AIP Journal of Applied Physics

Selective fibronectin adsorption against albumin and enhanced stem cell attachment on helium atmospheric pressure glow discharge treated titanium

Inho Han, Barbora Vagaska, Bong Joo Park, Mi Hee Lee, Seung Jin Lee, and Jong-Chul Park

Citation: Journal of Applied Physics **109**, 124701 (2011); doi: 10.1063/1.3599885
View online: http://dx.doi.org/10.1063/1.3599885
View Table of Contents: http://scitation.aip.org/content/aip/journal/jap/109/12?ver=pdfcov
Published by the AIP Publishing

Selective fibronectin adsorption against albumin and enhanced stem cell attachment on helium atmospheric pressure glow discharge treated titanium

Inho Han,[1] Barbora Vagaska,[1,2] Bong Joo Park,[1] Mi Hee Lee,[1,2] Seung Jin Lee,[3] and Jong-Chul Park[1,2,a)]

[1]*Cellbiocontrol Laboratory, Department of Medical Engineering, Yonsei University College of Medicine, Seoul 102-752, Republic of Korea*

[2]*Brain Korea 21 Project for Medical Science, Yonsei University College of Medicine, Seoul 102-752, Republic of Korea*

[3]*Department of Industrial Pharmacy, College of Pharmacy, Ewha Womans University, Seoul 120-750, Republic of Korea*

(Received 26 January 2011; accepted 10 May 2011; published online 23 June 2011)

Successful tissue integration of implanted medical devices depends on appropriate initial cellular response. In this study, the effect of helium atmospheric pressure glow discharge (He-APGD) treatment of titanium on selective protein adsorption and the initial attachment processes and focal adhesion formation of osteoprogenitor cells and stem cells were examined. Titanium disks were treated in a self-designed He-APGD system. Initial attachment of MC3T3-E1 mouse pre-osteoblasts and human mesenchymal stem cells (MSCs) was evaluated by MTT assay and plasma membrane staining followed by morphometric analysis. Fibronectin adsorption was investigated by Enzyme-Linked ImmunoSorbant Assay. MSCs cell attachment to treated and non-treated titanium disks coated with different proteins was verified also in serum-free culture. Organization of actin cytoskeleton and focal adhesions was evaluated microscopically. He-APGD treatment effectively modified the titanium surfaces by creating a super-hydrophilic surface, which promoted selectively higher adsorption of fibronectin, a protein of critical importance for cell/biomaterial interaction. In two different types of cells, the He-APGD treatment enhanced the number of attaching cells as well as their attachment area. Moreover, cells had higher organization of actin cytoskeleton and focal adhesions. Faster acceptance of the material by the progenitor cells in the early phases of tissue integration after the implantation may significantly reduce the overall healing time; therefore, titanium treatment with He-APGD seems to be an effective method of surface modification of titanium for improving its tissue inductive properties. © *2011 American Institute of Physics*. [doi:10.1063/1.3599885]

I. INTRODUCTION

Various biomaterials have been widely used in clinics, especially thanks to their mechanical properties and good biocompatibility. However, modern advanced materials should not only be inert or biocompatible, they need to be specifically designed to be "bioactive" for specific desired cellular responses, like selective protein adsorption, cell attachment, proliferation, and differentiation.[1,2] Except in drug releasing devices, these processes depend mainly on the surface properties of the materials. Therefore, the research has focused on surface modifications to control and enhance the initial response of cells.[3–5] Recently, long-term storage of titanium has been found to significantly decrease its ability of tissue integration, referred to as biological aging.[6] Surface carbon contamination, which affects its surface energy and capacity to bind proteins and, thus, also the following cell response, has been blamed for the decreased activity, and ultraviolet (UV) treatment was employed to eliminate it.[7]

Glow discharge treatments have been widely used for cleansing and surface processing in the microelectronics industry or for sterilization or surface modification in the biomedical field.[8,9] Also, enhanced cellular responses on plasma treated polymers have been well documented,[10–13] but there are very few studies about the plasma treatment of metallic biomaterials.[14–16] In our laboratory, we have constructed a typical planar type dielectric barrier discharge system (helium atmospheric pressure glow discharge, He-APGD), which can effectively modify metal surfaces with a large population of excited species.[17] In this study, we have hypothesized and studied that He-APGD treatment enhances the protein adsorption, such as fibronectin (FN), and so it influences the initial cell attachment through an integrin-mediated/focal adhesion mechanism.[18,19]

