

## Quantification of Enhanced Osteoblastic Adhesion to Ultraviolet-treated Titanium Plate

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**Abstract.** Although the advantage of ultraviolet (UV) irradiation of titanium plates for the attachment of osteoblast is known, the details of the experimental conditions have not been described in previous literature. We established optimal conditions of UV irradiation of titanium plate for the adhesion of mouse osteoblast MC3T3-E1 cells. The viable cell number was determined by MTT method. UV irradiation at two different wavelengths (253.7 and 365 nm) enhanced the cell attachment on titanium plate to comparable extents. The optimal UV exposure duration was 20 minutes and prolonged irradiation slightly reduced cell attachment. The attached cells proliferated during 24 hours, accompanied by the enhanced consumption of extracellular glutamine and arginine. The present study supports the previous reports of the efficacy of UV irradiation, and this simple and rapid assay system may be applicable for the study of the interaction of osteoblast and UV-activated titanium plates.

One of the most important oral reconstructions has been the use of dental implants as treatment option since the pioneering work by Branemark. The necessity of obtaining total osseointegration of dental implants has been a challenge to physicians. Although the improvement of surface properties of titanium implants by chemicals has evolved in the era of implantology (1-6), realistic surface treatment of titanium has not yet been achieved (1). The sterilization of

dental implants plays an important role in achieving the osseointegration of implants (7) and radiofrequency glow discharge or ultraviolet chamber irradiation yields a clean surface of titanium (8, 9). The UV sterilization of titanium surfaces produces a thinner oxide layer more efficiently than does steam sterilization (autoclave treatment) (8, 10). Exposure of titanium plate to UV irradiation reduces the carbon concentration of titanium surface by photocatalysis, and increases oxygen. Theoretically, the oxygen surfaces are hydrophilic (both amphiphilic and oleophilic) (11), binding structural water and forming -OH and -O<sub>2</sub> groups in the outer layer (12, 13). The surface energy and hydrophilicity of implant surfaces play a decisive role in its initial interaction with proteins and cells in bone (14-16).

We have newly established an assay system for the investigation of the interaction between metal and cultured cells. Using this technique, we recently reported that contact with hydroxyapatite-coated titanium plate significantly enhanced the proliferation of mouse osteoblastic MC3T3-E1 cells, based on the increased cell number and the enhanced consumption of amino acids, especially glutamine and arginine in the culture medium (2). Titanium surface roughness may affect gene expression (17) and osteoclastic differentiation (18). Recently, surface treatment of titanium plates with UV has been reported to significantly enhance the attachment of rat bone marrow-derived osteoblastic cells (19, 20), human mesenchymal stem cells (MSCs) (21) and pluripotent mesenchymal precursor cell line C2CI2 (22). Although this information is extremely valuable for the clinical application of titanium plates, the details of the experimental conditions have not been described so far. We report here a more simple and reproducible method of activating the titanium plate for the study of interaction between the cells and metals, using 3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) method and measurement of amino acid consumption.

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**Key Words:** Titanium, UV irradiation, cell attachment, MTT method, amino acid consumption.

## Materials and Methods

**Materials.** The following chemicals and reagents were used: alpha minimum essential medium ( $\alpha$ -MEM), Dulbecco's modified Eagle's medium (DMEM) (GIBCO BRL, Grand Island, NY, USA) fetal bovine serum (FBS) (JRH Bioscience, Lenexa, KS, USA); Type I pure titanium (Ti) (99.5%) (Tokuriki, Melters, Tokyo, Japan); MTT (Sigma-Aldrich, St. Louis, MO, USA); trichloroacetic acid (TCA), dimethyl sulfoxide (DMSO) (Wako Pure Chem Co., Osaka, Japan); culture polystyrene dishes and plates (6, 24 and 96-well) (Becton Dickinson, Franklin Lakes, NJ, USA).

