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Recombinant human IGF-1 produced by transgenic plant cell suspension culture enhances new bone formation in calvarial defects

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ABSTRACT

Transgenic plant cell suspension culture systems have been utilized extensively as convenient and efficient expression systems for the production of recombinant human growth factors. We produced insulin-like growth factor-1 using a plant suspension culture system (p-IGF-1) and explored its effect on new bone formation in calvarial defects. We also compared the bone regenerating potential of p-IGF-1 with commercial IGF-1 derived from *Escherichia coli* (e-IGF-1). Male C57BL/6 mice underwent calvarial defect surgery, and the defects were loaded with absorbable collagen sponge (ACS) only (ACS group) or ACS impregnated with 13 μg of p-IGF-1 (p-IGF-1 group) or e-IGF-1 (e-IGF-1 group). The sham group did not receive any treatment with ACS or IGFs after surgery. Live μCT and histological analyses showed critical-sized bone defects in the sham group, whereas greater bone formation was observed in the p-IGF-1 and e-IGF-1 groups than the ACS group both 5 and 10 weeks after surgery. Bone mineral density, bone volume, and bone surface values were also higher in the IGF groups than in the ACS group. Local delivery of p-IGF-1 or e-IGF-1 more greatly enhanced the expression of osteoblast-specific markers, but inhibited osteoclast formation, in newly formed bone compared with ACS control group. Specifically, p-IGF-1 treatment induced higher expression of alkaline phosphatase, osteocalcin, and osteopontin in the defect site than did e-IGF-1. Furthermore, treatment with p-IGF-1, but not e-IGF-1, increased mineralization of MC3T3-E1 cells, with the attendant upregulation of osteogenic marker genes. Collectively, our findings suggest the potential of p-IGF-1 in promoting the processes required for bone regeneration.

1. Introduction

Bone is an active tissue that is constantly being created and replaced in the process of remodeling via balanced interactions between osteoblasts and osteoclasts. Excessive bone damage that is beyond the self-regenerative capacity can be induced by congenital defects, trauma injury, infections, osteoporosis, and tumors. Therefore, patients with large defects or critical-sized defects of bone require multiple invasive surgeries and bone grafting over long periods of time [1,2]. While various therapies are used to achieve bone regeneration, implantation of autografts or allografts is considered a standard method in clinical practice [3–5]. However, bone grafting does not always provide satisfactory results because of donor and host site injury, deformity, morbidity, immune rejection, and/or cost issues [6]. Clinical use of bone-graft substitutes such as demineralized bone matrix and synthetic biomaterials, which contain bone-like biological and mechanical properties, is an alternative approach to bone grafting [7–9]. Further, combined treatment with growth factors has been considered to stimulate reconstruction of large bone defects. Growth factors including transforming growth factor β, fibroblast growth factors, vascular endothelial growth factor, platelet-derived growth factor, bone morphogenetic proteins (BMPs), and insulin-like growth factors (IGFs) have been used to synergistically stimulate the processes involved in bone healing [10–12].

Growth factors regulate multiple biological processes, including cell survival, differentiation, proliferation, and migration, and facilitate the recovery of biological and physiological functions of damaged tissues [13,14]. Of the IGFs, IGF-1 is known to stimulate the processes of cell

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growth and differentiation in various tissues and to modulate the growing skeleton in particular through endocrine/paracrine and autocrine mechanisms [15]. While conditional IGF-1 knockout mice showed reduced bone formation, its overexpression increased the volumes of long bones and calvarial bone [16]. IGF-1 is also essential for parathyroid hormone (PTH) action on bone; PTH did not have a positive effect on bone formation in the condition of osteoblast-specific deletion of IGF-1 [17]. In addition, IGF-1 signaling plays critical roles in maintaining the proliferation of chondrocytes and osteoblasts as well as in inducing adequate endochondral ossification [18]. All of these reports indicate that activation of IGF-1 signaling leads to enhancement of healing processes in bone defects, and that local delivery of IGF-1 facilitates bone regeneration in large or critical-sized bone defects.

Because the clinical use of growth factors has gradually expanded, it is of great importance to consider the efficacy with which these factors are produced and their pharmaceutical activity. DNA recombinant technology utilizing several heterologous expression systems, including bacteria, yeast, insect, and mammalian cell cultures, has long been applied to produce recombinant human growth factors. Of these systems, transgenic plant cell suspension culture systems have been utilized extensively to produce recombinant pharmaceutical proteins [19,20]. This is due to several advantages of plant cell culture systems: they are inexpensive and scalable alternative to other expression systems, there is reduced risk of contamination with viral and bacterial toxins, and purification of secreted proteins is convenient [21,22]. Clinical use of plant culture cell-derived IGF-1 (p-IGF-1) to stimulate bone formation may have various advantages over microbial and mammalian host systems. However, no studies have investigated the effects of p-IGF-1 on bone formation.

In this study, we examined the efficacy of p-IGF-1 in inducing bone regeneration using a calvarial defect mouse model. We also compared the capacity of p-IGF-1 to that of *Escherichia coli* (*E. coli*)-derived IGF-1 (e-IGF-1). To investigate the molecular mechanisms by which p-IGF-1 enhances bone formation, we analyzed expression patterns of genes and proteins specific to osteogenesis in newly formed bones. In addition, we estimated the effects of p-IGF-1 on proliferation, osteogenic marker expression, and mineralization of MC3T3-E1 pre-osteoblastic cells.

