

Morphological Features of Osteoblasts Cultured on Ultraviolet-irradiated Titanium Plates

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Abstract. *Background:* Although we have recently established optimal experimental conditions of ultraviolet (UV)-irradiation for titanium plates (e.g. wavelength and exposure time) which enhanced osteoblast adhesion to the plates, the effects of UV-irradiation on cell structure are still unclear. *Materials and Methods:* Digital stereomicroscopy was used to investigate morphological alterations of non-stained viable and hematoxylin-eosin (HE)-stained cells on UV-irradiated and non-UV-irradiated titanium plates for up to 24 hours. *Results:* In 24 hours, significant expansion of HE-stained cells (area, perimeter and sprouting processes) was observed on UV-irradiated plates. The sprouting processes appeared within 40 minutes of inoculation under both conditions, however, significant cell area expansion, which occurred in 5 minutes, was observed only on UV-irradiated plates. *Conclusion:* UV-enhanced cell attachment was related to morphological alteration which occurred immediately after inoculation. Digital stereomicroscopic evaluation was able to define and quantify morphological alterations of viable cells in an opaque environment.

There have been notable improvements in both diagnostics and procedures for dental implant treatments, however, osseointegration between implanted materials and alveolar bone is still a fundamental factor for recovering adequate functional occlusion (1, 2). To achieve complete osseointegration, enormous efforts have been applied to modify the surface of

dental implants, and hydroxyapatite coating has been widely accepted for this purpose (3). UV-irradiation of materials (e.g. glass, ceramics, plastics and metal) has been used for their surface modification (4-8). It has been reported that UV-irradiation of titanium, a common material for dental implants, enhances osteoblast cell attachment to the titanium surface (9-11). Consistent with these studies, we have established the optimal experimental conditions for UV-irradiation of titanium plates (e.g. wavelength and exposure time) which enhanced mouse osteoblast MC3T3-E1 cell adhesion to the plates (12). However, the effects of UV-irradiated titanium plates on osteoblast cell structures are still unclear. While Iwasa *et al.* (13) prepared thin (200 nm) titanium plates to observe cell morphology by light microscopy, the morphological investigation under opaque culture condition is challenging.

To gain further understanding, here we investigated morphological and functional alterations of mouse MC3T3-E1 cells which were incubated on UV-irradiated titanium plates for 24 hours, using digital stereomicroscopy with HE staining, and compared them with those observed for cells on non-UV-irradiated titanium plates. Scanning electron microscopy (SEM), which requires both fixation and ultra-thin electrically conducting material (e.g. gold) coating of cells, has been utilized to observe osteoblast cells on opaque culture plates (12-14). Recently, the dynamicity of morphological alterations in viable cells, although not osteoblast cells, was reported (15). We therefore also aimed to investigate the long- and short-term effects of UV-irradiated culture environment on morphological and functional alterations of mouse osteoblast MC3T3-E1 cell by digital stereomicroscopy.

Materials and Methods

Preparation and properties of titanium plates. Type I pure titanium (Ti: 99.5%; Tokuriki, Chiyoda-ku, Tokyo, Japan) sheet was used to prepare culture plates of 20×20×0.5 mm (n=12). Plates were embedded in epoxy resin and polished using an automatically rotated polisher (160-200 rpm; Buehler ECOMET

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Key Words: Titanium, UV-irradiation, cell attachment, digital stereomicroscopic study.

