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# Insulin-like growth factor binding protein-3 affects osteogenic efficacy on dental implants in rat mandible

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ABSTRACT

Insulin like growth factor binding protein-3 (IGFBP-3) in bone cells and its utilization in dental implants have not been well studied. The aim of this study was to determine the osteogenic efficacy of chitosan gold nanoparticles (Ch-GNPs) conjugated with IGFBP-3 coated titanium (Ti) implants. Ch-GNPs were conjugated with IGFBP-3 plasmid DNA through a coacervation process. Conjugation was cast over Ti surfaces, and cells were seeded on coated surfaces. For in vitro analysis the expression of different proteins was analyzed by immunoblotting. For in vivo analysis, Ch-GNP/IGFBP-3 coated implants were installed in rat mandibles. Four weeks post-implantation, mandibles were examined by microcomputed tomography (μCT), immunohistochemistry, hematoxylin & eosin and tartrate resistance acid phosphatase staining. In vitro overexpressed Ch-GNP/IGFBP-3 coated Ti surfaces was associated with activation of extracellular signal related kinase (ERK), inhibition of the stress activated protein c-Jun N-terminal kinase (JNK) and enhanced bone morphogenetic protein (BMP)-2 and 7 compared to control. Further, in vivo, Ch-GNP/IGFBP-3 coated implants were associated with inhibition of implant induced osteoclastogenesis molecules, receptor activator of nuclear factor kappa-B ligand (RANKL) and enhanced expression of osteogenic molecules including BMP2/7 and osteopontin (OPN). The μCT analysis demonstrated that IGFBP-3 increased the volume of newly formed bone surrounding the implants compared to control ($n = 5$; $p < 0.05$). These results support the view that IGFBP-3 overexpression diminishes osteoclastogenesis and enhances osteogenesis of Ti implants, and can serve as a potent molecule for the development of good implantation.



## 1. Introduction

Growth factors play an important role in wound healing and tissue regeneration [1]. They are naturally occurring proteins and essential in cellular signaling for growth, proliferation, and differentiation. In the field of tissue engineering, growth factors are a fundamental requirement [2,3]. Delivery of growth factors for tissue (e.g., bone, cartilage) or cell (e.g., nerves) has potential as a future therapeutic tool. Depending on the targeted cell type and its state of differentiation, growth factors can activate or regulate a variety of cellular functions. Furthermore, growth factors released from an implant surface can increase the osteoblastic activity of the bone tissue and helps to enhance bone regeneration in large animal model [4]. So, delivery of growth factor onto the implant surfaces is essential to improve implantation.

Various growth factors, like transforming growth factor β isoforms, bone morphogenetic proteins (BMPs), platelet-derived growth factor, and insulin-like growth factor influence bone healing [5]. Among insulin like growth factor binding proteins (IGFBPs; IGFBPs-1 to 6), IGFBP-3 is a major IGFBP species and binds with circulating IGF-I [6] and it is the third most abundant IGFBP found in osteoblasts [7]. Some evidence suggests that IGFBP-3 can have a positive effect on bone formation in some situations. For example, IGFBP-3 binds to type I collagen and participates in storing IGFs within the skeletal matrix [8] and it may also have a direct role in bone formation by acting on growth plate [9]. Moreover, recent studies also demonstrate the involvement of IGFBP-3 in the late stages of human tooth development [10]. However, the osteogenic effect of IGFBP-3 around the dental implant area has been still obscure.

Despite the fact that Ti and its alloys are among the most frequently used biomaterials in orthopedic and dental implants [11], Ti surfaces do not have an anti-oxidant activity on adherent cells in comparison with the normal cell culture condition on polystyrene surfaces [12]. Cells adhering to implant surfaces are subjected to oxidative stress during wound healing [13]. Signaling pathways involved in this process that

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regulate cell behavior in response to the implanted surfaces are still unclear.

Nanoparticles have been widely used in controlled delivery systems both in vitro and in vivo. The rate of release is mostly controlled by the degradation of the particles. However, unless nanoparticles can be immobilized on to the implant surfaces they freely move within the host system [14]. Therefore it is hard to localize the release of to the implant region with precision. Direct coatings of the surface of the implant and scaffolds with a drug or gene are options for local delivery. We have previously reported that the gold nanoparticles with plasmid DNA coated implant surfaces can easily carry the reporter gene onto the localized area [15]. Further, we have also showed that a plasmid containing the transcription factor c-myb and anti-inflammatory molecules PPARγ conjugated with Ch-GNPs enhances new bone growth and inhibit inflammation around implant surfaces [16,17]. However, in the same condition, the influence of IGFBP-3 in vitro and in vivo in a rat dental implant model is unclear. In this study, the role of IGFBP-3 in regulating osteoclastogenesis and bone regeneration at the bone implant surface was evaluated and the effect of IGFBP-3 on dental implants was determined.

## 2. Materials and methods

### 2.1. Cell culture

MC-3T3 E1 osteoblast-like cells (CRL-2593; American Tissue Type Collection, Manassas, VA, USA) were maintained at 37 °C in a humidified 5% $CO_2$ atmosphere in αMEM (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin and sub-cultured at a 1:4 ratio.

