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The enhanced integrin-mediated cell attachment and osteogenic gene expression on atmospheric pressure plasma jet treated micro-structured titanium surfaces

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ABSTRACT

The previous study has shown that the treatment of nitrogen-based non-thermal atmospheric pressure plasma jet (NTAPPJ) on titanium (Ti) surface resulted in promoted cell attachment. Nonetheless, essential understanding on enhanced cell–material interaction with NTAPPJ-treated surface and consequent differentiation of cell on the biomaterial is insufficient. Therefore in this study, effects of NTAPPJ on integrins, that play a fundamental role in regulating the activation of signaling pathways as well as adhesion of cell–material interactions, have been investigated. The Ti samples were exposed to NTAPPJ, where control samples were unexposed to NTAPPJ. The formations of chemical function groups on surface of Ti samples were investigated with X-ray photoelectron spectroscopy. Human plasma fibronectin (FN) adsorption was analyzed by protein assay kit and spectrophotometer. Also, for the investigation of specific gene expression of integrin subunits and osteoblast phenotype genes expression of MC3T3-E1 cells, quantitative PCR was carried out. The results showed that FN adsorption and gene expression levels of integrin subunits α_5, β_1 and β_3, and osteocalcin were significantly higher with cells cultured on NTAPPJ-treated samples. Therefore it was concluded that the cell differentiation along with the adhesion between cells and Ti biomaterials were promoted with NTAPPJ due to the improved integrin activation following the improved protein adsorption. Such results are expected to provide better understanding in application of NTAPPJ on biomaterials.

1. Introduction

Surface chemistry and topography of biomaterials affect protein adsorption and cellular response such as adhesion, cell cycle and gene expression [1–3]. A number of surface functional groups (hydroxyl groups: –OH, amine groups: $-NH_2$ and carboxyl groups: –COOH) are known to modulate affinity for proteins including fibronectin (FN) [4] which is the one of human blood serum and familiar to integrin $\alpha_5\beta_1$ and $\alpha_v\beta_3$, that regulate a cell adhesion, proliferation and differentiation [5].

Integrins are main receptors of cell surface and heterodimeric proteins of two subunits (α and β). Extracellular membrane (ECM) protein binding through extracellular domains of integrin is associated with focal adhesion formation and initiation of intracellular signaling such as ERK and MAPK [6]. These functions of integrin play an important role between cell and biomaterials.

Recently, numerous studies on the effects of non-thermal atmospheric pressure plasma jet (NTAPPJ)-treated biomaterial surface on cellular activity have been reported [7,8]. Nonetheless, the mechanisms for such effects on cell with plasma treated surface remain poorly understood. We previously showed that the NTAPPJ is capable of creating functional groups such as –OH, $-NH_2$ and –COOH on micro-structured titanium (Ti) surfaces without any change in unique topography. Also, it was shown that the nitrogen based-NTAPPJ treatment on Ti promotes preosteoblasts attachment and proliferation [7].

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In the present work, we elucidated the mechanism for increased cellular attachment and differentiation on the NTAPPJ-treated Ti through formation of chemical functional groups that consequently resulted in FN adsorption and integrin activation.

2. Materials and methods

2.1. Sample preparation and non-thermal atmospheric pressure plasma jet (NTAPPJ) treatment

Micro-structured Ti disks were prepared with sandblasted, large-grit and acid-etched (SLA) method according to the previous study (12 mm diameter and 1 mm thickness, commercially pure Ti, grade IV) [7]. Prior to the experiment, SLA-treated disks were sterilized with an ethylene oxide gas. Samples were treated with nitrogen-based NTAPPJ (5000 ccm, ~2.2 kV, ~1.0 mA, Plasma Bioscience Research Center, Kwangwoon University, Korea) [9] for 0, 0.5, 2 and 10 min which samples were referred to NP, P0.5, P2 and P10, respectively.

2.2. Cell culture

The murine osteoblast cell line of MC3T3-E1 was cultured on NP, P0.5, P2 and P10 samples with a culture medium (008-53, Welgene, Korea) containing 10% fetal bovine serum (16000, Gibco, USA) and 1% antibiotic–antimycotic (15240, Gibco, USA). The cells were cultured under a constant humidified condition of 5% CO_2 at 37 °C where the medium was changed every 2–3 days.

2.3. mRNA isolation and cDNA synthesis

For evaluation of integrins (α_1, α_2, α_5, α_v, β_1 and β_3) and osteogenic gene (osteocalcin and osteopontin) mRNA expression levels, the cells of 1×10^5 cells/cm^2 and 1×10^4 cells/cm^2 were cultured for 2/4/24 h and 7/14 d on samples, respectively. The TRIzol reagent (15596026, Ambion, USA) was used for RNA extraction and cDNA was synthesized using Omniscript RT Kit (205113, Qiagen, USA) according to the manufacturer's protocol. The reverse transcript reaction of mRNA was carried out at 37 °C for 90 min with a thermal cycler (2720, Applied Biosystems, USA).

