Effect of TiO2 Nanotube Layers Thickness on Periodontal Ligament Cells

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Abstract

Periodontal ligament cells (PDLCs) remaining in the tooth-extraction socket are involved in osseointegration after immediate implantation; however, their interaction with different implant surfaces has not been investigated. The aim of this study was to compare PDLC growth on substrates composed of differently sized titanium dioxide (TiO2) nanotubes with that on flat Ti. PDLC growth on Ti nanotubes was evaluated in terms of cell adhesion, proliferation, and osteogenic differentiation based on the expression of alkaline phosphatase (ALP), type 1 collagen (COL-1), osteopontin (OPN), and Runt-related transcription factor 2 (RUNX2). We found that TiO2 nanotubes of different diameters (30, 70, and 120 nm) manufactured on Ti surface by anodisation had a well-defined structure. PDLCs grown on nanotube layers demonstrated polygonal morphology with more filopodia than those on flat Ti, which was especially evident on the nanotubes of larger diameters. However, cell adhesion and proliferation was the highest on the smallest 30 nm nanotubes. Similarly, mRNA levels of the ALP, COL-1, OPN, and RUNX2 genes increased in PDLCs cultured on 30 nm TiO2 nanotube layers compared to those in the cells grown on 70 nm and 120 nm nanotubes. In conclusion, small diameter (30 nm) nanotube layers can support PDLC adhesion, proliferation, and differentiation better than larger sized nanotubes, and consequently, have higher potential to promote bone formation and integration of an immediate implant.

Keywords: TiO2 nanotubes; Periodontal ligament cells; Adhesion; Proliferation; Osteogenic differentiation

Introduction

Dental implants, which represent artificial tooth roots to support a dental prosthesis, have been successfully used as a popular approach to dental restoration [1]. Immediate implantation into fresh extraction sockets is an attractive alternative to delayed implant placement after alveolar healing because it presents several advantages such as reduction in rehabilitation time and number of surgical episodes, preservation of bone tissues, and improvement of the peri-implant gingival tissue aesthetics [2]. However, the difficulty in achieving primary stability leads to the early mobility of the implants thus limiting the reliability of this approach and its further application [3].

Soballe et al. have shown that micromovements (150 μm) of dental implants induce interposition and subsequent immobilisation of fibrous tissue leading to the formation of new bone tissue [4,5]; similar results were obtained in dogs and monkeys [6,7]. Immediate implantation, which is the placement of the implant in the periodontium before the healing of the tooth extraction site, is followed by the formation of connective tissue by residual periodontal ligament cells (PDLCs) in the fresh extraction socket. It is known that PDLCs have the ability for multipotent differentiation and could form collagen fibers, cementum, and bone [8,9]. These cells are also involved in the complex process of osseointegration, i.e., establishment of a direct bone-to-implant anchorage without intervening connective tissue, which is essential for the success of immediate implantation [10].

To avoid the interference of connective tissues, two main factors should be considered: the surface of the implant fixture, which should promote osteogenic differentiation, and faster healing process. Compared with other metals used for implantation, titanium (Ti) and its alloys have attracted attention because of their higher biocompatibility, corrosion resistance, and mechanical properties. In the past decades, many strategies have been used to improve the durability and function of Ti-based dental implants [11].

Recently, the modification of implant surface topography at the nanoscale level has becomes one of the major research strategies to promote wound healing, new osteoblast differentiation, and subsequent implant integration [12]. TiO2 nanotube layers obtained by anodisation have received considerable attention as implant biomaterials [13,14] because they could increase surface area and roughness, and improve nanoporous architecture and scaffold configuration of the implants though changes in the chemical composition and crystal structure [15-17]. As a result, TiO2 nanotubes grown on Ti surface enhance osteoblast adhesion, proliferation, gene expression [13,18] and improve osseointegration compared to flat Ti [19]. It has also been shown that the diameter of TiO2 nanotubes can significantly influence the behaviour of preosteoblasts and osteoblasts [20-22].

