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Protocols

Decreased fibrous encapsulation and enhanced osseointegration *in vitro* by decorin-modified titanium surface

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ABSTRACT

Orthopedic implants, using materials such as titanium, are extensively used in clinical surgeries. Despite its popularity, titanium is still inadequate to reliable osseointegration due to aseptic loosing. Fibrous encapsulation on the titanium implant interface prevents osseointegration and leads to the loosing of orthopedic implant. In this study, decorin was loaded on titanium surface by polydopamine film to examine fibrous encapsulation inhibition and bone growth acceleration. The coating of decorin was evaluated by X-ray photoelectron spectroscopy (XPS) and fluorescence microscopy. Quantitative analysis showed increased decorin coating on titanium surface when decorin in the loading solution increases. To test the effect of decorin modification, fibroblast and osteoblast cultures were utilized *in vitro*. The results showed that the functions of fibroblasts (proliferation, migration and collagen synthesis) were significantly attenuated on the decorin-modified surfaces and this anti-fibrous effect could be due to fibrotic gene suppression by decorin. In contrast, osteoblastic activities, such as calcium deposition and alkaline phosphatase (ALP) activity, were enhanced by the modified decorin. These results suggest that decorin coating on titanium surface inhibited proliferation and function of fibroblasts and improved that of osteoblasts. Therefore, this study is potentially useful for enhancing orthopedic implant.

1. Introduction

Internal fixation is a common treatment for most orthopedic diseases such as trauma and fracture. Titanium is among the most commonly used materials for these surgical fixation processes. However, the notable failure of titanium (Ti) implants after surgery raised great clinical challenges [1]. Aseptic loosening is a main reason for implant failure, which could cause huge subsequent financial burdens and disastrous trauma to patients [2,3]. In general, the loosened Ti implant is characterized by fibrous tissue encapsulation on the implant surface. Fibrous encapsulation on the implant-bone interfaces can prevent the osteoinductivity and osteoconductivity between the implant and the bone [4–6]. Successful implant osteoinductivity is determined by the interaction between the implant surface and the cells of the host tissue [7,8]. Bone formation on implant-bone interfaces depends on the recruitment of osteoblasts followed by osteoblast mineralization [9], while the fibrous tissue on the implant surface is formed by the increased fibroblasts [10]. Therefore, surface modification of Ti implants to suppress fibroblast proliferation and enhance osteoblast functions may be an appropriate strategy for potential orthopedic applications [11].

Different strategies have been attempted to modify the Ti surface suitable for the relevant cells [12,13]. Previous *in vitro* work by our group has shown that modified-curcumin onto the Ti surface can inhibit fibroblast growth and reduce fibrous encapsulation [12]. However, according to the experimental results, curcumin has no significant improvement on the effects of the proliferation and mineralization of osteoblasts [12,14]. Hu et al. reported that metal surfaces modified with alendronate can reduce the formation of fibrous tissue and increase osteoblast activity [13]. However, both drugs could not mimic the natural surroundings of cells, which could potentially hinder the applications of those modified surfaces. Recently, a promising method is to modify the selective

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extracellular matrix (ECM) components onto Ti surface to improve the surface microenvironment [15,16]. ECM has been reported to regulate cellular functions *in vivo*, such as osteoblast proliferation and mineralization [17,18]. Multiple ECM components have been applied to develop novel bioactive materials and surface modifications [19–23].

Decorin, a common ECM molecule, is a small leucine-rich proteoglycan (SLRP) composed of a protein core and glycosaminoglycan chains [24]. Recently, it has been reported that decorin can inhibit fibrous encapsulation by inducing fibroblast apoptosis and regulating fibril architecture [25,26]. In addition, some researches demonstrated that decorin can also improve osteoblast calcification *in vitro* [27,28]. Several studies reported a thin layer of "proteoglycan-rich" ground substance exists between the implant surfaces and the adjacent bone, therefore indicating that decorin may also play an important role in the osseointegration process on the implant surface [29–31]. However, whether decorin can be used as a potential modification of Ti surfaces in orthopedic implants is still not known.

ECM molecules have been immobilized on various surfaces by chemical reactions or physical adsorptions [32]. Our group previously constructed a polydopamine-treated Ti surface that can be utilized for drug immobilization [12]. In this study, we hypothesized that modification of the Ti surface with decorin may be a solution to combat fibrous encapsulation and enhance osseointegration. To verify this hypothesis and to shed light on its potential applications in orthopedic implants, decorin was immobilized on the titanium (Ti) surface with polydopamine as an anchor, and the biological effects of this decorin modification on fibroblasts and osteoblasts were investigated.