2. Materials and methods

2.1. Chemicals, laboratory equipment, and animals

e-IGF-1 (≥ 98% purity) was purchased from BioVision (Milpitas, CA, USA). Antibodies specific to BMP2 (BS3473) and osteocalcin (OCN; BS7961) were obtained from Bioworld Technology (St. Louis Park, MN, USA), while anti-osteopontin (ab8448; OPN) antibody was from Abcam (Cambridge, UK). Unless specified otherwise, other reagents were purchased from Sigma-Aldrich Co. LLC (St. Louis, MO, USA), and laboratory consumables were from Falcon Labware (Becton-Dickinson, Franklin Lakes, NJ, USA). Male C57BL/6 mice (six-weeks-old) were obtained from Orient Bio (Daejeon, South Korea) and assigned randomly to experimental groups. Mice were housed at 22 ± 1 °C and 55 ± 5% humidity on an auto-cycling 12 h light/dark cycle with free access to food and water. The animal care and use protocol implemented in this study was approved by the Chonbuk National University Committee on Ethics in the Care and Use of Laboratory Animals (CBU 2012-0039). All mice were acclimatized to the new laboratory environment for one week before surgical operation.

2.2. Preparation of p-IGF-1

Recombinant human IGF-1 protein was produced using a transgenic plant cell suspension culture system as described previously [23]. In brief, the PCR product of the IGF-1 gene was cloned into the pGEM T-Easy vector (Promega, Madison, WI, USA), fused with the signal sequence of rice amylase 3D (Ramy3D), and introduced into a plant expression vector containing hygromycin phosphotransferase as the marker for hygromycin B and the rice Ramy3D promoter expression system. Subsequently, rice calli (*Oryza sativa* L. cv. Dongin) were transformed by a particle bombardment-mediated transformation technique [24]. After bombardment, the explants were transferred to N6 selection medium supplemented with 2,4-dichlorophenoxyacetic acid (2 mg/l), sucrose (30 g/l), proline (0.5 g/l), glutamine (0.5 g/l), casein enzymatic hydrolysate (0.3 g/l), gelite (2 g/l), and hygromycin B (35 mg/l) as selection markers at 2–3 week intervals [25]. The KI001-3 transgenic cell line harboring the IGF-1 gene were selected in N6S medium and used to establish cell suspension cultures. Cultures were grown in 1000 ml N6S medium without gelite at 25 °C in a shaking incubator, and the inocula (200 ml) were transferred to new media every nine days. To induce the expression of the IGF-1 gene, media were removed from the suspension culture via aspiration, and N6S medium without sucrose was added to the cultures. The sample (1000 ml) of the culture supernatant, concentrated with 5.6 mg/l as determined by enzyme-linked immunosorbent assay kit (Endogene, Woburn, MA, USA), was harvested by filtration. The human IGF-1 produced from rice cell was recovered by hydrophobic interaction chromatography and anionic exchange chromatography. The concentration of human IGF-1 was determined by size-exclusion-high performance liquid chromatography (SE-HPLC). Purity is > 95% as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and HPLC. Finally, the recombinant IGF-1 protein (named p-IGF-1) was lyophilized (1 mg/vial) and stored at − 70 °C until use.

2.3. Preparation of absorbable collagen sponges (ACSs)

Local delivery of p-IGF-1 or e-IGF-1 was performed using ACSs. ACSs were prepared as described elsewhere with slight modification [26]. Briefly, type I atelocollagen powder (KOKEN Corp., Osaka, Japan) and chondroitin-6-sulfate were dissolved separately in 50 mM acetic acid to concentrations of 10 mg/ml and 5 mg/ml, respectively. Co-precipitation was performed by adding chondroitin-6-sulfate drop-wise over type I atelocollagen solution being stirred in a homogenizer, such that the final concentration of chondroitin-6-sulfate was nearly 5 mg/100 mg total dispersed solid. The resulting collagen-chondroitin-6-sulfate solution was lyophilized at − 80 °C for 6 h to yield an ACS. ACSs were incubated at room temperature for 30 min in 20 ml 40% (v/v) ethanol containing 50 mM 2-morpholineoethane sulfonic acid (MES; Fluka Chemie, Buchs, Switzerland; pH 5.5) and then further incubated for 12 h in a 20 ml solution of 40% (v/v) ethanol, 50 mM MES (pH 5.5), 24 mM 1-ethyl-3–(3-dimethyl aminopropyl) carbodiimide, and 5 mM *N*-hydroxysuccinimide. After several washing steps, ACSs were lyophilized, dissected into sections 4 mm in diameter, and sterilized by 10 kGy γ-irradiation.

2.4. Establishment of calvarial defects and local administration of IGFs

Critical-sized calvarial defects were created in mice according to methods described previously [26]; the surgical and experimental procedures are shown in Supplemental Fig. 1. Briefly, mice (n = 42, seven-weeks-old) received general anesthesia by intraperitoneal injection with a 1:1 mixture of zoletil (0.4 ml/kg, Virbac Laboratories, Carros, France) and rompun (10 mg/kg, Bayer Korea, Seoul, South Korea). After injection, the skulls of mice were shaved and sterilized using 10% betadine (Sam-II Pharm, Seoul, South Korea) on an operating table. Local anesthesia of the animals was performed by subcutaneous injection with 2% lidocaine containing 1:100,000 epinephrine (Lidocaine HCL Injs, Yuhan Corp., Seoul). For all mice, a 1 cm incision was made along the sagittal plane across the cranium, and a circular bone defect (4 mm in diameter) was created at the middle of the sagittal suture using a trephine. The stock solution of p-IGF-1 or e-IGF-1 was prepared by dissolving 1 mg of the factor in 1 ml of

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