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ORIGINAL ARTICLE

Responses of periodontal ligament stem cells on various titanium surfaces

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OBJECTIVE: Periodontal ligament has been reported to have adult stem cells (PDLSCs) which are responsible to regenerate the alveolar bone tissue after tooth is removed from its socket. Also PDLSCs may be the stem cells responsible for the osseointegration of titanium implants after installing the implant immediately in the fresh extracted socket. Here we tested cellular responses of PDLSCs on the various titanium surfaces to verify this notion.

MATERIALS AND METHODS: Titanium disc were prepared for the different surface textures; smooth machined, blasted with 75 and 125 μm Al_2O_3 particles, and anodized. PDLSCs were cultured on these titanium discs and tested their proliferation and gene expressions of osteocalcin, osteopontin, type I collagen, and GAPDH.

RESULTS: Proliferation of PDLSCs was higher on the rough surface blasted with 75 μm Al_2O_3 particles. Osteocalcin expression was increased on the Al_2O_3 particle treated-surface regardless of its particle size. Type I collagen expression was generally decreased with time in 6 days culture.

CONCLUSIONS: In this experiment, it was shown that cultured PDLSCs proliferate in higher rate on the rough surface especially at the 75 μm Al_2O_3 particle treated surface than other surfaces. Also, osteocalcin was highly expressed on the rough surfaces treated with 75 μm and 125 μm Al_2O_3 particles.

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Keywords: dental implant; periodontal ligament stem cell; titanium surface; osteocalcin

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Introduction

Titanium has been proven to be an effective implant material (Byon *et al*, 2008). Commercially pure titanium and titanium alloy are preferred for dental implants because of their corrosion resistance, biocompatibility, durability and strength (Li *et al*, 2008). In the atmosphere, titanium is spontaneously covered with 1.5–10 nm thickness of an oxide layer, forming titanium oxide or TiO_2 (Branemark *et al*, 1977). Several physiochemical properties of the oxide layer – low electrical conductivity, good thermodynamic stability and low ion-formation tendency in aqueous environments – are expected to be responsible for the excellent biocompatibility of titanium oxide implants (Kim *et al*, 2004).

Morphologic studies of titanium dental implants have shown that the surface roughness and compostion of implant and bone fixation are strongly correlated (Thomas and Cook, 1985). Moreover, osteogenic cells tend to form and attach more readily on the rough surface (Bowers *et al*, 1992; Cochran *et al*, 1994). When the surface of titanium implant is oxidized, its osseointegration capability is improved compared to pure titanium. When a positive voltage is applied to a titanium specimen immersed in an electrolyte, titanium is anodized forming a TiO_2 layer. This layer is porous and firmly adhered to the substrate, which is beneficial for the biological performance of implants (Choi *et al*, 2006).

A stem cell is able to generate many cells through mitotic cell division and differentiate into specialized cells (Shi *et al*, 2005). There are two types of stem cells, embryonic and adult stem cells. Embryonic stem cells are known as pluripotent, can give rise to three germ layer cells in fetus and adult (Park *et al*, 2008). On the other hand, adult stem cells have been shown to differentiate into various but limited types of specialized cells. It is also described that human periodontal ligaments contain stem cells which have potential to differentiate into osteoblasts, chondrocytes and adipocytes, comparable with previously characterized human

bone-marrow stem cells. This study showed that adult periodontal ligament stem cells could be utilized for periodontal ligament and cementum regeneration (Seo *et al*, 2004).

It is the first report about the response of human periodontal ligament stem cells to titanium surface that has been utilized for the dental implants to our best knowledge. The purpose of this study is to examine the response of periodontal ligament stem cells (PDLSCs) to variously treated titanium surfaces by measuring cell proliferation and gene expression pattern for the proteins which were known as key factors related with bone formation and maturation.

Materials and methods

Titanium disc preparation and characterization

Titanium disc were fabricated from a sheet of pure titanium board (Ti: medical grade 2). All samples were air-dried and sterilized with ethylene oxide gas for 24 h.

The experiment was composed of five groups. Group 1 (G1) consisted of 15 smooth machined surface disc with no treatment. Under 5 kgf cm^{-2} of pressure, group 2 (G2) and group 3 (G3) disc were sandblasted with 75 and 125 μm Al_2O_3, respectively. Group 4 (G4) contained 15 disc which were anodized at 300 V in an aqueous electrolytic solution of 0.02 mol l^{-1} calcium glycerophosphate and 0.15 mol l^{-1} calcium acetate. Group 5 (G5), a control group, contained no disc, but cells were directly seeded on culture dishes.

Representative disc from each group were subjected to surface analysis.

