Suppressed primary osteoblast functions on nanoporous titania surface

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Abstract: Titania nanotubes have large potential in medical implant applications but their tissue compatibility is still controversial. Considering that the biological behavior of primary osteoblasts is closer to the *in vivo* situation than other common cell lines, we investigate the response of primary osteoblasts on anodized nanotextured titania surfaces. Two nanotextured surface morphologies, namely the 5 V anodized surface with a pore diameter of 25 nm and the 20 V anodized surface with a tube diameter of 80 nm are chosen for this study. Initial cell adhesion is not obviously affected by the anodized surfaces. With the exception of slightly higher intracellular alkaline phosphatase activity and more extracellular

matrix deposition, cell growth, and cell differentiation represented by the expressions of osteogenesis-related genes are impaired on both anodized surfaces. This may be attributed to the compromised focal contact formation on the anodized surfaces. The difference in the phenotypes of the primary osteoblasts and the osteoblastic cell lines may partly account for the controversy in osteoblast cytocompatibility on titania nanotubes. © 2010 Wiley Periodicals, Inc. *J Biomed Mater Res Part A* 96A: 100–107, 2011.

Key Words: nanopopography, primary osteoblast, cell adhesion, alkaline phosphatase activity, real-time PCR

INTRODUCTION

The surface topography on biomedical implants plays a critical role in osseointegration and subsequent clinical success. Previous studies have shown that micrometer or submicrometer surfaces can enhance bone cell functions and promote implant osseointegration. Since bone tissues are composed of nanofibered hydroxyapatite dispersed in a mostly collagen matrix, it has been suggested that a nanopopography may more efficiently facilitate interactions with bone cell.^{1–3}

Many types of nanopopography have been adopted to study cellular response to the nanotextured surfaces, among which the titania nanotubular surface has drawn much attention. Vertically oriented and highly ordered titania nanotube arrays can be fabricated easily by simple anodization and the nanotube dimensions can be controlled by adjusting the experimental parameters.^{4–6} Titania nanotubes can induce the growth of nanodimensioned hydroxyapatite⁷ and it has been reported that titania nanotubes with a suitable size may enhance the functions of bone cells as well.^{6,8–10} Besides, the nanotubes can serve as carriers for drugs such as growth factors¹¹ or bacteriocides.¹² Titania nanotubes also have a smaller Young's modulus of ~36–43 GPa¹³ which is closer to that of bones compared with titanium. It may prevent bone resorption caused by stress shielding.

Although titania nanotubes have many favorable properties as bone implants, the biological performance of titania nanotubes is still not well understood. It has been reported that titania nanotubes with different sizes of 30–100 nm,⁶ 50 nm,¹⁰ 70 nm,^{8,11} and 80 nm⁹ enhance osteoblast cell functions^{6,8–11} and *in vivo* bone bonding.¹⁴ However, Park et al. have obtained contrary results that nanotubes with a smaller tube size (15 nm) strongly enhances cell activities, while cell functions which degrade with increasing tube size are severely impaired on nanotube layers with a tube diameter larger than 50 nm.^{4,5} We believe that the different cell types used in these experiments may be one of the reasons for the controversy on the cytocompatibility of titania nanotubes.^{15,16} Actually, Peng et al.'s work may support this hypothesis, showing that endothelial cell and vascular smooth muscle responds oppositely to titania nanotubes.¹⁷ We have also observed different response of fibroblast, epithelium and osteoblast to titania nanotubes in our ongoing experiments (unpublished data).

In nearly all the studies on the beneficial effects of titania nanotubes with different sizes on osteoblast/implant interactions, some osteoblastic cell strains such as MC3T3-E1,^{6,8,9,18} human osteoblast-like cell,¹¹ and human osteoblastic precursor cell line¹⁰ are used, because they are relatively easy to obtain and handle and generally offer better reproducibility

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FIGURE 1. The anodized nanotextured surfaces formed at 5 V (a) and 20 V (b), respectively.

of results than primary cell. However, the biological characteristics of cell strains are very different from those of cells *in vivo*. In this study, to study the osseointegration ability of titania nanotubes, we investigate the response of primary osteoblasts whose biological behavior is closer to that of cells *in vivo*. Our experiments are aimed at providing a better understanding of the cytocompatibility of titania nanotubes to hopefully resolve the current controversy.

