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Influence of the wettability of different titanium surface topographies on initial cellular behavior

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ABSTRACT

This study examined the influence of the time-dependent wettability of different surface topographies on initial cellular behavior. Titanium disks with smooth topography (SM) and three kinds of rough topography (sandblasted (SA), microtopography (M) and nanotopography (N)) were prepared. Time-dependent changes in surface wettability were observed in all surfaces as shown in previous studies. On SM surfaces, hydrophobic alteration influenced cell spreading and the activity of RhoA (a small GTPase protein of the Rho family), while no alterations were observed on rough surfaces except for the number of adherent cells. Serum adsorption could recover these functional deteriorations by hydrophobic alteration. These findings suggest that surface topography is a more potent regulator in initial cellular behaviors such as cell spreading and RhoA activation than surface wettability.

INTRODUCTION

The surface physicochemical properties of dental titanium implants play a critical role in establishing osseointegration¹⁾. Surface topography has been reported to improve cellular behavior in some kinds of cells^{2,3)}. In particular, micrometer- and nanometer-scale surface topographical cues have been known to have the specific capacities to alter cell morphology⁴⁻⁶⁾ in various cells and subsequent cellular behavior⁷⁾. Furthermore, the development of an implant surface with higher wettability is a relatively recent innovation. Several marketed dental titanium implants with different surface topographical cues also feature different surface wettability^{8,9)}, and a number of studies have found that a hydrophilic surface increased osteogenic gene expression¹⁰⁻¹²⁾ and formed a strong interface with bone¹³⁾. However, the previous review article have suggested that a hydrophilic surface is not apparently superior to a hydrophobic surface from a clinical point of view¹⁴⁾, suggesting that further research is necessary to elucidate the mechanisms involved in the variables of surface topography and wettability.

It has been demonstrated that the bioactivity of titanium degrades in a time-dependent manner¹⁵⁾. Previous studies have shown that the biological capabilities of titanium surfaces were attenuated on degraded surfaces compared with new surfaces^{15,16)}. Additionally, the time-dependent reduction of wettability has also been reported, which could

be attributed to alterations in the surface energy and adsorbed impurities from the ambient atmosphere¹⁷⁾. Although surface wettability is thought to affect biological capabilities, the detailed mechanisms underlying these interactions, such as the involvement of surface treatment and surface topography, remain elusive.

Previous studies indicated that surface wettability could play a critical role in initial cellular characteristics such as cell morphology and cell adhesion^{8,9)}. Surface topography, including microstructured and nanostructured surfaces, has also been known to modulate cell morphology and the activity of RhoA, a small GTPase protein of the Rho family, in cytoskeletal regulation^{7,18)}. RhoA is known to act as a molecular switch. In response to extracellular signals, RhoA induces coordinated changes in the organization of the actin cytoskeleton and in gene transcription to drive a large variety of biological responses including morphogenesis, chemotaxis, axonal guidance, and cell cycle progression.¹⁹⁾ However, these studies, including our previous study⁷⁾, did not take into consideration the effects of surface wettability. Another study showed that the RhoA activity in hydrophobic surface-adherent cells was significantly higher than in cells on hydrophilic surfaces, and that cell adhesion was affected by surface wettability and RhoA activity²⁰⁾. Although surface topography and surface wettability are considered to correlate with cell morphology, RhoA activity and cell adhesion, few studies have evaluated how the interaction with both of these surface properties modulates such initial cellular behaviors.

Most studies have found that surface wettability contributes to the early stage of cellular behaviors¹⁷⁾. Therefore, the purpose of this study was to demonstrate the effect of time-dependent surface wettability of four different surface topographies on subsequent initial cellular behaviors.

MATERIALS AND METHODS

Titanium disks

Commercially pure grade IV round titanium disks (diameter: 20 mm, thickness: 1 mm) were prepared as previously described, with some modifications⁷⁾.

- 1) Smooth (SM) surfaces: Disks were polished with silicone points (Shofu Inc., Kyoto, Japan), washed with distilled water (DW) and passivated with 30% HNO₃ for 5 min.
- 2) Sandblasted (SA) surfaces: Disks were polished using Si carbide papers starting from grade 400 to 600 grits, grit-blasted with 90- μ m aluminum oxide (Al₂O₃) particles, washed with DW, and passivated as described above.
- 3) Microstructured (M) surfaces: Disks were polished with Si carbide papers as described above, grit-blasted with 90- μ m Al₂O₃ particles, and washed with DW. These disks were treated with 5N HCl for 8 hours and washed and passivated as described above.
- 4) Nanostructured (N) surfaces: Disks were polished with Si carbide papers as described above, grit-blasted with 90- μ m Al₂O₃ particles, and washed with DW. These disks were treated with a 50/50 v/v% solution of 30% H₂O₂ and 2N H₂SO₄ for 2 hours and washed

and passivated as described above.