SEM analysis

By using a scanning electron microscope (S-800, Hitachi, Tokyo, Japan), the surface microtopography of the titanium disc from each group was examined. A focused electron beam was shone across the sample surface and images of the surface could be studied. The discs were examined at 500 × and 2000 × magnification.

Optical interferometer analysis

An optical interferometer is an instrument that provides numerical values for various surface roughness parameters. Incident flux is divided into reference flux and measurement flux and they are incident reference surface and measuring surface, respectively. Reference flux and measurement flux are combined to develop an interference pattern that represents the optical path difference. The pattern is captured by a CCD camera and analysed by an image process and a method that produces a three-dimensional image. The optical dimensional metrology center (Intek Engineering, Korea) was used in this experiment.

The mean of the absolute values of the surface departures from a mean plane within the sampling area is measured in μm and represented by R_a. The distance between the highest peak and the lowest valley within the sampling area is also measured in μm and represented by R_{max} or R_t. The measure of the symmetry of surface deviations about the mean plane is skewness and represented by R_{sk} (e.g. a positively skewed surface has more peaks than valleys).

X-ray diffraction analysis

X-ray diffraction (XRD) analysis was done in order to identify the surface chemical composition, physical properties and crystallographic structure of the samples. Rotaflex RTP 300RE with a thin film attachment (Rigaku Co., Akishima, Japan) was used on 2-θ mode (2θ: 10°–70°). The X-ray source was Cu-Kα; the tube voltage and current were 30 kV and 20 mA, respectively. The scanning speed was 4° min^{-1}.

Cell culture

Stem cells used in the experiments were collected from the periodontal ligament of a 26-year-old female, following the approved guidelines of the School of Dentistry, Seoul National University Institutional Review Board (IRB No. S-D20080014). Periodontal ligament cells were enzymatically digested for 1 h at 37°C in a solution of 3 mg ml^{-1} type I collagenase (BioBasic Inc., Toronto, ON, Canada) and 4 mg ml^{-1} of dispase (Gibco BRL, Grand Island, NY, USA). Single cell suspensions of periodontal ligament cells were obtained through 70 μm cell strainer. Controls consisted of cells cultured directly on the polystyrene surface of culture dishes. The cells were plated at 10 000 cells $disc^{-1}$ in a α-minimal essential medium (Welgene Inc., Daegu, South Korea) containing 15% fetal calf serum (Welgene Inc., Daegu, South Korea), 100 μmol l^{-1} of 1% L-ascorbic acid (Bio Basic Inc., Ontario, Canada), 2 mmol l^{-1} of 1% L-glutamine (Gibco BRL) and 100 U ml^{-1} of Antibiotic-Antimycotic (Gibco BRL). The cultures were incubated at 37°C in an atmosphere of 100% humidity and 5% CO_2 in the incubator (Forma Series II; Thermo Electron Corporation, Waltham, USA) and the media were changed every third day throughout the experiment.

Cell proliferation

The stem cells were obtained from the culture surface by 1 × Trypsin-EDTA (Gibco BRL) at 37°C for 5 min. Cells were evenly distributed so that each group ($n = 24$) contained 1×10^4 cells. At harvest, P1 cells were obtained from the subculture and the cell proliferation level was counted with a hemocytometer and 0.4% (w/v) trypan blue (Gibco BRL). Four randomly selected disc from each group were used for cell proliferation level, which was determined after 1 day, 3 days and 6 days.

Reverse Transcription PCR

RNA was isolated using RNeasy mini kit (Qiagen, Venlo, The Netherlands). First-strand cDNA was produced with the random hexamers using SuperScript III First-Strand kit (Invitrogen, Carlsbad, CA, USA). Specific primer sets (Table 2) for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), osteocalcin, osteopontin and type I collagen were used. PCR amplification for each primer sets consisting denaturation at 94°C for 2 min, 94°C for 45 s, annealing at 56–58°C for 45 s and an elongation step of 72°C for 45 s was performed for a