**Sample preparation.** The pure titanium sheet was used to prepare sample plates of 20×20×0.5 mm (n=10). Samples were placed in epoxy resin and polished by an automatically rotated polisher (160-200 rpm; Buehler, Lake Bluff, IL, USA) with different types of surface finishing, #400, 800, 1000, 1500 and 2000 waterproof abrasive paper (Fuji Star, Sankyo, Rikagaku, Okegawa, Japan) and 0.05-1  $\mu$ m diamond suspension with a buffing cloth (Chemomet, Buehler, Lake Bluff, IL, USA). Samples were removed from epoxy resin and cleaned by ultrasonic rinsing with distilled water, 99.5% ethanol and 99.5% acetone (23, 24) for 10 minutes (23-25) and then dried by blowing air. The titanium plates were divided into UV-irradiated (experimental) and non-UV-irradiated (control) groups (n=5 each). All of the titanium plates were reused across experiments after re-polishing and sterilization.

**Measurement of surface topography.** The polished surface of samples was assessed by scanning electron microscopy (SEM: JSM-6360LV, JEOL, Tachikawa, Japan) at a magnification of 300- and 1000-fold. Roughness measurement was carried on each sample by surface roughness tester (Surfcom, Seimitsu, Tokyo, Japan). Roughness parameters were obtained from six profiles (5 mm length) of the plate (four from the periphery and two in the center of plate), and then expressed as an average value, according to ISO 4287:1997. The analyses of results were considered in order of  $R_a$  (the arithmetic mean of the departures of roughness profile from mean line) (6, 26) and  $R_{max}$  (the vertical distance between the maximum peak to lowest valley within a single sample length) (6, 26).

**Sterilization and UV irradiation.** All samples were packed in standard plastic pouches and sterilized using standard steam sterilization (autoclave treatment). For UV irradiation, samples were placed in a UV chamber and were exposed to 253.7 nm (52  $\mu$ W/cm<sup>2</sup>) or 365 nm (3.40 mW/cm<sup>2</sup>). The UV exposure duration was 5, 20, 40 minutes or 1, 4 and 6 hours for 253.7 nm and 20 minutes for 365 nm, respectively (9).

**Cell culture.** MC3T3-E1 mouse cells from the calvaria of C57BL/6 mice (27) were sub-cultured as adherent cells in  $\alpha$ -MEM supplemented with 10% heat-inactivated FBS, 100 units/ml penicillin G and 100  $\mu$ g/ml streptomycin sulfate at 37°C under a humidified 5 % CO<sub>2</sub> atmosphere (2). Cells were washed with phosphate-buffered saline without Ca<sup>2+</sup> and Mg<sup>2+</sup> (pH 7.4) [PBS(-)] and detached by 0.25 % trypsin-0.025 % EDTA-2Na in PBS(-) for each experiment.

**Assay for cell attachment and proliferation.** Cells (0.8 ml of 2×10<sup>6</sup> cells/ml) were inoculated on each titanium plate. After incubation for 40 minutes at room temperature (25°C), each plate was washed

three times with PBS(-) to remove unattached cells and the viable cell number of the attached cells was determined by MTT method. Aliquots of the cells were incubated for a further 24 hours at 37°C in 5% CO<sub>2</sub> incubator, after the 40 minutes attachment before the MTT assay. In brief, attached cells on the plate were incubated for 1 hour with 0.2 mg/ml MTT in fresh DMEM with 10% FBS. We used DMEM plus 10% FBS as the incubation medium for MTT assay, since dissolving MTT reagent in  $\alpha$ -MEM produced a bluish coloring that made it difficult to accurately determine the viable cells. The use of DMEM was only for MTT assay, and all other experiments were carried out with  $\alpha$ -MEM with 10% FBS. The formazan formed during incubation was dissolved with 0.1 ml of DMSO, and the absorbance at 540 nm of the lysate was determined by using a microplate reader (Multiskan, Biochromatic, LabSystem, Osaka, Japan).

**Determination of free amino acids.** Culture supernatant (medium fraction) was mixed with an equal volume of 10% TCA and stood on ice for 30 minutes. After centrifugation for 5 minutes at 21,000×g, the deproteinized supernatant was collected and stored at -30°C. The supernatants (20  $\mu$ l) were subjected to a JLC-500/V amino acid analyzer (JEOL, Tokyo, Japan) and amino acids were detected using the ninhydrin reaction (28). The consumption of glutamine and arginine was determined by the difference of amino acid concentrations before and after incubation.