II. EXPERIMENTAL SETUP

Titanium samples were prepared from commercially pure grade 2 titanium sheets (CP-2, thickness 0.3 mm) in a shape of disks (diameter 13 or 20 mm). Before the plasma treatment, they were ultra-sonically cleansed consecutively in acetone, ethanol, and de-ionized water for 8 mins and then blow-dried with clean air. He-APGD treatment was performed by a dielectric barrier discharge type home-made plasma unit. The plasma head unit consisted of two alumina plates (thickness 1 mm) as dielectric barriers. The sample was placed between these plates, and the distance between

a)Author to whom correspondence should be addressed. Electronic mail: parkjc@yuhs.ac.

them was set to 1 mm. After sealing the dielectric barriers, helium was introduced into the narrow space between them at a rate of 200 mL/min. Aluminum electrodes were installed on the outer side of the alumina plates, and a high voltage pulse bipolar power supply (HPI500, FT-Lab Co., Korea) was employed as an energy source to fire plasma. The applied voltage, frequency, and duty were 2.0 kV, 20 kHz, and 20%, respectively. Treatment time was set to 3 seconds, 4 mins, and 8 mins, and non-treated disks (0 s) were used as a control. Before all cell experiments, the titanium disks were always sterilized by 30 mins incubation in 70% (v/v) ethanol and then washed twice in distilled water and dried.

Changes in hydrophilicity were probed by spraying deionized water mist onto control titanium and 8 min-He-APGD treated titanium at room temperature. The distance was about 40 cm. The pictures were taken by a Nikon D80 camera equipped with 105 mm extra-low dispersion lens (AF-S Micro Nikkor 105 mm, Nikon, Japan) and 5 times magnification filter (DHG Acromat Macro-200(+5), DHG, Japan). The elapsed time between He-APGD treatment, spraying, and taking pictures of drops was less than 15 mins and 5 mins, respectively.

For the initial attachment studies, 2 different types of osteoblast progenitor cells were used. MC3T3-E1 mouse pre-osteoblast cells (Riken, Japan) were maintained in minimum essential medium α-modification without ascorbic acid supplemented with 10% fetal bovine serum and 1% antibiotic antimycotic solution (α-MEM, all from WelGENE). The primary culture of human mesenchymal stem cells (MSCs, Lonza, USA) was maintained in mesenchymal stem cell Basal Media (MSC Basal Media) supplemented with mesenchymal stem cell growth medium (MSCGM) with Single Quots (Lonza). Cells were incubated at 37 °C in a 5% CO_2 humidified atmosphere incubator. Primary cultures were used up to 10^{th} passage and MC3T3-E1 cells up to 15^{th} passage for all experiments.

Cell attachment was determined at 2 hs after seeding using MTT assay. Briefly, cells were seeded onto titanium disks at the initial density of 3.0×10^4 cells/cm^2 and incubated for 2 hs in a CO_2 atmosphere incubator. The unattached cells were removed by gentle washing with phosphate-buffered saline (PBS). The specimens were then incubated with 3-(4,5-dimethylthiazol-2-yl)-diphenyltetrazolium bromide (MTT, final concentration 0.5 mg/mL, Amresco, Solon, OH, USA) for 2 hs. The formed formazan salts were dissolved in dimethyl sulfoxide (DMSO), and the optical density measured at wavelength of 570 nm by spectrophotometer.

For cell morphometric analysis, the cells were seeded on the titanium disks at the initial seeding density of 5.0×10^3 cells/cm^2 and, after 2 hs of incubation, they were fixed with ice cold 70% ethanol for 15 mins. Cell plasma membrane was visualized by staining with Texas Red C2-maleimide (Texas Red, 30 ng/mL in PBS, Invitrogen, USA). Cell nuclei were counterstained with Hoechst 33 258 (1 μg/mL in PBS, Sigma). Ten random pictures were taken by IX-70 microscope equipped with a DP-71 digital camera (Olympus, Japan). Cell attachment area, perimeter and Feret's diameter of 30 cells for each group were measured by Image J software (NIH, USA).