After passivation, disks cleaned with DW were put in 70% ethanol for sterilization for several hours and air-dried on a clean bench under UV light. The disks used immediately were defined as D0 disks. The other disks (D56 disks) were stored in culture dishes with a sealing film (Parafilm, Bemis Flexible Packaging, Chicago, IL, USA) and covered with aluminum foil on a clean bench for 56 days.

Surface analysis

Surface topography was examined under scanning electron microscopy (SEM; S-3400N, Hitachi High-Technologies Corporation, Tokyo, Japan) and surface roughness (Ra, the arithmetic average of the roughness profile) was measured under laser scanning microscopy (VK-9710, Keyence, Osaka, Japan) at each time point. The elemental composition of each D0 and D56 surface was examined using X-ray photoelectron spectroscopy (XPS; K-Alpha; Thermo Fisher Scientific, Waltham, MA, USA). The surface wettability of D0 and D56 disks in each surface group was also examined by using the sessile drop technique to measure the water contact angle (DM 500FK, Drop Master, Kyowa Interface Science, Saitama, Japan).

Cell culture

MC3T3-E1 cells from an osteoblast precursor cell line derived from murine calvaria were used in this study. These cells were cultured with alpha-minimum essential medium

(α -MEM; Gibco, Thermo Fisher, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco). At confluence, the cells were detached using 0.05% trypsin-EDTA phenol red (Gibco) and reseeded for further experiments.

Fluorescence staining for cell morphology analysis

The cells were seeded on D0 and D56 disks at a density of 2.0×10^4 cells/disk and cultured in α -MEM with 3% FBS for 6 hours. To visualize cell morphology, the actin filaments were labeled with Texas Red-X phalloidin (Invitrogen, Grand Island, NY, USA) and the nuclei were counterstained with DAPI (Vectashield HardSet Mounting Medium, Thermo Fisher) as described in our previous study⁷⁾. The stained cells were observed under a fluorescence microscope (BZ-9000, Keyence, Osaka, Japan).

RhoA activity assay

The level of activated RhoA in cells on each surface was measured using a commercially available kit (G-LISA RhoA activation assay kit; Cytoskeleton, Denver, CO, USA). The cells were seeded and cultured for 2 hours on D0 and D56 disks of all types, and the level of GTP-loaded RhoA in an equal amount of proteins from cell lysates was measured according to the manufacturer's instructions.

Cell adhesion assay

To evaluate the effect of surface topography and surface wettability on cell adhesion, the cells were seeded on D0 and D56 disks of each surface at a density of 1×10^6 cells/disk and

cultured in α -MEM with 3% FBS for 6 hours. Each disk was washed twice with phosphate-buffered saline (PBS; Gibco) to remove non-adherent cells, and the number of attached cells was counted using CellTiter 96 AQueous One Solution Reagent (MTS) (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Effect of protein adsorption on D56 disks of all surface types on cellular responses

The effects of protein-adsorption onto each D56 disk on subsequent cellular responses were also investigated. D56 disks of all types were pre-treated with FBS for 2 hours at 4°C and washed with PBS twice. The treated D56 disks were defined as D56(+). Cell morphology, RhoA activity, and cell adhesion were evaluated as described above.

Statistical analysis

All data were presented as the mean \pm standard deviation. To statistically analyze temporal changes in surface properties and the effect of different degrees of surface wettability on cellular behavior in each group, an unpaired t-test was used to compare D0 and D56 disks. Differences were considered significant at $p < 0.05$.

RESULTS

Surface characteristics and their temporal changes

The surface topography of each D0 disk is shown in Figure 1. SEM observation revealed that SM surfaces had a flat, smooth topography with a linear roughness pattern

(Fig. 1A and 1B). SA, M and N disks exhibited visible topographic alterations. SA surfaces showed 3D topography with the attachment of Al_2O_3 particles (Fig. 1C and 1D). M surfaces demonstrated the formation of 3D structures with clearly identifiable pits (Fig. 1E and 1F). Although N surfaces were similar to those of SA surfaces at low magnification (Fig. 1G), nanofeatures imparted to the surface by the $\text{H}_2\text{SO}_4/\text{H}_2\text{O}_2$ oxidative treatment were observed at high magnification (Fig. 1H). These features were consistent between D0 and D56 disks.

The surface roughness of each surface is depicted in Figure 2. The roughness values between D0 and D56 were not statistically different among all groups.

The XPS analysis demonstrated traces of chemical components on each D0 surface (Fig. 3). All surfaces presented traces of Ti, O and C. On SM surfaces, Si was detected as a result of the polishing procedure (Fig. 3A). Al and Si were detected on SA and N surfaces, resulting from the polishing and sandblasting procedures (Fig. 3B and 3D), although they were not detected on M surfaces because of the stronger acid-etching procedure (Fig. 3C). On each of the D56 disks, the traces of chemical components were quite similar and no marked changes were detected (data not shown).