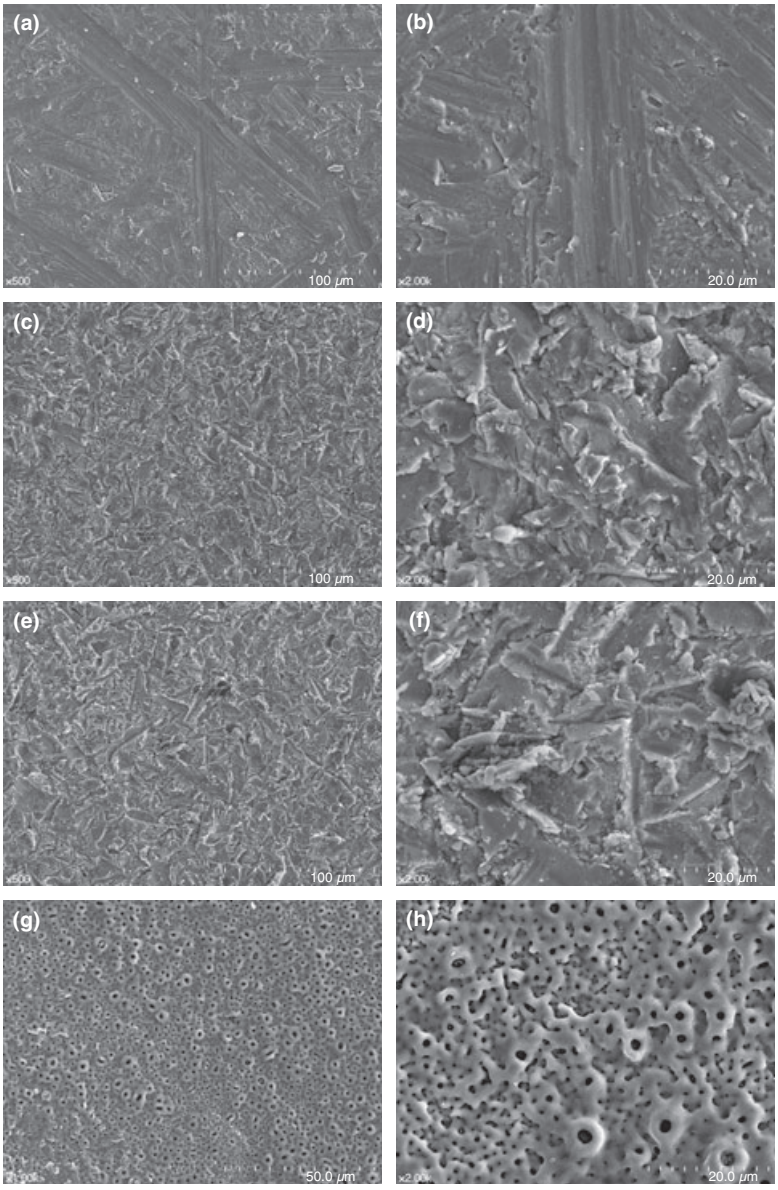

Figure 1 SEM micrographs of the modified titanium disks. (**a, b**): machined surface; (**c, d**): sandblasted with 75 μm aluminum oxide particles; (**e, f**): sandblasted with 125 μm aluminum oxide particles; (**g, h**): 300V anodized surface. Magnifications of 500 (left column) and 2000 (right column)

Table 1 Detorque values before and after 10^6 cycles of loading

Group	R_a (μm) (*mean ± s.d.*)	R_t (μm) (*mean ± s.d.*)	R_{sk} (μm) (*mean ± s.d.*)
Machined	1.091 ± 0.171^{A}	11.239 ± 2.389^{A}	-0.584 ± 0.91^{A}
75 μm blasted	1.132 ± 0.21^{A}	11.159 ± 2.624^{A}	0.38 ± 1.414^{A}
125 μm blasted	1.44 ± 0.172^{B}	10.849 ± 2.341^{A}	0.123 ± 0.777^{A}
Anodized	1.288 ± 0.186^{A}	10.843 ± 2.519^{A}	0.176 ± 1.097^{A}

The same superscripts denote no statistical difference between groups (one-way ANOVA and Scheffe test, A < B at $P < 0.05$).

Table 2 Primer sets

Primers	*Sequence(5′ → 3′)*
GAPDH	F: AGC CGC ATC TTC TTT TGC GTC
	R: TCA TAT TTG GCA GGT TTT TCT
Osteocalcin	F: GTC CAA GCA GGA GGG CAG
	R: TTG AGC TCA CAC ACC TCC C
Type 1 collagen	F: CAA AGA GTC TAC ATG TCT AG
	R: CAT GGG GCC AGG CAC GGA AA
Osteopontin	F: ATG AGA ATT GCA GTG ATT TGC
	R: CCT TTT ATT GAC CTC AGA AGA

total of 35 cycles. The ratios of osteopontin, osteocalcin and type 1 collagen mRNA expressions were compared and standardized by the expression level of housekeeping gene, GAPDH. These expression patterns were compared each experiment time sequence on days 1, 3 and 6.

Statistical analysis

The microtopographical values of the optical interferometer and cell proliferation levels among groups were compared by one-way ANOVA ($\alpha = 0.05$). When statistical differences were detected, Tukey's *post-hoc* test was used.

