Enhancing osteoblast-affinity of titanium scaffolds for bone engineering by use of ultraviolet light treatment

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ABSTRACT
Ultraviolet (UV) treatment immediately prior to use is attracting attention as an effective surface conditioning method for titanium to improve osteoblast-affinity. The affinity of titanium to osteoblasts in two-dimensional plate culture has been well studied, but that in three-dimensional cultures remains unclear. Here, we examined the effect of UV treatment on titanium scaffolds, comprising micro-thin titanium fibers, used in bone engineering. Titanium scaffolds, with and without UV treatment, were seeded with rat bone marrow derived osteoblasts, and the number of cells attached to scaffolds and osteoblastic phenotype in the cultures were examined. UV treatment improved the wettability of scaffolds and significantly reduced the percentage of surface carbon. Along with these physicochemical changes in the scaffolds, cell attachment increased by a factor of 1.3 as compared to that of the untreated control. In addition, alkaline phosphatase activity and calcium deposition significantly increased by a factor of 2.3 and 2.0, respectively. Robust formation of mineralized structures consisting of clear peaks of calcium and phosphorus was observed in the UV-treated scaffolds. The observed increase in osteoblast affinity and capability of mineralized matrix formation indicates the potential use of UV-treated titanium scaffolds for bone engineering.

Surface conditioning of titanium using ultraviolet (UV) treatment immediately prior to use effectively and efficiently establishes an osteoblast-affinity surface (3, 17, 19). Since the development of this technique, the technology has been applied to dental implants to facilitate satisfactory bone formation around the implants (7, 8, 22). Using this technology, the bone implant contact (BIC) index (an index to evaluate bone formation around the implants) of UV-treated implants was 98.2% compared to 51.7% in that of the untreated control after a four-week healing period in a rat study (1). The UV treatment induces various physicochemical changes to the surface of titanium as follows: 1) removing accumulating carbon, 2) converting the hydrophilicity from hydrophobic to hydrophilic, and 3) converting the electrical charge from electro-negative to electro-positive (2, 3, 17, 19). These surface properties of UV-treated titanium are considered advantageous for osteoblast affinity (4, 11, 15). Furthermore, UV treatment can be applied to all titanium devices of any design surface topography. Therefore, this effective and versatile technology potentially has wide applications to every titanium device that requires osteoblast affinity.

Titanium scaffolds are considered suitable biomate-
mials for bone engineering due to their high porosity, physical strength, biocompatibility, and osteoconductive properties (10, 25, 27). Different manufacturing methods and modifications of the titanium scaffolds have been studied to improve their capacity for bone engineering (10, 25); for example, coating of the surface with bioactive substrates is a common strategy used to enhance bone formation. Studies have shown that coating of the titanium surface with binding proteins, including phosphoproteins and with thin hydroxylapatite, achieved enhancement of bone formation and osteoblast activity, respectively (12–14, 16). The modification of titanium surface topography is also a possible option, because surface topography promotes the proliferation and differentiation of osteoblasts (20, 21). For instance, a micro-roughened titanium surface has an advantage over machined smooth surfaces, and results in enhanced osteoblastic differentiation and faster bone formation (20, 21). An advanced, innovative in vitro bone engineering technique uses a combination of titanium scaffolds along with a bone-like extracellular matrix and the flow perfusion culture system (5). However, these techniques sometimes require complicated processes and may still be incapable of ensuring predictable results and guaranteed success.

UV light treatment-induced osteoblast-affinity of titanium in two-dimensional plate cultures is well studied, but the utility of the technique in three-dimensional cultures remains unclear. Here, we examined the effect of UV treatment on three-dimensional cultures using titanium scaffolds to assess the possible use of surface conditioning for bone engineering. We hypothesize that the UV-treated titanium scaffold can efficiently attract osteoblasts and create bone effectively in vitro. In the present study, titanium scaffolds with and without UV treatment were seeded with rat bone marrow-derived osteoblasts, following which the number of cells attached to the scaffolds and osteoblastic phenotype of the cultures were assessed. In addition, the morphology and chemistry of the biological structures formed on the scaffolds were evaluated.

