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Titanium Delivery of Osteoblastic Cell Sheets: An In Vitro Study

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Abstract: We hypothesized that maximizing the number of cells on titanium implants would enhance its biological and bone-integration capabilities as a bone anchorage device. The purpose of this study was to determine the feasibility of fabricating osteoblastic cell sheets, combining them with titanium materials, and controlling their function. Rat femur-derived bone marrow cells cultured on poly(N-isopropylacrylamide) (PIPAAm) dishes were harvested in sheet form by exploiting temperature-responsive hydrophobic to hydrophilic conversion of the dish and subsequently transferring them to titanium disks. Cell sheets remained adherent and spread on micro-roughened titanium surfaces but not on machined surfaces. The post-transfer alkaline phosphatase (ALP) activity of the cell sheets responded to the presence or absence of dexamethasone and was increased by pre-treatment with the osteogenic amino-acid derivative N-acetyl cysteine (NAC) in a dose-dependent manner. Double-layering cell sheets on titanium enhanced ALP activity twofold compared to single sheets. Titanium implants enfolded with autologous osteoblastic cell sheets showed 2.5-times stronger bone-implant integration than controls in a rat femur model. We show that micro-roughened titanium materials can be combined with osteoblastic cell sheets to improve cellular supply at the implant interface. Furthermore, cell sheet function can be controlled and enhanced by biologic agents and technical modifications.

Key words: Titanium implants, Osseointegration, Bone-implant integration, Osteoblasts, Bone engineering

Introduction

Despite recent clinical successes and developments in titanium implant therapy, several issues related titanium’s biological capability remain unresolved. For dental implants, the protracted healing time and risk factors (including, but not limited to, host bone anatomy, diabetes, osteoporosis, smoking, and patient age) may limit their application and reduce success rates¹–³. A high proportion of orthopedic implants undergo revision surgery, for instance in between 5 and 40% (average 25%) of total hip replacements⁴. Therefore, rapid and secure bone-implant integration has been a clinical imperative in both fields.

However, it remains uncertain why bone tissue fails to form around the entire implant surface, with bone-implant contact percentages reported at 45% ± 16%⁵, or between 50% and 75%⁶–⁸, in the literature. Most implants fail due to incomplete bone formation at the surface or early/late destructive changes at the bone–implant interface⁹–¹⁰. Reduced bone-implant contact might be explained by the finite supply and recruitment of stem/osteoprogenitor cells around implant surfaces.

Cell sheet technology refers to the fabrication of sheets of cells using chemically-modified culture dishes¹¹–¹⁴ that allows the harvesting and delivery of a seamless mixture of cells and their extracellular matrix (ECM)¹⁵–¹⁷. Cell sheets can be delaminated by hydrophobic to hydrophilic conversion of poly(N-isopropylacrylamide) (PIPAAm), a temperature-responsive polymer¹⁸,¹⁹: PIPAAm-coated culture dishes are hydrophobic at 37°C and hydrophilic below 32°C. Therefore, cells cultured on PIPAAm-coated dishes at 37°C begin to dissociate from the dish in a intact layer when the temperature drops below 32°C. Since a cell sheet is harvested at full density (over-confl uency), this technique enables the delivery of a large number of cells. When the cell source is autologous, cell sheet technology provides opportunities to directly apply cells to damaged and diseased tissues. To date, cell sheet patches have been used to treat skin, heart, liver, cornea, ligament, and cartilage conditions¹⁶–²⁴. However, the use of cell sheet technology in bone tissue applications has been limited, and few attempts have been made to enhance biomaterials. This is perhaps unsurprising, since it is difficult to combine cell sheets and biomaterials due to the mechanical fragility of the sheets and difficulty in establishing contact and retention between cell sheets and biomaterials.