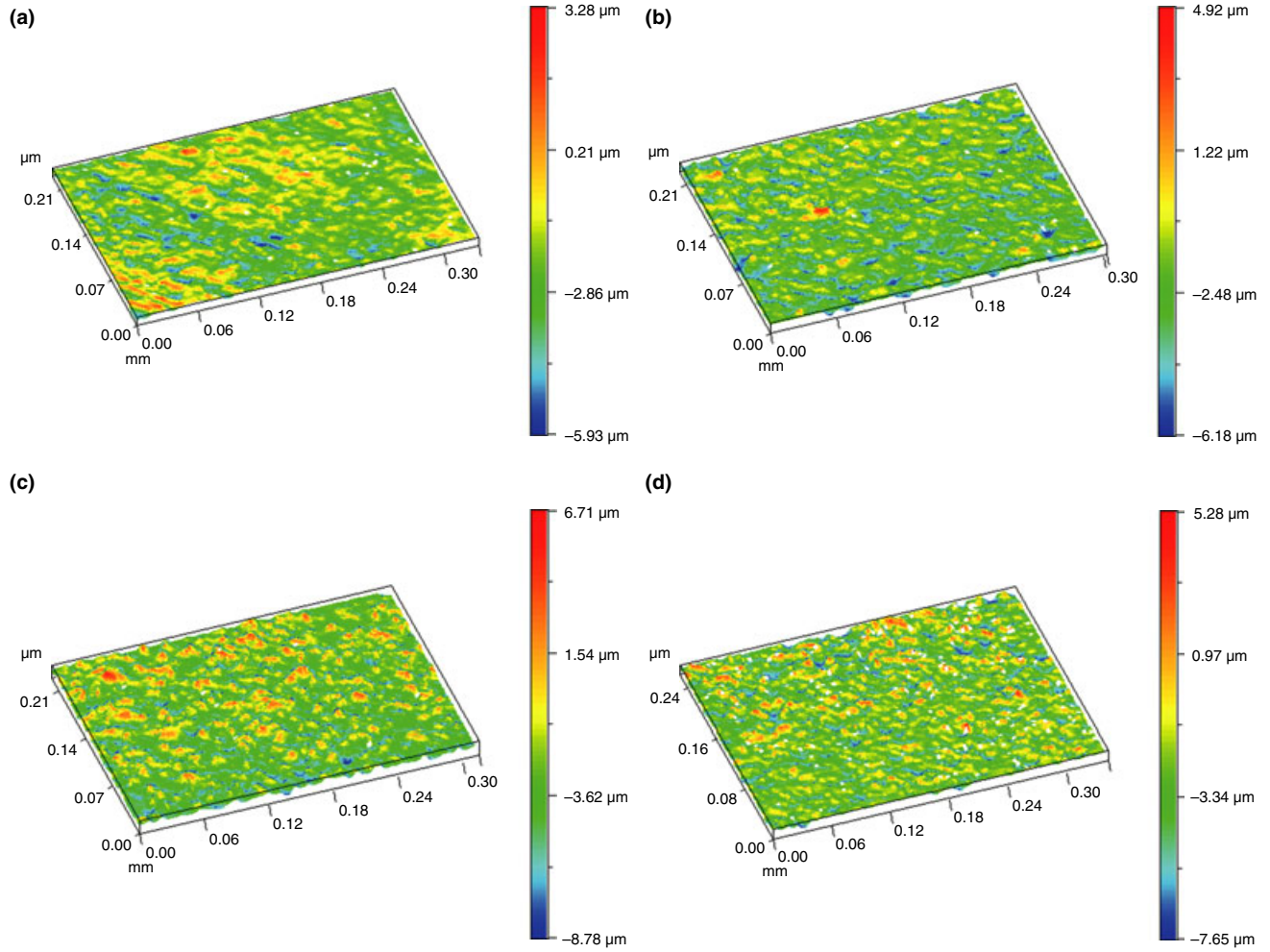

Figure 2 Microtopography of (**a**) the machined surface (highest peak: 3.28 μm, lowest valley: −5.93 μm); (**b**) the 75 μm blasted surface (range: 4.92∼−6.18 μm); (**c**) the 125 μm blasted surface (range: 6.71∼−8.78 μm); and (**d**) the anodized surface (range: 5.28∼−7.65 μm). The unit of *X*- and *Y*-axis is mm and that of Z-axis is μm

Results

On the SEM specimen of machined disc (G1), the machining grooves formed by the cutting machine of the manufacturing company were observed (Figure 1a and b). The surface had many depressions and indentations on 75 μm sandblasted disc (G2), making it very rough and irregular (Figure 1c and d). The 125 μm sandblasted disc (G3) surface seemed similar to that of 75 μm sandblasted disc; however, the depressions were larger in size (Figure 1e and f). On the anodized disc (G4), small pores that are 1–2 μm in size and look like craters were formed due to the anodic oxidation (Figure 1g and h).