The water contact angles of the D0 disks are presented in Figure 4A. On M and N surfaces, dropped water spread immediately and completely, and the water contact angle could not be measured (Fig. 4B), indicating superhydrophilicity. Among the D56 disks, M surfaces recorded the highest water contact angle, followed by N, SM and SA. Significant

differences between D0 and D56 disks in the SM and the SA groups were detected, although statistical analysis could not be performed because M and N D0 data could not be detected.

Cell morphology on D0 and D56 disks of each surface

Initial cell morphology was evaluated by fluorescence staining (Fig. 5). Well-spread cells were observed on SM D0 disks (Fig. 5A), although cells on D0 disks in SA, M and N groups exhibited multiple projections and irregular actin fibers compared with SM-adherent cells (Fig. 5B-5D). Morphological differences were not apparent among the rough groups. However, the cells on the SM D56 disks were shaped differently from the cells on the SM D0 disks. SM D56-adherent cells spread less and exhibited thinner and longer projections (Fig. 5E). However, the morphology of the cells on SA, M and N D56 disks were not markedly different from that of the cells on the respective D0 disks (Fig. 5F-5H).

Expression level of activated RhoA

To confirm the underlying mechanism of cell morphology or cytoskeletal regulation, the level of GTP-loaded RhoA during cell adhesion on all surfaces is shown in Figure 6. Significant upregulation of RhoA activity on SM D56 surfaces was observed compared with that on SM D0 surfaces, although no statistical differences were detected in the other groups.

Cell adhesion assay

The number of attached cells on each surface at 6 hours was determined using an MTS assay (Fig. 7). The number of adherent cells on hydrophobic D56 disks of each surface

was significantly lower than on the respective hydrophilic D0 disks.

Effects of protein adsorption of D56 disks on cellular responses

Although hydrophobic SM D56 disks exhibited altered cell morphology as shown in Figure 3E, FBS-coated SM D56 disks allowed improved cell spreading (Fig. 8A). However, differences in cell morphology were not apparent in rough groups (data not shown). Significantly lower RhoA activity on coated D56 disks was also detected compared with non-coated D56 disks in all groups (Fig. 8B). In SM-adherent cells, the association between cell morphology and RhoA activity was consistent with the data detected on SM D0 disks, in that well-spread cells expressed lower RhoA activity. Additionally, FBS coating was effective in downregulating RhoA activity on rough surfaces. The cell adhesion assay revealed that FBS-coated D56 disks significantly enhanced the number of adherent cells compared with non-coated D56 disks in all groups (Fig. 8C).

DISCUSSION

Several surface treatments of titanium or dental implants are in current use, and their surface physicochemical and biological properties have been evaluated^{8,9,21}). Surface topography is recognized as an important factor for better osseointegration and regulation of cellular behavior. Recent studies showed that surface topography modulates some cellular behaviors such as cell morphology, RhoA activation and osteogenic differentiation⁷). Although recent studies have suggested that surface wettability modulated by surface

treatments could enhance the early osseointegration process²¹⁾, it remains to be determined how the modulated surface properties reciprocally interact and how they regulate cellular behavior. Building on the findings of previous studies, the present study aimed to evaluate the effects of surface wettability of four different surface topographical cues on initial cellular behaviors.

SEM images showed distinct topographical cues of our surfaces as reported in our previous study⁷⁾, and surface roughness analysis showed that Ra values were higher in SA, M and N groups. Even with the surface treatments, surface topography and roughness were maintained and stable, at least, over the 56 days. However, time-dependent decreases in surface wettability were observed in all groups, although no marked differences were detected in the XPS analyses. A previous study indicated that adsorption of contaminants in the ambient atmosphere reduced surface wettability⁸⁾. However, that study also suggested that a reduction in surface wettability could be induced without contaminant adsorption, which was consistent with our data⁸⁾. Procedures such as grit blasting, acid etching, and UV light irradiation have been known to alter surface physicochemical properties including surface topography, roughness, surface energy and chemical composition, resulting in the enhancement of surface wettability^{8,21-23)}. In agreement with the findings of a previous study, a temporal reduction in surface wettability was observed in all groups¹⁷⁾.

Adherent cells on SM D0 disks were well-spread, although cells on rough surfaces

developed multiple projections, suggesting a modulation of cytoskeletal stretching by surface topography as previous studies reported^{4,7}). On hydrophobic surfaces of SM disks, adherent cells exhibited less spreading with thinner and longer projections compared with hydrophilic SM surfaces. On hydrophobic rough surfaces, however, no marked differences in cell morphology were observed. These findings imply that rough surface topographical cues can regulate cell morphology and play a more critical role in the regulation of cell morphology than surface wettability. Our findings of a significant upregulation of RhoA activation on SM D56 (hydrophobic surface) disks are supported by a previous study that reported higher RhoA activity on hydrophobic surfaces than on hydrophilic surfaces²⁰), while there were no significant differences in RhoA activity in rough surface groups. These results suggest that surface topography could also be a more potent factor in RhoA activation. Our previous study also showed that RhoA inhibition increased cell migration and the expression of osteogenic genes on SM surfaces⁷). However, these increases were not detected on rough surfaces, suggesting that surface topography can regulate RhoA activity and cellular behavior. Further studies will be necessary to elucidate the regulatory mechanisms of RhoA activity and cellular behavior by surface topography.

