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Enhanced cellular responses to titanium coating with hierarchical hybrid structure

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In this work, nano/micro hierarchical hybrid structured surface was prepared by fabricating a titania nanotube layer in plasma sprayed porous titanium coating (TC). In vitro human marrow stem cells (hMSCs) were employed for the evaluation of the biological properties of the anodized titanium coating with a hierarchical structure (HSTC). Significantly higher cell adhesion quantity (about 30% more) was found on the HSTC than that on the as-sprayed TC. The expressions of osteocalcin (OC) and osteopontin (OPN) for the HSTC were also detected to be about twice as high as those on the as-sprayed TC. The enhanced cell responses on the HSTC were explained by the improved protein adhesion resulted from the increased surface area and surface energy. Combining the advantages in the mechanical fixation and long-term stability of the plasma sprayed porous TC, the HSTC with a hierarchical structure may be a good candidate for hard tissue replacements, especially for load-bearing implants.

1. Introduction

A fast and firmer fixation of implants with natural bone tissue has been a persistent challenge to expand their therapeutic indications, reduce patient morbidity, and improve the success rate of the treatment [1–3]. Many topographic technologies, such as porous coatings fabricated by plasma spraying or titanium net zones formed by sintering, are widely used in clinical practice [4]. The porous layer can effectively alleviate the mechanical incompatibility between implants and host bone tissues. Meanwhile, it can encourage the bone tissue in-growth to ensure the fixation of implants. However, these surfaces with large roughnesses and pores always result in suppressed cell functions in the early implantation [5–7]. Sader et al. [6] found that osteoblasts could not spread completely on the Al_2O_3 blasted rough surfaces and cell proliferation rate was diminished at the beginning of incubation. A delayed viability and ALP activity were observed when cells were cultured on these rough surfaces. Boyan et al. also reported a better proliferation of osteoblasts on smooth (Ra 0.5 μm) surfaces than on rough one (Ra = 5 μm) [7]. Ponader et al. [8] found that highly rough surfaces (Ra ≥ 56.9 μm) significantly reduced the proliferation of hFOB cells, while osteogenic differentiation gene expressions were not significantly influenced by the surface roughness.

To improve the cell functions on the biomaterials and the early fixation of implants, nano-topographies were widely fabricated. In general, nanotopographic surfaces have been observed to promote the adhesion and proliferation of osteoblastic cells [9], bone matrix synthesis, and osseointegration [10,11]. Osteoblast adhesion has been shown to support over three times more cells on nano-phase alumina than on conventional alumina [12]. This increased adhesion was explained by the conformation of vitronectin adsorbed on the nano-phase alumina [13]. TiO_2-coatings possessing substantial "surface pores" at dimensions of 15–50 nm promoted the formation of calcium phosphate as well as induced tissue attachment [14,15]. Nanotopography was also a significant factor for the differentiation of mesenchymal stem cells (or osteoprogenitor cells) to osteoblastic phenotype [16]. Increased ALP activity and bone tissue growth were obtained for nanotopographic titanium implants [17].

Among the many types of nano-textured surfaces, titania nanotubular surface has drawn very much attention. Vertically oriented and highly ordered titania nanotube arrays can be fabricated easily by anodization and the nanotube dimensions can be controlled conveniently by adjusting the process parameters [18,19]. Nanotubular titania has been recognized as a promising biomaterial with proven biocompatibility, thermal stability, and corrosion resistance. Titania nanotubes have a small Young's modulus of ~36–43 GPa [20], which is closer to that of bones than titanium. It may prevent bone resorption caused by stress shielding. In addition, the possibility of creating unique nanotube architectures with high surface-to-volume ratio and controllable dimensions makes this material desirable for a range of biomedical applications [21–24]. Suitable size nanotubes can significantly enhance the functions of bone cells [25–28]. Besides, the nanotubes can serve as carriers for drugs, such as growth factors [29] or bactericides [30]. Additionally, the presence of this nanotubular layer can effectively improve the corrosion resistance of the titanium implants. An approximately

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30 mV increase in Ecorr and decreased Icorr (1.5 times less) and Ipass (3–6 times less) were obtained by anodized Ti6Al7Nb samples. [31].

To combine the good mechanical fixation of porous titanium coating by bone tissue growing in and early cell stimulation functions of titania tubular array surface, in this paper, nanotubular titania surface was fabricated onto widely used porous titanium coating. We hope to improve the early cell responses with the nanotubular titania array, and meanwhile, to preserve the porous structures of the Ti coatings for long-term fixation of implants.

2. Experimental processes

2.1. Titanium coatings preparation and characterization

Titanium coating (TC) on Ti-6Al-4V substrates (10 mm × 10 mm × 2 mm and Φ 34 mm × 2 mm) were fabricated by vacuum plasma spraying (VPS, F4-VB, Sulzer Metco, Switzerland) with the powder size at 50–150 μm. Coating samples after ultrasonic cleaning were applied for anodization in 0.5 wt.% hydrofluoric acid with a platinum electrode serving as the cathode. The anodizing potential was set at 5 V. The surface morphologies of the as-sprayed TC and hierarchical structured titanium coating (HSTC) were observed using a scanning electron microscopy (SEM, JEOL JSM-6700F, Japan).