MATERIALS AND METHODS

Specimen preparation

Pieces of cp titanium with 99.8% purity ($10 \times 10 \times 1 \text{ mm}^3$) were used as substrates for anodization. After polishing and ultrasonic cleaning, the substrates were anodized for 30 min in 0.5 wt % hydrofluoric acid using a DC power supply and a platinum electrode serving as the cathode. Two typical kinds of nanotextured surface were formed at different anodizing potentials, namely the 5 V anodized surface with a nanonet-like texture and the 20 V anodized surface with a nanotubular texture (Fig. 1). The polished surface (P) was used to observe the cellular behavior with the cell culture polystyrene (C) serving as the control. Immediately after anodization, the samples were rinsed with deionized water. After ultrasonic cleaning, the samples were sterilized by ultraviolet irradiation for 30 min on each side before cell seeding.

Surface characterization

A field emission scanning electron microscope (FE-SEM, JSM-6700F) was used to observe the topography of the prepared specimens. The crystallinity of the samples was evaluated by X-ray diffraction (XRD) using a Siemens D-500 diffractometer. Contact angle measurements were carried out by the sessile-drop method with a contact angle measuring system (EasyDrop Standard, KRUS, Germany) at room temperature. Two different liquids were used to carry out the contact angle measurements: distilled water and diiodomethane. The contact angles θ were measured by analyzing drop shape using the DSA1 software (KRUS). From

the contact angle results obtained with the two liquids, the surface free energy (SFE) of each surface was calculated.¹⁹

Cell culture

Primary rat calvarial osteoblasts were obtained by digestion of one day old Sprague-Dawley rats' calvarial bone.¹⁹ Ethical approval was granted by the University Research Ethics Committee of the Stomatological Hospital, FMMU (Xi'an, PR China). The cells were cultured in Dulbecco's minimum essential medium (DMEM, Gibco) supplemented with 10% bovine calf serum (BCS, Gibco) and 1% penicillin/streptomycin and incubated in a humidified atmosphere of 5% CO₂ at 37°C. Passages 2–5 were used in the experiments.

Cell adhesion

Osteoblasts were seeded on the substrates placed in 24-well culture plate at a density of 17,000 cells/cm² and were allowed to attach for 30, 60, and 120 min. At the prescribed time points, the nonadherent cells were removed by rinsing with a phosphate buffered saline (PBS) solution. The cells were then fixed and stained with 4',6'-diamidino-2-phenylindole (DAPI) and the cell numbers were counted in five random fields on each sample under a fluorescence microscope.

Cell viability

One milliliter of the cell suspension was seeded onto each specimen with a density of 2×10^4 cells/ml and were cultured in DMEM with 10% BCS. After 1, 4, and 7 days post-seeding, cell proliferation was assessed using the MTT assay. In brief, at the prescribed time points, specimens were rinsed gently three times with PBS and transferred to a new 24-well culture plate. The MTT solution was added and the specimens were incubated at 37°C to allow the formation of formazan. Afterwards, the formazan was dissolved using dimethyl sulfoxide (DMSO) and the optical density (OD) was measured at 490 nm using a spectrophotometer (Bio-tek, Germany).