There are several factors associated with cell adhesion. Mechanical anchorage to surface topographical cues, surface energy and wettability are known as factors influencing cell adhesion. In the present study, our results clearly revealed that the hydrophobic disks

(D56 disks) had a significantly reduced number of adherent cells compared with the hydrophilic disks (D0 disks), which was consistent with some previous studies^{20,24-26}. These results illustrate one of the positive characteristics of hydrophilic surfaces and suggest that the positive effects of hydrophilic surfaces shown in previous studies can be attributed to enhanced cell adhesion capacity and the resulting cellular interactions.

From the biological point of view, surface conditioning with adhesive proteins plays a pivotal role in cell adhesion that can be attributed to surface topography, surface charge and wettability^{24,27}. The influence of surface wettability on the adsorption of protein and subsequent cellular responses was also investigated. Although hydrophobic SM surfaces (SM D56) formed less well-spread cells with thinner and longer projections as shown in Fig. 5E, FBS-coated SM D56 disks restored spreading and induced lower RhoA activity in adherent cells. These results are consistent with our findings that well-spread cells on SM D0 (hydrophilic) disks expressed lower RhoA activity, suggesting that FBS coating causes a shift from hydrophobicity-derived properties to hydrophilicity-derived properties in cell spreading, RhoA activation and cell adhesion. Cell attachment, adhesion and spreading make up the first phase of cell/material interactions, and protein adsorption, especially the extracellular matrix, is required for integrin-receptor-based adhesion. FBS coating may facilitate this procedure and cellular behaviors may be restored^{24,28}.

This study clearly showed that the influence of surface wettability on initial cellular

behavior was more apparent on SM surfaces than on rough surfaces. In our previous study, rough surfaces induced higher RhoA activity compared with smooth surfaces, and this may have resulted from the mechanical stress caused by the 3D rough surface topography during adhesion⁷⁾. Consequently, cell morphology was regulated by the surface topography or the surface physical structure. However, these cellular behaviors were not influenced by surface wettability on each rough surface, while RhoA activity and cell morphology were influenced by surface wettability on smooth surfaces in the present study. It seems that rough surface topography may be a more potent factor in regulating initial cellular behavior than surface wettability. However, the present study also showed that hydrophilic surfaces were more capable of stimulating the number of adherent cells than hydrophobic surfaces, despite the surface topography. The results of our study suggest that the positive effects of hydrophilic surfaces shown in previous studies¹⁰⁻¹²⁾, especially relating to the expression of osteogenic markers, could be attributed to the increased numbers of adherent cells and the acceleration of subsequent cellular interactions. However, further investigation is required to validate this hypothesis.

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FIGURE CAPTIONS

Fig. 1: Scanning electron microscopy images of each surface.

A SM surface at low magnification, **B** SM surface at high magnification, **C** SA surface at low magnification, **D** SA surface at high magnification, **E** M surface at low magnification, **F** M surface at high magnification, **G** N surface at low magnification, **H** N surface at high magnification.

Fig. 2 Surface roughness analysis by laser scanning microscopy. No significant differences were observed between D0 and D56 in each surface group.

Fig. 3 X-ray photoelectron spectroscopy spectrum of **A** SM, **B** SA, **C** M and **D** N D0 surfaces.

Fig. 4 Water contact angle for the evaluation of surface wettability. **A** Significant increases in water contact angles in D56 disks of all groups compared with D0 disks. (t-test, ** $p < 0.01$) **B** Cross-sectional image of water droplet on M and N surfaces.

Fig. 5 Fluorescence images showing actin (red) and nucleus (blue) of **A** SM D0-adherent cells, **B** SA D0-adherent cells, **C** M D0-adherent cells, **D** N D0-adherent cells. D56-adherent cells are shown in **E** SM surface, **F** SA surface, **G** M surface and **H** N surface.

Fig. 6 Influence of surface wettability of each surface topography on RhoA activation. Comparisons between D0-adherent cells and D56-adherent cells on each surface were performed with a t-test. A statistical difference was detected on SM surfaces (t-test, ** $p < 0.01$).

Fig. 7 Cell adhesion assay. The number of adherent cells on D0 and D56 disks of each surface after 6 hours of culture was determined. Statistically significant differences between D0 and D56 disks of each surface were identified (t-test, ** $p < 0.01$).

