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The significance of differential expression of genes and proteins in human primary cells caused by microgrooved biomaterial substrata

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ABSTRACT

We demonstrate that etched microgrooves, with truncated V-shape in cross-section and subsequent acid etching, on titanium substrata alter the expression of various genes and proteins in human primary cells. Etched microgrooves with 30 or 60 μm width and 10 μm depth promoted human gingival fibroblast proliferation and significantly enhanced the osteoblast differentiation of human bone marrow-derived mesenchymal stem cells and human periodontal ligament cells by inducing differential expression of various genes involved in cell adhesion, migration, proliferation, mitosis, cytoskeletal reorganization, translation initiation, vesicular trafficking, proton transportation, transforming growth factor-β signaling, mitogen-activated protein kinase signaling, simvastatin's anabolic effect on bone, inhibitory guanine nucleotide binding protein (G protein)'s action, sumoylation pathway, survival/apoptosis, mitochondrial distribution, type I collagen production, osteoblast differentiation, and bone remodeling that were verified by the differential display PCR and quantitative real-time PCR. The most influential genes on the enhancement of fibroblast proliferation or osteoblast differentiation were determined by multiple regression analysis, and the expression of relevant proteins was confirmed by western blotting and protein quantitation.

1. Introduction

The connective tissue barrier, part of the soft tissue around the transmucosal area of oral implant or implant abutment surface, plays an important role in maintaining a proper seal between the oral environment and the peri-implant bone [1]. Soft tissue integration forms a strong barrier and may act as one of the factors that determine the clinical success of an oral implant [2]. *In vivo* examination of the soft tissue behavior around titanium (Ti) oral implants is problematic due to biological instabilities and technical difficulties; therefore, *in vitro* experiments are typically performed in order to examine the peri-implant soft tissue reaction [3]. Investigation of the effect of Ti surface microgrooves on *in vitro* cell behavior provides a model for determining how the peri-implant soft tissue reaction can be improved. By inducing contact guidance, Ti surface microgrooves of less than 10 μm in width alter both cell shape and gene expression of cultured fibroblasts [4]; but they do not enhance cell proliferation [5]. Alternatively, microgrooves exceeding 10 μm in width have also been reported to induce contact guidance [6], and these microgrooves, in combination with submicroscale acid-etched roughness and acid-etched ridges (hereafter referred to as etched microgrooves and ridges), enhance human gingival fibroblast (HGF) proliferation [7,8].

Previous studies comparing bone nodule formation on microgrooved Ti substrata were performed using differentiated osteoblasts obtained from rat calvarias. Bone nodule comparisons in these studies were performed between Ti-coated smooth silicone and Ti-coated silicone with micromachined grooves [9,10], and Ti-coated silicone with micromachined grooves and hydroxylapatite-coated silicone with micromachined grooves [11]. In our recent previous study, MG63 human osteoblast-like cells were used to investigate osteogenic maturation on microgrooved Ti surfaces [12]. Thus, the osteogenic response of cells on microgrooved Ti surfaces has only been described in cells already committed to become differentiated osteoblasts. However, osteoprogenitor cells respond and sense topographical cues better than mature cell types [13]. Therefore, it may be more relevant to use undifferentiated cells, rather than differentiated osteoblasts for determining the specific cell response to various biomaterial surfaces. Multipotent bone marrow-derived mesenchymal stem cells (MSCs) are the

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precursors to all bone forming osteoblastic cells, including osteoprogenitors, preosteoblasts, immature osteoblasts, and mature osteoblasts. Their ability to undergo osteoblastic or osteoblast differentiation is an essential component of osseointegration at the bone-implant surface, and this ability is important when investigating bone-implant surface interactions. Human periodontal ligament cells (PLCs), a progenitor cell type that has characteristics similar to stem cells, can also differentiate into specialized cells such as osteoblasts and chondrocytes [14]. Like undifferentiated MSCs, canonical Wnt/β-catenin signaling is an important stimulator of osteoblast differentiation and collateral activation of osteogenic transcription factors in PLCs [15]. PLCs rapidly proliferate and display a high level of osteoblast differentiation on rough Ti surfaces [16]. PLCs also regenerate alveolar bone at similar levels compared to MSCs [17] and may be an effective replacement to MSCs for various cell researches in the field of oral implantology.

The discovery that increased fibronectin mRNA expression occurs on titanium (Ti)-coated microgrooved silicone wafers versus smooth Ti [18] prompted subsequent research into the effect of biomaterial surface microgrooves on cultured cell gene expression. Microgrooved quartz slides strongly induce human fibroblasts to express genes involved in cell signaling, DNA transcription, RNA-protein translation, and extracellular matrix formation after 24 h in culture; however, after 5 days in culture, these same processes are inhibited [19]. Etched microgrooves and ridges on Ti surfaces upregulate the expression of genes involved in human fibroblast cell-matrix adhesion and adhesion-dependent cell cycle progression [7], which ultimately leads to enhanced cell adhesion and proliferation [8]. In addition, gene expression profiling of human osteoblastic cells cultured on a polymethylmethacrylate (PMMA) surface containing periodic surface microgrooves using microarray analysis and signaling pathway analyses revealed significant alterations in gene expression [20]. In the study, 100-μm wide and 330-nm deep microgrooves induced upregulation of genes involved in integrin signaling pathways such as α-actinin, Rho, receptor tyrosine kinase (RTK), and phospholipase Cγ (PLCγ) compared to cells on a planar PMMA surface, whereas 30-μm wide and 330-nm deep microgrooves induced non-altered or reduced expression levels of these genes [20]. Skeletal stem cells cultured on PMMA containing 100-μm wide microgrooves upregulate expression of extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) signaling genes such as RTK, PLCγ, and protein kinase C (PKC), as well as an osteospecific transcription factor, E twenty six (Ets), and signal transduction and activator of transcription 1/3 (Stat1/3) [21]. Alternatively, smaller 30-μm wide microgrooves are associated with a downregulation of similar ERK/MAPK signaling genes and an upregulation of an adipospecific transcription factor, proliferator-activated receptor γ (PPARγ) [21]. Although individual genes have been studied, results from large-scale gene expression studies in human primary cells in osteogenic culture on Ti surfaces with etched microgrooves and ridges have not been reported. Such a study is necessary to obtain a better understanding of how these surfaces affect cells in osteogenic culture, and for identifying the genes regulated by these surfaces.