A number of studies have investigated the growth of osteoblast cell lines on TiO2 nanotube layers; however, there are few reports on PDLCs, which are important for the outcome of immediate implantation. This is the first investigation of PDLCs grown on TiO2 nanotubes of different diameters. Here, we evaluated cell morphology, actin cytoskeleton, adhesion, proliferation, and gene expression of PDLCs cultured on TiO2 nanotube layers formed on Ti substrate. The findings of this study can provide the parameters for further optimisation of implant design.
Materials and Methods

Fabrication of TiO₂ nanotube layers

TiO₂ nanotubes were prepared by anodisation technique described earlier [22,23]. Ti thin foil (0.25 mm thick) with the purity of 99.5% (Alfa Aesar) was soaked in a mixture of 2 ml 48% HF; 3 ml 70% HNO₃, and 100 ml deionised water for 5 min to remove the naturally formed TiO₂ layer, then washed with deionised water, and dried by nitrogen flow. TiO₂ nanotubes of 30 nm, 70 nm, 120 nm in diameter were formed on the anodised Ti surfaces using the voltages of 5 V, 15 V, 25 V, respectively, in the electrolyte containing 1 M H₃PO₄ and 0.5 wt% HF for 3 h; platinum was used as a counter electrode. After anodisation, the samples were washed with deionised water, dried by nitrogen flow, and the nanotubes were then sintered at 450°C for 3 h. The experimental samples of 1 cm × 1 cm were sterilised in a steam autoclave at 120°C for 30 min.

Substrate surface characterisation

All samples were sputter-coated with gold using a Hummer I Sputter-coater for 3 min, and surface microstructure of TiO₂ nanotube layers was then examined by scanning electron microscopy (SEM; FEI Sirion 200, Hillsboro). The images were acquired at the magnification of 1,000× or 100,000×.

Isolation and culture of human PDLCs

Human PDLCs were isolated from adult healthy premolars extracted for orthodontic reasons, and their use in this study was approved by the Institutional Review Board after obtaining patients’ informed consent. Teeth were washed with phosphate-buffered saline (PBS), and periodontal ligament tissue was obtained from the middle third of the tooth root and cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS, Gibco), 100 U/ml penicillin and 100 mg/l streptomycin [24]. The cells were grown to confluence at 37°C in a 5% CO₂ humidified incubator.

PDLCs were seeded on flat Ti and TiO₂ nanotubes of 30 nm, 70 nm, and 120 nm in diameter at the density of 2 × 10⁴ cells/ml for the attachment and proliferation assays, and at a higher density of 4 × 10⁴ cells/ml for the osteogenic differentiation assay; in the latter case, the cells were cultured in osteogenic medium (50 μg/ml ascorbic acid, 10 mM β-glycerophosphate, and 10 mM dexamethasone).

Cell morphology

PDLCs of the third passage were seeded on the sample surface for 24 h, washed twice with PBS, fixed in 2.5% glutaradehyde in PBS for 1 h, and washed three times with PBS for 10 min. Then, the samples were dehydrated in a graded series of alcohol (35, 50, 75, 90, and 100%) for 10 min and dried by supercritical fluid CO₂. Sample surfaces were sputter-coated in gold, and cell morphology was examined by the field emission SEM; each group (n=3) was evaluated at five spots: top, bottom, right, left, and middle.

Actin staining

After 24 h of culture, PDLCs were washed three times with PBS, fixed in 4% paraformaldehyde for 30 min, and washed again with PBS. The cells were then permeabilised with 0.1% Triton X-100 in PBS for 5 min, gently washed with PBS, incubated with 1% BSA for 20 min to block non-specific binding sites, and actin filaments were stained with phalloidin-TRITC (20 μg/ml, Sigma) for 30 min. After washing with PBS, the cells were counter-stained with DAPI (Sigma) to visualise the nuclei, washed with PBS, and placed on glass slides using Fluoromount (Sigma). Actin formation was examined by confocal laser scanning microscopy (CLSM) using a TLS SP2 instrument (Leica, Wetzlar, Germany); the images were acquired with the red (actin) and blue (DAPI) filters.

Cell adhesion

To evaluate cell adhesion, PDLCs were seeded on the substrates for 2 h, washed with PBS, and fixed in 4% paraformaldehyde for 15 min; cell nuclei were labelled by DAPI for 5 min. The images were obtained by CLSM, and adhered cells were counted using the Image-Pro 5.0 software (Media Cybernetic; Silver Springs, MD, USA).

