Biomaterials 32 (2011) 366–373

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

The effect of immobilization of heparin and bone morphogenic protein-2 (BMP-2) to titanium surfaces on inflammation and osteoblast function

Sung Eun Kim [a], Sang-Hun Song [b], Young Pil Yun [a], Byung-Joon Choi [b], Il Keun Kwon [c], Min Soo Bae [c], Ho-Jin Moon [c], Yong-Dae Kwon [b,*]

[a] Department of Orthopedic Surgery and Rare Diseases Institute, Korea University Medical College, Guro Hospital, #80, Guro-dong, Guro-gu, Seoul, 152-703 Korea
[b] Department of Oral and Maxillofacial Surgery, School of Dentistry, Kyung Hee University, 1 Hoegi-dong, Dongdaemun-gu, Seoul, 130-701 Korea
[c] Department of Maxillofacial Biomedical Engineering and Institute of Oral Biology, School of Dentistry, Kyung Hee University, 1 Hoegi-dong, Dongdaemun-gu, Seoul, 130-701 Korea

ARTICLE INFO

Article history:
Received 6 July 2010
Accepted 3 September 2010
Available online 28 September 2010

Keywords:
Titanium
Heparin
Bone morphogenic protein-2 (BMP-2)
Osteoblasts
Inflammation
Peri-implantitis

ABSTRACT

The aim of this study was to investigate biologic function of bone morphorgenic protein-2 (rhBMP-2) immobilized on the heparin-grafted Ti surface. Ti surfaces were first modified by 3-amino-propyltriethoxysilane (ATPES), followed by grafting of heparin. BMP-2 was then immobilized on the heparin-grafted Ti surfaces. Pristine Ti and functionalized Ti surfaces were characterized by X-ray photoelectron spectroscopy (XPS), measurement of water contact angles, and protein adsorption. The biological activity of MG-63 cells on pristine and functionalized Ti surfaces was investigated by cell proliferation assays, measurement of alkaline phosphate (ALP) activity, and determination of calcium deposition. Anti-inflammatory effects were assessed by RT-PCR to measure the transcript levels of IL-6 and TNF-α. XPS revealed that heparin and BMP-2 were successfully grafted and immobilized on the Ti surfaces, respectively. In addition, Ti surfaces with BMP-2 immobilized were more hydrophilic than pristine Ti. Furthermore, BMP-2 immobilized Ti promoted significantly higher ALP activity and calcium deposition by MG-63 cells than pristine Ti. The inflammatory response was also decreased when cells were grown on heparin-grafted, BMP-2-immobilized Ti surfaces. The results of this study suggest that by grafting heparin and immobilizing BMP-2 on Ti surfaces, inflammation can be inhibited and osteoblast function promoted.

1. Introduction

Titanium (Ti) is widely used in the field of orthopedic and dental implants because of its good biocompatibility, superior mechanical properties, and excellent corrosion resistance. Because titanium prevents direct bone–to–bone contact, a variety of surface modifications have been developed to facilitate bone tissue responses toward titanium surfaces including hydroxyapatite coating, biomolecular immobilization, and control of the surface topography [1–3]. In recent years, another approach to stimulate bone–titanium interactions has been developed; this approach involves immobilizing peptides, such as Arg–Gly–Asp (RGD) and lysine–arginine–serine–arginine (KRSR), on the Ti surface [4–6]. However, the adhesion layer between the titanium and these peptides is weak and tends to crack. To solve these shortcomings, another approach involving surface modification with bone morphogenic proteins (BMPs) has been developed. BMPs play important roles in bone and cartilage regeneration. Among BMPs, BMP-2 has very strong osteoinductive activity. Previous studies have shown that BMP-2 can induce the osteogenic differentiation of mesenchymal cells and de novo orthotopic or ectopic bone formation [7–10]. Although BMP-2 has successfully been used to stimulate bone regeneration, use of BMP-2 is expensive, a high dose is required (1 mg BMP-2/mL defect), and BMP-2 has a short half-life *in vivo* [11]. To overcome these problems, BMP-2 delivery systems such as collagen gels, sponges, scaffolds, hyaluronic acid, and fibrin gels for prolonged, local release of BMP-2 have been studied [12–19]. However, these systems have problems such as uncontrolled release rates, release of BMP-2 for only a short period, and a high initial burst of release [20]. In this study, we used heparin to control the release of BMP-2. Heparin, a highly sulfated and liner natural polysaccharide, has been shown to have binding affinities to various growth factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and transforming

* Corresponding author. Tel.: +82 2 958 9440; fax: +82 2 966 4572.
E-mail address: yongdae.kwon@gmail.com (Y.-D. Kwon).