Specific adsorption of fibronectin was evaluated also by a modified enzyme-linked immunosorbant assay (ELISA) method, as described before.[16] Two protein solutions were used in this experiment: bovine plasma fibronectin (FN) solution (25 μg/mL in PBS, Sigma) or mixed solution of fibronectin and bovine serum albumin (BSA, 1 mg/mL in PBS, Invitrogen, USA) at a mass ratio FN:BSA 1:100, which corresponds to the physiological ratio of these two proteins in blood plasma in order to evaluate the competitive adsorption of these two proteins. He-APGD treated and control disks were placed in a 24-well plate, loaded with 200 μL of protein solution and incubated for 1 h at 37 °C in a 5% CO_2 atmosphere incubator. After 2 washings with PBS, the samples were blocked with 5% (w/v) BSA solution for 1 h at 37 °C and then incubated with monoclonal anti-fibronectin antibody overnight at 4 °C (dilution 1:1000, Takara, Japan) followed by secondary antibody (goat anti-mouse immunoglobulin G conjugated with alkaline phosphatase, dilution 1:2000, Sigma). Colorimetric detection was performed by incubation of the samples with phosphatase substrate, p-nitrophenyl phosphate (pNPP, Sigma), at 37 °C for 30 mins, and the absorbance of the yellow product was measured spectrophotometrically at 405 nm.

In serum free cell culture experiments, the disks were coated with fibronectin (25 μg/mL in PBS) for 1 h at 37 °C and then washed twice in PBS. To prevent unspecific binding, the disks were then blocked with 5% (w/v) BSA (in PBS, 30 mins at 37 °C) and washed again. Disks coated with 5% BSA (in PBS, 1 h at 37 °C) were used as negative controls. Cell adhesion to non-coated disks was also observed. The MSC cells were pretreated with cyclohexamidine 4 hs before cell seeding (20 μg/mL in serum free media, Sigma) and the attachment time was shortened to one hour in order to prevent the effect of matrix proteins secreted by the cells, which would compromise the analysis of cell adhesion to the pre-coated proteins. After detaching the cells by trypsinization and centrifugation, they were resuspended in serum free media and seeded to the prepared disks at density of 2.5×10^4 /cm^2. After the incubation, cell adhesion was determined by MTT assay, as described before.

For immunostaining of cytoskeleton and focal adhesions, the MSCs were seeded at the initial seeding density of 5.0×10^3 cells/cm^2 and, after 3 hs of incubation, they were fixed with 3.7% (v/v) neutral buffered formalin. The cells were then permeabilized with 0.25% (v/v) Triton X-100 solution and incubated with 1% (w/v) BSA solution to block unspecific antibody binding. To visualize the focal adhesions, the cells were then incubated overnight at 4 °C with monoclonal anti-vinculin (dilution 1:60, Santa Cruz Biotechnology, USA). The samples were then incubated for 1 h at room temperature in a solution of secondary antibody, goat anti-mouse immunoglobulin G conjugated with Texas Red (dilution 1:100, Santa Cruz) mixed with Alexa (488)-conjugated phalloidin (5 U/mL, Invitrogen) to visualize actin cytoskeleton. The nucleus was counterstained with Hoechst 33 528, as mentioned above, and the samples were mounted in aqueous mounting medium (Dako Faramounts, Dako North America Inc., CA, USA) and evaluated under fluorescence microscope.

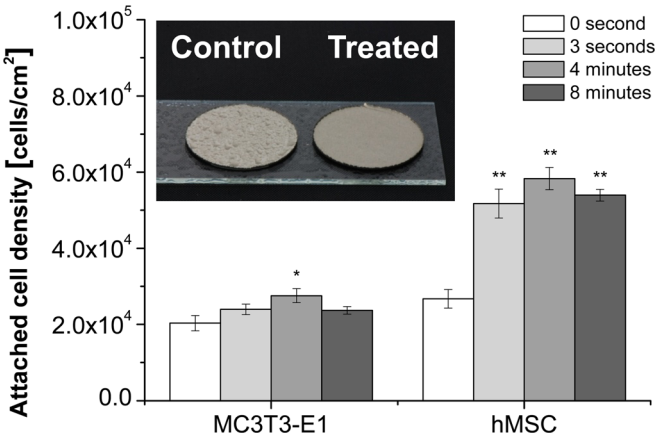

FIG. 1. (Color online) **Initial attachment of osteoblast progenitor cells on titanium with or without He-APGD treatment.** Cell density (cells/cm^2) on the non-treated and He-APGD treated disks was evaluated by MTT assay 2 hs after seeding. The results are shown as mean ± standard error of mean (n = 4). The data were analyzed by Student t-test (*$p < 0.05$, **$p < 0.01$ in comparison with the control, 0 second). Inset: Surface wettability of titanium before and after He-APGD treatment. Left: non-treated titanium; right: 8-minute He-APGD treated titanium.