MTT assay

Cell viability was evaluated 2, 4, and 7 days after PDLC seeding on the substrates using the MTT assay. The samples were washed in PBS and transferred to a new 24-well polystyrene culture plate; 20 μl of 5 mg/ml MTT (Sigma) in 200 μl culture medium was added into each well. After 4 h of incubation at 37°C, the medium was removed and 200 μ1 DMSO was added to each well and mixed for 10 min. DMSO extracts were transferred into a new 96-well polystyrene plate, and the absorbance of each solution was measured at the wavelength of 590 nm using a spectrophotometer (Safire2®, Tecan, Männedorf, Switzerland).

Gene expression analysis

Gene expression in PDLCs grown on flat Ti and TiO₂ nanotubes of different diameters was evaluated by real-time quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from PDLCs cultured for 7 and 14 days using Trizol (Sigma), and 1 μg RNA was reverse transcribed using the Reverse Transcription Kit (TaKaRa, Japan) at 37°C for 15 min followed by 85°C for 5 s. RT-qPCR analysis of ALP, COL-1, OPN, and RUNX2 expression was performed using a real-time PCR system (Bio-Rad, Hercules, CA, USA) at the following cycling conditions: 95°C for 10 min followed by 40 cycles of 95°C for 30 s, 60°C for 1 min, and 72°C for 1 min; PCR specificity for each gene was determined by the dissociation curve analysis at 95°C for 15 s, 60°C for 1 min and 95°C for 15 s. The primer sequences are listed in Table 1.

The expression of the tested genes was normalised to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) used as a house-keeping gene and compared to that of the cells grown on flat Ti using the 2-ΔΔCt method [25].

Statistical analysis

All experiments were conducted in triplicate and statistical analysis was performed using the SPSS 11.5 software. One-way ANOVA followed by the Student-Newman-Keuls post hoc test were used to evaluate differences between the samples; p-value of less than 0.05 was considered statistically significant.
Gene Primers (F=forward; R=reverse) Accession Numbers Product Size (bp)
OPN F: 5’CATGAGAATTGCAGTGTTTGCT3’ R: 5’CTTGCAAGGGTCTGTGGGG3’ NM_000582.2 186
ALP F: 5’TCAAACCGAGATACAAGCAC3’ R: 5’CCACCAGCAAGAAGAAGC3’ NM_000478.4 219
COL-1 F: 5’TCCAACGAGATCGAGATCC3’ R: 5’AAGCCGAATTCCTGGTCT3’ NM_000088.3 191
RUNX2 F: 5’GCCTTCAAGGTGGTAGCCC3’ R: 5’CGTTACCCGCCATGACAGTA3’ NM_001015051.3 67
GAPDH F: 5’TGGACAGTCAGCCGCATCTT3’ R: 5’ATCCGTTGACTCCGACCTTCA3’ NM_002046.3 90

Table 1: Primer sequence used for Real-time PCR.

Results

Sample characterisation

Surface microstructure of flat Ti and TiO\textsubscript{2} nanotubes of different diameters was examined by SEM. The surface of flat Ti exhibited randomly positioned shallow grooves due to mechanical polishing (Figure 1a), whereas that of the nanotubes formed on the anodised Ti substrates had well-defined structurally ordered morphology (Figures 1b-1d).

Figure 1: Physical characterization of different sized nanotube surfaces. The images show (a) flat Ti and highly ordered nanotubes with three different diameters (b) 30 nm, (c) 70 nm, and (d) 120 nm.

We randomly selected 30 nanotubes in each group and calculated their diameter using the Image-Pro 5.0 software. The diameter of nanotube layers anodised at 5 V, 15 V, and 25 V was 30.33 ± 4.35 nm, 74.53 ± 9.28 nm, and 124.25 ± 17.98 nm, the thickness was ~ 100, 300, and 500 nm, and surface roughness was 9.29 ± 0.68, 18.02 ± 0.98, and 45.56 ± 1.20 nm respectively (for flat Ti, surface roughness measured in our previous study was 4.63 ± 0.39) [23].

PDLC morphology

Morphological analysis of PDLCs grown on differently sized TiO\textsubscript{2} nanotubes for 24 h was performed by SEM (Figure 2). The cells cultured on flat Ti exhibited elongated shape (Figure 2a), whereas those grown on 30 nm nanotube surface were spread freely and developed extensive filipodia (Figure 2b), which facilitated cell anchorage to the nanotubular structures potentially stimulating PDLC differentiation. The cells cultured on 70 nm (Figure 2c) and 120 nm (Figure 2d) nanotube layers displayed polygonal shape with minimal spreading and increased number of filopodia compared to PDLCs grown on flat Ti or 30 nm nanotube layers.

