Osteoblast Differentiation with Titania and Titania–Silica-Coated Titanium Fiber Meshes*

VILLE V. MERETOJA, M.Sc.,¹ ANJA E. DE RUIJTER, B.Sc.,² TIMO O. PELTOLA, Ph.D.,¹
JOHN A. JANSEN, D.D.S., Ph.D.,² and TIMO. O. NÄRHI, D.D.S., Ph.D.¹

ABSTRACT

Two surface-reactive sol–gel coatings, namely titania (TiO₂) and a mixture of titania and silica (TiSi), were applied to titanium fiber meshes. Differentiation of rat bone marrow stromal cells toward an osteogenic phenotype with coated and uncoated (cpTi) substrates was compared. The amount of DNA in cpTi and TiSi matrices did not increase after day 3, but with TiO₂ matrices the amount increased for 7 days. The prolonged period of proliferation with TiO₂ scaffolds resulted in a delay in alkaline phosphatase induction. However, osteocalcin incorporation into extracellular matrix by day 14 was greater with TiO₂ scaffolds than with cpTi scaffolds. Calcium deposition was also greater with TiO₂-coated substrates than with uncoated substrates. With the TiSi scaffolds osteocalcin production and mineralization were lower than with the cpTi scaffolds. The current study confirms our previous findings that titanium fiber mesh supports attachment, growth, and differentiation of rat bone marrow stromal cells. Furthermore, the osteogenic capacities of cell–scaffold constructs under cell culture conditions were increased with a sol–gel-derived titania coating, but not with a titania–silica coating.

INTRODUCTION

THE STANDARD TREATMENT of large bone defects involves transplantation of autologous or allogeneic bone grafts. However, new approaches to manage such defects are needed because of, for example, insufficient supplies, donor site morbidity in relation to autografts, risk of immune reactions, and transmission of disease in relation to allografts. Tissue engineering is a rapidly developing area of reconstructive medicine in which tissue-inducing factors and/or cells are combined with a scaffold material to regenerate the structure and function of the original tissue.^{1,2}

Various growth factor–delivery system combinations have been applied to enhance bone regeneration.^{3,4} However, the main body of work has concentrated on the

members of the transforming growth factor- β superfamily and their carrier matrices.^{5,6} These growth factors can induce bone formation at a defect site through recruitment of mesenchymal stem cells for proliferation and differentiation toward an osteogenic lineage. Accordingly, bone repair can also be enhanced through direct application of osteogenic cells (possibly together with growth factors) to tissue-engineering scaffolds before implantation.^{7,8} A good source of such cells is bone marrow containing mesenchymal stem cells/osteoprogenitor cells that can be expanded under cell culture conditions to achieve sufficient numbers of cells for clinical applications (see Derubeis and Cancedda⁹ and references therein).

An implanted bone-regenerative material should form a direct contact with surrounding tissues, with no inter-

¹Department of Prosthetic Dentistry and Biomaterials Research, Institute of Dentistry, University of Turku, Turku, Finland.

²Department of Periodontology and Biomaterials, University Medical Center, Nijmegen, The Netherlands.

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vening fibrous capsule. It has been suggested that this requirement can be met through use of bioactive materials, on the surfaces of which deposition of calcium phosphate takes place and specific proteins are incorporated *in vivo*.^{10–12} Usually passive titanium surfaces can be rendered bioactive by various means such as alkali-heat treatment,¹³ anodic oxidation,^{14,15} or sol-gel-derived titania coatings.¹⁶ Our previous study showed that, in addition to bone contact, the sol-gel-derived TiO₂ coatings can also facilitate direct soft tissue attachment, with no fibrous capsule formation.¹⁷ In many applications the latter can be as important as direct bonding to bone. We also found that formation of a calcium phosphate mineral layer was not required for enhancement of soft tissue contact.

Reactive ceramic coatings such as various calcium phosphates and bioactive glasses have also been used to improve attachment of titanium implants to tissues.^{18,19} However, such coatings are ultimately resorbed *in vivo*, exposing underlying metal surfaces and perhaps resulting in loss of existing contact with tissue.²⁰ On the other hand, silica-containing dissolution products themselves can have beneficial osteopromoting functions.^{21,22} Stable TiO₂ surfaces that release silica can be achieved through use of titania-silica films derived from sol-gels. Such mixed oxide films have been widely used in fields such as catalysis,²³ but there have been only a few studies of their use as biomaterials.^{24–26} Thin films of this kind seem to enhance fibroblast proliferation and osteoblast differentiation *in vitro*.