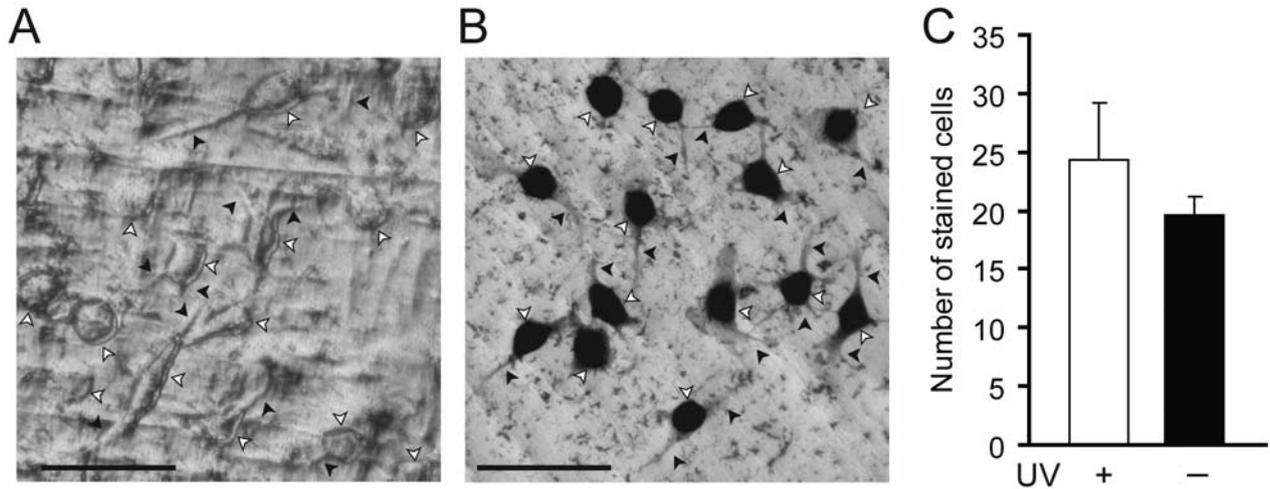


Figure 1. Non-stained viable MC3T3-E1 cells (A: with differential interference contrast (DIC)) and HE-stained MC3T3-E1 cells (B: without DIC) observed under digital stereomicroscopy ($\times 1,000$) at 24 hours after incubation. Open arrowhead: soma of osteoblast. Closed arrowhead: sprouting processes of osteoblast. Scale bar: 50 μm . C: Number of HE-stained cells on UV-irradiated and non-UV-irradiated titanium plates.

3, Lake Bluff, IL, USA) with a series of waterproof abrasive papers (#400, 800, 1000, 1500 and 2000; Fuji Star, Sankyo Rikagaku, Okegawa, Saitama, Japan) followed by 0.05-1 μm diamond suspension with a buffing cloth (Chemomet; Buehler). Plates were carefully removed from the epoxy resin and cleaned by ultrasonic rinsing with distilled water, 99.5% ethanol and 99.5% acetone for 10 minutes and then dried by blowing air (12, 16, 17).

The titanium plates were divided into UV-irradiated and non-UV-irradiated groups (n=6 each). All plates were sterilized by autoclaving. For UV irradiation, plates were placed in a UV chamber and were exposed to 253.7 nm (52 $\mu\text{W}/\text{cm}^2$) for 20 minutes (12). All of the plates were reused across experiments after re-polishing and sterilization. The polished surface of plates was observed by differential interference contrast (DIC) digital stereomicroscopy (VHX-1000; Keyence, Yodogawa-ku, Osaka, Japan) at a magnification of 1,000-fold. Since the surface roughness of culture plates is associated with cell function (e.g. attachment and differentiation) (2, 18), we confirmed roughness parameters (R_a : arithmetic mean of departures of roughness profile from mean line, and R_{max} : vertical distance between maximum peak to lowest valley within a single sample length) of polished titanium plates as previously described (12, 19, 20) and obtained similar values to those of a previous report ($R_a=0.19\pm 0.08 \mu\text{m}$ and $R_{max}=1.27\pm 0.43 \mu\text{m}$) (12).

Experimental design and assay for cell morphology. Mouse MC3T3-E1 cells from the calvaria of C57BL/6 mice (20) were sub-cultured as adherent cells in alpha minimum essential medium (α -MEM; GIBCO BRL, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; JRH Bioscience, Lenexa, KS, USA), 100 units/ml penicillin G and 100 $\mu\text{g}/\text{ml}$ streptomycin sulfate at 37°C under a humidified 5% CO_2 atmosphere (14). Cells (0.4 ml of 6×10^5 cells/ml) were inoculated onto each group of titanium plates (n=3 each) and incubated for 40 minutes at room temperature (RT: 25°C; see below), then incubated further (24 hours at 37°C in 5% CO_2).

After 24 hours' incubation, cultured osteoblasts were photographed at 1,000-fold magnification by DIC microscopy (Figure 1A). Subsequently, cells were fixed with 4% paraformaldehyde (PFA) and stained with hematoxylin and eosin (HE) on titanium plates then were photographed at 1,000- and 2,000-fold magnifications (Figure 1B and 2A, respectively; without DIC). To evaluate the effect of UV on cell attachment, the number of HE-stained cells in the region of interest (ROI: $200\times 200 \mu\text{m}^2$) in each 1,000-fold magnified photograph was counted. Cells whose area exceeded the boundary line of ROI more than 50% were not counted. To evaluate the effect of UV on cell morphology, 10 HE-stained cells were selected in each 2,000-fold magnified photograph, and then the area and perimeter of the cell, the length of major and minor axes of cell expansion (sprouting extension), the length of major and minor axes of soma, and the number and length of sprouting processes were measured by ImageJ software (NIH ImageJ, Version 1.43u; Figure 2B).