The objectives of this study were to fabricate and characterize osteoblastic cell sheets and determine the feasibility of transferring them to titanium surfaces with different surface features. We also determined whether biologic agents or technical modifications could enhance the phenotype of transferred cell sheets. We postulate that successful in vitro cell sheet transfer to titanium materials will pave the way for more advanced in vivo applications such as the enhancement of titanium mesh and scaffolds for bone regeneration/engineering and titanium screws and plates for bone immobilization. As a pilot in vivo experiment, osteoblastic cell sheets were fabricated using an autologous cell source (to avoid an immune reaction) and enfolded around titanium implants. Bone-implant integration strength with or without osteoblastic cell sheets was compared in a rat femur model.

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Materials and Methods

Titanium sample preparation and characterization

Commercially pure titanium was machined in disk (20 mm diameter, 1.5 mm thickness) and cylindrical (1 mm diameter, 2 mm length) forms. Relatively smooth machined surfaces and micro-roughened titanium surfaces were tested. To micro-roughen the surface, the disks and cylinders were acid-etched with 67% (w/w) sulfuric acid (H$_2$SO$_4$) at 120°C for 75 s. Surface morphology was examined by scanning electron microscopy (SEM) (XL30, Philips, Eindhoven, Netherlands). Titanium surface hydrophilicity or hydrophobicity was evaluated by measuring the contact angle of 10 µl of ddH$_2$O placed on titanium disks.

Osteoblast culture

Bone marrow cells isolated from the femurs of 8-week-old male Sprague–Dawley rats were placed into alpha-modified Eagle's medium supplemented with 15% fetal bovine serum, 50 µg/ml ascorbic acid, 10 mM Na–β-glycerophosphate, 10–8 dexamethasone, and antibiotic–antimycotic solution. Cells were incubated in a humidified atmosphere of 95% air, 5% CO2 at 37°C. At 80% confluency, cells were detached using 0.25% trypsin 1 mM EDTA-4Na and seeded onto temperature-responsive PIPAAm dishes (UpCell, CellSeed, Tokyo, Japan) or regular 12-well cell culture-grade polystyrene dishes (Costar 3513, Corning Inc., Corning, NY) at a density of 3 × 104 cells/ml. The culture medium was renewed every three days.

Osteoblastic cell sheet fabrication

Osteoblastic cell sheets were prepared non-enzymatically as recommended by the manufacturer. Briefly, osteoblasts were cultured on temperature-responsive PIPAAm dishes (UpCell) for seven days. After confirming confluency, the dishes were removed from the incubator. Then, a polyvinylidene difluoride (PVDF) membrane (CellShifter) was placed on the osteoblasts and the temperature of the dish allowed to fall from 37ºC to 25ºC (for 5-10 minutes), at which point the cells started to dissociate from the dish. The PVDF membrane serves as a support for cell sheets during dissociation and transfer. The cell sheet-PVDF membrane complex was removed from the dish using tweezers and placed on a titanium disk. After 15 min incubation at 37ºC, the PVDF membrane was removed, leaving the cell sheet adherent to the titanium disk surface. To examine the response of cell sheets to biological agents, dexamethasone was removed from the culture medium in some experiments. Furthermore, some cell sheets were treated with N-acetyl cysteine (NAC), an amino-acid derivative known to enhance osteogenesis, to investigate possible biological enhancement of osteoblastic cell sheets.

Cell metabolism and density quantification

The metabolic activity of cells in sheets was evaluated by measuring mitochondrial dehydrogenase enzyme production using WST-1-based colorimetry (WST-1, Roche Applied Science, Mannheim, Germany). The culture well was incubated with 100 µl tetrazolium salt (WST-1) reagent at 37°C for 4 h. The amount of formazan produced as a byproduct of mitochondrial dehydrogenase activity was measured using an ELISA reader at 420 nm. The WST-1 value was also used to quantify the number of cells (cell density), because the number of cells and metabolic activity are correlated.