Table 1 and Figures 2 and 3 show the results of the optical interferometer analyses. The highest surface roughness was observed on the 125 μm sandblasted disc (G3; R_a = 1.44 ± 0.172 μm). The lowest surface roughness was observed on the machined disc (G1; R_a = 1.091 ± 0.171 μm). The sandblasting procedure increased surface roughness (ANOVA, Tukey $P <$ 0.05). Even though the R_t and R_{sk} values vary, there is no significant difference among four groups.

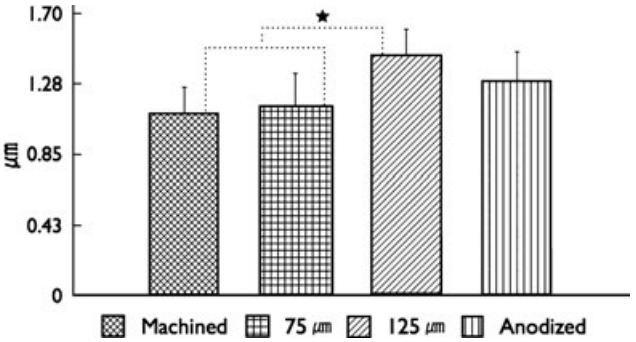

Figure 3 The surface roughness according to various surface treatments (R_a, μm). The roughness of 125 μm sandblasted disc was significantly higher than those of machined and 75 μm sandblasted disc (marked with an asterisk). Tukey's *post-hoc* test was used for the statistic analysis

From the XRD analysis, the anodized surface (G4) showed a strong peak near 25°, indicating the presence of crystals of anatase in the oxide layer. There was

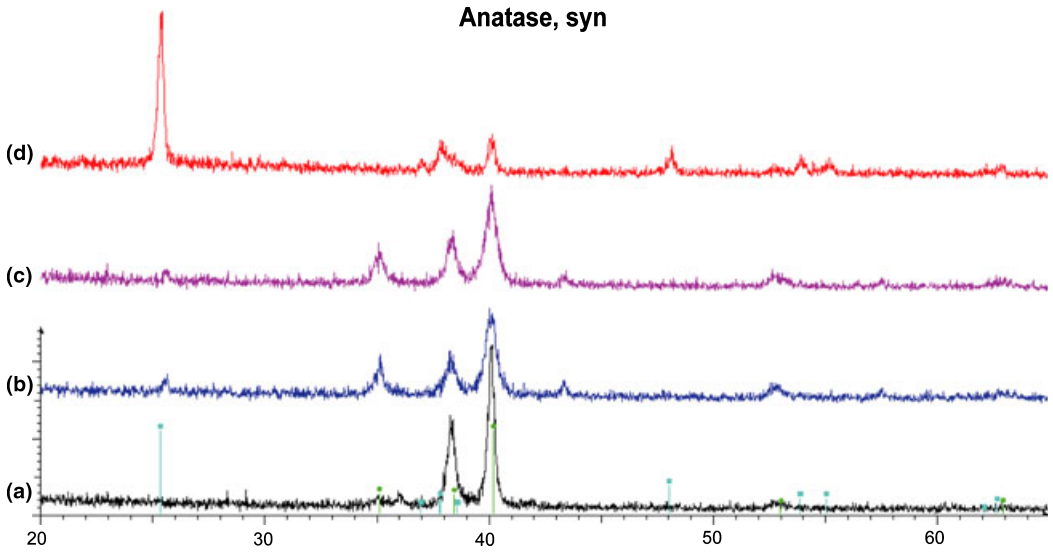

Figure 4 XRD profiles for the surfaces of (**a**) machined, (**b**) 75 μm blasted, (**c**) 125 μm blasted and (**d**) anodized titanium disc. The anodized surface (d) shows a strong peak near 25° of 2θ (2-Theta) presenting the anatase crystals. The other surfaces (**a–c**) show only the pure titanium peak near 40°

another peak – weaker than the one at 25° – at 48°, which is another indicator of anatase structure. The other surfaces (G1, G2 and G3) presented only the pure titanium peak near 40° (Figure 4).

On day 1, the cell proliferation levels were very low compared to those on days 3 and 6. Both of the sandblasted groups (G2 and G3) had higher cell proliferation value than the other groups and it was significantly different (Figure 5). On day 3, although it was statistically not significant ($\alpha = 0.05$), anodized disc (G4) and the 125 μm sandblasted disc (G3) showed higher cell proliferation levels than the machined disc. However, on day 6, the 75 μm sandblasted group (G2) was the highest again. Additionally, the group with no disc was statistically lower than the other groups ($P < 0.05$).