Fig. 8 Effects of protein adsorption in fetal bovine serum (FBS) on cellular behavior. Each D56 disk was pre-incubated in FBS for 2 hours at 4°C. **A** Cell morphology, **B** RhoA activity and **C** cell adhesion were evaluated and compared with non-treated D56 disks. Statistically significant differences between D0 and D56 disks of each surface were identified (t-test, ** $p < 0.01$).

Fig.1A

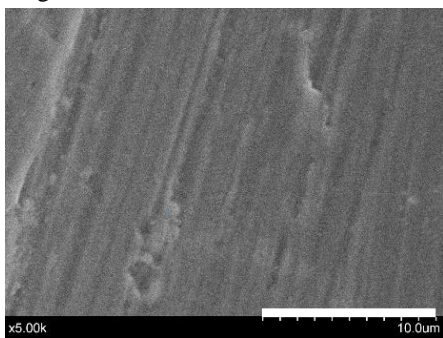


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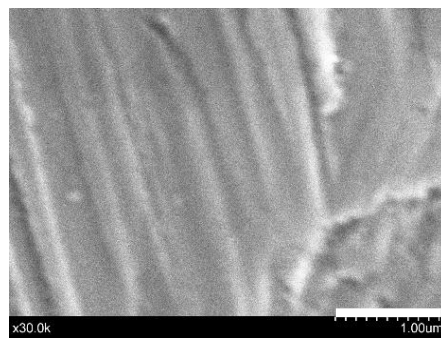


Fig.1C

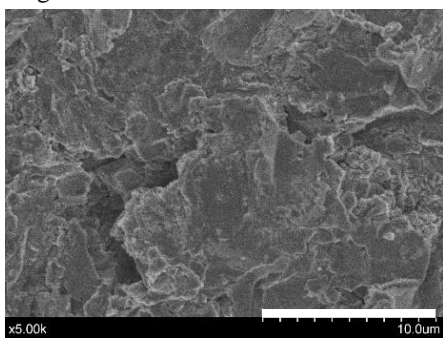


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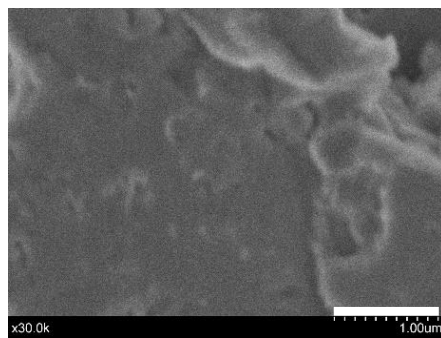


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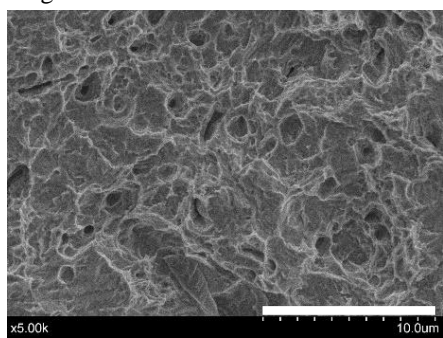


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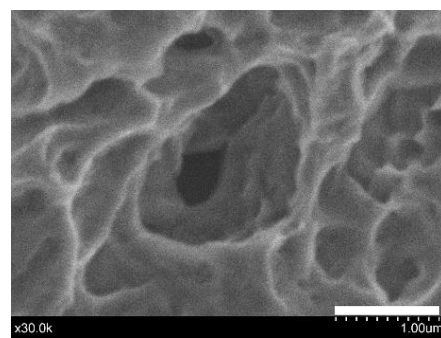