Figure 2: SEM micrographs of PDLCs seeded on (a) flat Ti; (b) 30 nm nanotube layers; (c) 70 nm nanotube layers; and (d) 120 nm nanotube layers after 24 h. The PDLCs on the nanotube layers were displayed polygon shape with more filopodias than flat Ti, especially on the 70 nm and 120 nm nanotube layers.
The analysis of PDLC actin cytoskeleton by immunofluorescence revealed similar morphological trends observed by SEM, i.e., polygonal shape and increased extension of filopodia for the cells cultured on nanotubes of larger diameter (Figure 3). In addition, actin fibers in PDLCs cultured on flat Ti and 30 nm nanotubes looked longer and directionally organised compared to those in the cells grown on 70 nm and 120 nm nanotubes.

Figure 3: Phalloidin staining of the actin cytoskeleton of adherent PDLCs after 24 h of incubation on the different samples: (a) flat Ti; (b) 30 nm nanotube layers; (c) 70 nm nanotube layers; and (d) 120 nm nanotube layers.

Cell adhesion

Figure 4 shows PDLC adhesion to nanotubes of different diameters after 2 h. Cell adhesion to 30 nm nanotubes was the highest compared to other tested surfaces. The number of PDLCs attached to 30 nm nanotubes significantly exceeded that detected on flat Ti (p<0.05), as well as on 70 nm and 120 nm nanotube surfaces (p<0.01).

Cell proliferation

The analysis of PDLC proliferation on different substrates indicated that on day 2, cell multiplication did not significantly vary between the groups (Figure 5). On day 4, however, cell proliferation rate on 30 nm and 70 nm nanotubes was statistically higher than that on flat Ti and 120 nm nanotubes (p<0.05), and on day 7, it was significantly higher for the 30 nm group than for other cells (p<0.01). In addition, PDLCs grew significantly slower on 120 nm nanotubes than on other tested surfaces (p<0.01 for day 7) (Figure 5).

Gene expression analysis

The expression of ALP, COL-1, OPN, and RUNX2 in PDLCs grown on different Ti surfaces was analysed on days 7 and 14 of culture by qPCR (Figure 6). At day 7, the ALP gene associated with early osteogenic differentiation and the COL-1 gene encoding a matrix protein synthesised by osteoblasts, showed higher expression in the cells cultured on 30 nm nanotubes compared to other groups (p<0.05; Figures 6a and 6b). The OPN gene coding for the most abundant non-collagenous matrix protein in the bone and associated with bone mineral metabolism, was upregulated in PDLCs grown on 30 nm nanotubes at day 7 and especially at day 14, when its expression increased 2.5 fold (p<0.01; Figure 6c). The RUNX2 gene, which is essential for osteoblastic differentiation, showed a similarly tendency in PDLCs grown on 30 nm nanotubes (p<0.05; Figure 6d).

Figure 4: Comparative study of the adhesion of PDLCs on (a) flat Ti; (b) 30 nm nanotube layers; (c) 70 nm nanotube layers; and (d) 120 nm nanotube layers. The bar graphs show the average ± standard error bars. **p<0.01 compared to flat Ti, ##p<0.01 compared to 30 nm nanotube layers.

Figure 5: MTT assay data showing the proliferation of PDLCs on flat Ti and TiO$_2$ nanotubes. The error bars in the figure represent the standard deviation for three samples for each data. *p<0.05 compared to flat Ti, **p<0.01 compared to flat Ti.

Discussion

The ability of PDLCs for osteogenic differentiation has been demonstrated in many previous studies [25,26]. The improvement of PDLC adhesion to and proliferation on implant surfaces could facilitate osseointegration and healing leading to the success of immediate implantation. In this study, we evaluated the effects of differently sized TiO$_2$ nanotube layers on PDLC adhesion, proliferation, and expression of the genes involved in osteoblastic differentiation. Our findings demonstrate that the diameter of nanotubes could influence the behaviour and metabolic activity of PDLCs.