In this study, we determined the effect of Ti surface etched microgrooves and ridges on cell proliferation, osteoblast differentiation, and gene and protein expression in human primary cells. We hypothesized that some of the differential gene expression caused by this surface topography would significantly influence cell proliferation and osteoblast differentiation. Thus, the purpose of this study was two-fold: 1) to verify the effect of Ti surface etched microgrooves and ridges on human primary cell response-related gene expression and 2) to determine the influential gene expression on the cell proliferation and osteoblast differentiation caused by the corresponding substrata. To address our hypothesis and purposes, we first analyzed HGF proliferation and osteoblast differentiation of MSCs and PLCs on the Ti substrata at various microgroove widths and depths. Following a large-scale gene expression analysis using the differential display PCR to determine whether etched microgrooves and ridges induced altered gene expression, we confirmed the results by quantitative real-time PCR. Expression of the influential genes on cell responses determined by multiple regression analysis was confirmed by the western blotting and protein quantitation.

2. Materials and methods

2.1. Fabrication of titanium substrata

Commercially pure Ti sheets (0.14-mm-thick, grade-2; TSM-TECH Co. Ltd., Ulsan, Korea) were washed and dried in acetone, buffered with emery powder compounds, and ground using a cloth wheel (Yougar Enterprise Inc., Incheon, Korea) at 1800 rpm on an angle grinder (GWS 20–230; Robert Bosch GmbH, Stuttgart, Germany). The ground Ti with a Ra $\leq$ 0.1 μm was used as a control substratum (NE0). The NE0 control was then acid-etched using 1% hydrofluoric acid (HF) for 10 s to be used as another control (E0). Truncated V-shaped surface microgrooves, with their widths identical to the ridge widths, were created on the NE0 control by photolithography as previously described [22]. An acid-etching process that generated etched microgrooves and ridges followed this process. Ti substrata with a combination of etched microgrooves 15, 30, and 60 μm wide, and 3.5 and 10 μm deep were prepared and used as the experimental substrata: E15/3.5, E30/10, and E60/10 (Table 1). In all experiments, fabricated Ti substrata were cleaned three times in an ultrasonic device with sterile distilled water for 30 min, washed another three times using distilled water, and dried at room temperature overnight before use.

2.2. Scanning electron microscopy

The surfaces of the fabricated Ti substrata were imaged with a scanning electron microscopic (SEM) (JSM-6700F, JEOL, Tokyo, Japan) (Fig. 1).

2.3. Cell culture

Healthy gingival tissues were acquired from patients undergoing oral surgery for the removal of impacted wisdom teeth at the Department of Oral and Maxillofacial Surgery according to informed consent guidelines as prescribed in an approved Institutional Review Board protocol of Kyung Hee University Hospital at Gangdong. HGFs were cultured from the tissues as previously described [22]. MSCs were purchased from Lonza (Lonza Inc., Walkersville, MD, USA) and grown in MSC-specific growth medium (MSCGM™; Lonza Inc.) at 37 °C under 5% CO_2. PLCs were acquired from periodontal ligament tissues of the root surfaces of freshly extracted bicuspid teeth in patients undergoing orthodontic treatment at the Department of Orthodontics, following informed consent guideline protocol approved by the Institutional Review Board at Kyung Hee University Hospital in Gangdong. Following extraction, the teeth were placed in Dulbecco's Modified Eagle Medium (DMEM; WelGene, Daegu, Korea) containing 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) and 1% antibiotic/antimycotic solution (Invitrogen). Periodontal ligament tissue was obtained from the middle third of the bicuspid root using a #15 surgical blade. Tissues were treated with 1.1 units/ml dispase (Invitrogen) and 264 units/ml collagenase (Invitrogen) at 37 °C under 5% CO_2 for 1 h. The tissue samples were then washed with DMEM (WelGene) and cultured in DMEM (WelGene) containing 10% FBS (Invitrogen) and 1% antibiotic/antimycotic solution (Invitrogen). The cells at passages from 3 to 5 were used in this study.

Table 1
Fabrication of titanium (Ti) substrata with surface microgrooves and subsequent acid etching.

	NE0	E0	E15/3.5	E30/10	E60/10
Groove width	0	0	15	30	60
Groove depth	0	0	3.5	10	10
Bottom width	0	0	8	10	40
Subsequent acid-etching	non-etched	acid-etched	acid-etched	acid-etched	acid-etched

NE0, ground Ti; E0, NE0 with subsequent acid etching; Eα/β, Ti substrata with surface microgrooves of α μm width and β μm depth and with subsequent acid etching.