Sintered titanium fiber meshes loaded with osteogenic cells have previously shown potential as mechanically strong bone graft substitute *in vivo*.^{27,28} In this study, we applied titania and titania-silica sol-gel coatings to such meshes and compared osteogenic activities under cell culture conditions. To our knowledge, this is the first study of these types of coatings in a three-dimensional cell culture model.

MATERIALS AND METHODS

Scaffold preparation

The cell culture scaffolds were cut from a sintered titanium fiber mesh (Bekaert, Zwevegem, Belgium) with a volumetric porosity of 86%, a density of 600 g/m², and

a fiber diameter of 40 μ m. The average distance between fibers was 250 μ m. Disk-shaped specimens (6 mm in diameter and 0.8 mm thick) were used as such (cpTi) or coated with titania (TiO₂) or titania-silica (TiSi), using the sol-gel technique described by Jokinen *et al.*²⁶ with slight modifications.

The titania sol was produced by dissolving tetraiso-propyl orthotitanate (TIPOT) in ethanol and mixing this with a solution containing ethylene glycol monoethyl ether, deionized water, hydrochloric acid, and ethanol. The sol was aged at 0°C for 24 h before dipping. The titania-silica sol was produced by mixing the TiO₂ and SiO₂ sols described below to obtain a molar ratio of 30% TiO₂ and 70% SiO₂. The SiO₂ sol was prepared by mixing tetraethyl orthosilicate, ethanol, and water at room temperature, and adding nitric acid as a catalyst for hydrolysis. The sol was aged at 40°C for 60 min before use. The TiO₂ sol was prepared by mixing TIPOT, ethanol, nitric acid, and water at room temperature. It was aged at 40°C for 30 min before use. The final TiO₂-SiO₂ sol was further aged at 40°C for 24 h and then cooled to 0°C before the dipping process. The compositions and aging times of the prepared sols are shown in Table 1.

The titanium fiber mesh substrates were cleaned ultrasonically in acetone (5 min) and ethanol (5 min) and dried in air before each coating cycle. Coating was achieved by dipping substrates into a sol and then withdrawing them at a speed of 0.3 mm/s. The substrates were then heated at 500°C for 10 min. The dipping and heating cycle was repeated two to five times. Uncoated and coated scaffolds were finally cleaned ultrasonically and sterilized with γ radiation (minimum, 25 kGy).

Cell culture

Rat bone marrow osteoblast-like cells were obtained and cultured as described by Maniopoulos *et al.*²⁹ Briefly, the femurs of four young male adult Sprague-Dawley rats (weight, 140 to 160 g) were isolated for each experiment. The bones were wiped with 70% alcohol and immersed twice in α -MEM (Sigma, St. Louis, MO) culture medium containing penicillin-streptomycin (100 units/mL, GIBCO; Invitrogen, Breda, The Netherlands). The condyles were cut off and bone marrow was flushed out with complete cell culture medium (α -MEM, antibiotics supplemented with 15% fetal bovine serum [GIBCO], ascorbic acid (50 μ g/mL; Sigma), 8.5 mM sodium β -

TABLE 1. COMPOSITION AND AGING OF COATING SOLS

TiO ₂ :SiO ₂ (mol%)	Ethanol:alkoxide	H ₂ O:alkoxide	Acid:alkoxide	Oxide content (g/100 mL)	Aging
100:0	8.20	1.00	0.018	9.52	24 h at 0°C
30:70	6.72	1.06	0.100	9.97	24 h at 40°C

glycerophosphate [Merck, Darmstadt, Germany], and 10 nM dexamethasone [Sigma]). The resulting suspension was passed through a 20-gauge needle and plated cells were cultured in a humidified 5% CO₂ atmosphere at 37°C.

After 7 days of primary culture, the adherent cell population was enzymatically detached (0.25% trypsin–0.02% EDTA [Sigma]) and a stock of 1.0×10^6 cells/mL was prepared in complete culture medium. Cell suspension (~0.1 mL per titanium mesh) was added to each type of substrate in 15-mL polypropylene tubes. Cells were allowed to adhere at 37°C for 3 h, with manual shaking every 30 min. After seeding, the substrates were transferred to the wells of 24-well culture plates, with one mesh per well containing 1 mL of fresh culture medium. Culture was continued for up to 21 days, with replacement of medium every 2 to 3 days. On the basis of light microscopy of the culture plates, the scaffolds were transferred to clean culture wells every 7 days to ensure that no extensive cell growth and/or mineral deposition took place outside the titanium meshes. The whole cell culture process was repeated in two independent experimental runs.