For western blot analysis of integrin β_1 and focal adhesion kinase (FAK) expression, MSCs were seeded at disks of diameter 20 mm in a 12-well plate at initial density of 1.5×10^5 cells/well. After 24 h incubation in a CO_2 incubator, the cells were rinsed twice in ice cold PBS and lysed with Radio-Immunoprecipitation Assay lysis buffer. The proteins were separated by electrophoresis and then transferred to polyvinylidene fluoride membranes. After blocking the membranes in 5% skim milk (Difco Laboratories, BD, France), they were incubated overnight at 4 °C with mouse monoclonal anti-integrin β_1 (dilution 1:100, Santa Cruz) or mouse monoclonal anti-FAK antibody (dilution 1:100, Santa Cruz). Finally, the membranes were incubated with a secondary antibody (goat anti-mouse immunoglobulin G conjugated with horse radish peroxidase, dilution 1:2000, Santa Cruz). Chemiluminescent detection of membranes was performed using the chemiluminescence detection kit and high-performance chemiluminescence film (Amersham, GE Healthcare Limited, UK).

All data are expressed as mean ± SEM of 4 samples (3 samples for serum-free cell attachment studies) and were statistically analyzed by Student's t-test. Significant differences in comparison with the control samples were considered to exist when $p < 0.05$.

III. RESULTS AND DISCUSSION

Hydrophilicity of the titanium surface after He-APGD treatment was characterized by spraying of de-ionized water mist. Dispersed and agglomerated water drops were formed from the mist on the non-treated titanium surface, while the water drops did not agglomerate but wetted the He-APGD treated titanium surface (8-minutes) completely (inset of Fig. 1).

For the initial attachment studies, 2 different types of osteoblast progenitor cells were used - MC3T3-E1 and human mesenchymal stem cell (MSC). Plasma treatment of titanium increased the number of attached cells in both tested cell types as determined by MTT assay (Fig. 1). The number of attached cells increased by 20 to 35% and more than 100% for the 2 hs attachment assay with MC3T3-E1 cells and MSCs, respectively. Moreover, morphometric analysis of the attached cells has shown that cells growing on plasma treated samples had significantly ($p < 0.05$) higher Feret's diameter, perimeter, and attachment area (Table 1). The initial interaction of cells with the biomaterial is one of the most crucial steps for an early acceptance of the implant.[20] This study has shown that He-APGD treatment of titanium has a substantial positive effect on initial cell attachment and spreading of two different osteoprogenitor cell types. It has not only increased the number of attached cells, but also affected their morphology and promoted faster spreading and better attachment of cells to the plasma treated surface in comparison with the non-treated controls.

Interaction of cells with the titanium surface is mediated by a layer of proteins that adsorbs to the implant surface from plasma or other physiological fluids. This layer mediates integrin-receptor based cell attachment and proliferation and determines the cellular response to the biomaterial.[21,22] It is known that bovine serum albumin (BSA) is a protein present at the largest amounts in blood plasma and, if adsorbed to biomaterials surface, it consumes other protein adsorbable sites and prevents cell adhesion. However, fibronectin (FN), collagens, and other trace proteins (osteopontin, laminin, and vitronectin) are adhesive proteins and ligands for the integrin receptors supporting cell attachment to the surface.[23–25] FN is a soluble protein, and it is well-

TABLE I. **Cell morphometric measurements.** Morphology of cells on titanium disks 2 hours after seeding was visualized by staining the plasma membrane with Texas Red maleimide C2 and nucleus with Hoechst 33 258. Ten random pictures were taken by IX-70 microscope equipped with a DP-71 digital camera (Olympus, Japan). Cell attachment area, perimeter, and Feret's diameter of 30 cells from each group were measured from micrographs by Image J software (NIH, USA). The results are shown as mean ± standard error of mean (n = 30). The data were analyzed by Student t-test (*$p < 0.05$ in comparison with the control, 0 second).