To investigate the short-term effects of UV on cell attachment and cell morphology, cells were inoculated on each group of titanium plates (n=3 each). After 40 minutes' incubation, cells were photographed at 1,000-fold magnification with DIC, then fixed and stained on the plates. Photographs were taken at 1,000- and 2,000-fold magnifications (Figure 2B and C, respectively) and the number of stained cells and morphological features were analyzed. All of the acute experimental group (n=3 each) and two experimental pairs of plates incubated for 24 hours were observed by DIC microscopy (1,000-fold magnification) for the initial 40 minutes of RT incubation. The observation area was selected and a photograph was taken (photograph 0), then the same selected area was photographed for 40 minutes at 5-minute intervals (photographs 1-8) and saved for off-line analysis. In photograph 0, 10 cells were selected and their major and minor axis lengths and area of soma were measured. In photographs 1-8, the same cells were analyzed to investigate the effects of UV on cell morphology. The morphological features obtained during 40 minutes observation period were standardized based on those obtained from photograph 0 for comparison.

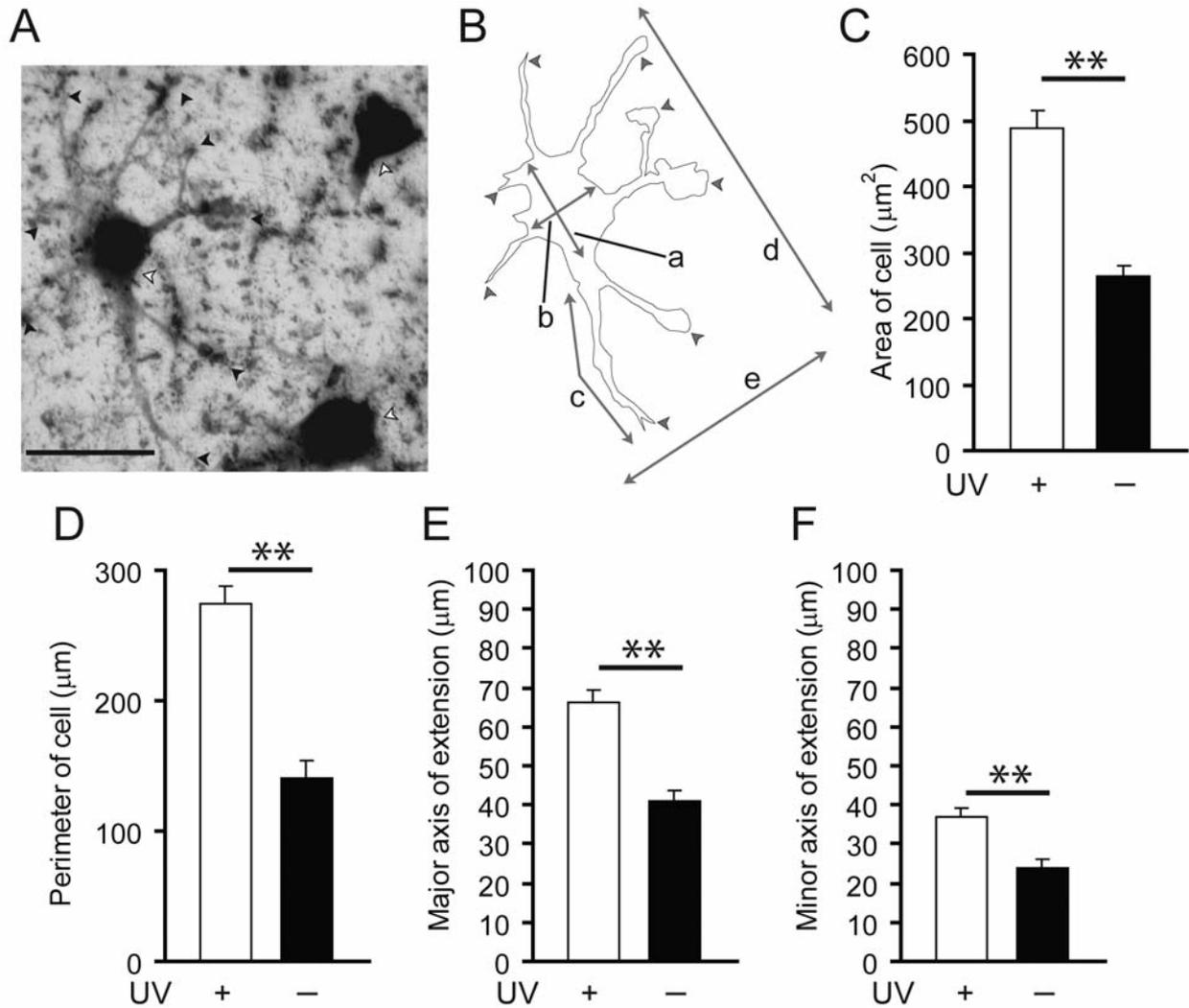


Figure 2. HE-stained MC3T3-E1 cells observed under digital stereomicroscopy (A: $\times 2,000$ without DIC) at 24 hours after incubation. Open arrowhead: soma of osteoblast. Closed arrowhead: sprouting processes of osteoblast. Scale bar: 25 μm . B: Schematic drawing of cell contour (area and perimeter): a: major axis of soma, b: minor axis of soma, c: length of sprouting process, d: major axis of sprouting extension, e: minor axis of sprouting extension. Area of cell included sprouting processes (C), perimeter length of cell included sprouting processes (D), length of major axis of sprouting extension (E), length of minor axis of sprouting extension (F) of HE-stained MC3T3-E1 cells on UV-irradiated and non-UV-irradiated titanium plates. $**p < 0.01$, Student's *t*-test.

