The Biological Behavior of Osteoblast on the TiO_2 Nanotube Array Coating

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***Abstract*-TiO_2 nanotube arrays formed by anodic oxidation on titanium surface can effectively promote osteoblast' migration, proliferation and other biological behavior, and it has potentials of loading drugs and biological agents. In this study, TiO_2 nanotube arrays with different diameters were prepared by anodic oxidation and their effects on the biological behavior of osteoblast were also investigated. It's found that the osteoblasts exhibit a certain size-dependent effects at the nanoscale. TiO_2 nanotube arrays with small diameter can promote cell proliferation, migration and biological activity. The interactions between nanotubes with adhesion proteins integrin are the main factors contributing to this effect.**

Keywords-TiO_2 nanotube; osteoblast; size-dependent effect; osteopontin protein

I. INTRODUCTION

With the development of nanotechnology biomaterials, it's found that the cells are sensitive to the nanoscale morphology of the material surface and able to perceive different nano-sized structure and have a series of response behavior [1-4]. Titanium and its alloys are the preferred materials for orthopedic implants due to the good mechanical properties and biocompatibility. The TiO_2 nanotube array (TNT for short) coating on titanium surface formed by anodic oxidation can be used as an ideal cell culture platform that had potentials of loading of drugs and biological agents. In addition, TiO_2 nanotubes can promote the adhesion, pr oliferation and differentiation of bone remodeling-related cells in vitro, and increase the rate of implant osseointegration in vivo, showing its promising application in the field of planting material [5-7].

It was found in the studies that different types of cells incubated on TiO_2 nanotube array coating exhibit similar size dependent effect [8]. Although the cell behavior on TiO_2 nanotubes has been investigated for many years, the optimal nanotubes size for cell growth remains controversial, meanwhile, the influence factors such as different TiO_2 nanotube preparation methods, disinfection methods and cell incubation time also make the research that nanotube could enhance cell activity controversial [9].

Therefore, in order to reveal the functional mechanism of the TiO_2 nanotubes on cell and promote its application in the field of bone grafting, we took the osteoblast (CRL-11372) as the study object and investigated its biological behavior on TiO_2 nanotube array with different diameter. According to the effect of TiO_2 nanotubes on osteoblast cell proliferation rate, biological activity and the expression of osteopontin, we investigated the perception of osteoblasts for the change of microenvironment at the nanometer scale and provided theoretical support and evidence for the applications of TiO_2 nanotube in the field of bone transplantation.

II. EXPERIMENTAL

A. Preparation and Characterization of TiO_2 Nanotube Array

The titanium sheet (Φ30*3mm) was polished and washed with acetone and deionized water respectively. The Ti sheet was oxidized in the glycol containing 0.5%wt ammonium fluoride at 60V for 2h and 60V for 4h with a platinum sheet as the cathode. Both platinum and Ti were connected to a DC power supply (SKD-1105A, SAKO). After the oxidation, samples were cleaned and annealed in the vacuum furnace at 450°C for 1h. The morphology of TiO_2 nanotube was detected by field emission scanning electron microscopy (FE-SEM, ULTRA PLUS, Zeiss, Germany). The crystalline phase of sample was characterized by X-ray diffraction analysis (XRD, Rigaku D/Max 2500) with Cu Kα and scanning range of 20-90°.

B. Culture of Osteoblast CRL-11372

The human osteoblasts (HOB, American Type Culture Collection) were cultured in the DMEM medium (Hyclone) containing 10% fetal bovine serum (Hyclone), 100U/ml penicillin and 100μg/ml streptomycin. The experimental environment was maintained at 37°C in a thermostat incubator with 5% CO_2. DMEM Medium was replaced with a period of two days. After 2 weeks, the cells were treated with 0.25% trypsin (EDTA-free) and then passaged according to 1: 3. Finally, the osteoblasts were incubated on the TiO_2 nanotube samples with density of 10^4 times of initial density.

C. MTT, AO and Immunofluorescence Experiments

The cells were taken from experimental culture samples and placed in 2ml DMEM culture medium. The

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cell sample of 200ul was added to 96-well plate (set five wells) by pipette. 20ul MTT solution (5mg/ml) was added to each hole and then the sample was cultured for 4h. Then 150ul dimethyl sulfoxide was added to each well and oscillated for 10 min. The absorbance for each well was measured at 490nm using enzyme-linked immunosorbent detector. The blank wells (medium, MTT, dimethyl sulfoxide) and control wells (cells, drug dissolution medium, culture broth, MTT, dimethyl sulfoxide) were also set (3 wells for each group). The cell activity value was calculated in accordance with Equation 1:

$$[\text{Cell viability } (\%)=(\text{Abs}_{\text{test}} / \text{Abs}_{\text{control}})\times 100\%] \quad (1)$$