Gel electrophoresis result and the graphs of the ratio of GAPDH, osteopontin, osteocalcin and type I collagen mRNA expression throughout the experimental periods are shown on Figures 6 and 7. By comparing the gene expression to GAPDH, the ratio of osteopontin mRNA expression on all the groups showed no difference between groups and between day 1, day 3 and day 6 after incubation. Osteocalcin expression relative to GAPDH was various. Especially, at day 6, the expression of osteocalcin dramatically decreased on plastic surface (G5) and increased on Al_2O_3 sandblasted surfaces (G2 and G3). The ratio of type I collagen mRNA expression on the anodic oxidized surface (G4) increased throughout the entire experimental period. In contrast, the level of type I collagen decreased dramatically in polystyrene culture dish (G5) and slightly in sandblasted disk (G2 and G3). mRNA expression of osteocalcin and type I collagen of the osteoblast-like cells showed a tendency to be lower on control polystyrene surface (G5) than the other surfaces.

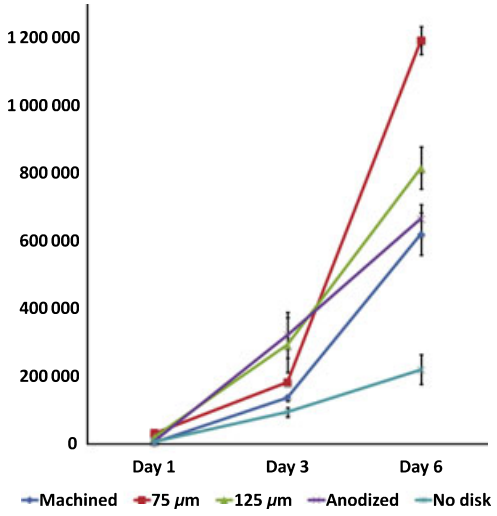

Figure 5 Cell proliferation ($n = 4$) on machined surface (G1), 75 μm blasted surface (G2), 125 μm blasted surface (G3), anodized surface (G4) and plastic plate with no disc (G5). PDL (periodontal ligament cell) were cultured on the machined surface, aluminum sandblasted surface with different thickness, titanium surface with anodic oxidation and plastic surface during the 6 days. Groups in which titanium disc were sandblasted with Al_2O_3 showed more proliferation between day 1 and 3. Control group with no disc had low cell proliferation. On day 6, Group of sandblasted with 75 μm Al_2O_3 showed higher cell proliferation than any other groups. ($P < 0.05$)

Discussion

During the osseointegration, rough titanium implant surface provides a good adherent place for osteoblasts and the initiation of new bone formation begins from the titanium surface other than the adjacent alveolar bone. In this situation, the contact osteogenesis can be anticipated rather than distant osteogenesis (Davies, 2003). Various physical, chemical and physicochemical