Statistical analysis. Results are presented as means \pm standard error of the mean (SEM). Statistical analysis was performed using Student's *t*-test for comparison between two groups, and Bonferroni *t*-test or two-way ANOVA for multiple comparisons as appropriate. Differences were considered significant at $p < 0.05$.

Results

Cell morphological alterations under 24-hour incubation. The DIC photograph of viable MC3T3-E1 cells incubated for 24 hours on UV-irradiated and non-UV-irradiated plates showed variety in the shapes (round, ellipse and *etc.*) and the sizes of the cell contour and the extent of sprouting processes

(Figure 1A), and this was also observed in HE-stained cells on each plate (Figure 1B). After HE staining, only the cells attached to the titanium surface remain; the number of stained cells on UV-irradiated plates was greater than those observed on non-UV-irradiated plates, but not significantly (Figure 1C). The detailed analysis of morphological features for HE-stained cells at higher magnification (Figure 2A) showed that UV-irradiation significantly ($p < 0.01$, Student's *t*-test) increased the cell area and perimeter, the length of major and minor axes of sprouting extension, and the number and length of sprouting processes of incubated cells compared with those observed on non-UV-irradiated plates

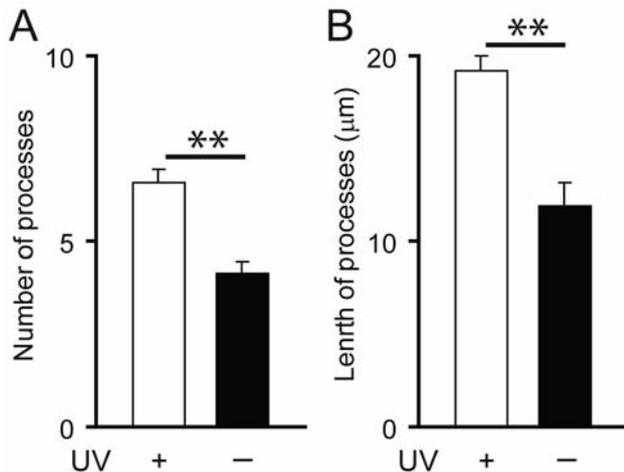


Figure 3. Number (A) and length (B) of sprouting processes after 24 hours' incubation. ** $p < 0.01$, Student's t -test.

(Figure 2C-F and 3A and B). On the other hand, there were no significant differences in the length of major (UV: $16.5 \pm 0.9 \mu\text{m}$ and non-UV: $15.3 \pm 0.4 \mu\text{m}$) and minor (UV: $11.9 \pm 0.5 \mu\text{m}$ and non-UV: $11.6 \pm 0.3 \mu\text{m}$) axes of soma between cells under different conditions.