	Attachment area [μm^2]		*Cell perimeter [μm]*		*Feret's diameter [μm^2]*	
He-APGD treatment conditions	MC3T3-E1	hMSC	MC3T3-E1	hMSC	MC3T3-E1	hMSC
0 second	5815.0 ± 399.5	5348.7 ± 367.8	261.2 ± 9.9	289.1 ± 12.1	89.2 ± 2.8	88.8 ± 3.6
3 seconds	9377.7 ± 873.0*	8191.9 ± 582.2*	347.0 ± 15.7*	371.3 ± 15.6*	111.8 ± 4.3*	109.3 ± 3.4*
4 minutes	9641.9 ± 770.1*	8452.1 ± 709.0*	324.0 ± 12.5*	363.4 ± 15.1*	107.5 ± 3.3*	110.0 ± 3.8*
8 minutes	10 796.9 ± 727.6*	9533.0 ± 826.3*	363.7 ± 15.1*	435.1 ± 30.4*	119.6 ± 3.6*	118.3 ± 4.6*

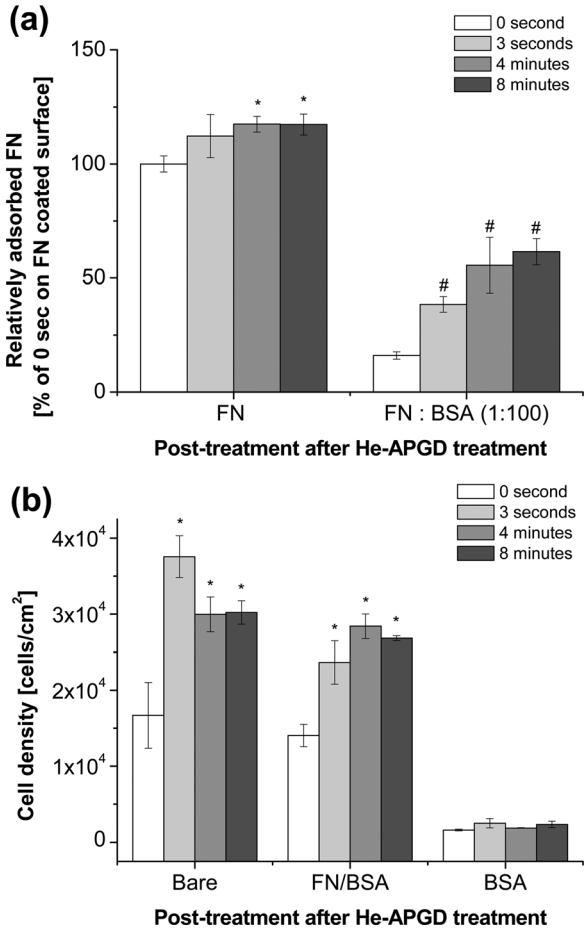

FIG. 2. **Adsorbed protein density on He-APGD treated titanium. a) Competitive adsorption of fibronectin (FN) to He-APGD treated titanium disks.** Adsorption of FN from FN solution or FN/BSA solution mixture (FN:BSA = 1: 100) was determined after 1 h incubation at 37 °C by a modified ELISA method. The results are shown as mean ± standard error of mean (SEM) (n = 4). The data were analyzed by Student t-test (*$p < 0.05$ in comparison with the FN treated condition without He-APGD treatment; # $p < 0.05$ in comparison with FN:BSA mixture treated condition without He-APGD treatment). **b) Attachment of MSC cells to He-APGD treated titanium in serum-free culture.** Cell attachment of cyclohexamidine pretreated MSC cells to FN (25 μg/mL) and BSA (5%) pre-coated or to non-coated He-APGD treated titanium disks within 1 h was determined by MTT assay. The number of attached cells is expressed as cell density (cells/cm^2) on the non-treated (0 second) FN-coated disks. The results are shown as mean ± SEM (n = 3,*$p < 0.05$ in comparison with the control, 0 second by Student t-test).

established that it has a critical role in osteoblast adhesion to biomaterials, their survival, and the following differentiation.[26–28] Therefore, we have examined its adsorption with a modified ELISA method using FN-specific antibodies.

The modified ELISA method[16] showed relative increase 10-16% in FN adsorption from the single protein solution to He-APGD treated disks in comparison with the non-treated control samples in Fig. 2(a). In the following study, we have conducted a competitive FN adsorption experiment from a protein mixture of FN and BSA in a ratio 1:100, which corresponds to the physiological ratio of these two proteins in human blood plasma and so it simulates the real process of protein adsorption more reliably. Presence of BSA in the protein mixture dramatically inhibited FN adsorption to only approximately 20% of the amount on the non-treated disks coated with pure FN. However, FN adsorption increased gradually on the plasma treated specimens with the increasing treatment time, up to a threefold enhancement on 8 min treated samples in Fig. 2(a). Although the effects of He-APGD on titanium could not be elucidated based on the promoted cell attachment and FN adsorption results on He-APGD treated titanium, the plausible effect of He-APGD treatment could be assumed as schematically prepared in Fig. 3. The employed ELISA method using anti-FN antibodies is only a semi-quantitative method. Therefore, it remains unclear whether the enhancement is caused only by the increase in the total amount of FN adsorbed on the titanium, as shown in Fig. 3(b), or also by a conformational change of the FN molecule resulting in a better exposure of the epitopes for antibody binding, as shown in Fig. 3(c). Further FN conformational studies would be needed to confirm this hypothesis, but it can be suggested that the enhanced cell attachment on He-APGD treated titanium is supported by higher density of the cell binding ligands (RGD sequences in FN) as a result of higher FN adsorption and/or potentially also thanks to its more favorable conformation.

