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Influence of various superhydrophilic treatments to titanium on initial attachment, proliferation and differentiation of osteoblast-like cells

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ABSTRACT

The purpose of this study was to investigate the influence of various superhydrophilic treatments to titanium on behavior of osteoblast-like cell. Roughened surfaces of Ti were produced by alumina-blasting and acid-etching. Superhydrophilic surface groups comprised application of immediately after blasting and acid-etching (DW), oxygen plasma (Plasma), ultraviolet light (UV). Specimens stored in air at 3weeks were used as a control (Air). Initial cell attachment, proliferation, alkaline phosphatase activity and osteocalcin secretion of mouse osteoblast-like cells MC3T3-E1 were enhanced more on superhydrophilic surface groups than Air specimens. On confocal laser scanning microscopic images of cell morphology, expression of actin filaments was observed on the superhydrophilic surface groups, whereas there was relatively fewer actin filaments expression on the Air surfaces at all culture time. These results indicate that DW, Plasma, or UV treatment has potential in the creation and maintenance of superhydrophilic surfaces and enhancing initial attachment, proliferation and differentiation of osteoblast-like cells.
INTRODUCTION

Initial attachment, proliferation and differentiation of osteoblasts at the implant-bone interface play an important role in the early stages of osseointegration. In general, these cell behaviors are influenced by the surface topography and physicochemistry of the implants to which they attach\(^1\). The surface topography has marked influence on cell adhesion, extension and sequence\(^2\). Furthermore, the surface physicochemistry involves the surface charge (zeta potential, isoelectric point) and surface energy (surface wettability, contact angle). Surface wettability is an important physicochemical property on protein adsorption and subsequent cell behavior\(^3\). High surface wettability, that shows high surface energy, is generally reported to promote greater cell adhesion than low surface energy\(^4\). Sand-blast and acid-etching, oxygen plasma, or ultraviolet light is commonly suggested as method for the modification of the surface topography and surface physicochemistry.

The previous study showed that the superhydrophilicity resulted from storage in distilled water immediately after blasting and acid-etching of titanium(Ti)\(^5\), and this surface enhanced cell response\(^6\). In addition, a more differentiated phenotype of cells grown on superhydrophilic surface was attested by a remarkable increase of alkaline phosphatase activity, osteocalcin and osteoprotegerin production\(^7-9\).

Cold plasma treatments have been proposed as means of modifying the hydrophilicity\(^10-13\). Surface modification with cold plasma is an effective way of enhancing the surface wettability due to the removal of hydrocarbon and introducing hydroxyl groups. Several studies have reported changes in surface biocompatibility in terms of cell attachment and protein adsorption with cold plasma treatment\(^14,15\). It is also reported that the glow discharge plasma treatment, one kind of cold plasma treatments, to titanium plates enhanced the hydrophilicity as well as the initial attachment and differentiation of osteoblast-like cells\(^16\). Earlier studies confirmed that the greater the hydrophilicity of the surface, the greater the level of early-stage cell adherence, especially on superhydrophilic surfaces\(^10\). Cells spread much more widely on hydrophilic surfaces than on hydrophobic surfaces\(^11\).
Ultraviolet (UV) light-induced superhydrophilicity of TiO$_2$ was discovered in 1997\textsuperscript{17}. It was reported that the higher wettability was resulted from alteration of surface chemistry by removal of hydrocarbon due to the photocatalytic activity of TiO$_2$\textsuperscript{18,19}.

Concerning with the relationship between cell behavior and the superhydrophilic treatment of Ti, many \textit{in vitro} studies have investigated individually as above-mentioned. However, to our knowledge, no studies have reported about this relationship at the same time, i.e., under the same experimental condition. Therefore, the purpose of this study was to investigate the influence of various superhydrophilic treatments to titanium on behavior of osteoblast-like cell.