Cell morphological alterations induced by short-term UV effects. The DIC photograph of viable cells incubated for 40 minutes on UV-irradiated and non-UV-irradiated plates showed mainly a round shape of the cell contour and varying cell sizes, without sprouting processes (Figure 4A). HE-stained cells also showed round shape of cell contour and varying cell sizes (Figure 4B). Interestingly, the cells which already expressed sprouting processes were observed (Figure 4Ci and ii) and there was no significant difference in the incidence of cells with sprouting processes between UV (0.77) and non-UV (0.6) -irradiated plates. After HE stain, the number of stained cells on UV-irradiated plates was significantly ($p < 0.01$, Student's t -test) greater than those observed on non-UV-irradiated plates (Figure 4D). The detailed analysis of morphological features for HE-stained cells at higher magnification (Figure 4C) showed UV-irradiation significantly ($p < 0.05$, Student's t -test) increased the area of incubated cells compared with those observed on non-UV-irradiated plates (Figure 4E), however, there were no significant differences in the perimeter of cells or the number of sprouting processes (Figure 4F and G).

Cell morphological alterations occurred immediately after inoculation. In general, cells inoculated on non-UV-irradiated plates shrunk, reducing the cell contour (Figure 5A and B). The significant ($p < 0.05$ or $p < 0.01$, Bonferroni t -test) reduction in the area of cells on non-UV-irradiated plates

compared with those observed at time 0 was detectable within 5 minutes of inoculation and was maintained for 40 minutes (Figure 5A). The reduction in the major axis length of cells was also observed across 40 minutes, however, significant ($p < 0.01$, Bonferroni t -test) reduction was observed 15-40 minutes after the inoculation. The length of the minor axis of cells was reduced, but not significantly (Figure 5B). On the other hand, cells on UV-irradiated plates showed an expansion of their cell contour (Figure 5A and B). Significant ($p < 0.05$ or $p < 0.01$, Bonferroni t -test) increase in the area and major axis of cells compared with those observed at time 0 was detected at 10 minutes after inoculation and reached a plateau in 15 minutes then cell size was maintained over the subsequent observation period (Figure 5A). Significant ($p < 0.05$ or $p < 0.01$, Bonferroni t -test) increase in the major axis length of cells was also observed from 5 minutes after inoculation. The length of the minor axis of cells showed a significant ($p < 0.05$ or $p < 0.01$, Bonferroni t -test) increase, however, at only 25, 30 and 40 minutes after inoculation (Figure 5B). Comparison of morphological features between cells on UV-irradiation vs. non-UV-irradiation gave significant differences in the area ($p < 0.01$, $F(1,17)=616.1$, two-way ANOVA), and the length of the major axis ($p < 0.01$, $F(1,17)=547.2$, two-way ANOVA) and the minor axis ($p < 0.01$, $F(1,17)=242.1$, two-way ANOVA) of cells. There were also treatment \times time effects observed for the area ($p < 0.01$, $F(8,17)=12.0$, two-way ANOVA), the length of the major axis ($p < 0.01$, $F(8,17)=9.9$, two-way ANOVA) and the minor axis ($p < 0.01$, $F(8,17)=6.2$, two-way ANOVA) of cells.

Discussion

This study investigated the effects of UV-irradiation on morphological alterations of mouse MC3T3-E1 cells. The observation of both non-stained viable cells (with DIC) and HE-stained cells (without DIC) was performed with digital stereomicroscopy, which allows extra-long observation distance (25 mm at $\times 1,000$) on UV-irradiated and non-UV-irradiated titanium plates. Although DIC observation allowed description of the contour of the cell and confirmed the existence of sprouting processes of cells incubated for 24 hours on each type of titanium plate (Figure 1A), HE-staining observation without DIC was able to provide a more detailed picture of sprouting processes at the same ($\times 1,000$, Figure 1B) and higher ($\times 2,000$, Figure 2A) magnifications. Since this comparison indicated that the accuracy of DIC observation is sufficient for analysis of cell contour, but not sprouting processes, the detailed cell morphological alteration was analyzed with higher magnified HE-stained cells without DIC.