MATERIALS AND METHODS

Preparation of titanium scaffolds and their characterization. Rectangular titanium scaffolds (5 mm × 5 mm and 3 mm thick) comprising thin fibers of 20 μm diameter with 89% porosity (Hi-Lex, Kobe, Japan) were prepared for this experiment (Fig. 1A). All scaffolds were etched with 67% H2SO4 (Sigma-Aldrich, Missouri, USA) at 120°C for 10 s to establish a micro-roughed surface. The pore structure and surface topography were examined using a scanning electron microscope (SEM; Nova 230 NanoSEM; FEI, Hillsboro, Oregon). The elemental composition on titanium surfaces was evaluated by electron spectroscopy for chemical analysis (ESCA). ESCA was performed using an X-ray photoelectron spectroscopy (XPS) (ESCA3200; Shimadzu, Tokyo, Japan) under high vacuum conditions (6 × 10−7 Pa). The wettability of the titanium scaffolds was evaluated by measuring the contact angle of 10 μL of double distilled water (ddH2O) droplets. UV treatment was performed by treating the titanium scaffolds with UV light for 12 min using a photo device (TheraBeam SuperOsseo; Ushio Inc., Tokyo, Japan) immediately prior to use in cell culture experiments.

Osteoblast culture and seeding. The bone marrow-derived mesenchymal stromal cells of 8-week old male Sprague-Dawley rats (Charles River Laboratories, California, USA) were cultured in an osteogenic induction medium containing alpha-modified Eagle‘s medium (Invitrogen, California, USA) supplemented with 15% fetal bovine serum (Invitrogen),

Fig. 1 (A) Titanium scaffolds comprising thin fibers were 5 mm × 5 mm and 3 mm thick, with a porosity of 89%. (B, C) Scanning electron microphotographs of the titanium scaffolds and the surface topography of the titanium wire.
50 mg/mL ascorbic acid (Sigma-Aldrich), 10 mM Na-β-glycerophosphate (Sigma-Aldrich), 10^{-8} M dexamethasone (Sigma-Aldrich), and antibiotic-antimycotic solution (Invitrogen). Cells were incubated in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. Titanium scaffolds with and without UV treatment were placed in 24 well plates, following which scaffolds in the cells of passage 2 were seeded with a 1 mL cell suspension including 5 × 10⁴ cells/mL. The culture medium was renewed every 3 days.

**Statistical analyses.** All culture studies were performed in triplicates (n = 6). One-way analysis of variance (ANOVA) was used to examine differences between the un-treated control and the UV-treated scaffolds. P < 0.05 was considered statistically significant.

**RESULTS**

**Characterization of UV-treated titanium scaffolds**
1) Surface topography, 2) elemental composition of the surface, and 3) wettability were assessed to confirm the UV-treatment-induced physicochemical changes on titanium scaffolds. Low magnification SEM imagery of the titanium scaffolds showed randomly arranged titanium fibers distributed evenly. The distance between each titanium fiber was between 150 μm and 200 μm and the pore sizes were approximately uniform (Fig. 1B). High magnification SEM imagery of the titanium scaffolds represented a uniform roughness feature comprising sharp ridges and pits at a micron-scale (Fig. 1C). No topographical change occurred because of UV treatment (Data not shown here). UV treatment significantly decreased the percentage of surface carbon on titanium scaffolds. The percentage of occupancy of carbon on titanium scaffolds was 31.8 ± 3.9% before UV treatment and 10.5 ± 1.6% after UV treatment (Fig. 2A). UV-treated scaffolds showed good wettability. The droplets of 10 μL of ddH₂O placed on an untreated scaffold were reflected and took a spherical shape. In contrast, the water drops were immediately absorbed into UV-treated scaffolds (Fig. 2B). The contact angle of water drops were 123.5° ± 2.5° in the untreated control and 0° in the UV-treated scaffolds.

**Biological structure formed on the scaffold.** The morphologies and elemental composition of the biological structure formed on the scaffold on day 10 were evaluated by SEM and energy dispersive x-ray spectroscopy (EDS) (UltraDry EDS Detector and NORAN™ System 6; Thermo Fisher Scientific, Inc., Massachusetts, USA). The cultures were fixed and dried following a previously described method (28). In brief, the specimens were fixed with 2.5% glutaraldehyde solution (Sigma-Aldrich) and 1% osmium tetroxide (Sigma-Aldrich), and subsequently dehydrated by sequential immersion in 30%, 50%, 75%, 90%, 95%, and 99% ethanol (Sigma-Aldrich). The immersion in 99% ethanol was repeated thrice. Samples dehydrated with ethanol were thoroughly substituted with t-butanol (Sigma-Aldrich) and subjected to drying under vacuumed conditions at 4°C. After drying, the samples were observed by SEM using the low vacuum mode. The elemental composition was analyzed by EDS. The EDS spectrums were randomly measured and average Ca/P ratio and Ca/titanium ratio were calculated (n = 6).
The amount and quality of the biological structures formed on the scaffolds in the culture were assessed using SEM and EDS after 10 days. The mapping image of untreated and UV-treated scaffolds showed distinct elemental localization and element composition. The mapping image of the untreated control scaffold represented the elements of titanium as fibrous shapes and overlapping localization of calcium and phosphorus (Fig. 4A). In contrast, almost all titanium fibers within the UV-treated scaffolds were covered by calcium and phosphorus (Fig. 4A). The calcium/phosphorus ratio was calculated to evaluate the maturation of mineralized structures, and no significant difference was observed between the untreated control and the UV-treated scaffolds (Fig. 4B). UV-treated titanium scaffolds showed substantially higher values of the calcium/titanium ratio, representing the occupancy of calcium as compared to that of titanium on the surface of the specimens (Fig. 4C).