**MATERIALS AND METHODS**

\textit{Sample preparation and surface treatment}

Pure titanium (Ti, Grade 2, Kobe Seiko, Tokyo, Japan) were used in this study. All Ti disks (13 mm in diameter, 1.0 mm in thickness) were perpendicularly sand-blasted from a distance of 10 mm with 150 μm Al$_2$O$_3$ at 0.4 MPa air pressure. After sandblasting, the disks were etched with an equal mixture of hydrochloric and sulphuric acid (Wako Pure Chemicals, Tokyo, Japan) at 70ºC for 5 min. The surface roughness (Ra), as measured with Handysurf E-30A (Tokyo Seimitsu, Tokyo, Japan), with a length of 4 mm and a cut-off of value of 0.8 mm, was 1.52±0.09 μm.

The surface roughened specimens were subjected to various types of physicochemical treatment that were not altered the surface topography, as shown in Table 1 according to the results of previous study\textsuperscript{5}. Air specimens (control) were stored in air for 3 weeks. DW specimens were stored in distilled water for 3 days immediately after blasting and acid-etching. Plasma specimens were prepared with oxygen plasma treatment using a plasma-surface modification apparatus (VEP-1000, ULVAC, Kanagawa, Japan). Briefly, the specimens were introduced into the chamber of the apparatus and exposed to low-energy oxygen plasma treatment (200W, 1.5 Pa, gas flow rate 50 sccm) at room temperature for 30 s (Plasma). Ultraviolet treatment was performed using a UV ozone cleaner
Bioforce Nanosciences, Sweden) for 1 h (UV). This equipment creates UV radiation with a total power of 19 mW/cm², and excitation wavelengths of 185 and 254 corresponding to ultraviolet C (UV-C), and 365 nm corresponding to ultraviolet A (UV-A). The Plasma and UV specimens were stored in distilled water immediately after plasma and UV treatment for 3 days, respectively.

Contact angle measured against double-distilled water using a contact angle meter (Phoenix α, Meiwaforces, Tokyo, Japan) were 100(±7)° on Air specimen, whereas, the other specimens (DW, Plasma and UV) showed superhydrophilicity that had a water contact angle of almost zero degree.

**Cell culture**

Mouse osteoblast-like MC3T3-E1 were purchased from the RIKEN Bioresource Center (Wako City, Japan). Cells were cultured at 37°C in an atmosphere of 5% CO₂ and 100% humidity in an alpha modification of Eagle’s minimal essential medium (α-MEM, Life Technologies, MD, USA) containing 10% fetal bovine serum (FBS, Life Technologies, MD, USA). After reaching 80% confluence, the cells were trypsinized and seeded onto the test substrates. The medium was changed every 3 days.

**Cell attachment ability**

Cells were seeded onto all Ti disks (n=10) placed in 24-well tissue culture plates at a density of 5×10⁴ cells/cm² (in 1 mL). Initial attachment of cells was determined using WST-1 based colorimetry (WST-1, Roche Applied Science, Mannheim, Germany) after remaining in the culture for 3, 6 and 12 h. At each time period, the discs were washed 3 times with phosphate-buffered saline (PBS, pH7.4, Life Technologies, MD, USA) to remove unattached cells and transferred to new culture plates. The culture wells were incubated at 37°C for 1 h with 50 μL reagent containing tetrazolium salt (WST-1) and 500 μL culture medium, after which 110 μL reaction solution was moved to 96-well plates to measure absorbance. Formazan production was measured using a microplate reader (SpectraMax M5, Molecular Device, Tokyo, Japan) at 450 nm.
**Cell morphology**

After 3, 6 and 12 h culture, the cells were washed twice in PBS, fixed for 30 min in 4% paraformaldehyde phosphate buffer solution (Wako Pure Chemicals, Tokyo, Japan), and permeabilized in 1% TritonX-100 in PBS for 5 min. The cells were then incubated in PBS containing 3% BSA for 30 min. Subsequently, Ti disks were washed 3 times in PBS, after which they were dyed with DAPI (1:200, Life Technology, Gaithersburg, MD, USA) and Phalloidin (1:100, Alexa Fluor488, Life Technology, Gaithersburg, MD, USA) for 30 min. A confocal laser scanning microscope (LSM5DUO, Carl Zeiss, Oberkochen, Germany) was used to examine cell morphology and cytoskeletal arrangement. Areas of actin were also quantified using an image Analyzer (ImageJ, NIH, Bethesda, MD, USA) at 5 randomly selected areas per each of 3 disks.