Cell proliferation

Cell proliferation was measured using the BrdU incorporation assay. 100 µl of 100 mM BrdU solution (Roche Applied Science) was added to the culture wells on day 2 followed by incubation for a further 10 h. After trypsinizing the cells and denaturing DNA, cultures were incubated with a peroxidase-conjugated anti-BrdU antibody for 90 min and reacted with tetramethylbenzidine for color development. Absorbance was measured at 370 nm using a microplate reader (Synergy HT, BioTek Instruments, Winooski, VT).

Alkaline phosphatase activity

The ALP activity of cells or cell sheets was examined using colorimetry- and image-based assays. For colorimetry, cultures were rinsed with ddH$_2$O before adding 250 µl p-nitrophenylphosphate (Lab Assay ATP, Wako Pure Chemicals, Richmond, VA) and incubating at 37°C for 15 min. ALP activity was evaluated as the amount of nitrophenol released through the enzymatic reaction and measured at 405 nm using an ELISA reader (Synergy HT). For image analysis, cells were washed twice with Hanks’ solution and incubated with 120 mM Tris buffer (pH 8.4) containing 0.9 mM naphthol AS-MX phosphate and 1.8 mM Fast Red TR for 30 min at 37°C. The ALP-positive area was calculated as: [(stained area/total dish area) x 100]% using image analysis software (ImageJ, NIH, Bethesda, ML).

Post-transfer cell sheet sustainability on titanium

The area of a cell sheet placed on a titanium disk was measured at transfer and 24 h after transfer using image analysis software (ImageJ). Cell sheet sustainability was calculated as: [(cell sheet area after 24 h)/(cell sheet area at transfer)] x 100 (%).

Microscopic observation of post-transfer cell sheets

Confocal laser scanning microscopy was used to examine the intactness of cell sheets after transfer to titanium disks. 24 h post-transfer, cells were fixed in 10% formalin and stained using the fluorescent dye rhodamine phalloidin (actin filament, red color; Molecular Probes, OR). Cell sheets were also stained with mouse anti-vinculin monoclonal antibodies (Abcam, Cambridge, UK) followed by FITC-conjugated anti-mouse secondary antibodies (Abcam) to visualize the expression of vinculin, a focal adhesion protein. Intercellular continuity, cytoskeletal development and adhesion properties, and potential damage or voids within cell sheets were examined by confocal laser microscopy.

Animal surgery

Autologous osteoblastic cell sheets were fabricated to enfold implants; autologous cells were chosen to avoid adverse immune reactions. First, surgery was performed to fabricate and harvest the cell sheet. Eight-week old male rats were anesthetized by inhalation of 1-2% isoflurane. After their legs were shaved and scrubbed with 10% povidone-iodine solution, the distal aspect of the right femur was carefully exposed via skin and muscle incisions. The knee was exposed and, through a small hole drilled with a 0.8 mm-diameter bur 15 mm from the knee joint, 0.1 cc bone marrow was extracted by aspiration. Surgical sites were then closed in layers. The collected bone marrow cells were expanded and seeded on temperature-responsive dishes, as above. Osteoblastic cell sheets were ready to harvest 10 days after extraction. A cell sheet was prepared for each individual animal.
Figure 1. Surface properties of the titanium disks used in this study. SEM images of machined (A) and acid-etched (B) titanium surfaces. (C) 10 μl of ddH₂O placed on machined (left) and acid-etched (right) titanium disks. (D) The contact angle of ddH₂O placed on titanium disks. ** p<0.01.

Figure 2. Characterization of osteoblasts on regular culture-grade polystyrene dishes and temperature-responsive PIPAAm dishes. (A) The number of attached cells 24 h after seeding evaluated by the WST-1 assay. (B) The number of propagated cells 48 h after seeding evaluated by the WST-1 assay. (C) BrdU incorporation into DNA evaluated 48 h after seeding. (D) ALP activity measured on days three and seven. NS, not significant.

