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Small intestine submucosa sponge for *in vivo* support of tissue-engineered bone formation in the presence of rat bone marrow stem cells

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ABSTRACT

The aim of the current study was to visualize new bone formed *in vivo* on a small intestine submucosa (SIS) sponge used as a tissue-engineered scaffold for the repair of damaged bone. The SIS sponge provided a three-dimensional pore structure, and supported good attachment and viability of rat bone marrow stem cells (rBMSCs). To examine bone regeneration, we prepared full-thickness bilateral bone defects in the rat crania, and then treated the defects with an implanted SIS sponge or PGA mesh without or with rBMSCs, or left the defects untreated. Bone defects were evaluated by micro-CT and histologically after 2 and 4 weeks. Micro-CT demonstrated a trend toward a decrease in bone void in both the SIS sponge and SIS sponge/rBMSCs groups compared to the control and PGA mesh groups. At 4 weeks, bone formation in defects containing SIS sponge/rBMSCs was significantly greater than in all other groups. A histological analysis after 2 and 4 weeks of implantation showed localized collagen and osteocalcin deposition on SIS sponges and SIS sponges with rBMSCs. These *in vivo* results indicate that the SIS sponge, implanted at bone-removal defects, facilitated bone regeneration.

1. Introduction

Bone tissue engineering seeks to develop strategies to repair and regenerate bone at sites of bone defects and damage caused by trauma or disease without the limitations and drawbacks of current clinical autografting and allografting treatments [1–3]. A key component of the bone tissue engineering paradigm is a suitable scaffold, which functions as a structural support and delivery vehicle, providing osteoprogenitor cells and osteoinductive factors necessary for the formation of new bone tissue [4,5]. The ideal scaffold should possess an environment capable of supporting growing bone tissue and demonstrate good biocompatibility. In addition, the scaffold must have high porosity with extensive pore interconnectivity to allow for uniform penetration of the biologic medium and enable bone tissue ingrowth. Various biomaterials have been developed to fulfill these bone tissue engineering requirements [6].

Small intestine submucosa (SIS), derived from the submucosal layer of porcine intestine, is an acellular, naturally occurring collagenous extracellular matrix material [7–11]. SIS consists of types I and III collagens, which together comprise greater than 90% of the total collagen content, and small amounts of types IV, V and VI collagens. It also contains a wide variety of cytokines, including basic fibroblast growth factor (bFGF), transforming growth factor-β (TGF-β), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF) and insulin-like growth factor-1 (IGF-1), as well as glycosaminoglycans, fibronectins, chondroitin sulfates, heparins, heparin sulfates and hyaluronic acids [12–14]. These constituents are well known to play important roles in tissue remodeling.

SIS is a biomaterial that can be easily produced for use as an experimental scaffold for tissue engineering applications. It has been used primarily as a scaffold for the repair of soft tissues, and has been used successfully in vascular graft applications and for bladder wall and diaphragm repair [15–20]. SIS has also been explored for use in orthopedic soft tissue applications to repair ligaments, tendons and menisci [21–24]. In addition, a few studies using animal models have shown that bone defects could be treated with SIS [25–27]. The SIS scaffold has been shown to act as a regenerative matrix for grafting bone, guiding the attachment of host cells and forming new bone in a predefined shape. These

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studies demonstrate the potential of SIS as a bone graft biomaterial. Recently, we have investigated the fabrication of SIS sponges for use as a supporting scaffold for the growth of infiltrating cells [28]. The fabrication of SIS sponges are subjected to a variety of processing steps including mechanical manipulation, digestion, chemical-crosslinking, and sterilization. Ideally, the fabrication of SIS sponges would minimize processing steps that could remove or destroy the bioactive molecules in the SIS. Even though such processing and manufacturing steps may affect SIS material and structural properties, previous studies have shown that the SIS after fabrication can be produced that is able to support the growth and differentiation of a variety of cells [29–31].

To our knowledge, no previous study has examined SIS sponges as an *in vivo* bone-regenerative scaffold for rat bone marrow stromal cells (rBMSCs), which are the most commonly used seed cells for bone tissue engineering. To this end, we applied three-dimensional SIS sponges as a scaffold using an experimental rat cranial bone-defect model, investigating whether the implantation of an SIS sponge could conduct newly forming bone across full-thickness, rectangular bone defects. In addition, we evaluated tissue-engineered bone formation using microcomputed tomography (micro-CT) and histology.

2. Materials and methods

2.1. Preparation of SIS sponge

Sections of porcine jejunum were harvested from market pigs (Finish pig, F_1; Land race + Yorkshire, around 100 kg at 6 months) within 4 h of sacrifice and prepared according to the method of Badylak et al [7]. Briefly, to separate SIS in porcine jejunum, fat firstly removed from porcine jejunum, followed by carefully washing with water. The porcine jejunum cut in lengths of approximate 10 cm and then washed with a saline solution. SIS was obtained by mechanical removal of the tunica serosa and tunica muscularis. Finally, the obtained SIS washed again with a saline solution and freeze-dried at −55 °C for 48 h using freeze dryer (FD 8505, Ilshinlab, Daejeon, Korea). The dried SIS was pulverized using freezer mill (6700, SPEX Inc., USA) at −198 °C to give 10 ~ 20 μm size of SIS powder. The obtained SIS powders (1% concentation) were added in 10 ml vial with aqueous solution consisting of 3% acetic acid and 0.1% pepsin, and then stirred for 48 h to cleave only non-triplehelical domains of collagen. Accordingly, the collagen molecules which have native triplehelical structure were solubilized from tissue. The SIS solution was carefully poured into homemade silicone molder (10 mm × 10 mm) to give SIS sponge shape, followed by freeze-drying. SIS sponge was crosslinked with 1-ethyl-(3,3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) in 10 mM concentrations using solution of a mixture of deionized water (DW) and ethanol (5/95, v/v) for 24 h. The crosslinked SIS sponge in the molder were dipped in water at 40 °C for 1 h, followed by freeze-drying to give finally SIS sponge.