The AO (acridine orange staining) method was used to test the cell activity. The osteoblasts were incubated on each samples with initial density of $1*10^4$ per square centimeter and cultured for 24 hours. Then samples were fixed 20 minutes by PBS buffer with 4% paraformaldehyde. The fixed samples were stained for five minutes with AO method, washed with PBS and finally observed by a laser confocal scanning electron microscope. The osteoblasts were cultured on TiO_2 nanotubes for 7 days and fixed with 4% paraformaldehyde for 30 minutes at room temperature. Then the samples were washed with PBS for 5 minutes. The osteoblasts were treated with 1% Triton X-100 for 30 minutes. Then the samples were soaked in PBS solution containing 1% BSA and primary antibodies (1:200, Anti-osteopontin) and incubated at 4 °C for 12 hours. After washing with PBS, IgG-TRITC (1:500, Abcam biotech company) was added as secondary antibody and the cells were incubated for 1 hour at 37 °C. After washing with PBS, DAPI (1:1000, chemicon) was used to stain the osteoblast nuclear for five minutes at room temperature and confocal microscopy was used to observe the cells.

D. Statistical Significance

All quantitative data in the research was shown as mean value ± standard deviation. The t-test of statistically analysis was made by SPSS 10.0 software, and it was considered to have statistically significant when P<0.05.

III. Result and Discussion

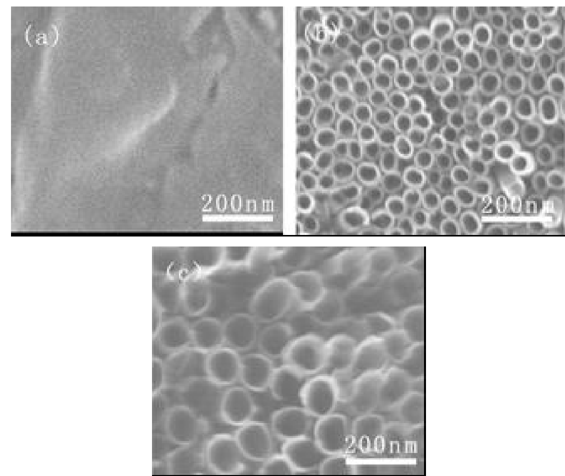

Figure 1. Morphology of different samples, (a) titanium substrate, (b) TiO_2 nanotube with diameter of 20nm, (c) TiO_2 nanotube with diameter of 100nm

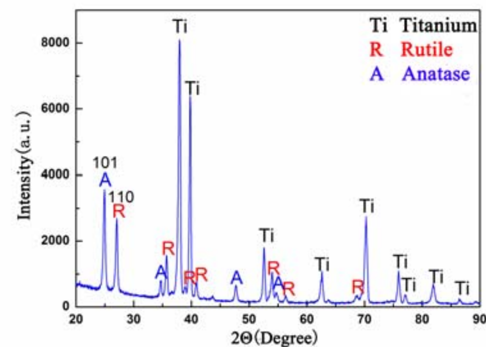

Figure 2. The XRD pattern of TiO_2 nanotube coating annealed at 450°C for 1h

The morphologies of titanium substrate and TiO_2 nanotube with different diameters were shown in Figure 1. The TiO_2 nanotubes prepared by anodic oxidation were tightly packed and highly ordered which had separate tubular morphology. The different diameters of TiO_2 nanotube array can be obtained by controlling the oxidation voltage and time. Wherein, the diameter of TiO_2 nanotube prepared at 20V, 4h was approximately 40nm and the tube wall thickness is about 5nm. And TiO_2 nanotube prepared at 60V, 4h had the diameter of 100nm and wall thickness of 10nm. According to the XRD pattern of TiO_2 nanotube array coating annealed at 450 °C for 1h (Figure 2), the sample showed mixed crystalline structure of anatase and rutile.

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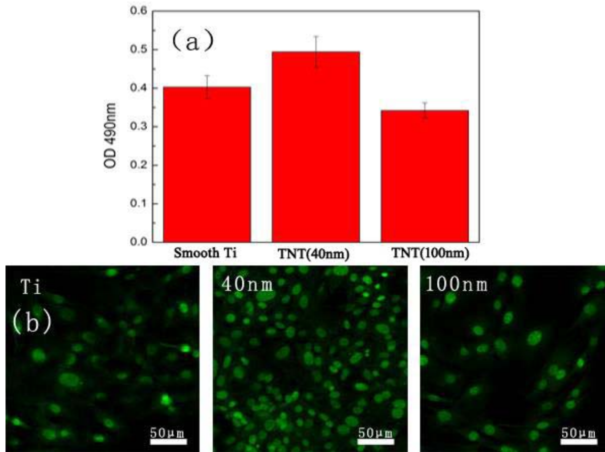