Figure 3. Osteoblastic response to dexamethasone (Dex) on temperature-responsive PIPAAm dishes. Bone marrow cells expanded on regular polystyrene dishes were seeded onto PIPAAm dishes and cultured for three days with or without dexamethasone. An entire culture well (top) and cells (bottom) after ALP staining. A histogram showing ALP-positive areas is also presented. ** p<0.01.
Figure 4. Schematic of osteoblastic cell sheet fabrication and transfer to titanium disks. (A) Bone marrow cells reach confluency seven days after seeding onto temperature-responsive PIPAAm dishes. (B) A polyvinylidene difluoride (PVDF) membrane was placed onto osteoblasts for 5-10 min and the temperature allowed to drop from 37°C to 25°C. (C) The PVDF membrane and the cell sheet being picked up with a pair of tweezers. By this time, the cell sheet has dissociated from the PIPAAm dish and attached to the PVDF membrane. (D) The cell sheet-PVDF membrane complex placed on a titanium disk. (E) After 15 min incubation at 37°C, the PVDF membrane can be removed, leaving the cell sheet on the titanium.

Figure 5. Post-transfer characterization of osteoblastic cell sheets. (A) Images of a cell sheet 24 h after transfer to titanium disks and polystyrene dishes. The cell sheet remained adherent and spread only on acid-etched titanium. Its configuration is marked with arrows. Cell sheet sustainability is presented in the histogram as the area % between transfer and 24 h later. (B) Metabolic activity of cell sheets 24 h post transfer evaluated using the WST-1 assay. (C) ALP activity in the cell sheet three and six days post transfer. (D) Confocal microscopic images of post-transfer cell sheets stained with antibodies to detect actin (red) and vinculin (green). Bar:200μm. * p<0.05, ** p<0.01.
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and delivered to the same animal after enfolding the axial surface of an implant with a fabricated cell sheet trimmed to a 2 x 3 mm rectangle with a scalpel.

Ten days after the first surgery, the second surgery (implant placement) was performed. Based on the in vitro results, acid-etched cylinder implants were used. Implant sites were prepared 10 mm from the distal edge of the left femur by drilling with a 0.8 mm round bur and enlarging with reamers. As a control, implants without cell sheets were placed in the same way. The Chancellor’s Animal Research Committee at the University of California at Los Angeles (UCLA) approved the protocol and all experimentation was performed in accordance with the United States Department of Agriculture (USDA) guidelines on animal research.

**Biomechanical implant push-in test**

The established biomechanical implant push-in test was used to assess the biomechanical strength of bone-implant integration \(^{26,27}\). At week two of healing, the femurs containing cylindrical implants were harvested and embedded in autopolymerizing resin with the top surface of the implant horizontal. The testing machine (Instron 5544 electro-mechanical testing system; Instron, Canton, MA) equipped with a 2000N load cell and a pushing rod (diameter 0.8 mm) was used to load the implant vertically downward at a crosshead speed of 1 mm/min. The push-in value was determined by measuring the load-displacement curve peak.

**Statistical analysis**

In vitro experiments were performed in triplicate \((n=3)\), while six animals were used in in vivo experiments \((n=6 \text{ cell sheet-enfolded implants and } n=6 \text{ control implants})\). Differences between experimental groups were examined by one-way ANOVA with ad hoc Bonferroni multiple comparison correction performed as needed. \(p<0.05\) was considered statistically significant.

**Results**

**Surface features of titanium disks**

Scanning electron microscopy imaging of machined titanium disks showed indistinct, parallel marks from the lathe-turning process without a clearly recognizable three-dimensional structure (Fig. 1A). The acid-etched surface had a micro-scale compartmental structure consisting of sharp peaks and valleys, typical of acid-etched titanium disks (Fig. 1B). Although both surface types were hydrophobic (contact angle of ddH2O greater than 60º), the contact angle was significantly greater on acid-etched than machined titanium, indicating that the acid-etched titanium was more hydrophobic (Fig. 1C and D).