**Cell proliferation**

Cells were seeded onto all Ti disks (n=5) placed in 24-well tissue culture plates at a density of $1 \times 10^4$ cells/cm$^2$ (in 1 mL). Proliferation of cells was determined using WST-1 based colorimetry after remaining in the culture for 1, 3, 7 and 14 days in the same manner as the cell attachment ability assay.

**Cell differentiation**

For the cell differentiation assay, the cells were seeded onto Ti disks (n=5) at a density of $1 \times 10^4$ cells/cm$^2$ (in 1 mL) in medium supplemented with 50 μg/mL ascorbic acid and 10 mM β-glycerophosphate.

1. Alkaline phosphatase activity

   Alkaline phosphatase (ALP) activity in the MC3T3-E1 cells on each specimen after incubate for 7, 14, 21 and 28 days was assayed using the LabAssay™ ALP kit (Wako Pure Chemicals, Tokyo, Japan) according to the manufacturer’s instructions. The cell layers were first washed with PBS, after
which 1mL of 0.1% Triton X-100 was added and detached using a cell scraper, followed by sonication for 30 s on ice (Branson, USA). Cell debris was removed by centrifugation at 15,000 rpm for 15 min at 4°C. The levels of ALP activity was normalized by the amount of total protein using BCA protein assay reagent (Pierce Chemical, Rockford IL, USA) according to the manufacturer’s instructions.

2. Osteocalcin secretion

Osteocalcin (OCN) was quantified in the cell culture supernatant for 7, 14, 21 and 28 days incubation using the Mouse Gla-Osteocalcin High Sensitive EIA Kit (Takara, Shiga, Japan) according to the manufacturer’s instructions.

Statistical analysis

The statistical significance of the data was assessed with an analysis of variance (ANOVA) followed by the Bonferroni multiple comparison test (α=0.05).

RESULTS

Cell attachment ability

The initial cell attachment ability after 3, 6, and 12 h of cultivation is shown in Fig.1. Cell attachment in the superhydrophilic surface groups (DW, Plasma and UV) was greater than that in the Air group at all culture time. In addition, cell attachment on in the DW specimens was greater than that in the other groups.

Cell morphology

Confocal laser scanning microscopic images of cell morphology at 6 h of cultivation are shown in Fig.2. Expression of actin filaments was observed on the superhydrophilic surface groups, whereas there was relatively fewer actin filaments expression on the Air surfaces at all culture time. The cytomorphometric parameter of cell area (μm²/cell) is shown in Fig. 3. The cell area in the superhydrophilic surface groups was significantly larger than that in the Air specimens at 6 h, and the
DW and Plasma specimens was significantly larger than that in the Air specimens at 12 h.

**Cell proliferation**

The cell proliferation ability at 1, 3, 7 and 14 days of cultivation are shown in Fig.3. Cell proliferation in the superhydrophilic surface groups was greater than that in the Air specimens at 1 and 3 days. At 7 days, cell proliferation on DW was significantly higher than that on the Air specimens. After 14 days of cultivation, cell proliferation ability tended to decrease compared to 7 days of cultivation, and there was no significant difference in cell proliferation ability among specimens.

**Alkaline phosphatase activity**

Alkaline phosphatase (ALP) activity on each specimen at each time period is shown in Fig. 5. ALP activity in the superhydrophilic surface groups was greater than that in the Air specimens at 14 days, ant that on DW was higher than that on Air specimens at 7 and 14 days. In addition, ALP activity in Plasma specimens was higher than that in UV specimens at 14 days.

**Osteocalcin secretion**

Osteocalcin secretion on each specimen at each time period is shown in Fig.6. Osteocalcin secretion was clearly recognized at 21 and 28 days, and the osteocalcin secretion in Plasma specimen was greater than that in the other specimens at 28 days.