Figure 3. (a) Proliferation rate of osteoblast on different samples (mean ± SD, n=3). *$p<0.05$ compared with smooth Ti and annealed Ti, (b) AO staining evaluation of live cells and the membrane integrity

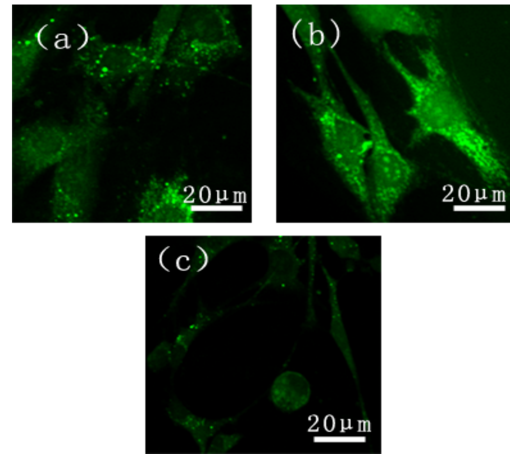

Figure 4. Immunofluorescence of osteopontin protein of cells cultured on different substrates. (a) Ti, (b) TiO_2 nanotube (40nm), (c) TiO_2 nanotube (100nm)

Figure 3a showed the MTT assay results of osteoblasts incubated on different substrates with the smooth titanium as control group. The cells incubated on TiO_2 nanotubes with different diameters of 40nm and 100nm showed the opposite proliferation trend. The osteoblasts on 40nm TiO_2 nanotube had the highest survival rate ($P<0.05$) with the OD value of 0.494. The cell viability of smooth titanium sample was in second place. The cell viability of the sample with larger diameter (100nm) is only 0.342. The results of AO method for osteoblasts on different substrates were shown in Figure 3b. Based on the selective inhalation of nucleic acid of cells for dye, the AO method can be used to evaluate the biological activity of cells. When the dye is absorbed into the double-stranded nucleic acid, cells are able to emit green fluorescence with fluorescence excitation, which can be used to evaluate the cell viability by observing the number and completeness of cell membrane and nucleus. It was shown that the osteoblasts on smooth titanium and 40nm TiO_2 nanotube coating had larger cell number and clear cell membrane and nuclear structure, which indicating the better biological activity of osteoblasts on 40nm TiO_2 nanotube coating. Nevertheless, the osteoblasts on 100nm TiO_2 nanotube coating had low biological activity proved by the significantly reduced number, the narrow cell structure and weak fluorescent display. The immunofluorescence assay results (osteopontin protein expression) of osteoblasts on different culture substrate were shown in Figure 4. The OPN protein expression effect of osteoblasts ranked as 40nm TNT> smooth Ti> 20nm TNT. The osteopontin is an important functional protein of the bone cells and the increased protein expression indicated that the cells have enhanced migration trend.

As the above results showed, the cells on TiO_2 nanotubes with different diameters exhibited different biological behavior. It seems that there is a "threshold" of nanotubes size that plays a role in mediating the cells. Brammer [4] reported that the TiO_2 nanotubes with smaller diameter (about 30nm) can maximize the cell adhesion, thereby promoting cell proliferation and migration, however, the nanotube with greater diameters (70-100nm) had less cell adhesion which indicated the inhibited cell proliferation to a certain extent. Our experiment also had a similar conclusion. TiO_2 nanotube with the larger diameter inhibited the biological activity of osteoblasts, while the nanotube with small diameter osteoblasts could increase the osteopontin expression that indicated high cell mobility. The phenomenon that TiO_2 nanotube with small diameter (<50nm) could enhance cell activity can be attributed to the following mechanism. The interaction between cells and incubate interface is controlled by the extracellular matrix and the integrin in extracellular matrix is the receptor which control the cell adhesion. The size of integrin is about 10nm, thereby, suitable geometric morphology of incubate surface would affect the signal of integrin to cell nuclei and then affect the adhesion, proliferation and migration of cells. When the nanotube size is less than 50nm, the integrin can enter the tube and promote the cell stretching out a lot of filopodia that could promote cell migration. However, when the diameter size is larger than a certain limit, the cell signal transfer of integrin will no longer be supported by the nanotubes, therefore, cell migration and growth will be subject to certain restriction. More seriously, some specific cell apoptosis would occur on the large-diameter nanotube surface. Of course, the experimental factors such as fluoride residues on nanotube surface [10] and the incubation time [11] of the cells would also affect the biological behavior of bone cells. Related investigations should be implemented in the future studies.

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IV. Conclusion

We studied the influence of TiO_2 nanotube arrays with different diameters on the biological behavior of osteoblasts in this paper. It's found that the osteoblasts exhibit a certain size-dependent effects at the nanoscale. TiO_2 nanotube with smaller diameter (40nm) is conducive to promoting the proliferation and migration of bone cells, while the larger diameter would suppress the biological activity.

Acknowledgements

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