Alkaline phosphatase activity and amount of DNA

At predetermined time points, four replicate substrates were washed with phosphate-buffered saline (PBS) and transferred to microcentrifuge tubes containing 1 mL of sterile water. The tubes were then incubated at 37°C for 10 min and stored at –70°C. After thawing, the samples were treated ultrasonically in an ice–water bath for 10 min and the released amount of DNA and alkaline phosphatase (ALP) activity were measured from supernatant.

Amounts of DNA were measured in 100 μ L of supernatant transferred to microtiter plates. Equal amounts of PicoGreen double-stranded DNA (dsDNA) quantitation reagent (Molecular Probes Europe, Leiden, The Netherlands) were added to each well and incubation took place for 10 to 15 min at room temperature, with protection from light. Fluorescence from three replicate wells was measured with a Victor multilabel counter (PerkinElmer Life and Analytical Sciences/Wallac, Turku, Finland), at excitation and emission wavelengths of 490 and 535 nm, respectively. Amounts of DNA were read from a λ phage dsDNA standard curve.

To measure ALP activity, 50 μ L of supernatant was transferred to a microtiter plate and 200 μ L of *p*-nitrophenyl phosphate substrate solution (Sigma) were added. The plate was incubated at 37°C for 1 h and 50 μ L of a 3 M NaOH solution was added to each well to stop the enzymatic reaction. Mean readings of ab-

sorbance from three replicate wells were recorded at 405 nm, using an enzyme-linked immunosorbent assay (ELISA) plate reader (Multiskan MS; Labsystems, Helsinki, Finland). Amounts of converted substrate were read from a *p*-nitrophenol standard curve. ALP activities measured were normalized in relation to amounts of DNA determined.

Quantitation of matrix mineralization

At predetermined time points, four replicate substrates were washed in PBS and transferred to 1 mL of 0.5 N acetic acid. After 24 h of incubation at room temperature, with mild shaking, amounts of dissolved calcium in the supernatant were determined. For each assay, fresh reagent containing *o*-cresolphthalein complexone (50 μ g/mL) and 8-hydroxyquinol (1 mg/mL) in an ethanolamine–boric acid buffer was prepared. Five microliters of standard or sample was pipetted onto a microtiter plate and 300 μ L of reagent was added. After 10 min of incubation at room temperature, absorbances from three replicate wells were recorded at 560 nm, using an ELISA plate reader. Calcium concentrations were read from a CaCl₂ standard curve.

Osteocalcin production

Amounts of osteocalcin (OC) produced by the cultured cells were determined with a rat osteocalcin enzyme immunoassay (EIA) kit (Biomedical Technologies, Stoughton, MA). At predetermined time points, four replicate substrates were washed in PBS and transferred to microcentrifuge tubes containing 1 mL of assay buffer and stored at –70°C. After thawing, the samples were treated ultrasonically in an ice–water bath for 10 min, and the OC released into the supernatant was measured in accordance with the manufacturer's instructions.

Scanning electron microscopy

Substrates to be subjected to scanning electron microscopy (SEM) were washed in PBS and fixed with 2% glutardialdehyde in a 100 mM cacodylic acid buffer, pH 7.4. The fixed substrates were dried in a rising ethanol series (70 to 100%) and sputter-coated with ~20 nm of gold. The samples were examined with a JSM-5500 microscope (JEOL, Tokyo, Japan). The presence of calcium and phosphorus within mineral deposits was verified by energy-dispersive X-ray spectroscopy (EDS) analysis (Princeton Gamma-Tech, Rocky Hill, NJ).

Statistics

Statistical analyses were performed with the SPSS version 11.0 software package (SPSS, Chicago, IL).

FIG. 1. Amounts of cellular DNA released from culture scaffolds. Columns represent means and error bars represent standard deviations; *statistically significant difference ($p < 0.05$) between the time points indicated.

Data were analyzed by one-way analysis of variance (ANOVA) followed by the Tukey post hoc test.