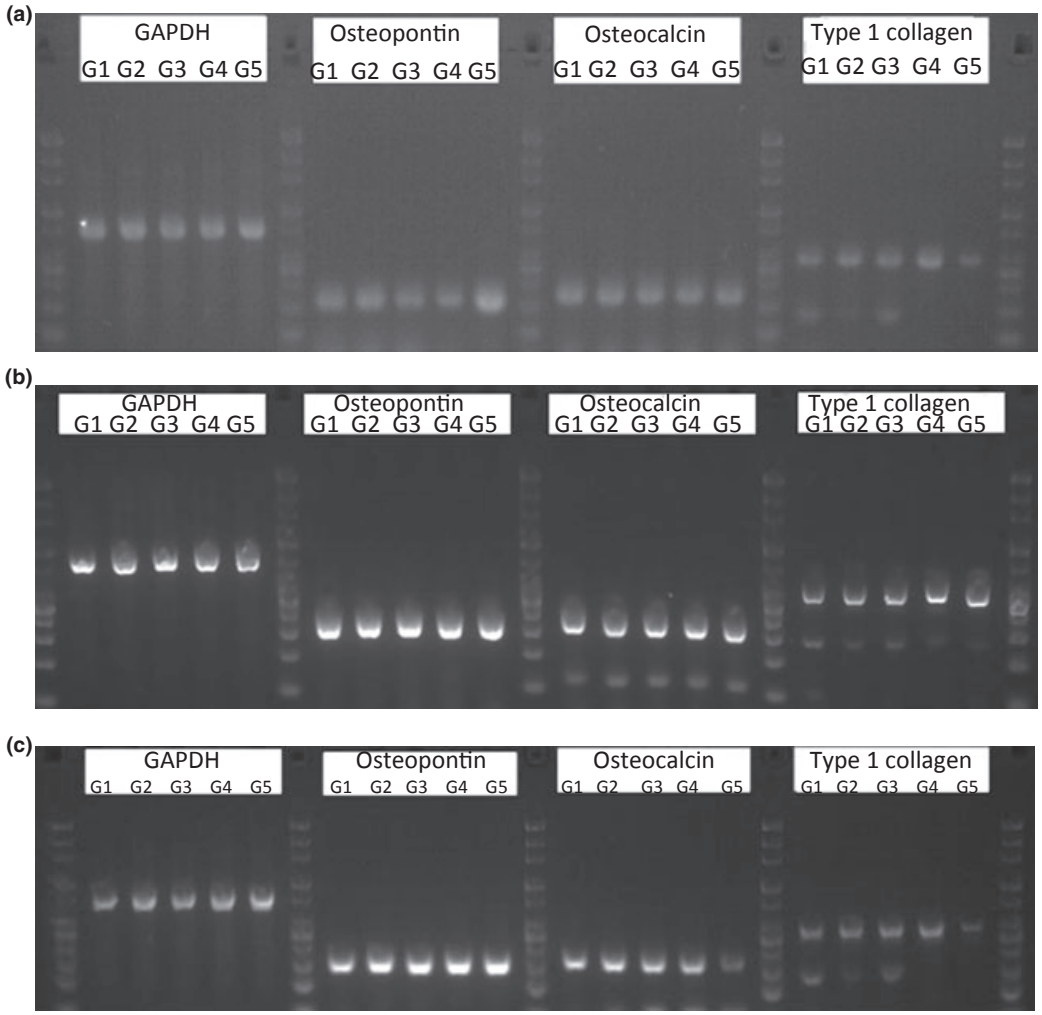

Figure 6 Gel electrophoresis of GAPDH, osteopontin, osteocalcin, and type 1 collagen mRNA expression on (**a**) day 1, (**b**) day 3 and (**c**) day 6. Gene expression was detected with RT-PCR for osteopontin, osteocalcin, type I collagen and GAPDH. The expression pattern was variable with different surface treatment. Among those genes, osteocalcin was increased in the rough titanium surfaces treated with both 75 and 125 μm Al_2O_3 particles

methods for the titanium surface treatment were developed for higher biocompatible rough surface implants. In previous studies about the biological response of anodized titanium implants under different voltages, roughness (R_a) values were measured by means of optical interferometer analysis. One group that was anodized with 270 V had the roughness of 0.88 ± 0.13 μm and another with 400 V had 1.7 ± 0.37 μm. Given these results, it was confirmed that the discs in the anodized group (G4, anodized at 300 V) of this study were prepared successfully. These studies showed that as the applied anodizing voltage increased, rougher surface was formed with the resultant rapid increase of R_a value (Choi *et al*, 2006; Park *et al*, 2007). Larsson *et al* (1996) also reported that the bone to implant contact extends with the increasing surface roughness and the oxide thickness. However, at the voltage above 500 V, surface roughness decreased even though oxide layer thickness increased and this change did not introduce better biologic response study (Choi *et al*, 2006). In this reason, anodizing voltage of 300 V was chosen in this study. The R_a values of the sandblasted discs were also in the same range in the previous studies (Kim *et al*, 2003; Roh *et al*, 2003; Na and Koak, 2005).

The three mineral structures that TiO_2 can form on the titanium surface are anatase, brookite and rutile. The most abundant and most extensively studied is the rutile phase. At lower temperatures the anatase and brookite phases are more stable, but both will revert to the rutile phase when subjected to high temperatures. Compared to rutile phase, anatase structure shows stronger bond strength between titanium metal and tissue. As anatase structure absorbs more hydroxyl and phosphoric ion in the tissue fluid, anatase phase is favourable for the deposition of bone-like apatite (Park *et al*, 2007; Neupane *et al*, 2009). In XRD analysis, the anatase peak was only found in the anodized disc while the other disc had very thin oxide layer in which only pure titanium peak was detected.