In order to examine the mechanism by which cells attach to plasma treated titanium, an attachment assay to FN pre-coated or non-coated samples in serum free culture media was performed. Attachment of MSC cells onto He-APGD treated disks coated with FN just one hour after seeding was significantly ($p < 0.05$) higher than to non-treated disks coated with FN under the same conditions (Fig. 2(b)). There was a twofold increase in cell attachment to 4 and 8 min treated samples. Coating of the disks with albumin prevented cell attachment comparably on all samples, allowing only around 15% attachment of the control sample. The increase in number of attached cells was consistent with the increasing amount of adsorbed FN, and, moreover, the enhancement of cell attachment on He-APGD treated titanium was higher than in normal cell culture media, which supports our hypothesis that the effect of He-APGD on the

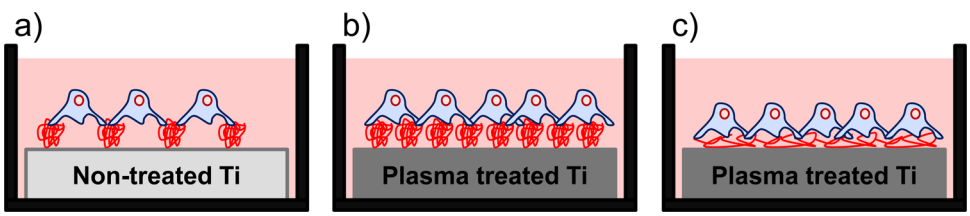

FIG. 3. (Color online) **Schematically prepared plausible effect of He-APGD treatment on FN adsorption and cell attachment.** FN adsorption and cell attachment on non-treated titanium (a) and promoted cell attachment on He-APGD treated titanium caused by increased population of adsorbed FN (b) or conformational change of the adsorbed FN (c).

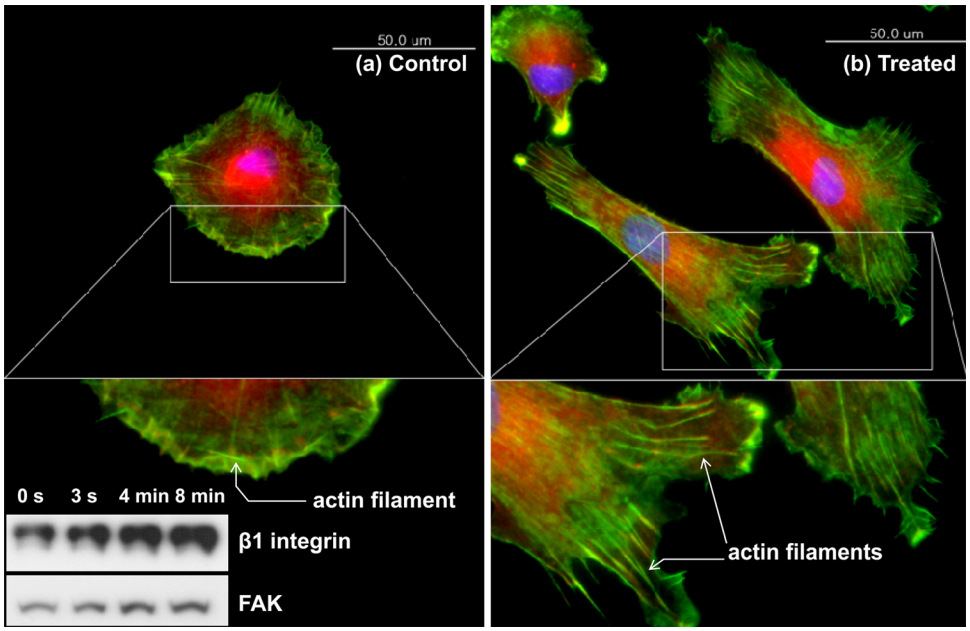

FIG. 4. (Color online) **Focal adhesion formation and actin cytoskeleton organization in MSCs.** Representative fluorescence microscopic images of MSC cells 3 hs after seeding on non-treated (a) and 8 mins He-APGD treated (b) titanium disks. Actin filaments (green and arrowed) were stained with Alexa 488-conjugated phalloidin. Cell plasma membrane (red) was visualized with Texas Red C2-maleimide staining and Cell nuclei (blue) were counter-stained with Hoechst 33 258. Scale bar = 50 μm. Inset: **Western blot analysis of focal adhesion proteins expression in MSCs.** Representative western blot images of β1-integrin and FAK from MSC lysates obtained 24 hs after seeding on non-treated or He-APGD treated titanium disks.