Fig.1G

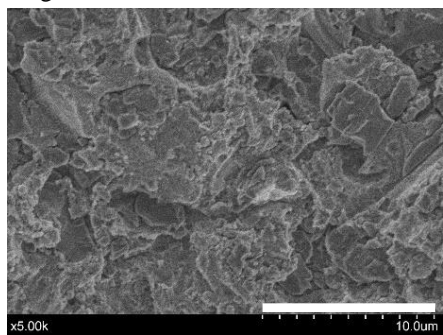


Fig.1H

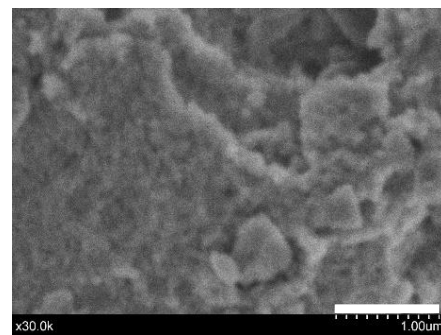


Fig.2

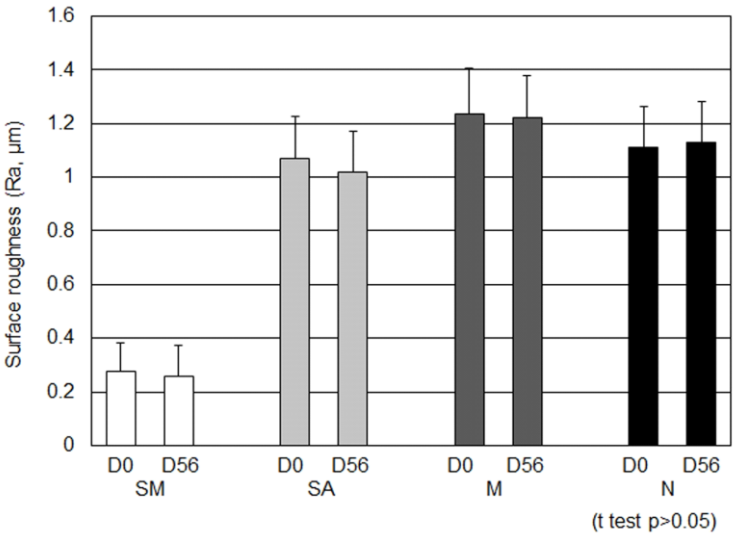


Fig.3A

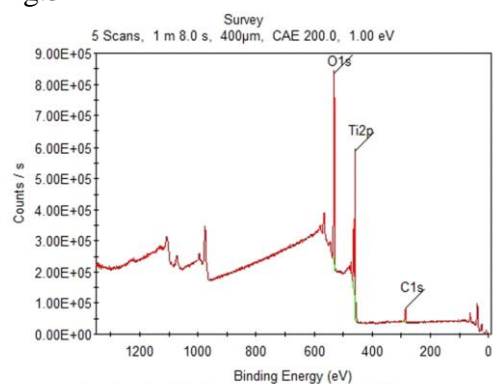


Fig.3B

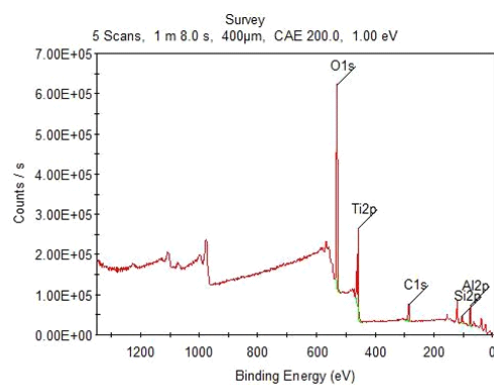


Fig.3C

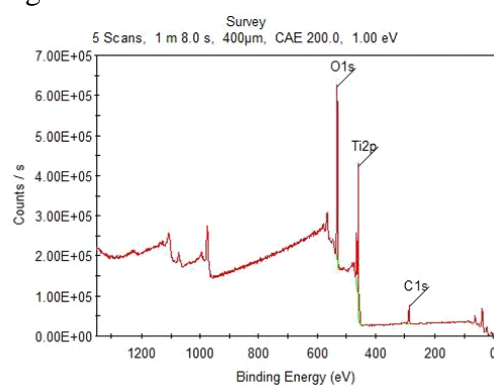


Fig.3D

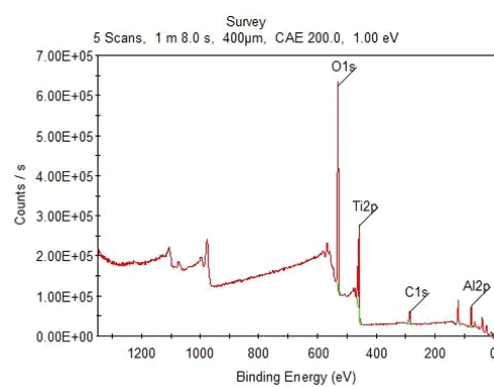