It has been established that UV-irradiation forms an oxide network (21-23) which provides a hydrophilic (both

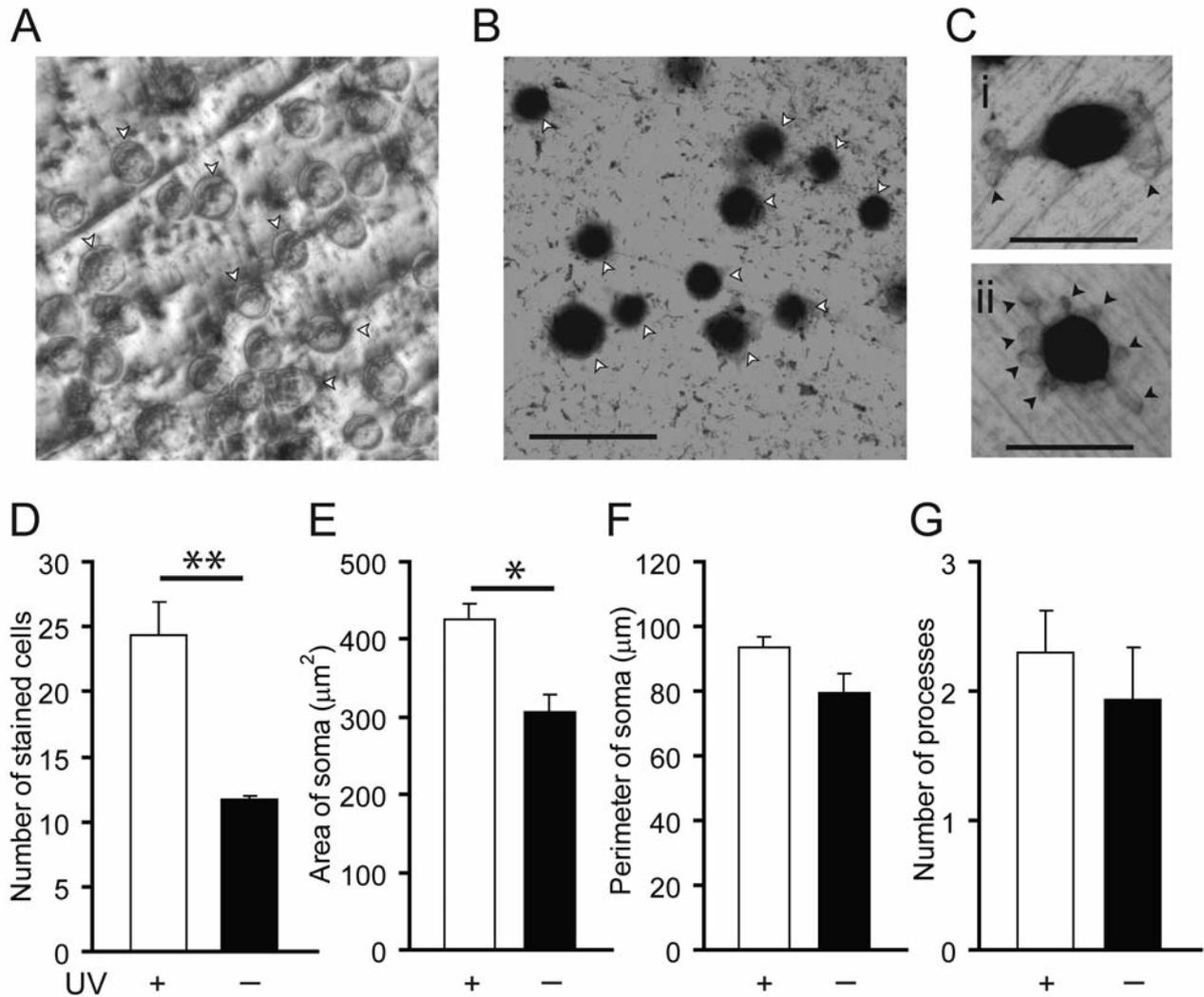


Figure 4. Non-stained viable MC3T3-E1 cells (A: with DIC $\times 1,000$) and HE-stained MC3T3-E1 cells (B: without DIC $\times 1,000$, C: without DIC $\times 2,000$) observed under digital stereomicroscopy at 40 minutes after inoculation. Open arrowhead: soma of osteoblast. Closed arrowhead: sprouting processes of osteoblast. Scale bar: 50 (A and B) and 25 (C) μm . D: Number of HE-stained MC3T3-E1 cells on UV-irradiated and non-UV-irradiated titanium plates. Area of soma (E), perimeter of soma (F) and number of sprouting processes (G) of HE-stained MC3T3-E1 cells on UV-irradiated and non-UV-irradiated titanium plates. * $p < 0.05$, ** $p < 0.01$, Student's *t*-test.