Cell adhesion and surface spreading influences such cellular functions as proliferation, migration, and extracellular matrix production [26]. Therefore, we evaluated PDLC attachment to the surface of TiO$_2$ nanotubes with different diameter by SEM. Our observation that cell adhesion to 30 nm nanotubes was the highest among the tested surfaces is consistent with the results obtained for other cell types [14,15]. The enhanced adhesion may be related to the increased adsorption from the culture medium of serum proteins such as fibronectin and vitronectin, which play an important role in cell adhesion [21]. Brammer et al. have reported that small-diameter (30 nm) TiO$_2$ nanotubes provide the highest cell adhesion rate because of the increased surface for protein adsorption, whereas on large diameter nanotubes, proteins were rarely adsorbed on the upper wall surface because of large empty pores [20].

The proliferation of PDLCs cultured on 30 nm nanotubes was higher than that on 70 nm and 120 nm nanotubes, which was consistent with a previous study reporting that the adhesion, proliferation, migration, and differentiation of mesenchymal stem cells and osteoblast-like cells were maximal on 15 nm nanotubes and minimal on 100 nm nanotubes [27]. The cells seeded on the surface of small diameter TiO$_2$ nanotubes showed higher formation of focal contacts and actin stress fibers, as well as upregulation of paxillin phosphorylation compared to those grown on TiO$_2$ nanotubes of larger sizes [28]. However, different results were obtained for mouse calvarial MC3T3-E1 cells and bone marrow stromal cells (BMSCs), indicating that the response may be cell type-specific [22,23].

The effect of nanotube layers on promoting osteogenic differentiation of PDLCs was a major focus of this study. Quantitative PCR analysis of osteogenic differentiation markers ALP, COL-1, OPN, and RUNX2 showed that the expression of these genes was increased in PDLCs grown on 30 nm nanotubes, which was in contrast to gene

Figure 6: The genes (a) ALP, (b) COL-1, (c) OPN, and (d) RUNX2 were assayed by Real-time PCR at days 7 and 14, after PDLCs seeding on different samples. The results were normalized by GAPDH and shown as fold change (baseline=day 7 cells on flat Ti; *p<0.05 compared to flat Ti, **p<0.01 compared to flat Ti).
expression in osteoblast-like cells reported previously [20,23]. These studies have shown that MC3T3-E1, osteoblasts, and BMSCs demonstrated much higher expression of osteogenic genes when grown on larger size TiO$_2$ nanotubes, which contributed to cell cytoskeletal tension and formation of stress fibers resulting from a dramatic stem cell elongation. Such conflicting results can probably arise from the differences in cell type and origin.

PDLCs are useful cells for the reconstruction of periodontal tissues because they contain osteogenic and fibrogenic progenitor cells [29,30], which differentiate into fibroblasts, cementoblasts, or osteoblasts depending on the surrounding conditions. A recent study on stem cells derived from the periodontal ligament has reported increased osteogenic differentiation of the cells seeded on rough surfaces coated with Al$_2$O$_3$ particle-treated Ti [31]. Furthermore, another study investigating MC3T3, human bone marrow stromal cells, murine femoral stromal cells, and canine bone marrow cells have reported significant variations in their mineralisation and proliferation response to Ti surfaces [32]. Overall, these results emphasise the existence of cell-specific differences in the osteogenic differentiation between osteoblast-like cells and PDLCs. However, the underlying mechanisms and the corresponding signalling pathway are unclear and require further investigation.

In the fresh socket after tooth extraction, the residual periodontal ligament tissue remained in the cervix and tooth root tip, and can critically influence immediate implantation. Therefore, to further optimise implant design, the interaction of PDLCs with the implant surface should be considered. Given that the osteogenic differentiation of PDLCs on TiO$_2$ nanotubes is distinct from that of osteoblasts, it can be suggested that to achieve better osseointegration, different surface sections of the immediate implants can be covered with TiO$_2$ nanotubes of various diameter. Further studies are needed to test this hypothesis.

Conclusions

In summary, our study demonstrates that PDLCs cultured on small diameter (30 nm) TiO$_2$ nanotubes showed higher adhesion and proliferation, as well as expression of osteogenic genes compared to those grown on flat Ti or large diameter (70 nm and 120 nm) nanotubes. Our results indicate that PDLCs have a potential for bone formation on TiO$_2$ nanotube surfaces and that their impact should be considered when manufacturing the materials to be used for immediate implantation.

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