stem Cell Growth Medium (Lonza). All cells were cultured at 37 °C with 5% CO_2 and 100% humidity.

2.5. Cell attachment

MG63 cells were plated at a density of 20,000 cells per well on tissue culture polystyrene (TCPS), PT, graphitic carbon-coated PT (G-PT), SLA, or graphitic carbon-coated SLA (G-SLA). After 4 h, non-adherent cells were aspirated. Attached cells were fixed in 10% neutral buffered formalin for 10 min. Cells were incubated with 1 μg/mL Hoechst 33342 in PBS for 10 min. Cells were rinsed twice with PBS and fluorescence measured.

2.6. Protein production

MG63 cells or human MSCs were plated on tissue culture polystyrene (TCPS), PT, graphitic carbon-coated PT (G-PT), SLA, or graphitic carbon-coated SLA (G-SLA) using the seeding densities and culture methods as described above. When cultures reached confluence on TCPS, cells were incubated with fresh medium for 24 h. At harvest, conditioned medium was collected. Osteocalcin levels were measured by radioimmunoassay following manufacturer's instructions (Biomedical Technologies Inc., Stoughton, MA). Levels of osteoprotegerin (OPG), vascular endothelial growth factor-A (VEGF-A), active transforming growth factor beta 1 (TGF-β1), and latent TGF-β1 were measured by commercially available ELISA per manufacturer's instructions (R&D Systems, Minneapolis, MN). Cells were released from the surface by two sequential 10 min incubations in 0.25% trypsin-EDTA (Life Technologies) and cell number determined using a Z2 Cell and Particle Counter (Beckman Coulter, Brea, CA). Cells were then lysed and alkaline phosphatase specific activity measured [22] in the cell lysate and normalized to total protein content (Pierce BCA Protein Assay, Rockford, IL).

2.7. Gene expression

MG63 cells or human MSCs were plated on TCPS, PT, G-PT, SLA, or G-SLA using the cell densities and culture methods described in "Cell Culture Methods". When cultures reached confluence on TCPS, cells on all surfaces were incubated with fresh medium for 12 h. At harvest, total RNA was extracted using TRIzol® (Life Technologies, Carlsbad, CA). RNA quantity was determined using a NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA). cDNA templates were created by reverse transcribing 250 ng of RNA (High Capacity Reverse Transcription cDNA kit, Life Technologies). The resulting cDNA was used for real-time qPCR with gene-specific primers using the StepOnePlus Real-time PCR System and *Power* Sybr® Green Master Mix (Life Technologies). Fluorescence values were quantified as starting quantities using known dilutions of MG63 cells or MSCs cultured on TCPS. Gene expression is presented as normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Primers for integrin subunits (Table 1) were designed using Beacon designer software (Palo Alto, CA) and synthesized by Eurofins MWG Operon (Huntsville, AL).

2.8. Integrin silencing

MG63 cells were transduced with shRNA lentiviral transduction particles (Mission®, Sigma Aldrich, St. Louis, MO) targeting integrin α1 (ITGA1, TRCN0000057748), integrin α2 (ITGA2, TRCN0000308081), integrin αv (ITGAV, TRCN0000003240), or integrin β1 (ITGB1, TRCN0000029644). MG63 cells were plated at 20,000 cells/cm^2 and cultured overnight. Particles were added to the cells at a multiplicity of infection of 7.5. After 18 h incubation, transduced cells were selected with 0.25 μg/ml of puromycin. Silencing was confirmed using real-time qPCR as described above. MG63 and integrin silenced MG63 cells were plated and protein secretion analyzed as described above.

2.9. Statistics

All data presented are mean ± standard error of $n = 6$ independent cultures per variable. Data were examined by ANOVA with post-hoc Bonferroni's modification of Student's t-test. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Surface characterization

Substrate surface morphology was retained after film deposition due to the conformational growth of the amorphous films. Both SEM (Fig. 1A) and AFM (Fig. 1B) images showed that the films follow the same topography, with only insignificant variations in average surface roughness, confirming that chemical modification of the surface was achieved without variations in topography. Under the deposition conditions used, graphitic-carbon films were

Table 1
Primer sequences used for real-time qPCR analysis of gene expression.

Gene	Primer sequence	
GAPDH	F	GCTCTCCAGAACATCATCC
	R	TGCTTCACCACCTTCTTG
ITGA1	F	CACTCGTAAATGCCAAGAAAAG
	R	TAGAACCCAACACAAAGATGC
ITGA2	F	ACTGTTCAAGGAGGAGAC
	R	GGTCAAAGGCTTGTTTAGG
ITGA5	F	ATCTGTGTGCCTGACCTG
	R	AAGTTCCCTGGGTGTCTG
ITGAV	F	GTTGCTACTGGCTGTTTTGG
	R	CTGCTCCCTTTCTTGTTCTTC
ITGB1	F	ATTACTCAGATCCAACCAC
	R	TCCTCCTCATTTCATTCATC
ITGB3	F	AATGCCACCTGCCTCAAC
	R	GCTCACCGTGTCTCCAATC
ITGA6	F	CCCACATCACAAGACTATGC
	R	GAAACAGGAAAAGACGGTAGG
ITGA8	F	CTTCTTGGATTGTTGGTTCTCG
	R	TCAGGGGTCTTGTCATTTGTC

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