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doi:10.1016/j.biomaterials.2010.09.008

growth factor-β (TGF-β) [21]. Moreover, biomaterial systems with heparin have been shown to have advantages for the controlled release of these growth factors [22,23]. However, inflammatory responses from the patient's own skin and/or mucosa and the implant materials sometimes occur during surgical insertion. With the popularization of dental implants, the incidence of peri-implant disease is now a growing problem causing local bone destruction resulting in failure of the implants. For the above reasons, implant surfaces should have both anti-inflammatory activity and facilitate biomolecular adhesion to enhance osteoblast function. Therefore, in this *in vitro* study, we modified the surface of titanium using heparin. Heparin has well characterized anti-inflammatory and anticoagulant properties [24]. According to Heana et al., low-molecular-weight heparin (enoxaparin) reduced the high glucose-induced activation of endothelial cells by inhibiting monocyte adhesion [25]. In this study, the free amino groups of 3-amino-propyltriethoxysilane (ATPES) were first anchored to the titanium surface to create regions of high positive charge, and then heparin was covalently grafted. Heparin was grafted to the titanium surface using a 1-3thyl-3-dimethylaminopropyl carbodiimide (EDC)-mediated coupling reaction between the primary amine groups of rhBMP-2 and the carboxyl groups of heparin.

We hypothesized that a Ti surface with grafted heparin and immobilized rhBMP-2 would inhibit inflammation and enhance osteoblast function.

2. Materials and methods

2.1. Amino-functionalized titanium

Titanium (Ti) discs were kindly supplied by Dio Implant Co., Ltd (Busan, Korea). Ti discs were washed in an ultra-sonicator containing absolute ethanol for 1 h prior to use. Amine-functionalized Ti discs were produced in anhydrous toluene containing 3-aminopropyltriethoxysilane (ATPES, Sigma–Aldrich, MO, USA). In brief, 50 Ti discs were immersed in 250 mL anhydrous toluene and 10 mL (v/v) of ATPES. The reaction was performed at 120 °C under an N_2 atmosphere with a reflux condenser for 24 h. Ti discs were then washed with toluene for 20 min to remove unreacted silane. This washing procedure was repeated five times. After washing, the amino-functionalized Ti discs were dried at 60 °C for 24 h.

2.2. Grafting of heparin and immobilization of recombinant human bone morphogenic protein-2 (rhBMP-2) to titanium surfaces

Heparin was grafted to the surfaces of the amine-treated Ti discs by the 1-ethyl-3-dimethylaminopropyl carbodiimide (EDC)-mediated reaction between the primary amine groups of the Ti surface and the carboxyl groups of heparin. Briefly, 1 mg/mL heparin was dissolved in 0.59 mg EDC/0.18 mg NHS in 0.1 M MES buffer (pH 5.6). The Ti discs were then immersed in the aforementioned solution for 24 h at room temperature. After the reaction, the Ti discs were thoroughly washed with 0.1 M Na_2HPO_4 (1 h), 4 M NaCl (three times in 24 h), and distilled water (three time in 24 h), respectively. After washing, the Ti discs were frozen at −80 °C for 24 h and lyophilized for 3 days. rhBMP-2 at a concentration of 10 or 50 ng/mL was immobilized on the surface of the heparin-grafted Ti discs. In brief, the heparin-grafted Ti discs were immersed in 0.1 M MES buffer solution (pH 5.6). rhBMP-2 at a concentration of 10 or 50 ng/mL was then added to the 0.1 M MES buffer solution (pH 5.6) and the reaction was allowed to proceed for 24 h at room temperature.

2.3. Determination of the amount of heparin-grafted to the titanium surface

The amount of heparin-grafted on the surface of the titanium discs was determined using the toluidine blue method. Briefly, Ti discs were placed into 1 mL phosphate buffer saline (PBS, pH 7.4) solution containing 1 mL of 0.005% toluidine blue solution. After 30 min under gentle shaking, 2 mL of hexane was added. After the Ti discs were removed from solution, the absorbance of the aqueous phase was measured at 620 nm. The amount of heparin coated on the surface of titanium was calculated from a calibration curve that was constructed using various concentrations of heparin.

2.4. In vitro rhBMP-2 release study

To evaluate the release kinetics of rhBMP-2 immobilized on the surfaces of heparin-grafted Ti discs, the Ti discs were soaked in a 15 mL conical tube (Falcon, USA) containing 3 mL PBS (pH 7.4) at 100 rpm at 37 °C. At predetermined time intervals of 1 h, 2 h, 8 h, and 1, 3, 5, 7, 14, 21, and 28 days, the supernatant was collected and replaced with fresh PBS solution. All samples were stored at −20 °C until analysis. The absorbance of samples was determined with an enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions using a microplate reader (Bio-Rad, Hercules, CA, USA) at a wavelength of 495 nm.