2.2. Cell adhesion and proliferation

The human marrow stem cells (hMSCs) were isolated and expanded as previously described [32]. For adhesion and proliferation analysis, cells were cultured in α-MEM culture medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) and antibiotics at 37 °C in a humidified incubator of 5% CO_2 and 95% air. The medium was changed every 2 days. For differentiation study, the medium was supplemented with 50 μM L-ascorbic acid, 10 mM glycerophosphate, and 100 nM dexamethasone. The morphologies of hMSC were observed by SEM with cells after cultured on the samples for 12 h. The sample treating processes included cell fixing overnight in 2% glutaraldehyde, dehydrating in a graded series of alcohol/hexamethyl disilazane at various proportions and drying in an oven at 37 °C overnight.

The quantitative analyses of attachment and proliferation of hMSC were performed using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, Sigma, St. Louis, MO) assay. For attachment analysis, cell suspension 1 μl (about 5×10^4 cells) was seeded in a well and cultured in a humidified 37 °C/5% CO_2 incubator. After 4, 8, and 12-h incubation, 0.1 ml of MTT solution was added. Another 4 h incubation was employed to form formazan. Then, half milliliter of dimethylsulfoxide (DMSO, Sigma-Aldrich) was added to dissolve the formazan. The optical density (OD) was measured at 570 nm using an automated plate reader (Bio-tek, USA).

For cell proliferation measurements, the cultured cell density was 1×10^4 cells/well, and the predetermined time point was assigned to 1, 4, 7, and 14 days. Similar MTT assay was used to measure the proliferation of cells. The number of hMSCs was expressed as the ratio of the OD value relative to the value for day 1 of the same specimen.

2.3. Alkaline phosphatase (ALP) activity assay

hMSCs were seeded at a density of 5×10^4 cells/well in an osteogenic medium for ALP activity evaluation using p-nitrophenylphosphate (*p*NPP, Sigma, St. Louis, MO). After incubation for 4, 7, and 14 days, cells were collected by a 0.2% Triton X-100 solution through four freeze-thaw cycles. Cell lysate (50 μl) was mixed with 50 μl *p*NPP (1 mg/ml) in 1 M diethanolamine buffer (pH 9.8) and incubated at 37 °C for 45 min on a platform shaker. Then, 25 μl of 3 N NaOH was added to 100 μl of the mixture to stop the reaction. Absorption at 405 nm of the mixed solution was measured (Bio-Tek, USA) for the quantification of ALP.

2.4. Protein absorption assay

One sample was incubated in 1 ml medium (α-MEM, Gibco, USA) containing 10% fetal calf serum (FCS, Gibco, USA) at 37 °C for 2 h, and then, washed with PBS for 3 times. The adsorbed proteins were detached into 500 μl of 1% sodium dodecyl sulfate (SDS) solution and measured by a Micro-BCA kit (Pierce, USA).

2.5. Quantitative real-time PCR

To quantify the osteogenic-associated gene expression of hMSCs, cells were cultured in an osteogenic medium at a density of 5×10^4 cells/well in a 6-well plate. After incubation for 4, 7, 14, or 21 days, the total RNA was isolated from hMSCs using a TRIZOL (Invitrogen, Carlsbad, CA), and 1 μg of the RNA solution was converted to complementary DNA (cDNA). Quantitative real-time PCR was performed using an ABI 7500 Real-Time PCR System (Applied Biosystems, USA) with a PCR kit (SYBR Premix EX Taq, TaKaRa). The comparative Ct-value method was used to calculate the relative quantity of alkaline phosphatase (ALP), type I collagen (COL1), osteocalcin (OC), and osteopontin (OPN) expressions.

2.6. Statistical analysis

Results are expressed as the means ± SEM. Statistical differences were determined by an analysis of Mann–Whitney. Values of $P < 0.05$ were considered to be statistically significant.

3. Results

3.1. Surface characterization of the coatings

SEM surface micrograph depicted in Fig. 1A shows the rough surface of the as-sprayed TC. From Fig. 1A, it could be observed that the as-sprayed coating was composed by melted and flattened particles. A higher magnification showed that the melted particle surfaces were relatively smooth (Fig. 1B). After anodization, no apparent changes were found in the low magnification picture (Fig. 1C). The average surface roughness (Ra) of TC before and after anodization was 27.6 ± 2.5 and 26.2 ± 1.7 μm, respectively. At a higher magnification (Fig. 1D), a nano-net-like texture was found in the surface and the average diameter of the tubules was about 30 nm. XRD results show that the nanotube thin film was amorphous, and only Ti peaks could be found in the XRD pattern (not shown here). The thickness of the nanotube wall was about 10 nm.

3.2. Adhesion and proliferation of hMSCs

Cell morphologies viewed by SEM are shown in Fig. 2. After 12-h incubation, a lot of cells were found to attach on the two kinds of coating samples. Most of the cells on the HSTC surfaces exhibited a polygonal morphology with a relatively large size, while those on the as-sprayed TC showed an elongated body with long filopodia. This elongated body may be explained by the relatively lack of anchoring sites on the coatings. The long filopodia was ascribed to the poor focal contact formation on the sample surfaces [33–35] and trying to find a more suitable site to attach and proliferate [36]. The quantitative evaluation of the attached cells on the two kinds of coatings is shown in Fig. 3. The number of attached cells increased with increase of culture time, and apparently much more cells were found to adhere on the HSTC samples after 4, 8, and 12-h incubation.

The comparison of the cell proliferation on the two kinds of coatings is shown in Fig. 4. The proliferation during the first 4-day culture showed no apparently differences between the two kinds of samples, while an obviously higher proliferation was observed after 7-days culture on the HSTC, which remained to the day 14.