RESULTS

Proliferation

The highest initial DNA amounts were measured from cpTi scaffolds ($p < 0.05$), whereas there was no significant difference between DNA release from TiO₂ and TiSi scaffolds by day 1 (Fig. 1). On cpTi and TiSi substrates proliferation had already ceased by day 3, and the amounts of DNA did not increase after that. On the other hand, DNA release from TiO₂ substrates increased until

day 7. The maximal amount of DNA per scaffold was similar with all substrate types.

Differentiation markers

ALP activity, an early marker of osteoblast differentiation, was measured after 1, 3, and 7 days of culture (Fig. 2). The maximal ALP activities for cpTi and TiO₂ scaffolds were observed on days 3 and 7, respectively. With TiSi substrates peak activity occurred by day 3, but without reaching statistical significance ($p = 0.06$). The levels of maximal ALP activities were similar with all substrate types.

A later differentiation marker, OC, was studied after 2 and 3 weeks of culture (Fig. 3). The highest amount of

FIG. 2. Alkaline phosphatase activities of cultured cells. Columns represent means and the error bars represent standard deviations; *statistically significant difference ($p < 0.05$) between the time points indicated.

FIG. 3. Osteocalcin protein accumulation in scaffolds during cell culture. Columns represent means and error bars represent standard deviations; *statistically significant difference ($p < 0.05$) between scaffold types.

protein secreted by day 14 was found with TiO_2 substrates, the lowest with TiSi substrates ($p < 0.05$). By day 21 the lowest OC secretion was still observed with TiSi substrates, but there was no significant difference between protein levels with cpTi and TiO_2 substrates.

Matrix mineralization

Mineral deposition was first observed, by means of SEM, on day 5 with TiO_2 scaffolds. With the other scaffolds mineralization did not occur before day 7. There were no other perceptible differences between cell layers grown on the various substrates (Fig. 4). The extent of matrix mineralization by osteoblasts was measured via amounts of calcium deposited on the scaffolds (Fig. 5). At both the 8- and 16-day time points, calcium deposition was greatest with the TiO_2 -coated substrates and least with the TiSi-coated substrates ($p < 0.05$).

DISCUSSION

It has been shown that cell-loaded titanium fiber scaffolds have bone-forming potential in ectopic³⁰ and orthotopic rat models.^{27,28} Titanium meshes that were not loaded with cells had only minor osteoconductive properties in these studies. *In vivo* data suggest that sol-gel TiO_2 coating can increase both bone and soft tissue attachment to titanium implants.^{16,17} Under two-dimensional culture conditions, cellular response has been found to be further enhanced by incorporation of a soluble silica phase in the coating.²⁴ We therefore felt that nonresorbable bioactive coatings based on titania, especially ones that released silica, might enhance the performance of titanium fiber scaffolds. Why release of silica from a coating should have a favorable effect is not

clear. The silica concentrations in the cell culture medium were approximately 10 times lower than those resulting from dissolution of bioactive glasses (2 to 10 ppm of silica versus 35 to 85 ppm of silica). Such dissolution has been shown to have osteopromotional effects.^{31,32} The nanoscale structures of thin titania and titania-silica coatings can be beneficial in relation to osteoblasts.³³ However, whereas the chemical compositions of the sols used in this work were similar to those in our previous studies, the topographies of the coatings on the scaffolds was not similar. Investigation of the fiber meshes by SEM showed that the coating layer was thick and extensively cracked. The coatings on planar surfaces were much thinner and crack free. The coatings were not characterized further in this study.

Marked cellular expansion was observed with cpTi and TiSi scaffolds between 1 and 3 days of culture, but only minor proliferation was observed after further incubation. With TiO_2 -coated meshes there was cellular expansion for up to 7 days. However, maximal DNA release was no greater than with the other types of matrix. An overall short proliferation phase corroborates our previous results,^{34,35} and can be explained on the basis of the high concentration of cells ($1 \times 10^6/\text{mL}$) used to seed the scaffolds. This would have resulted in numerous cell-cell interactions, and allowed rapid differentiation into the osteogenic lineage.³⁶ Enhanced differentiation after high-density seeding has also been reported with polymeric scaffolds.³⁷ The prolonged proliferation with TiO_2 substrates was reflected in later attainment of maximal activity of the early osteoblast marker ALP in TiO_2 matrices than in the other matrices. Similar results have been obtained when cell differentiation has been delayed because of an absence of osteogenic supplements during osteoblast preculture. In the experiments concerned, pro-

FIG. 4. Morphological evaluation of cells cultured for 5 days on cpTi (A and D), TiO₂ (B and E), and TiSi (C and F) scaffolds. The lower magnification scanning electron micrographs (A–C) show confluent cell layers covering individual fibers with outgrowths over scaffold pores at fiber intersections. At higher magnification (D–F) multiple cell layers spanning the fibers are visible. Only with the TiO₂ scaffolds was there commencement of mineral deposition (arrows) at this time. Original magnification: (A–C) $\times 100$; (D–F) $\times 750$. Scale bars: (A–C) 200 μm ; (D–F) 20 μm .