In addition, the biological structures formed on the scaffolds represented clear morphological differences between untreated control and UV-treated scaffolds. The number of cells attached onto the scaffold in the culture after 24 h was observed by a laser microscope and quantified using a WST-1 colorimetric assay to evaluate the ability of titanium scaffolds to attract cells. The micrograph stained for actin showed the existence of cells on the scaffold with the UV-treated scaffolds attracting more cells on their surfaces compared to that of the control (Fig. 3A). The cells on the UV-treated scaffolds extended cell projections to a wider extent and displayed cell spreading along titanium fibers (Fig. 3A). The WST-1 result supported the finding of the microscopic observation. Colorimetrically measured cell numbers in the UV-treated scaffolds were 30% higher than that of the untreated control (Fig. 3B). In addition, ALP activity and the amount of calcium deposition were assessed as osteoblastic phenotypes. UV-treated titanium scaffolds demonstrated a significant enhancement in the osteogenic phenotypes to a factor of 2.3 times in the ALP activity and 2.0 times in the calcium deposition as compared to those in the untreated control (Fig. 3C, D).

**Enhanced in vitro bone formation on the UV-treated scaffolds**

The in vitro biological structures formed on the titanium scaffolds were assessed to evaluate the ability of titanium scaffolds to facilitate substantial bone formation. The amount and quality of the biological structures formed on the scaffolds in the culture were assessed using SEM and EDS after 10 days. The mapping image of untreated and UV-treated scaffolds showed distinct elemental localization and element composition. The mapping image of the untreated control scaffold represented the elements of titanium as fibrous shapes and overlapping localization of calcium and phosphorus (Fig. 4A). In contrast, almost all titanium fibers within the UV-treated scaffolds were covered by calcium and phosphorus (Fig. 4A). The calcium/phosphorus ratio was calculated to evaluate the maturation of mineralized structures, and no significant difference was observed between the untreated control and the UV-treated scaffolds (Fig. 4B). UV-treated titanium scaffolds showed substantially higher values of the calcium/titanium ratio, representing the occupancy of calcium as compared to that of titanium on the surface of the specimens (Fig. 4C).

In addition, the biological structures formed on the scaffolds represented clear morphological differences between untreated control and UV-treated scaffolds. Within the spaces between titanium fibers on the untreated control scaffolds, small amounts of biological structures were formed (Fig. 5A). These structures were not enough to occupy spaces between fibers in untreated titanium scaffolds (Fig. 5A). High magnification image showed matrix vesicles that were secreted by osteoblasts, and intact bare titanium surface was observed on untreated control titanium fibers (Fig. 5B). In contrast, substantial bio-
logical structures filling the spaces between titanium fibers were formed on UV-treated scaffolds after the same period (Fig. 5C, D). UV-treated titanium fibers were fully covered by bone without a bare titanium surface (Fig. 5E).

DISCUSSION

The osteoblast affinity of UV-treated titanium surfaces is well studied in two-dimensional plate culture and has an impact in the research field of titanium implants, particularly in regards to the two-dimensional interface of biomaterials. For instance, the established BIC in UV-treated implants is approximately 100%, which is a result that has not been achieved by any other surface treatment method. The benefits of UV light treatment on titanium disks so as to achieve excellent bone formation with activated behavior of osteoblasts are as follows: 1) increased osteoblast migration; 2) increased attachment of osteoblasts; 3) facilitated osteoblast spread; 4) increased proliferation of osteoblasts; and 5) promoted osteoblastic differentiation (1). In the present study, UV light treatment in addition significantly enhanced cell attachment and osteoblastic phenotype on the three-dimensional culture with titanium scaffolds (Fig. 3A–D). This study revealed for the first time the biological effect of UV treatment on the activation of osteoblast behavior in the three-dimensional culture. The substantial in vitro bone formation strongly suggested potential use of this UV treatment for the titanium scaffold-based bone formation.