2. Materials and methods

2.1. Surface modification

Pure Ti substrates (10 × 10 mm) were washed with a solution containing H_2SO_4 and H_2O_2 (2:1, v/v), ultrasonically cleaned with deionized water, and dried in air [20]. Then, polydopamine was coated on to Ti substrates (Ti-DOPA) by immersion in a 2 mg/ml dopamine solution (10 mM Tris buffer, pH 8.5) overnight in dark [33]. The substrates were subsequently washed with ultrapure water to remove excess dopamine and dried. Each Ti-DOPA substrate was then immersed in 0.1 ml of decorin (100 μg/ml and 200 μg/ml) loading solutions (distilled water, pH 8.5) for 24 h at room temperature followed by triple washing with distilled water (washing solutions) and drying in air. The prepared substrates were called Ti-DOPA-decorin100 and Ti-DOPA-decorin200, respectively. The freshly prepared decorin-modified Ti substrates were used for subsequent experiments.

2.2. Surface characterization

The chemical composition of the surfaces (Ti, Ti-DOPA, Ti-DOPA-decorin100 and Ti-DOPA-decorin200) was checked by X-ray photoelectron spectroscopy (XPS) on an ESCALAB 250Xi X-ray Photoelectron spectrometer microprobe (Thermo Scientific) with a monochromatized Al Kα X-ray source (1486.7 eV). The C 1s (C—C bond) peak at 284.6 eV was used as the reference of all binding energies [34]. Immobilized decorin was detected using an anti-decorin antibody (Sigma-Aldrich). The plate was rinsed with PBS-Tween (PBS-T) (0.1%) and blocked by incubation in an aqueous solution of 1% nonfat milk for 30 min. The plate was subsequently incubated with an anti-decorin antibody (1:200 dilution) for 2 h at room temperature and washed 3 times with PBS-T (0.1%) before being incubated with an Alexa Fluor 488-conjugated secondary antibody (1:500 dilution)(Beyotime)for 1 h at room temperature [21]. After washing 3 times with PBS-T (0.1%), the absorption of decorin on the polydopamine substrates was observed under confocal laser scanning microscopy (CLSM) (Zeiss LSM 710).

2.3. Surface density and release test

The surface density of decorin on the substrates (Ti-DOPA-decorin100 and Ti-DOPA-decorin200) was determined by bovine decorin ELISA kit. In brief, the combined solution was composed of loading solution after decorin immobilization and the washing solution (as mentioned above). The decorin concentration on the substrates was calculated from the difference between the amount of decorin in the initial loading solution and the remaining in the combined solution. The optical density of the solution at 450 nm was measured, and the remaining concentrations in the combined solution were obtained using a standard curve. Tests to monitor the possible release of decorin from the substrates were conducted by immersing the substrates in 1 ml of phosphate-buffered saline (PBS) over 10 days [13]. At each time point, the decorin concentration in the PBS was measured by ELISA.

2.4. Cell adhesion and proliferation

Fibroblasts NIH3T3 and osteoblasts MC3T3-E1 (ATCC) were cultured in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) and Alpha Minimum Essential Medium (α-MEM, Invitrogen), respectively, both supplemented with 10% fetal bovine serum (FBS, Invitrogen) and 1% penicillin/streptomycin (Invitrogen). Cell adhesion assay on different substrates (Ti, Ti-DOPA, Ti-DOPA-decorin100 and Ti-DOPA-decorin200) was investigated by counting the number of attached cells after 30,000 cells were seeded on each substrate for 6 h. The adherent cells were then detached by trypsin and counted with a Scepter 2.0 handheld automated cell counter (Millipore). For the proliferation assay, cells were seeded on each substrate at a density of 10,000 cells/cm^2. After 1, 3, and 7 days of culture, the number of cells on the substrate was counted as described above and cell metabolic activity was tested by Cell Counting Kit-8 (CCK-8) assay (Keygen) [35]. Cell proliferation results are presented as optical density measured at 450 nm using a micro-plate reader (BioTek).

2.5. Fibroblast migration assay

30,000 fibroblasts were seeded on each substrate (Ti, Ti-DOPA, Ti-DOPA-decorin100 and Ti-DOPA-decorin200) for 7 days. Then, cells were harvested in DMEM without FBS and counted. Migration assay was performed by transwell chambers (8 μm; Corning Inc), which were placed in the 24-well plate. The suspended cells were separately seeded in the upper chamber containing 20,000 cells, and 600 μl of DMEM containing 15% serum was added to the lower chamber. After incubation for 24 h at 37 °C in 5% CO_2, the cells on the upper surface of the membrane were removed, and then the migrated cells were fixed with 4% paraformaldehyde and washed with PBS. Fibroblasts were stained with 0.1% crystal violet (Keygen) for 20 min and then washed gently several times with ultrapure water to remove excess stain. The number of migrated cells was determined by a Scepter 2.0 handheld automated cell counter (Millipore).

2.6. Collagen type I staining

Fibroblasts were cultured on the substrates (Ti, Ti-DOPA, Ti-DOPA-decorin100 and Ti-DOPA-decorin200) in the medium supplemented with 50 μM ascorbic acid (MP Biomedicals) for 7 and