Fig.4A

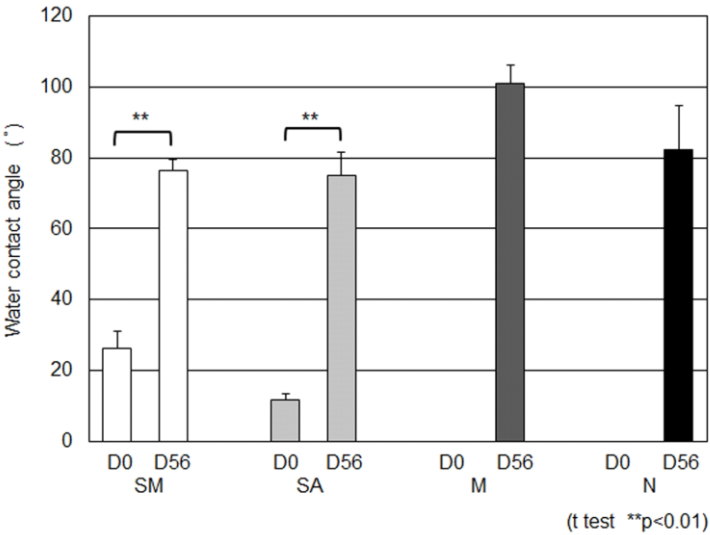


Fig.4B

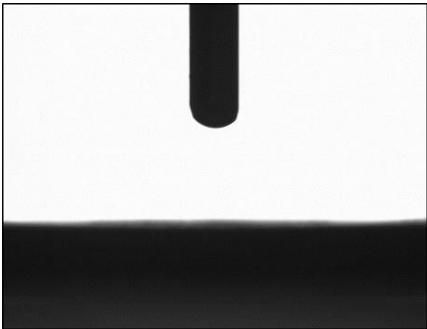


Fig.5A

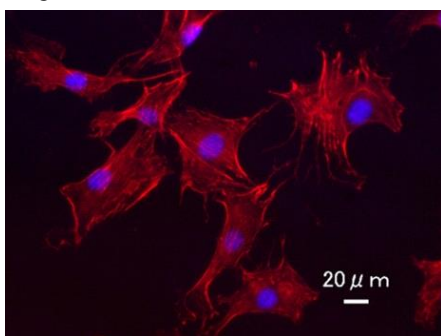


Fig.5B

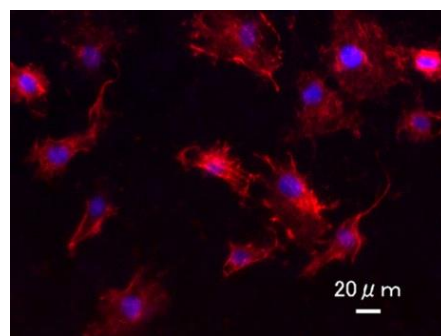


Fig.5C

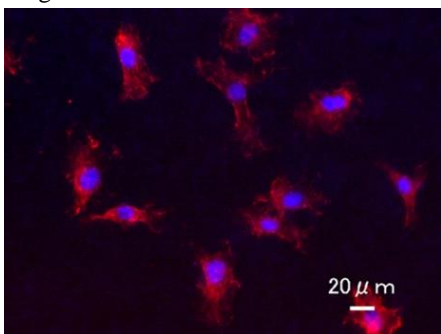


Fig.5D

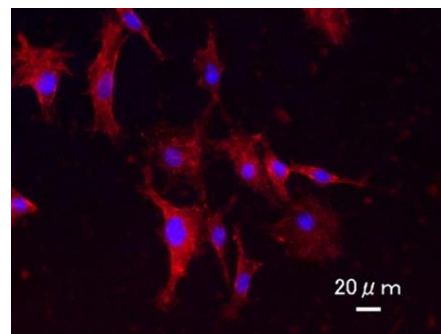


Fig.5E

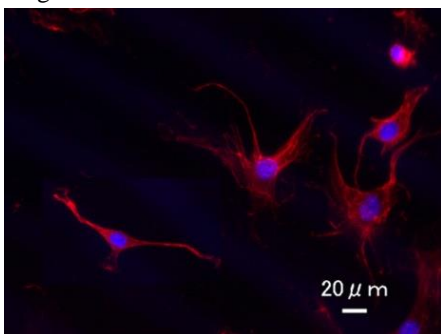


Fig.5F