2.5. Characterization of the Ti discs

To determine the surface morphologies of pristine and functionalized Ti discs (heparin-grafted Ti discs and BMP-2-immobilized Ti discs), a scanning electron microscope (SEM, S2300, Hitachi, Japan) was used. The substrates were coated with gold using a sputter-coater (Eiko IB, Japan). The SEM was operated at 15 kV. The surface composition of pristine Ti discs, heparin-grafted Ti discs, and rhBMP-2-immobilized Ti discs was analyzed by X-ray photoelectron spectroscopy (XPS) on a K-Alpha spectrometer (Thermo Electron, USA) with an Al Kα X-ray source (1486.6 eV photons). The C1s hydrocarbon peak at 284.84 eV was used as the reference for all binding energies. The area of each peak was normalized to the total peak area of all atomic elements to calculate surface atomic percentages. To evaluate the hydrophilic properties of pristine Ti and the functionalized Ti surfaces, contact angles were measured using the sessile drop method and a video contact angle instrument (Phoenix 150, SEO, Korea) at room temperature.

2.6. In vitro cellular responses

MG-63 cells (human osteosarcoma cell line, Korean Cell Bank Line, Seoul, Korea) were used to characterize the biocompatibility of the pristine and functionalized Ti discs (heparin-grafted Ti discs, rhBMP-2 (10 ng)-immobilized Ti discs, and rhBMP-2 (50 ng)-immobilized Ti discs) by measuring cell proliferation, alkaline phosphatase activity, and calcium deposition. Cells were cultured in Φ-100 culture plates at 37 °C in a humidified atmosphere supplied with 5% CO_2. Cells were maintained in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% FBS, 50 μg/mL ascorbic acid, 10 nM dexamethasone, and 10 mM β-glycerolphosphate in the presence of 100 U/mL penicillin and 100 μg/mL streptomycin. Prior to cell-seeding, specimens were sterilized with 70% EtOH for 10 min and rinsed twice with phosphate-buffered saline (PBS).

2.7. Cytotoxicity test

Cytotoxicity tests for pristine and functionalized Ti discs were carried out according to the ISO/EN 10993 Part 5 guidelines. To obtain extraction media, DMEM medium was incubated with pristine, amine-treated Ti, and functionalized Ti discs, respectively, for 24 h at 37 °C. NIH3T3 Fibroblasts and MG-63 cells were seeded into 96-well plates at a concentration of 5×10^4 cells/well and incubated for 24 h at 37 °C with DMEM supplemented with 10% FBS, and 1% 100 U/mL penicillin and 100 μg/mL streptomycin. After 24 h of culture, the DMEM medium was removed from the 96-well plates, the cells were washed with PBS, and extraction media was added. Cells were incubated for 24 and 48 h. At each time point, the extraction medium was aspirated and CCK-8 proliferation kit (Dojindo, Japan) reagents were added to cells. Cells were then incubated for 1 h at 37 °C, and the optical density of live cells was measured using a microplate reader at a wavelength of 450 nm.

2.8. Live/dead assay

The viability of cells on the surface of the pristine and functionalized Ti discs was assessed by live/dead staining. In brief, MG-63 cells were seeded at a density of 5×10^4 cells/mL on the surface of a series of Ti discs in 48-well plates. After a 48 h incubation, cells/specimens were rinsed three times with PBS and then incubated with live/dead stain (2 μM calcein AM and 4 μM ethidium homodimer-1) for 30 min at room temperature (RT). Viable cells (green) and dead cells (red) were counted under a confocal laser scanning microscope (CLSM, EZ-C1, Nikon, Japan).

2.9. Protein adsorption assay

To evaluate protein adsorption on the surfaces of pristine and functionalized Ti surfaces, fibronectin and bovine serum albumin (BSA) were used as representative proteins. Five hundred microliters of BSA (1 mg/mL BSA/PBS) or fibronectin (1 mg/mL fibronectin/PBS) were pipetted onto each surface, respectively. After 3 h, non-adherent proteins were removed and collected. Bradford solution (Bio-Rad, Hercules, CA, USA) was added to each surface for 1 h at 37 °C. Protein concentrations were determined using the Bradford assay according to the manufacturer's protocols, and absorbance was measured using a microplate reader at a wavelength of 595 nm.

2.10. Cell proliferation

MG-63 cells were seeded on pristine and functionalized Ti discs at a density of 1×10^5 cells and incubated for 7 days. At predetermined time intervals (1, 3, and 7 days), specimens were rinsed with PBS and CCK-8 proliferation kit reagents were added to the specimens. After a 1 h incubation, reagents were carefully transferred