TABLE I. Primers Used for Real-Time PCR

Gene	Forward Primer Sequence (5'-3')	Reverse Primer Sequence (5'-3')
ITG	TGCGATAGGTCCAACGGCTTA	CATTTGTCGCTACGCATGGAAC
RUNX2	CCATAACGGTCTTCACAAATCCT	TCTGTCTGTGCCTTCTTGGTTC
ALP	AACGTGGCCAAGAACATCATCA	TGTCCATCTCCAGCCGTGTC
BMP	CAACACCGTGCTCAGCTTCC	TTCCCACTCATTCTGAAAGTTCC
OPN	TCCTGCGGCAAGCATTCTC	CTGCCAACTCAGCCACTTTCA
OCN	GGTGCAGACCTAGCAGACACCA	AGGTAGCGCCGGAGTCTATTCA
GAPDH	GGCAGAGTCAAGGCTGAGAATG	ATGGTGGTGAAGACGCCAGTA

Cell morphology

The cell morphology was observed using fluorescent staining and SEM. After 3 h of culture, osteoblasts were fixed in 2% paraformaldehyde, and stained using Hoechst (nuclei blue color, sigma) and rhodamine phalloidin (actin filament green color, sigma) before fluorescence microscopy. After 4 days of culture, the samples with attached cells were washed with PBS and distilled water, fixed, and sputter coated with carbon. The cell morphology was observed using the FE-SEM.

Alkaline phosphatase activity

The cells were seeded on the substrates in 24-well culture plates at a density of 2×10^4 cells/well. After 7 and 14 days of culture, the cells were washed three times with PBS and lysed in 0.1% Triton X-100 using four standard freeze-thaw cycles. The alkaline phosphatase activity (ALP) activities of the samples were determined by a colorimetric assay using an ALP reagent containing p-nitrophenyl phosphate (p-NPP) as the substrate. The absorbance of p-nitrophenol formed was measured at 405 nm. The intracellular total protein content was determined using a commercial BCA protein assay kit and the ALP activity was normalized to the total protein content.

Osteogenesis-related gene expressions

To investigate the influence of the nanoscale titania surfaces on the gene expressions of seeded osteoblasts, the osteogenesis-related genes were analyzed using the real-time polymerase chain reaction (Real-time PCR). The cells were seeded at 2×10^4 cells/well and cultured for 3 and 10 days. The total RNA was isolated using the TRIzol reagent (Gibco). One microgram of RNA from each sample was reverse transcribed into complementary DNA (cDNA) using the PrimeScriptTM RT reagent kit (TaKaRa). The forward and reverse primers for the selected genes are listed in Table I. Expression of osteogenesis-related genes including integrin- β 1 (ITG), runt-related transcription factor 2 (RUNX2), ALP, bone morphogenetic protein-2 (BMP), osteopontin (OPN),

and osteocalcin (OCN) were quantified using the Real-time PCR (Bio-Rad iQTM5 Multicolor Real-Time PCR Detection System) with SYBR[®] Premix ExTM Taq II (TaKaRa). Data analysis was carried out using the iQTM5 Optical System Software Version 2.0 (Bio-Rad). The relative expression levels for each gene of interest were normalized to that of the housekeeping gene GAPDH.

Statistical analysis

The data were analyzed using SPSS 14.0 software (SPSS, USA). A one-way ANOVA followed by a Student-Newman-Keuls *post hoc* test was used to determine the level of significance. $p < 0.05$ was considered to be significant and $p < 0.01$ was considered to be highly significant.

RESULTS

Titania nanotube surface characterization

In the SEM micrographs depicted in Figure 1, the nanostructured surface is evident on the anodized titanium. A nanonet-like texture is formed at 5 V and the average diameter of the holes is about 25 nm. At 20 V, a nanotubular texture is formed and the typical dimension of the hollow titania nanotubes is about 80 nm in inner diameter. The XRD patterns show that the titania nanotube surface after sterilization have a amorphous nature (data not shown). The contact angles and values of surface free energy are listed in Table II. The anodization treatment dramatically decreases the water contact angle on titania and enhances the surface free energy, especially the polar component. Anodization at 20 V results in a little smaller water contact angle and higher surface energy than at 5 V.