**DISCUSSION**

In this study, we examined the influence of various superhydrophilic treatments to titanium on the behavior of osteoblast-like cell. The results showed that all superhydrophilic groups enhanced initial attachment, proliferation and differentiation of osteoblast-like cells regardless of kind of the superhydrophilic surface treatment including DW, Plasma and UV specimens. Among the superhydrophilic surface groups, there no apparent differences in the cell responses except the DW
specimens slightly enhanced the initial cell attachment compared with the other superhydrophilic groups.

The surfaces of all Ti specimens were subjected to the alumina blasting and acid etching in this study. The surface topography has marked influence on cell adhesion, extension and sequence. Numerous experimental reports from in vitro and in vivo studies have pointed to a more rapid bone response to roughened surfaces than to smoother polished or turned surfaces\textsuperscript{20,21}. In addition, the importance of the nano-structure of the surfaces on biological response has been recently recognized\textsuperscript{22}. Kubo et al. showed that the addition of nano-structures to a micro-roughened implant surface stimulated the proliferation and differentiation of osteoblast cells with synergetic effect\textsuperscript{22}. Furthermore, Cochran et al. showed that the ratio of bone contact on the SLA implant with micro- and nano-surface was greater than that of TPS implant with only the micro- surface\textsuperscript{23}. Accordingly, in the present study, the micro- and nano-topographies was created on Ti surface by blasting and acid etching to expect the enhancing the cell response\textsuperscript{6}.

In the present study, the Air specimens with hydrophobic surfaces that were stored in air for 3 weeks were used as a control. In general, titanium constantly adsorbs organic impurities such as hydrocarbons from the air atmosphere. This leads to an increase in hydrophobicity, which is referred to as an aging phenomenon\textsuperscript{24,25}. It is also reported that the atomic percentage of carbon reaches 50 at\% in hydrophobic titanium surfaces stored in air atmosphere\textsuperscript{12,26}.

The superhydrophilicity on the DW specimens may have resulted from storage in distilled water immediately after the following surface preparation in the previous report\textsuperscript{5}. First, acid-etching can clean the surface, and if immediately followed by storage in distilled water prevent adsorption of hydrocarbon and also enhance the formation of hydroxyl groups. Second, hydrophilicity may enhance by the cleaning the surface and the large surface area created by roughening. This phenomenon has been described in the Wenzel model\textsuperscript{27}, which predicted that the contact angle on a flat surface, which is less than \(90^\circ\), will decrease if the surface is roughened. The resulting increase in surface area appears
to have enhanced hydrophilicity. It is considered that other blasting and acid etching procedure such as commercially available SLActive (Straumann) might show same superhydrophilicity with same mechanism as increase in hydrophilicity in the DW specimens\textsuperscript{28).

The superhydrophilicity was also obtained on the Plasma and UV specimens in this study. Cold plasma-surface modification with various gases generated in a high-voltage electric field at a low pressure is suitable for change in surface physicochemistry\textsuperscript{1}. Wei \textit{et al.}\textsuperscript{11} showed that plasma polymerization with hexamethyldisiloxane followed by oxygen plasma treatment modified surfaces. In their study, the water contact angle of sample surfaces varied from 106° (hydrophobicity) to almost 0° (superhydrophilicity), and oxygen-functional groups were introduced on polymer surfaces during oxygen plasma treatment. On the titanium, hydroxylation of the TiO\textsubscript{2} on the Ti substrate by oxygen plasma exposure is similar to that of water photo-oxidation with UV irradiation. The hydroxylated TiO\textsubscript{2} substrate is constructed in the form of Ti-OH via oxidation by oxygen plasma\textsuperscript{29).

On the UV treatment, the higher wetting is caused by surface physicochemistry alteration with TiO\textsubscript{2} photocatalytic activity that was associated with the removable of hydrocarbon from the surface. In the case of Ti, a UV light energy of greater than 3.2 eV, corresponding to a wavelength of less than 387 nm, is needed to induce TiO\textsubscript{2} photocatalytic activity to excite an electron from the valence band to the conduction band. In addition, organic substances of the titanium surface are decomposed by UV irradiation in an excitation wavelength of less than 260nm\textsuperscript{17}. These surfaces of Plasma and UV specimens also maintained superhydrophilicity with immediate storage in distilled water after treatment in the previous study\textsuperscript{5).