enhanced cell attachment is dependent on the higher adsorption of FN.

Morphological change of cells, such as spreading, is an active process depending on cell adhesion molecules, namely integrin adhesion receptors, which act as the central regulators of cell-biomaterial interaction.[29] After binding their ligands, integrin subunits cluster into supramolecular complexes, focal adhesions. Other proteins, such as cytoskeletal and signaling molecules, are recruited. Thus, activation of integrins affects signal cascades similar to those triggered by growth factor receptors. Focal adhesions provide anchorage signal, and all these structures directly support migration, cell cycle progression, and expression of differentiation-related genes.[30–33]

The extent of focal adhesion formation and cytoskeleton organization is therefore one of the reliable markers of biocompatibility and cellular perception of the material.[34] Actin cytoskeleton organization and focal adhesion formation observations in MSCs 3 hs after cell seeding, as shown in Fig. 4, have revealed that the shape and size of cell body was clearly different on He–APGD treated samples. Cells attached to non-treated titanium surface were smaller, round-shaped, and their cytoskeleton was not well-developed yet. Actin was diffuse in cell cytoplasm and only short, thin fibers could be observed, especially in the cell periphery. Similarly, vinculin staining showed that, even though all cells expressed vinculin, it was diffused evenly in the cell cytoplasm, and only small, not very distinct focal adhesions could be found on the cell periphery. In contrast, cells attached to the plasma treated samples were larger, had elongated shape, and formed filopodia-like projections that were enforced with clear stress fibers. Vinculin-positive focal adhesions were observed, especially at the ends of these actin filaments, in the elongated projections. Western blot analysis of other crucial focal adhesion proteins has also revealed enhancement of their expression by He-APGD treatment, as shown in the inset of Fig. 4. Specifically, expression of β_1 integrin, the major FN-binding integrin subunit, was up regulated in MSCs plated on He-APGD treated titanium disks in a dose dependent manner. Expression of focal adhesion kinase (FAK), the first kinase in the signaling pathway triggered by integrin binding, was enhanced in a similar pattern. These results show that the expression was upregulated on He-APGD treated samples in comparison with control disks, suggesting strong positive signaling from focal adhesions and better attachment of cells to the modified substrate.

IV. CONCLUSIONS

He-APGD treatment of titanium has significantly improved its bioactivity, as shown by the enhanced number of attaching cells, their morphology, and the attachment area. He-APGD treatment creates a superhydrophilic surface which promotes selectively higher adsorption of fibronectin, a protein of critical importance for cell/biomaterial interaction. Therefore, the improved cell response appears to be dependent on the fibronectin-integrin mediated mechanism of cell attachment. Faster acceptance of the material by the progenitor cells in the early phases of osseointegration after the implantation may significantly reduce the overall healing time; therefore, titanium treatment with He-APGD seems to be an effective surface modification method.

ACKNOWLEDGMENTS

This work was supported by a National Research Foundation (NRF) of Korea Grant funded by the Korean Government (MEST) (Grant number 2010-0002089) and was supported by a grant of the Korea Healthcare technology R&D Project, Ministry for Health & Welfare, Republic of Korea (A100834). This article is dedicated to I. Han's lovely wife (Jeehae Choi) and pretty children (Jisu Han and Sangyoon Han).

[1]L. L. Hench and J. M. Polak, Science **295**, 1014 (2002).
[2]T. Albrektsson and C. Johansson, Eur. Spine J. **10**, 96 (2001).