**Statistical analysis.** The mean values and standard deviations were calculated. The average value were compared by one-way ANOVA and Bonferroni-type multiple *t*-test. The value of statistical significance was set at the 0.05 level.

## Results

**Surface roughness measurement.** The mean value of  $R_a$  and  $R_{max}$  of titanium plate samples were 0.20±0.08  $\mu$ m and 1.25±0.39  $\mu$ m, respectively (Figure 1A). SEM images of polished plate surfaces showed that the titanium plates had almost a flat surface, with some grooves, at magnifications of 300 and 1000 (Figure 1B, C).

**Cell attachment and proliferation.** UV irradiation of titanium plate at two different wavelengths (253.7, 365 nm) enhanced the adhesion of MC3T3-E1 cells to comparable extents (Figure 2). UV irradiation significantly improved the cell attachment, compared to UV-untreated plates. The optimal UV-exposure duration was 20 minutes and prolonged irradiation slightly diminished the attachment. It should be noted that UV-treated plates had cell attachment ratios similar to or slightly higher than polystyrene plates commonly utilized in tissue culture, although the difference between these was not statistically significant (Figure 3). When the attached cells were incubated for 24 hours, a much higher number of cells (reflected by high value of absorbance at 540 nm) were obtained from each plate compared with 40 min incubation, indicating the occurrence of active proliferation on all plates. It should also be noted

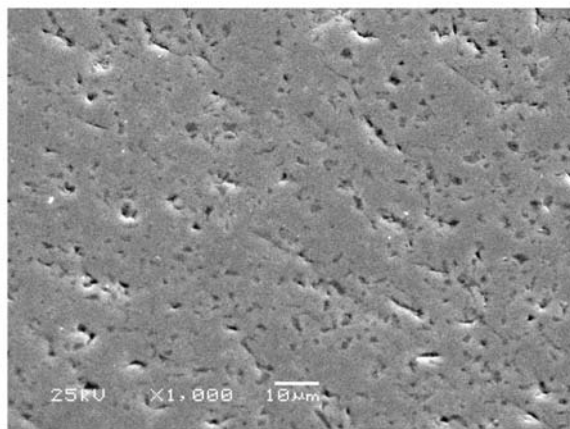
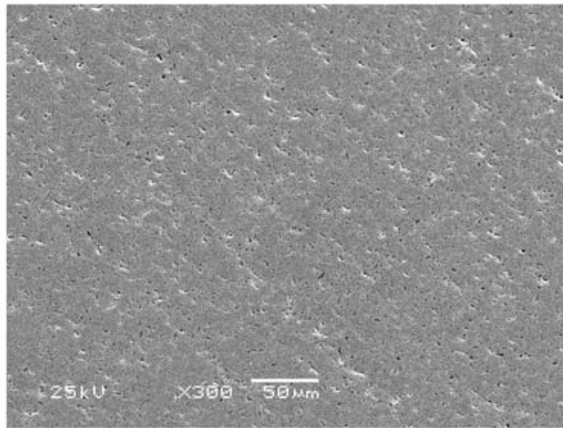
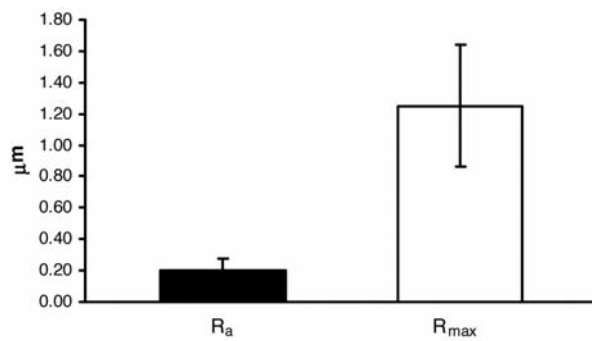


Figure 1. Surface topography of polished titanium plate. A: Parameter measurement from six profiles of 10 titanium plate samples. Each point represents mean $\pm$ S.D. B-C: SEM images of the titanium plate surface at 300- and 1000-fold magnification.

that the cell numbers from UV-treated titanium plates were much higher than those from the unirradiated plates and even polystyrene plates (Figure 4). During 24 hours, the consumption of glutamine and arginine in the cells attached to UV-irradiated plates was 16.9% [(485-415) $\times$ 100/415] and 32.7% [(73-55) $\times$ 100/55] higher than that of the cells attached to the non-irradiated plates (Table I).