### 2.2. Preparation of chitosan stabilized GNPs and complexes with DNA

Chitosan stabilized GNPs were prepared using a simple graft-on technique, as described previously [15]. Briefly, 1.0 ml freshly prepared 10 mM $HAuCl_4$ solution was added to 2.0 ml of 0.33% chitosan solution in 0.1 M HCl and stirred for 1 h. Subsequently, 0.4 ml (0.1 M) of freshly prepared ice-cold sodium borohydride was added to this solution with constant stirring. A rapid color change to red wine indicated the formation of Ch-GNPs. The Ch-GNPs were collected by ultracentrifugation at 35,000 *g* for 30 min at 4 °C. Surface plasmon band (SPB) of Ch-GNPs was analyzed by UV–visible spectroscopy centered at 520 nm. Particle sizes were determined to be 10–20 nm by transmission electron microscopy (TEM). cDNA of LacZ and IGFBP-3 was cloned in plasmid DNA (pcDNA3.1 His/Myc; Invitrogen, Carlsbad, CA, USA). Nanoparticle characterization and DNA complexes were prepared as previously described [16].

### 2.3. Loading of Ch-GNP/DNA on Ti surfaces and mini-screw

For in vitro analysis of DNA loading on Ti surfaces (6 × 6 × 0.1 cm), DNA was conjugated with Ch-GNPs and administration was achieved by direct adsorption on the Ch-GNP/DNA-coated surfaces. Ch-GNP/DNA solution was mixed with 500 μl serum and antibiotic-free medium, and applied to the surfaces by dipping of the surfaces in the mixture. The applied solution was dried at room temperature. Ti surfaces coated with Ch-GNP/IGFBP-3 were placed into 100 mm-diameter cell culture plates. Equal numbers of cells ($5 \times 10^5$) were seeded on the plates, and 1 ml of the cell culture medium was added. After 2 h, the cell culture medium was replaced with a fresh medium. Aseptic conditions were maintained throughout the adsorption process. For in vivo analysis, loading of DNA and implant size were described previously [17]. Briefly, cylindrically-shaped, commercially pure Ti square thread screws with a length of 4.5 mm and a diameter of 0.85 mm were used to model a dental implant. For coating with Ch-GNP/IGFBP-3 and LacZ, the implants were immersed 10 times in a nanoparticle-DNA solution and frozen at −40 °C. Ch-GNP/LacZ coated implants were used as a control.

### 2.4. Cell viability

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) based assay was used to determine cell viability. MC-3T3 E1 cells were seeded on normal culture and Ti (1 × 1 × 0.1 cm) and Ti surfaces coated with Ch-GNP/LacZ and Ch-GNP/IGFBP-3 (n = 3 for each). The coated and uncoated surfaces were placed into 24-well tissue culture plates and cultured for 24 and 48 h. At the end of the incubation period, the samples were rinsed twice with sterile phosphate buffered saline (PBS) and the attached cells were incubated in a medium containing 100 μg/0.1 ml of MTT (Sigma-Aldrich, St. Louis, MO, USA) for 4 h. After carefully removing the medium, the purple formazan product was dissolved in dimethyl sulfoxide and the optical density values were recorded using a Synergy 2 spectrophotometer (Bio-Tek, Winooski, VT, USA) at 570 nm.

### 2.5. Immunoblotting

Immunoblotting was done as previously described [15,16]. Briefly, primary antibody to IGFBP-3 (sc-9028) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), phospho-extracellular signal-regulated kinase (ERK; #9251) and c-Jun N-terminal kinase (JNK; #4377) were purchased from Cell Signaling Technology (Beverly, MA, USA) and BMP-7 (BS3674) and BMP-2 (BS3473) were purchased from Bioworld (Minneapolis, MN, USA). The signals were visualized by chemiluminescence detection according to the manufacturer's protocol (Amersham Pharmacia Biotech, London, UK) and detected using a LAS-4000 scanner (Fuji Film, Tokyo, Japan)

### 2.6. Animals and surgical procedures

Ten, 8-week-old, Sprague Dawley male rats were used. The protocol was approved by the Ethical Committee of Chonbuk National University (CBU 2012-0031). All surgical procedures were performed under general anesthesia induced intra-muscularly with Zolazepam (Zoletil 50; Virbac, Carros, France) and Xylazine hydrochloride (Rompun; Bayer Korea, Seoul, Korea) under veterinary supervision. In control groups, Ch-GNP/LacZ coated implants (n = 5) were used. Ch-GNP/IGFBP-3 (n = 5) coated implants were used in the experimental group. Each rat was reared in an individual cage where the temperature was maintained at 20–25 °C and the relative humidity at 30–50%. The lower first molar was extracted carefully to avoid damaging the extraction socket and it was verified that no dental root remained. After checking that the alveolar bone healed well one month after the extraction, implant placement was performed. The implant site was prepared using a 0.8 mm-diameter drill. The implants were inserted after making a hole with a drill at low speed (900 rpm) with saline irrigation to avoid heating. Post-operative antibiotic treatment based on body weight was carried out by injection twice daily into the rats for 4 days.

### 2.7. Microcomputed tomography (μCT) analysis

μCT was done using a model 1076 apparatus (SkyScan, Kontich, Belgium) operating with an anode electrical current of 100 kV at a resolution of 18 μm. After implantation, the rats in the Ch-GNP/LacZ (n = 5) and Ch-GNP/IGFBP-3 (n = 5) groups were anesthetized and scanned by local μCT to evaluate the dynamic changes in peri-implant tissue at 4 weeks. The regions of interest (ROIs) that included the mandible compartment around implants were selected. After scanning, three-dimensional models were generated by CTVol (Skyscan) and bone volume around implants was analyzed by the CTAn program (Skyscan), which was also used to examine the μCT data sets for new bone growth. The volume of interest (VOI) consisted of the collective