FIG. 5. Calcium deposition on culture scaffolds. Columns represent means and the error bars represent standard deviations; *statistically significant difference ($p < 0.05$) between scaffold types.

liferation of cells inside porous scaffolds continued for up to 2 weeks³⁸ and 4 weeks,³⁹ as long as ALP activity was increasing. All of the findings are in accordance with the notion that there is a reciprocal relationship between proliferation and differentiation of osteogenic cells derived from bone marrow and calvaria.^{40,41}

In contrast to ALP measurements, the TiO₂ scaffolds exhibited enhanced osteocalcin protein incorporation into the extracellular matrix. The amounts of OC with TiO₂ scaffolds were greater than with cpTi scaffolds in early cultures. However, OC levels did not increase between 14 and 21 days of culture, probably because protein release was compromised from highly mineralized samples. OC incorporation with TiSi scaffolds was significantly less than with the other matrices. Our results are consistent with those of Nishio *et al.*,⁴² who found enhanced osteoblast differentiation but not proliferation on bioactive titania surfaces. Greater osteoblast proliferation has been found with titania coatings than with uncoated titanium alloy by Faust *et al.*⁴³ They did not study cell differentiation.

Marked mineralization of the scaffolds was observed after 8 days of culture. At this time, the levels of calcium were highest with TiO₂-coated matrices, and lowest with TiSi-coated matrices. TiO₂ coatings are known to form bonelike apatite layers on their surfaces in simulated body fluid,⁴⁴ and also to absorb calcium and phosphate ions in the presence of proteins.¹⁷ However, the marked calcium deposition with cpTi and TiSi scaffolds suggests that the mineralization observed had a biological basis. The mineralization seen in the SEM micrographs was always associated with cells and the extracellular matrix. SEM showed that there were mineral deposits on TiO₂ scaffolds even on day 5. With the other types of scaffold mineralization was not perceptible until day 7. Mineraliza-

tion subsequently proceeded with equal effectiveness with all of the materials. In those cultures, calcium deposition on the scaffolds corresponded to a final Ca²⁺ concentration of ~0.8 mM after each change of medium. This would seem to be a limit concentration that cannot be exceeded under the standard culture conditions used in this study (our unpublished observations).

The medium used for osteoblast culture routinely contains sodium β -glycerophosphate as an osteogenic supplement.⁴⁵ This is rapidly hydrolyzed by the ALP produced by the cells, with release of inorganic phosphate into the medium.^{46,47} High phosphate concentrations can then lead to spontaneous precipitation of calcium phosphate, even in the absence of cells.⁴⁸ However, only minor amounts of mineral precipitation were observed by means of light microscopy at the peripheries of culture wells, and it was associated mostly with outgrowth of cells from scaffolds. The calcium deposition on the substrates corresponded more closely to levels of osteocalcin, a component of the extracellular matrix, than to levels of ALP activity. This would indicate that most of the calcium was deposited by mineralizing osteogenic cells within the scaffolds rather than as a result of simple chemical reactions in the solution.

The study described in this article included two independent cell culture experiments. However, only one set of data is reported in detail. The repeated study gave similar relative results, with minor quantitative differences.

The current study confirms our previous findings that titanium fiber mesh supports attachment, growth, and differentiation of rat bone marrow stromal cells. Furthermore, the osteogenic capacities of cell-scaffold constructs under cell culture conditions were increased with a sol-gel-derived titania coating, but not with a titania-silica coating.

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Address reprint requests to:

Ville V. Meretoja, M.Sc.

Department of Prosthetic Dentistry and

Biomaterials Research

Institute of Dentistry, University of Turku

Lemminkäisenkatu 2

FIN-20520 Turku, Finland

E-mail: ville.meretoja@utu.fi