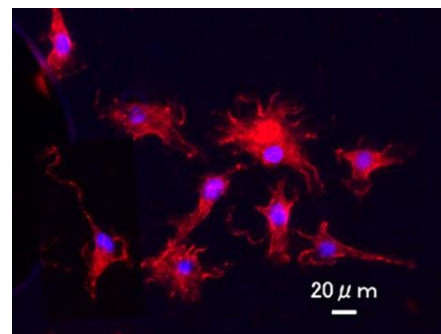


Fig.5G

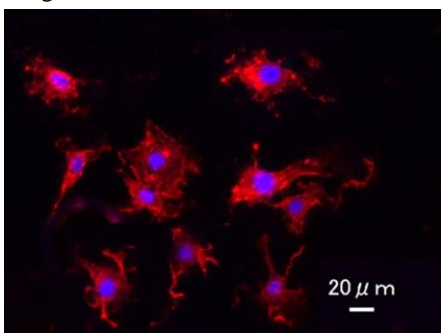


Fig.5H

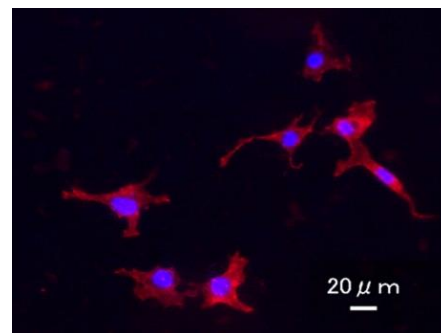


Fig.6

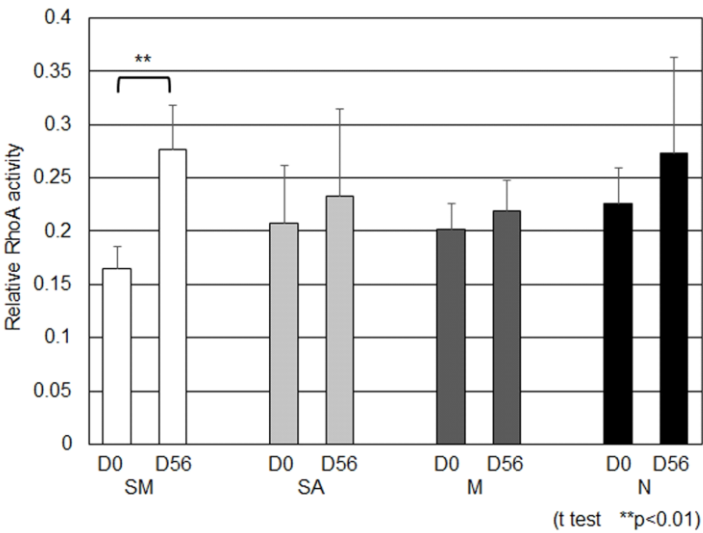


Fig.7

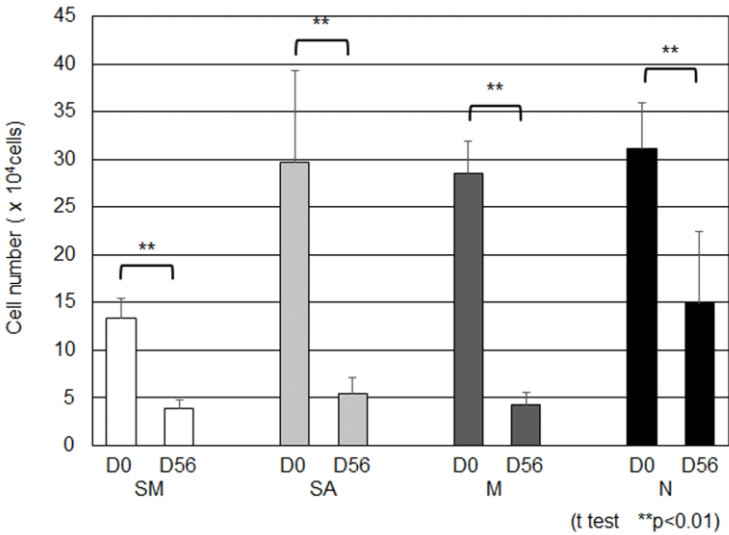


Fig.8A

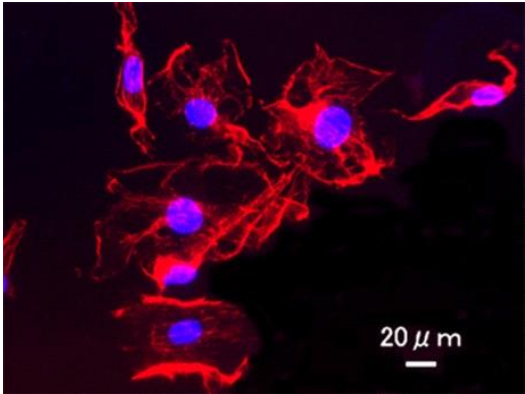


Fig.8B

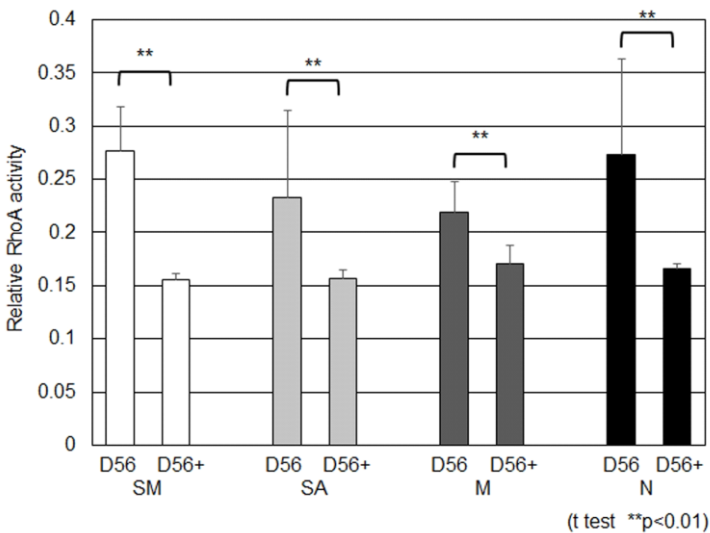


Fig.8C