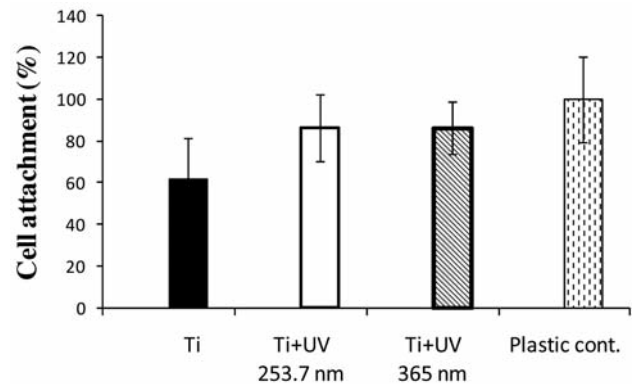


Figure 2. Effect of UV irradiation at different wavelength on cell attachment. Titanium plates were exposed for 20 minute to UV radiation at 253.7 and 365 nm. Cells were inoculated on unirradiated titanium plates, UV-irradiated plates or polystyrene plates, and incubated for 40 minutes. After washing three times with PBS(–) to remove the loosely bound cells, the attached cells on the plates were incubated for 1 hour with MTT reagent to determine the relative viable cell number. Irradiation of the plates by two different wavelengths of UV produced no statistically significant differences. Each value represents the mean  $\pm$  S.D. obtained from four determinations. Absorbance at 540 nm of control cells was 0.981-1.359.

Table I. Amino acid consumption by MC3T3-E1 cells attached to UV-treated or untreated (control) titanium plate.

		Concentration (μM)		Change
		0 h	24 h	
UV	Gln	1488 $\pm$ 32	1003 $\pm$ 27	(–485)
	Arg	469 $\pm$ 6	396 $\pm$ 15	(–73)
Control	Gln	1457 $\pm$ 60	1042 $\pm$ 68	(–415)
	Arg	464 $\pm$ 14	409 $\pm$ 23	(–55)

Each value represents the mean $\pm$ S.D. of 3-4 determinations.

## Discussion

The present study demonstrated that UV irradiation of titanium plates by short duration (20 minutes) exposure enhanced the attachment of MC3T3-E1 mouse osteoblastic cells to the titanium plates, as judged by the increased viable cell number recovered from the plates and enhanced consumption of glutamine and arginine by the attached cells (2). Since the method relies on cellular metabolic activity, the present results may be much more reliable than those of previous direct cell counting with SEM (19) or detachment by trypsin with vibrational force (29). We have confirmed that MC3T3-E1 cells were actually attached to the UV-irradiated plates under SEM observation after fixation of the cells with 2% glutaraldehyde in 0.1 M cacodylate buffer (data not shown). However, the