amphiphilic and oleophilic) environment (24) and electrostatic force (13) on the metal surface. These physical alterations of metal surfaces enhanced cell activities (12, 22, 25-27) and, consistent with previous studies, the effects of UV-irradiation on cell morphology was observed as a significant ($p < 0.01$) increase in the cell extension (Figure 2C-F) and the number and length of sprouting process (Figure 3A and B) in this study. We also observed an increase in the cell number on UV-irradiated titanium surface after 24 hours' incubation, however, the probability value was not statistically significant (Figure 1C). This discrepancy between the present and previous results (12) might be due to the lower number (30%) of inoculated cells

used here to investigate individual cell morphological alterations. Another possibility is that the conditions we used in the present study allowed the cells to be grown to the saturation level of cell density, thus making the difference between UV-irradiation and non-UV-irradiation very small. On the other hand, significant ($p < 0.01$) short-term effects of UV-irradiation were observed regarding an increase of the cell attachment and the area of cells, but not features related to sprouting processes (Figure 4). In particular, continuous observation of viable cells with DIC revealed that the UV-irradiation of the plates induced significant ($p < 0.01$) expansion of the cell area in 15 minutes (Figure 5). These results suggest that environment of the

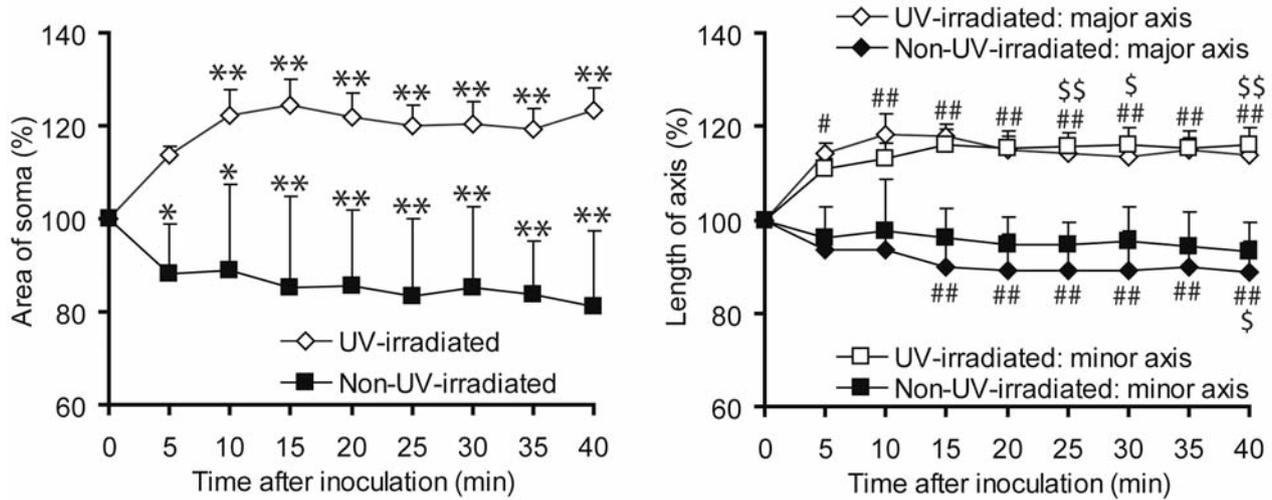


Figure 5. Short-term effects of UV-irradiated culture environment on alteration of cell morphology. Alteration of area (A) and major and minor axes (B) of MC3T3-E1 cells during 40 minutes after inoculation. * $p < 0.05$, ** $p < 0.01$, vs. time 0 (area); # $p < 0.05$, ## $p < 0.01$, vs. time 0 (major axis); \$ $p < 0.05$, \$\$ $p < 0.01$, vs. time 0 (minor axis), Bonferroni t-test.

UV-irradiated plates induced rapid cell attachment to the metal surface and facilitated the extension of sprouting processes. While further studies are needed to delineate specific morphofunctional relationships, this system is a useful supplement for quantifiable investigation of viable cells in an opaque culture environment.

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