Cell adhesion and cell viability

No significant difference among the adherent cell numbers on the 5 and 20 V anodized surfaces and polished surface is observed after 30, 60, and 120 min of culture (Fig. 2). At days 1, 4, and 7, there is still no large difference for the cell viability on the 5 and 20 V anodized surfaces and the polished surface, except that the cell proliferation on the 5 V

TABLE II. Contact Angles (°) and Values of Surface Free Energy (mJ/m²) of Specimens

Samples	Contact Angle (°)		Surface Free Energy (mJ/m ²)		
	Distilled Water	Diiodomethane	Total	Polar	Dispersive
5 V	31.18 ± 3.92	19.70 ± 3.26	72.22	24.35	47.87
20 V	25.39 ± 4.31	14.57 ± 2.84	75.36	26.18	49.18
P	61.54 ± 4.46	33.44 ± 1.36	53.31	10.58	42.74

FIGURE 2. (a) Fluorescence micrographs of osteoblasts (nuclei in blue) incubated on samples for 60 min of culture and (b) Osteoblasts adhesion measured by cell counting at 30, 60, and 120 min. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

anodized surface is a little lower than that on the other two titania surfaces at days 1 and 4 (Fig. 3).

Cell morphology

Osteoblasts grow and spread well on all three surfaces after 3 h or 4 days of culture (Figs. 4 and 5). The SEM pictures show that the cells spread well and attach closely onto all three substrates (Fig. 4). In particular, on the 20 V anodized surface, the cells seem to show a larger amount of thin filopodia [Figs. 4(b,e)]. This phenomenon is already obvious after 3 h of culture as shown by the fluorescence pictures. On the 20 V surface, many more cells show a large amount of microfilament compared with other two surfaces (Fig. 5). The higher magnification SEM pictures disclose that the nanoscale extracellular matrix (ECM) deposited on the ano-

dized surfaces follows the surface texture [Figs. 4(g,h)], showing forms of nanopartilces on the 5 V surface with a diameter of about 30 nm and nanobelts on the top edge of nanotube with a width of about 20 nm. On the polished surface, less ECM is deposited [Fig. 4(i)].

Intracellular alkaline phosphatase activity

Even without the osteogenic medium, the primary osteoblasts on the samples still differentiate and synthesize ALP (Fig. 6). At day 7, the ALP activity of the osteoblasts on the 20 V surface is higher than that on the polished surface, while that on the 5 V surface is lower than that on the control. After 14 days of culture, the osteoblasts on the substrates synthesize more ALP. The ALP activity of the osteoblasts on the 5 V and 20 V anodized surfaces is higher than that on the polished surface but with no statistical significance.

FIGURE 3. Cell viability/proliferation on samples after 1, 4, and 7 days of incubation using colorimetric MTT assay. * $p < 0.05$ and ** $p < 0.01$ compared with the polished surface and ## $p < 0.01$ compared with the 20 V surface.

Osteogenesis-related gene expression

The gene expression on different substrates is quantified using Real-time PCR and the results are compared in Figure 7. In general, the osteoblasts cultured on the anodized surfaces exhibit down-regulated gene expressions. After 3 days of culture, both anodized nanotextured surfaces induce down-regulation expressions of nearly all the genes concerned in this study, although statistical differences are found only in the expressions of RUNX2 and OPN. On the three surfaces, the expressions of all the genes show a time-dependent increase. After incubation for 10 days, there are no obvious differences between the osteoblast gene expressions on the 5 V anodized surface and the polished control surface. However, the expressions of ITG, RUNX2, BMP, and OPN on the

FIGURE 4. Osteoblast cell morphology and ECM deposition on the 5 V anodized surface (a, d, and g), the 20 V anodized surface (b, e, and h) and the polished surface (c, f, and i) after 4 days of culture.