**Osteoblast function on regular polystyrene dishes and temperature-responsive PIPAAm dishes**

Prior to testing the feasibility of osteoblastic cell sheet fabrication, we examined whether culturing osteoblasts on temperature-responsive dishes had an impact on their function. The number of cells attaching during the first 24 h of culture was similar between regular polystyrene and temperature-
and/or rolled up on polystyrene dishes and machined titanium surfaces, whereas cell sheets on acid-etched titanium remained adherent and spread (Fig. 5A). The cell sheet area was reduced to 4 and 5%, respectively, on polystyrene and machined titanium after 24 h (Fig. 5A). However, cell sheets maintained over 75% coverage of acid-etched titanium disks (Fig. 5A). Accordingly, the metabolic activity at 24 h of cell sheets on machined titanium was low (Fig. 5B). Osteoblastic cell sheet transfer was only successful on acid-etched titanium.

Post-transfer cell sheet sustainability was further examined on acid-etched titanium by evaluating cellular ALP activity. ALP activity increased between days three and five, with normal expression of the osteoblast functional phenotype (Fig. 5C). Finally, confocal microscopy showed that, 24 h after transfer, cell sheets were intact without voids or discontinuities in cellular aggregation, although variability in cell density was noted within cell sheets (Fig. 5D). Cytoskeletal actin and vinculin were intensely expressed over the entire cell sheet, further confirming stable settlement and healthy cell function.

Functional control and pre-enhancement of osteoblastic cell sheets

We next determined whether biologic agents or technical modifications could alter the osteoblastic phenotype of cell sheets. Cell sheets fabricated with or without dexamethasone were transferred to acid-etched titanium disks. After transfer, both cell sheets were cultured under the same conditions with dexamethasone. Three days after transfer, the ALP activity was significantly higher in cell sheets fabricated with dexamethasone than without dexamethasone (Fig. 6A). Next, the effect of NAC pre-treatment, a known osteogenesis enhancer, was examined. NAC treatment was performed prior to transfer of the cell sheets to titanium disks, with cell sheets treated the same after transfer. After three days on titanium, the cell sheets showed a NAC dose-dependent increase in ALP activity (Fig. 6B). Finally, single and double cell sheet layers were compared. A second cell sheet layer was placed on the first using the same procedure as the first layer. After 24 h on titanium, the metabolic activity of double-layer cell sheets was nearly twice that of single-layer cell sheets (Fig. 6C). Similarly, ALP activity increased with the second layer (Fig. 6D).

Strength of bone-implant integration with cell sheet-enfolded implants

After two weeks of implant healing, the femur was exposed and the wound carefully observed. There were no differences in appearance between implants with and without osteoblastic cell sheet enfolding. There were no signs of inflammation, infection, granulation tissue formation, or other pathologies, and no implants were misplaced. Biomechanical push-in testing showed that the implants enfolded with osteoblastic cell sheets showed a 2.5-fold higher value than control implants without cell sheets (Fig. 7).

Discussion

Here we report the successful combination of osteoblastic cell sheets and biomaterials. Titanium was selected as a biomaterial because of its established biocompatibility and use for temporary or long-term/permanent anchorage, fixation and regeneration. Although osteoblastic cell sheets settled on both smooth machined surfaces and micro-roughened acid-etched surfaces in the short term, cell sheets were only retained for 24 h or more on acid-etched surfaces. Once retained on the roughened surfaces, the cell
sheets remained adherent and spread during the entire follow-up period of five days in this study. Actin and vinculin expression, which are critical proteins for cell attachment and settlement, were clearly detected in cell sheets on acid-etched titanium. Cellular metabolic activity and alkaline phosphatase activity were normal, indicating that the initial phenotype and subsequent differentiation of osteoblasts were as expected.