UV light treatment effectively removed carbon from the surface of the titanium scaffold used in this study (Fig. 2A). UV treatment significantly decreased the percentage of surface carbon to one third compared with that of untreated control. Previous study reported the influence of carbon accumulation on the osteoblast affinity (11). In that study, behavior of osteoblasts cultured on titanium with different degrees of hydrocarbon contamination was observed. The suppression of initial cellular activities, such as cell attachment and

Fig. 3 Increased number of attaching cells and enhanced osteoblastic phenotype on the UV-treated scaffold. (A) Micrograph of osteoblasts cultured on scaffold 24 h after seeding (actin filaments are represented in red). Untreated control titanium scaffolds (UT) and UV-treated scaffold (UV). (B) The number of cells was measured colorimetrically with WST-1 reagent 24 h after seeding. (C) The activity of alkaline phosphatase (ALP) was quantified colorimetrically. (D) The amount of calcium deposition was quantified colorimetrically (differences between UV treatment and untreated control scaffolds: * P < 0.05).
of osteoblasts (20, 21), this surface topography emphasizes the hydrophobic status of untreated titanium. As the Wenzel Equation indicates, hydrophilic surfaces become more hydrophilic, whereas hydrophobic surfaces become more hydrophobic with an increase in roughness (26). The combined use of a rough surface and UV treatment might be the reasonable solution to achieve both promoted differentiation of osteoblasts and a hydrophilic property.

Scaffold materials suitable for bone engineering are required to possess the ability to attract cells to their surface because osteoblasts are anchorage-dependent cells that need an appropriate adhesion signaling to proliferate (4, 6, 23). UV treatment promotes cell attraction and adhesion through electrostatic mechanisms (15). The mechanisms convert the electrical charge of titanium from electro-negative to electro-positive. An established electro-positive titanium surface is able to attract more cells because most cells are negatively charged (2, 3, 17, 19). In the present study, UV-treated scaffolds attracted 30% more cells to their surface as compared to the untreated control scaffolds.

Hydrophilicity is an important property for the scaffold, because it related with the efficiency with which liquid can move to the inside of scaffolds. In contrast, scaffolds with hydrophobic properties inhibit the movement of liquid into the scaffold. Penetration of cell suspension to the inside of the scaffolds is important in in vitro and penetration of blood and bone marrow to the inside of the scaffolds is important in in vivo. The UV-treated titanium scaffolds showed hydrophilic properties that were suitable for bone engineering (Fig. 2B, C). In addition, UV treatment is strongly recommended for application to a rough surface. While a rough surface has an advantage of promoting differentiation of osteoblasts (20, 21), this surface topography emphasizes the hydrophobic status of untreated titanium. As the Wenzel Equation indicates, hydrophilic surfaces become more hydrophilic, whereas hydrophobic surfaces become more hydrophobic with an increase in roughness (26). The combined use of a rough surface and UV treatment might be the reasonable solution to achieve both promoted differentiation of osteoblasts and a hydrophilic property.

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**Fig. 4** Enhanced bone formation on the UV-treated scaffolds. (A) The mapping images and spectrums of energy dispersive x-ray spectroscopy surface element analysis of biological structures formed on untreated control scaffolds (UT) and UV-treated scaffolds (UV) [titanium (cyan), calcium (red), and phosphorus (yellow)]. Scale bar represents 100 μm. (B) Ca/P ratio of biological structures formed on the scaffolds. (C) Ca/Ti ratio of biological structures formed on the scaffolds (differences between UV treatment and untreated control scaffolds: *P* < 0.05).
untreated control (Fig. 3B). It is probable that the electrostatic mechanism might affect the cell attraction within this three-dimensional scaffold.

Robust formations of mineralized structures were observed in UV-treated scaffolds. EDS spectrum of this structure on UV-treated scaffolds showed a clear peak of calcium and phosphorus, and the average Ca/P ratio was 1.3 : 1 (Fig. 4C). This Ca/P ratio is close to 1.66 : 1 of mature bone (9, 18). The structures were almost filling the space between titanium fibers of the scaffold. Previous studies using similar titanium scaffolds reported calcified structures with large and small globular accretions and collagen bundles covered the fibers (14, 24), but the substantial mineralized structure filling the spaces between each fiber were not established. The substantial formation of mineralized structures in UV-treated scaffolds indicates the potential use of UV-treated titanium scaffolds for bone engineering.

The present study revealed the effect of UV treatment on the three-dimensional titanium scaffold structure and indicated the potential application of UV light treatment for bone engineering. However, there are questions, including porosity, physical strength, thickness of fibers, pore size, and pore design, that are remaining to be resolved. Future discussion and extra in vivo studies are warranted for the development of titanium scaffold based bone engineering.

REFERENCES