20 V anodized surface are statistically lower than those on the other two surfaces.

DISCUSSION

Titania nanotubes have attracted much attention recently for their potential in implant application. However, the biological behavior of titania nanotubes is still controversial.^{5,6,8,9} Considering that the biological behavior of primary osteoblasts is closer to the situation *in vivo* and that different phenotypes of osteoblasts used in previous studies may account for the controversial results, we monitor the behavior of primary osteoblasts on the nanoscale titania surfaces fabricated by anodization. It should be noted that these samples are not annealed but they are sterilized by ultraviolet irradiation. These factors may affect the experimental results.

Two distinct surfaces, the 5 V anodized surface with a pore diameter of 25 nm and the 20 V anodized surface with an inner tubular diameter of 80 nm are formed by anodization. These two surfaces are chosen to observe the pore size effect on primary osteoblasts. The XRD results show that the surfaces have an amorphous nature. The different

phases of titania, amorphous, anatase, and rutile, influence the cell functions on them.²⁰ The surface free energy is considered to play an important role in the cell/implant interaction²¹ and so it is monitored in this study. The anodization treatment enhances the surface free energy of titanium and the surface energy of the 20 V surface is a little higher than that of the 5 V surface. However, in our study, the cell behavior cannot be explained well by the surface free energy difference. Higher surface free energy is believed to benefit cell function, but in this study the anodized surfaces with higher surface free energy induce down-regulated gene expressions. The surface nanotopography may thus play a more important role than the surface free energy, which has also been indicated by other study.²² It is worth mentioning that there is residual fluoride in the nanotubes resulting from the preparation procedure.²⁰ Proper content of fluoride is widely reported to encourage osteoblast function,²⁰ so the down-regulation of gene expressions cannot be attributed to the fluoride residual.

Initial adhesion of anchorage dependent cells is critical for their subsequent functions.²³ We observe no obvious difference in the initial cell adhesion on the different

FIGURE 5. Fluorescence microscopy images of the cells with dual staining with phalloidin for actin filaments (green) and Hoechst for nuclei (blue). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

substrates. Our observation is somewhat similar to those reported previously that no difference of initial cell attachment can be found in the initial 2 h.^{6,8,18} However, after one week, controversial results have been reported. Some have observed enhanced growth of MC3T3-E1 on titania nanotubes,^{6,8} while others have reported inhibited growth of primary human osteoblast-like cells on titania nanotubes except the 15 nm ones.⁵ Besides the substantially different nature of the titania nanotubes in these studies, we think

that the different phenotypes of the osteoblasts used in their studies may also be responsible for the different results. Our studies are partially consistent with Park et al.'s results showing that growth of primary osteoblasts on the anodized nanotextured surfaces with tube diameters larger than 15 nm is a little slower than that on the control.⁵ The differential growth behavior of the primary osteoblasts and osteoblastic cell lines may be explained by the differences in their biological behavior. The osteoblastic cell lines show faster proliferation speed and abnormal differentiation phenotype in contrast with the primary cells during culture.²⁴ It has been reported that the groove texture MC3T3-E1 cells are only slightly affected by the groove texture, whereas primary rat bone marrow cells are affected more significantly.¹⁵ Hence, it is reasonable that the osteoblastic cell line and the primary osteoblast may react differently to the nanoscale textures.

SEM reveals that the cells attach closely to the polished surface and are connected with each other nearly fully covering the entire substrate. The cells spread out with robust lamellipodia on the 25 nm nanonet-like surface whereas the cells on the 80 nm nanotubes show a large amount of thin filopodia. This phenomenon has been observed in previous study for osteoblasts^{5,6,8,18} and other cells such as mesenchymal stem cells^{4,25} and endothelial cells.²⁶ This long filopodia may be ascribed to the poor focal contact formation on the nanotubular surfaces.^{4,5,25} On the titania nanotubes with a larger dimension, the ECM is mainly deposited on the edge of the nanotubes [Fig. 4(h)], thus providing insufficient ECM attachment sites for the cells. This has been partly verified by Lee's study, in which cells form long filopodia when they are on a substrate not suitable for focal contact formation (silicon dioxide nanorods in that study) and trying to find a more suitable area to attach and proliferate.²²