In this study, we hypothesized that there would be different intensities of the same gene expressions, which

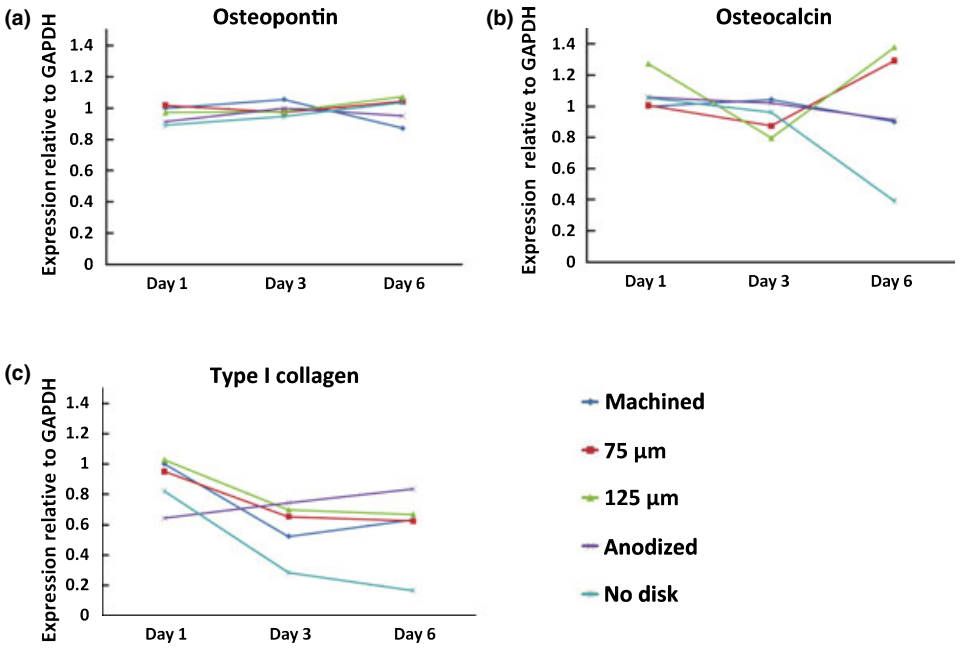

Figure 7 Normalized gene expression pattern on the various titanium surfaces. Gene expression was normalized with GAPDH per each group during the 6 days culture. (**a**) By comparing to gene expression to GAPDH, osteopontin shows no difference between groups and between day 1, day3 and day 6. (**b**) Osteocalcin expression relative to GAPDH is various. Especially, at day 6, the expression of osteocalcin decreases dramatically in control group. (**c**) Level of collagen type I decreases continuously relative to GAPDH in Al_2O_3 sandblasted disk. However, in the titanium oxide disk, the expression of collagen type I seems to increase during 3–6 days

are osteopontin, osteocalcin and type I collagen. As shown in Figure 6, the intensity of GAPDH bands was the same throughout the five groups on the same given day. GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) is generally assumed that its expression is unaffected by experimental conditions. GAPDH was used as a control gene and GAPDH expression was assumed as a baseline to normalize expression of Osteopontin, Osteocalcin and type I collagen (Ogawa and Nishimura, 2003; Matsuzaka *et al*, 2004).

Osteopontin and osteocalcin, both synthesized by osteoblasts, are indicators of formation and maturation of mineralized tissues. Osteopontin is also found in osteoclasts and epithelial cells of several organs. Osteocalcin is known to be expressed in bone or dentin forming cells (Bellows *et al*, 1999). Similarly, type I collagen is also synthesized by osteoblasts and is a reliable indicator of bone matrix synthesis (Kim *et al*, 2006). Other than the machined surface (G1), all the disc showed slight gradual increase in the osteopontin expression throughout the experiment. Similarly, both of the sandblasted disc (G2 and G3) and the anodized disc (G4) had increases in osteocalcin and type I collagen expressions, respectively. Moreover, the dishes that contained no disc (G5) had remarkably low levels of osteocalcin and type I collagen. All of these data show that having disc (G1, G2, G3 and G4) – no matter how the disc were treated – is better than not having disc (G5) in terms of bone forming capability. It seems that human periodontal ligament stem cell has affinity to the rough titanium surface other than the smooth surface as was expected. In considering previous report of hard tissue forming ability of PDLSCs (Seo *et al*, 2004), these characteristics may be helpful to understand the healing mechanism of immediate implantation in the freshly extracted socket.

The exact surface characteristics necessary for optimal osseointegration by human periodontal ligament stem cells remain to be elucidated. Future studies should be designed to investigate the response of stem cells to osseointegration of dental implants in the specific animal models.

Conclusion

Overall, the gene expression on the differently treated titanium surfaces shows different patterns during the 6 days and the following conclusions can be drawn. The PDLSCs proliferate in higher rate on the rough surface especially at the 75 μm Al_2O_3 particle treated surface than other surfaces. Also, osteocalcin was highly expressed on the rough surface treated with 75 and 125 μm Al_2O_3 particles. Considering the resultant expression of bone forming proteins, PDLSCs seem to have potential for the bone formation on various titanium surfaces.

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Author's contributions

Authors Heo YY and Um SY contributed equally to this work.

Conflict of interest

The authors have no conflict of interest.

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