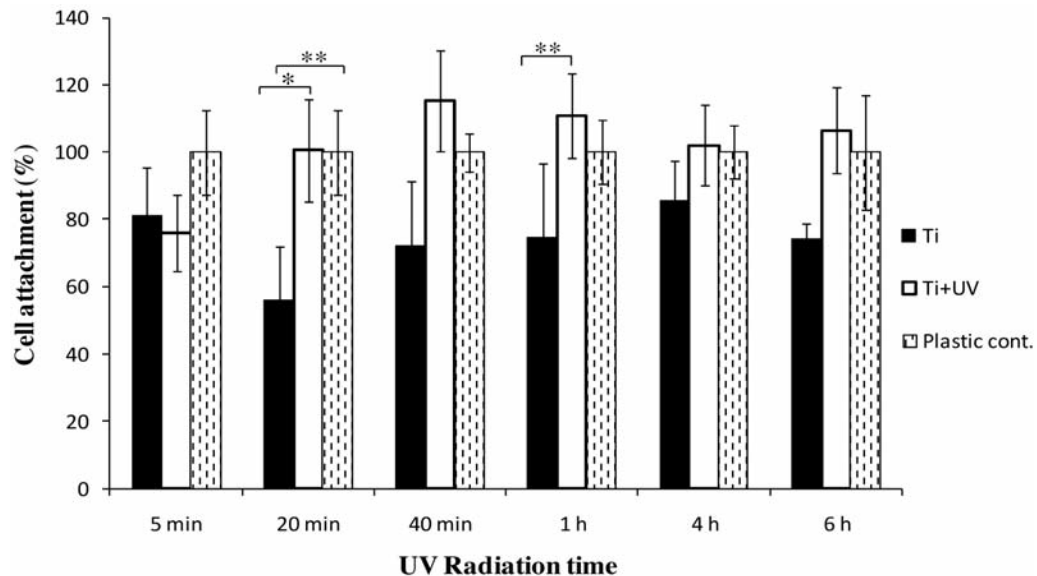


Figure 3. Effect of exposure duration of UV irradiation on cell attachment. Titanium plates were exposed to UV (253.7 nm) for 5, 20, 40 minutes, 1, 4 or 6 hours. Cells were inoculated on these plates, or unirradiated titanium plates or polystyrene plates, and incubated for 40 minutes. After washing three times with PBS(–), the relative number of attached cells were determined by MTT method. Each value represents the mean±S.D. obtained from four determinations. Absorbance at 540 nm of control cells was 0.311-0.808. \* $p < 0.05$ , \*\* $p < 0.01$ .

present MTT method has one pitfall in that the formazan was not completely recovered by DMSO, suggesting that a minority of the cells still remained on the plate. Since more cells seem to be unrecoverable from the UV-treated plate, our data underestimate the actual number of cells present. To overcome this problem, the measurement of amino acid consumption by actively proliferating cells may be a good choice for monitoring cellular activity, since this can still be monitored even when the cells are tightly bound to the plate. The present study is preliminary, and therefore more detailed kinetics study (time course and irradiation dose response) of changes in the viable cell number and cellular amino acid utilizing capability are necessary. In order to apply the UV-irradiated plate clinically, it is also necessary to investigate whether MC3T3-E1 cells grown on the UV-irradiated titanium plate can differentiate into more mature cells upon treatment with appropriate stimuli, monitoring the expression of differentiation-associated genes.

The first report of photocatalysis exemplified by  $\text{TiO}_2$  dates back to the 1970s (30). Several studies have focused on antibacterial and sterilization effects of UV-irradiation (31, 32). The present study further substantiates that UV irradiation of pure titanium plates improves the attachment of osteoblasts to the plate surface, achieved by as little as 20 minutes' exposure to UV.

## Acknowledgements

The surface polish and analysis were assisted by Dr. Taniichiro Yamaga, and SEM observation was assisted by Mr. Shiro Kunii. This research was supported by 'The Exchange Program between Meikai University, Japan and Universidad Autónoma del Estado de México, México'.

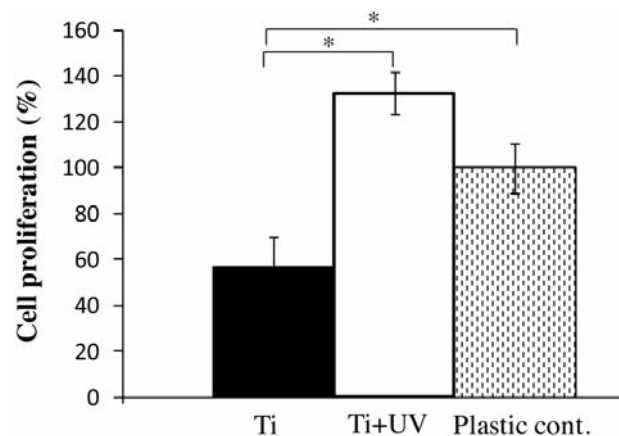


Figure 4. Proliferation of cells attached to various types of plates during 24 hours. Titanium plates were exposed to UV irradiation at 253.7 nm for 20 minutes. Cells were inoculated on the plates, and incubated for 40 minutes. After washing with PBS(–), the attached cells were incubated for a further 24 hours in fresh medium. The relative numbers of viable cells were determined by MTT method. Each value represents the mean±S.D. obtained from four determinations. Absorbance at 540 nm of control cells was 1.471-1.866. \* $p < 0.05$ .

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Received March 2, 2010

Revised May 26, 2010

Accepted June 4, 2010