A higher ALP activity is observed on the anodized surfaces even though a lower activity is seen on the 5 V surface during the first 1 week of culture. This is more or less in accordance with ALP gene expression pattern obtained by real-time PCR. Others have reported an elevated ALP activity of MC3T3-E1 on the nanotubular surfaces compared

FIGURE 6. ALP activity of osteoblasts on samples after 7 and 14 days of culture. * $p < 0.05$ and ** $p < 0.01$ compared with the polished surface and ## $p < 0.01$ compared with the 20 V surface.

FIGURE 7. Expression of osteogenesis-related genes by primary osteoblasts cultured on titanium surfaces after incubation for 3 and 10 days: (a) Integrin- β 1, (b) RUNX2, (c) ALP, (d) BMP, (e) osteopontin, and (f) osteocalcin. The data were generated by Real-time RT-PCR and were shown as mean expression relative to the housekeeping gene GAPDH $\pm$ SD. * p < 0.05 and ** p < 0.01 compared with the polished surface, # p < 0.05 and ## p < 0.01 compared with the 5 V anodized surface.

with titania surfaces with the complete media.⁹ The exact reason for the elevated ALP activity on the nanotubular surface as well as the tendency with MC3T3-E1 is still unclear but it is believed that the higher cell mobility on the titania nanotube may play a part.

To the best of our knowledge, it is the first time that down-regulated expression of osteogenesis-related genes of primary osteoblasts incubated on anodized nanotextures is reported. Gene expressions are severely impaired on the 20 V anodized surface compared with the 5 V one. Cell adhesion on biomaterials and associated signal transduction are mediated by focal adhesion involving integrin clustering. More and more evidences indicate that clustering of integrins requires that the spacing between ligated integrins be less than 50–70 nm.²⁷ Park et al. suggest that a spacing of

15 nm may provide the optimum length scale for integrin clustering and focal contact formation and spacings larger than 50 nm severely hamper them.⁴ The down-regulated gene expressions on the anodized surfaces may be related to hampered focal adhesion formation and it is more severely impaired on the 20 V anodized surface for the larger tube size of 80 nm, leading to much lower gene expressions. The down-regulated expression of Integrin- β 1 may confirm this hypothesis. Integrin- β 1 is associated with the integrins expressed by osteoblasts representing the dominant adhesion mechanism to ECM.²⁸ The signals from the ECM transduced through the focal contacts into the cells are very important for cell proliferation, differentiation, or apoptosis.²⁹ The shortage of signals from ECM for inadequate focal contact formation on the anodized surfaces, especially the

20 V one, results in checked cell differentiation and associated gene expressions. Thereafter, it is reasonable that the expression of RUNX2 which is an important transcription factor that contributes to the regulation of the expression of many osteoblast genes³⁰ and the downstream genes such as BMP, OPN, and OCN are down-regulated. Recently Biggs et al. have shown that the nanopit arrays (100 nm deep and 120 nm in diameter possessing a centre-centre spacing of 300 nm) disrupt adhesion formation and cellular spreading in primary human osteoblasts preventing osteospecific differentiation in human osteoprogenitors.³¹ It supports our results to a certain degree.

CONCLUSION

We observe depressed functions of primary osteoblasts on the anodized nanotextured surfaces. Initial cell adhesion is not obviously affected by the anodized surfaces. A little higher intracellular ALP activity and more ECM deposition are found on the anodized surfaces, while cell growth and osteogenesis-related gene expression are both impaired on them, especially the 20 V anodized surface. This may be attributed to the compromised focal contact formation on the anodized surfaces. The difference in the phenotypes of the primary osteoblasts and osteoblastic cell lines may account for previous conflicting reports in osteoblast cytocompatibility of titania nanotubes. It should be considered when evaluating the biocompatibility of titania nanotubes as well as comparing results from different sources.

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