A number of reports showed that cell responses were promoted on hydrophilic surface than on hydrophobic surface. Concerning with DW specimens, Lei \textit{et al.} suggest that SLActive that was subjected to similar treatment as DW specimens was likely to enhance cell attachment in the early stage. They also reported that the higher surface energy enhanced the cell response by regulating the expression of the cell adhesion-associated molecules such as FAK, and the surface energy influences
integrin-mediated pathways\textsuperscript{30}). It is reported that the more differentiated phenotype of cells grown on SLActive was noticed compared to SLA\textsuperscript{9),} and osteoblasts grown on SLActive surfaces exhibited a differentiated phenotype characterized by increased ALP activity and OCN synthesis and generated an osteogenic microenvironment through higher production of PGE\textsubscript{2} and TGF-\textbeta\textsubscript{1}\textsuperscript{7,31).} Furthermore, Zhao et al.\textsuperscript{7) suggested that enhancement of ALP activity on hydrophilic Ti surfaces is due to production of matrix vesicles, indicating an enhancement of osteoblastic differentiation. Thus, DW specimens with superhydrophilicity enabled to enhance of cell adhesion, proliferation and differentiation, compared to Air specimens with hydrophobicity.

Concerning with the Plasma specimens, Wei et al. investigated the influence of surface wettability by oxygen plasma treatment on competitive protein adsorption and initial attachment of osteoblasts, because the efficiency of cell adhesion and growth may depend on the balance between adhesion-promoting and adhesion-inhibiting proteins, which competitively adsorb to the surface\textsuperscript{10).} In general, a cell adhesion protein such as fibronectin (Fn) and a cell adhesion-inhibiting protein such as albumin (Alb) are both included in the serum used for cell culture. They demonstrated that Fn was prone to adsorb preferentially on superhydrophilic surfaces, resulting in high cell attachment, whereas Alb preferentially adsorbs on hydrophobic surfaces, interfering with cell attachment. Accordingly, in the present study, Fn adsorption may have been responsible for the observed increase in cell response on the Plasma specimens in culture media.

Aita et al. reported that the UV-treated titanium surface offered osteoblast-affinity environment, as demonstrated by enhanced attachment, spread, proliferation and differentiation of osteoblasts\textsuperscript{12).} In addition, their study revealed that osteoblastic gene expression and ALP activity were increased on the UVC-treated titanium disks, and the increased rate of osteoblastic differentiation may be attributed to the increased cell-to-cell interaction or promoted cell signaling pathways stimulated by attachment and adhesion molecules such as vinculin\textsuperscript{13).}

There are possible explanations for enhancing the cell responses on superhydrophilic surface;
decreasing the hydrocarbon, formation of basic hydroxyl group and increasing the polar component on the Ti surface. Progressive accumulation of organic molecules onto titanium surfaces is considered unavoidable under ambient conditions. The level of hydrocarbon strongly correlated with rates of protein adsorption and cell attachment. When hydrocarbons covering of Ti surfaces are expected to be removed by superhydrophilic treatments in this study, which may promote the interaction between the proteins and cells\textsuperscript{12}.

Another reason for enhancement of the cell response on the superhydrophilic groups was considered due to the introducing the basic hydroxyl groups on the Ti surface. There are two kinds of hydroxyl group on Ti outermost surfaces, negatively charged acidic hydroxyl group and positively charged basic hydroxyl group, respectively, playing an important role in protein adsorption\textsuperscript{32}. Feng \textit{et al}..\textsuperscript{33} showed that at the initial stage of the cell culture the basic hydroxyl groups promote the chemical interaction between the osteoblasts and the Ti surfaces, indicating that the basic hydroxyl group probably played a more important role than the acidic hydroxyl groups in the bioactivity of Ti. In general, most of proteins are negatively charged under the neutral environment. Accordingly, it was considered that protein adsorption related to cell attachment was promoted on the superhydrophilic groups with increasing mainly the basic hydroxyl group that was charged positively.