In addition to assessing the feasibility of transferring osteoblastic cell sheets to titanium surfaces, we also demonstrated that cell sheets can undergo phenotypic enhancement prior to transfer with the use of chemical agents or modification of the procedure. Metabolic activity was enhanced with NAC, an antioxidant amino-acid derivative and recently described osteogenesis-enhancing molecule (39). The ALP activity of osteoblastic cell sheets after transfer was dependent on the NAC dose prior to transfer. NAC is thought to enhance osteogenesis by promoting cellular differentiation. Therefore, NAC-mediated enhancement of osteogenic cell sheets may be an effective strategy for improving the cell sheet-titanium complex and accelerating osteoblastic differentiation to expedite bone formation.

As a technical modification, layering multiple cell sheets was attempted with the intention of increasing cell volume rather than promoting differentiation since, as noted above, limited numbers of osteogenic cells may hamper bone formation around titanium implants. In considering how best to increase cell number, we were aware that the number of cells in a single cell sheet was finite, since cells do not proliferate after reaching confluency. Furthermore, the cell sheets detached from the temperature-responsive dishes even at 37°C after reaching confluency. Therefore, we increased the cellular volume beyond the capacity of a single sheet by layering multiple single osteoblastic cell sheets. The double-layered cell sheets were stable for three days of follow-up and resulted in a doubling of ALP activity.

Successful cell sheet transfer was restricted to acid-etched titanium, assumed to be due to the surface roughness enabling cell sheet-titanium mechanical interlocking. The osteoblastic cell sheets had inherent “rolling up” and contractile properties and, therefore, did not remain in functional sheet form without retention forces; even regular polystyrene dishes, which are considered to have high affinity for cells, were incapable of retaining the cell sheet for 24 h. Fortunately, most currently used titanium implant products are micro-roughened, similar to the acid-etched surface tested here, and the requirement for surface roughness may not be a significant barrier in future applications. The impact of hydrophilicity or hydrophobicity of biomaterials on cellular attachment and retention remains contentious (39), with biological outcomes differing depending upon the biomaterial type and surface morphology and the cell type (29-31). With respect to titanium, hydrophilicity may positively affect the attachment and proliferation of osteoblasts under certain conditions, but there is no significant correlation between the degree of hydrophilicity and cellular attachment (29-31). In fact, the difference in hydrophobicity between machined and acid-etched surfaces was unremarkable in this study, and both surfaces were hydrophobic. Therefore, it is unlikely that the minor differences in hydrophobicity between the two surfaces significantly altered cell sheet retention.

This study demonstrated the feasibility of fabricating osteoblastic cell sheets and transferring them to titanium. Surface micro-roughness was required for retention and maintenance of cell sheets on titanium. Modifying the technique or chemical enhancement can be used to enhance osteoblastic cell sheet function. Finally, we examined the strength of bone-implant integration after enfolding titanium implants with osteoblastic cell sheets. The fabrication and use of autologous osteoblastic cell sheets was technically feasible and resulted in a substantial increase in the strength of bone-implant integration. Although this was a pilot in vivo experiment and further studies are required, the technique holds promise for use with other titanium-based therapeutic devices including titanium implants, screws, plates, mesh, cages, and other scaffolding devices.

In conclusion, here we explored the construction of osteoblastic cell sheet-titanium complexes. Osteoblastic cell sheets were successfully transferred to acid-etched micro-roughened titanium surfaces but not to relatively smooth machined surfaces. Post-transfer osteoblastic cell sheet function was enhanced by pre-treatment with the osteogenic enhancer NAC in a dose-dependent manner and by multi-layering the cell sheets. A pilot in vivo experiment showed that titanium implants enfolded with autologous osteoblastic cell sheets showed significantly stronger bone-implant integration than controls. These results establish the viability of constructing osteoblastic cell sheet-titanium complexes to improve cellular supply at titanium interface and the cell number and degree of osteoblastic differentiation can be controlled.

Acknowledgment

The authors have no disclosures of interest.

Conflict of Interest

The authors have declared that no COI exists.

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