2.4. Quantitative PCR (qPCR)

To quantify the mRNA expression of integrins, osteocalcin (OCN) and osteopontin (OPN), the qPCR using SYBR green PCR master mix (4367659, Applied Biosystems, USA) on a real-time PCR system (7300, Applied Biosystems, USA) was performed. The primers used in qPCR experiments were listed in Table 1. The housekeeping gene of GAPDH was used as the reference to other gene expression and all qPCR results were elucidated as the fold change of the target gene expression in the experimental groups compared to the control group (NP; no NTAPPJ treatment) with $2^{-\Delta\Delta CT}$ method [10].

2.5. Fibronectin (FN) adsorption assay

Soluble human plasma FN (F2006, Sigma, USA) was allowed to be adsorbed on NP, P0.5, P2 and P10 samples for 2 h, 4 h and 24 h under standard cell incubation environment. After each incubation time, all samples were gently washed twice with phosphate buffer saline (PBS, Gibco, USA) and transferred into new well. The adsorbed FNs on sample surfaces were measured with a protein assay kit (23235, Thermo, USA) at 562 nm using a spectrophotometer (Epoch, BioTek, USA) according to the manufacturer's instruction. The quantitative analysis of FNs in reagent solution was calculated with extrapolation from a standard protein linear slope.

Table 1
Target gene primer sequences used for quantitative PCR analysis.

Mouse gene	Primer sequences	
Integrin α_1	Forward	5′-ACA CTC GGT GAC CTT GTG GAT-3′
	Reverse	5′-ACA ATT CCA GCA ACC ACG CCT-3′
Integrin α_2	Forward	5′-GGA CTG CAG AAC CAC TTC CT-3′
	Reverse	5′-AGC GGC AGA GAT CGA TAC AC-3′
Integrin α_5	Forward	5′-GGC AGA AGG CAG CAA TGG TG-3′
	Reverse	5′-AGG CAT CTG AGG TGG CTG GA-3′
Integrin α_v	Forward	5′-CAT CTT GGC AGT TCT CGC AG-3′
	Reverse	5′-GCG CCA CTT AAG AAG CAC CT-3′
Integrin β_1	Forward	5′-TTA TTG GCC TTG CCT TGC TG-3′
	Reverse	5′-CCG CCT GAG TAG GAT TCA TT-3′
Integrin β_3	Forward	5′-ATG AAT GCG CAG CAC AGA GC-3′
	Reverse	5′-CAG GAA GGC GCG TAA GCA AT-3′
Osteocalcin	Forward	5′-AGG GAG GAT CAA GTC CCG-3′
	Reverse	5′-GAA CAG ACT CCG GCG CTA-3′
Osteopontin	Forward	5′-GAC CAC ATG GAC GAC GAT G-3′
	Reverse	5′-TGG AAC TTG CTT GAC TAT CGA-3′
GAPDH	Forward	5′-CCC TGT TGC TGT AGC CGT A-3′
	Reverse	5′-CCG GTG CTG AGT ATG TCG-3′

GAPDH: glyceraldehydes-3-phosphate dehydrogenase.

2.6. Surface chemical change analysis

The surface chemical change analysis was carried out using the X-ray photoelectron spectroscopy (XPS, K-alpha, Thermo VG, UK) using monochromatic Al (Al Kα line: 1486.6 eV) X-ray sources and a sampling area of 400 μm in diameter for surface chemical composition changing on each sample with/without NTAPPJ treatment. Spectra were recorded with a 200 eV pass energy (step size: 1 eV) for the survey scan and 50 eV pass energy (step size: 0.1 eV) for the C 1s, O 1s and N 1s regions. Binding energies were calibrated against the C 1s binding energy at 284.8 eV.

2.7. Statistical analysis

The statistical differences of the resulting data were analyzed with one-way ANOVA. The * symbol indicated significance of p value less than 0.05 by Tukey's test for a multiple comparison test. The FN adsorption and gene expression experiments were performed in at least four replicates.

3. Results and discussion

3.1. Integrins gene expression

We recently reported that cell attachment and proliferation were stimulated with NTAPPJ treatment on SLA-treated Ti surfaces [7]. Therefore integrins gene expressions were analyzed in this study for the better understanding of why cellular attachment was enhanced by NTAPPJ treatment. For the P2 and/or P10 samples, mRNA expression of integrin α_5, β_1, and β_3 were significantly increased at after 4 h, 2 h, and 4 h and 24 h, respectively ($p < 0.05$, Fig. 1). The expressions of other integrins were also generally increased, though there was no significant difference, possibly due to the small sample size.

The integrins on cell membrane are activated by binding of ECM-derived ligand and the 'activated' integrins begin to aggregate together in cluster forming focal adhesion and associate with the actin cytoskeleton. In fact, the promoted level of vinculin and actin filament as focal adhesion components were already been confirmed in the previous study [7]. These cytoskeleton structures activate discrete signaling pathways (MAPK, JNK and ERK) through accumulation and activation of signaling mediator such as FAK and Src, that can ultimately modulate direct critical cell function including differentiation [5,11,12].