The increasing in the polar component on the Ti surface was also one reason for enhancing the cell response on superhydrophilic groups. Surface energy affects cell behavior, with cells spreading and attaching more easily on surfaces with high surface energy. Earlier studies found that the higher the surface energy resulted in greater numbers of adhered osteoblasts and higher cell activity\textsuperscript{34,35}. Particularly, the polar component of surface energy influenced the behavior of osteoblasts on Ti surfaces more strongly than dispersion component, which was attributed to the interaction between cells and Ti\textsuperscript{36}.

As discussed above, the factors responsible for the remarkable cell response in the superhydrophilic groups over the Air specimens were the result of the synergistic effects of the
following: elimination of hydrophobic hydrocarbons, the introduction of basic hydroxyl groups, and the increase in the polar component.

On comparison between superhydrophilic groups, DW specimen was greater than in the cell attachment compared to the Plasma and UV specimens at all culture time, and in the ALP activity compared to the UV specimens at 14 days culture time in this study. In addition, Plasma specimen was slightly higher in ALP activity and osteocalcin secretion compared to the UV specimen at 14d and 28d, respectively. The interaction between titanium substrate and cell adhesion proteins such as fibronectin as well as other osteogenic proteins may be responsible for these phenomena. It is necessary to clarify the further mechanism about these facts.

It is necessary to clarify the further mechanism about the fact that the DW specimen was slightly greater than the Plasma, UV specimens on the initial cell attachment.

In conclusion, the present study revealed that the superhydrophilic surfaces are effective for enhancing the osteoblast-like cells regardless of the superhydrophilic surface treatment including DW, Plasma and UV specimens, and no apparent differences in the cell responses were recognized among the superhydrophilic groups except the superiority of DW specimen in the cell attachment. These results indicate that the superhydrophilic treatments to the Ti implants may play an important role for enhancing the bone formation in early stages of osseointegration.

REFERENCES


**Figure legends**

Fig. 1 Initial attachment of MC3T3-E1 cells on Ti surface after 3, 6, or 12 h of cultivation. Identical letter shows no significant difference on each culture time \((p>0.05)\).

Fig. 2 Confocal laser scanning microscopic images of cell morphology at 6 h cultivation with dual staining with DAPI for nuclei (blue) and phalloidin for actin filaments (green).

Fig. 3 Cell area of MC3T3-E1 cells on Ti surface after 3, 6, or 12 h of cultivation.
Identical letter shows no significant difference on each culture time ($p>0.05$).

Fig. 4 Proliferation of MC3T3-E1 cells on Ti surface after 1, 3, 7 or 14 days of cultivation. Identical letter shows no significant difference on each culture time ($p>0.05$).

Fig. 5 ALP activity of MC3T3-E1 cells on Ti surface after 7, 14, 21 or 28 days of cultivation. Identical letter shows no significant difference on each culture time ($p>0.05$).

Fig. 6 Osteocalcin secretion of MC3T3-E1 cells on Ti surface after 7, 14, 21 or 28 days of cultivation. Identical letter shows no significant difference on each culture time ($p>0.05$).
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<th>Code</th>
<th>Treatment</th>
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<tr>
<td>Air</td>
<td>Stored in air for 3 weeks after blasting and acid-etching (control)</td>
</tr>
<tr>
<td>DW</td>
<td>Stored in distilled water for 3 days immediately after blasting and acid-etching</td>
</tr>
<tr>
<td>Plasma</td>
<td>Treated with oxygen-plasma (200W, 1.5Pa) for 1 min, and stored in distilled water for 3 days</td>
</tr>
<tr>
<td>UV</td>
<td>Treated with ultraviolet radiation (19 mW/cm², λ=185, 254, 365 nm) for 1 h, and stored in distilled water for 3 days</td>
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</table>
Fig. 1  Initial attachment of MC3T3-E1 cells on Ti surface after 3, 6, or 12 h of cultivation. Identical letter shows no significant difference on each culture time ($p > 0.05$).
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