[3]L. L. Guehennec, A. Soueidan, P. Layrolle, and Y. Amouriq, Dent. Mater. **23**, 844 (2007).
[4]Y. W. Lim, S. Y. Kwon, D. H. Sun, H. E. Kim, and Y. S. Kim, Clin. Orthop. Relat. Res. **467**, 2251 (2009).
[5]A. Gupta, M. Dhanraj, and G. Sigavami, Internet J. Dent. Sci. **7**, 1 (2009).
[6]N. Hori, W. Att, T. Ueno, N. Sato, M. Yamada, L. Saruwatari, T. Suzuki, and T. Ogawa, J. Dent. Res. **88**, 663 (2009).
[7]F. Iwasa, N. Hori, T. Ueno, H. Minamikawa, M. Yamada, and T. Ogawa, Biomaterials **31**, 2717 (2010).
[8]F. Poncin-Epaillard and G. Legeay, J. Biomater. Sci., Polym. Ed. **10**, 1005 (2003).
[9]B. O. Aronsson, J. Lausmaa, and B. Kasemo, J. Biomed. Mater. Res. **35**, 49 (1997).
[10]F. Teraoka, M. Nakagawa, and M. Hara, Dent. Mater. J. **5**, 560 (2006).
[11]G. Khang, S. J. Lee, J. H. Lee, Y. S. Kim, and H. B. Lee, Biomed. Mater. Eng. **9**, 179 (1999).
[12]D. Briemi, S. Strametz, K. Schroder, N. M. Meenen, W. Lehmann, W. Linhart, A. Ohl, and J. M. Rueger, J. Mater. Sci.: Mater. Med. **16**, 671 (2005).
[13]I. Han, B. Vagaska, D. H. Lee, B. J. Park, S. W. Shin, J. K. Kim, S. J. Lee, and J. C. Park, "Pre-wetting process with helium atmospheric pressure glow discharge for three-dimensional porous scaffold" Macromol. Res. (to be published).
[14]C. C. Lin, H. C. Cheng, C. F. Huang, C. T. Lin, S. Y. Lee, C. S. Chen, and K. L. Ou, Jpn. J. Appl. Phys. **44**, 8590 (2005).
[15]H. Yamamoto, Y. Shibata, and T. Miyazaki, J. Dent. Res. **84**, 668 (2005).
[16]Y. Shibata, M. Hosaka, H. Kawai, and T. Miyazaki, Int. J. Oral Maxillofac Implants **17**, 771 (2002).
[17]S. Tang and H. S. Choi, J. Phys. Chem. **112**, 4712 (2008).
[18]T. A. Horbett, *Biomaterials Science: An Introduction to Materials in Medicine*, 2nd ed., edited by B. Ratner, A. S. Hoffman, F. J. Schoen, and J. E. Lemons (Elsevier, Amsterdam, 2004).
[19]C. H. Thomas, C. D. McFarland, M. L. Jenkins, A. Rezania, J. G. Steele, and K. E. Healy, J. Biomed. Mater. Res. **37**, 81 (1997).
[20]J. E. Davies, J. Dent. Educ. **67**, 932 (2003).
[21]T. A. Horbett, BMES Bulletin **23**, 5 (1999).
[22]B. Kasemo and J. Lausmaa, Environ. Health Perspect. **102**(Suppl 5), 41 (1994).
[23]A. S. G. Curtis and J. V. Forrester, J. Cell Sci. **71**, 17 (1984).
[24]S. R. Sousa, M. Lamghari, P. Sampaio, P. Moradas-Ferreira, and M. A. Barbosa, J. Biomed. Mater. Res. Part A **84A**, 281 (2008).
[25]Y. Z. Yang, R. Cavin, and J. L. Ong, J. Biomed. Mater. Res. Part A **67**, 344 (2003).
[26]R. K. Globus, S. B. Doty, J. C. Lull, E. Holmuhamedov, M. J. Humphries, and C. H. Damsky, J. Cell Sci. **111**, 1385 (1998).
[27]T. Velling, J. Risteli, K. Wennerberg, D. F. Mosher, and S. Johansson, J. Biol. Chem. **277**, 37377 (2002).
[28]K. Anselme, Biomaterials, **21**, 667 (2000).
[29]R. O. Hynes, Cell, **110**, 673 (2002).
[30]C. F. Lai and S. L. Cheng, J. Bone Miner. Res. **20**, 330 (2005).
[31]J. A. Garcia, Biomaterials, **26**, 7525 (2005).
[32]E. H. J. Danen and A. Sonnenberg, J. Pathol. **200**, 471 (2003).
[33]G. R. Owen, D. O. Meredith and ap I. Gwyn and R. G. Richards, Eur. Cells Mater. **9**, 85 (2005).
[34]B. Geiger, A. Bershadsky, R. Pankov, and K. M. Yamada, Nat. Rev. Mol. Cell Biol. **2**, 793 (2001).