2.2. rBMSC isolation

The rBMSCs were harvested from the femurs and tibias of four week-old female Fischer rats. Briefly, the rats were subjected to ether euthanasia and the bones of the hind limbs were aseptically excised. The soft tissues were removed, and the femurs and tibias were placed in 50 ml DMEM. The proximal end of each femur and the distal end of each tibia were removed using sterile scissors. A hole was then created in the knee joint end of each bone, using a 26-gauge needle, and PBS (pH 7.4) was used to flush the marrow from the shaft. The flushed marrow was fully suspended in PBS, and the cell suspensions from all bones were combined and centrifuged at 400 g for 5 min. The resulting pellet was resuspended in fresh primary medium [DMEM supplemented with 10% fetal bovine serum (FBS; Gibco BRL, NY, USA) and 100 U/ml penicillin] and seeded to tissue culture flasks at 1×10^5 cells. After five days of expansion, the cultures were rinsed three times with PBS for removal of non-adherent cells. The medium was exchanged every two days throughout the studies. For use in the experiments, adherent cells were rinsed thoroughly with PBS and then detached by trypsinization.

2.3. PKH67 cell labeling

The rBMSCs were labeled using the PKH67 Fluorescent Cell Linker Kit (Sigma, St Louis, MO, USA) according to the manufacturer's instructions. In brief, the cultured rBMSCs were washed with serum-free media and centrifuged for 5 min at 2000 rpm. The provided diluent C (0.5 ml) was added to 2×10^7 rBMSCs and immediately mixed with 0.5 ml of PKH67/diluent C (2 μl/498 μl). After incubation for 5 min at room temperature with gentle tapping, 1 ml of serum was added and samples were incubated for 1 min to stop the labeling reaction, followed by addition of 2 ml complete media. Finally, the rBMSCs were centrifuged for 10 min at 2000 rpm, transferred to a fresh tube and washed three times with complete media.

2.4. Cell culture on SIS sponge and PGA mesh

SIS sponge or PGA nonwoven mesh (Albany International, NY, USA) was sterilized by EO gas at Hansbiomed Company. For cell culture experiments, the SIS sponge (5 mm × 5 mm) and PGA mesh (5 mm × 5 mm) were prepared and placed individually into the wells of a 24-well tissue culture plate (Falcon, USA) and then incubated for 1 h under culture media. After the suction of media, the rBMSCs (3×10^4 cells/well) were transferred to each well and incubated for 7 and 14 days. The culture media was changed every 2 days throughout the studies. Fluorescence images were visualized under an Olympus IX81 fluorescent microscope (Olympus, Tokyo, Japan) equipped with the Meta Image Series software (MetaMorph, Molecular Devices Corporation, Pennsylvania, USA). For SEM measurement, at 7 and 14 days, the SIS sponge and PGA mesh without or with rBMSCs were fixed with 0.5% glutaraldehyde for 24 h, followed by ethanol dehydration. The fixed SIS sponge and PGA mesh were coated with a conductive layer of gold using a plasma-sputtering apparatus (Emitech, K575, Kent, UK), and scanning electron microscopy (SEM, S-2250N, Hitachi, Japan) images were obtained. For MTT assay, cell viability of three SIS sponges or three PGA mesh performed individually and then calculated as average value. In brief, 100 μl of PBS solution of the MTT tetrazolium substrate (5 mg/ml) was added after 1, 2, 3, 7 and 14 days. After incubation for 4 h at 37 °C, the resulting violet formazan precipitate was solubilized by the addition of 1 ml of DMSO and shaken for 30 min. An aliquot from each well (100 μL) was transferred to a 96-well plate. The solutions were then read using a plate reader of an ELISA (EL808 ultra microplate reader; Bio-Tek Instrument, Vermont, USA). The optical density of each well determined at 590 nm.

2.5. Animal implantation surgery

The rats were housed in sterilized cages with sterile food and water and filtered air, and were handled under a laminar flow hood following aseptic techniques. All animals were treated and all surgical procedures followed protocols approved by in accordance with the Korea Research Institute of Chemical Technology's Council on Animal Care Guidelines. Twenty-four Fischer rats (140–160 g, 9 weeks), divided into four groups of three rats each, were used in the animal tests for 2 and 4 weeks. The four experimental groups were designed as followed; SIS sponge only, PGA mesh only, SIS sponge + rBMSCs (5×10^5); PGA mesh + rBMSCs (5×10^5). Full-thickness rectangular hole of 5 mm × 5 mm was created in both the left and right the cranial bone of each rat that had been anesthetized by using ketamine and rompun (1:1 ratio, 1.5 ml/kg). The implants were only inserted into hole created in the right cranial bone. The left cranial bone used as control. A more elaborate description of the implantation procedure can be found in the Fig. 3. The resulting implants were then allowed to develop and biopsy in vivo over a 2 and 4 weeks.

2.6. Microcomputed tomography

The scaffolds ($n = 3$) were analyzed by using microcomputed tomography (micro-CT). Micro-CT was carried out with a high-resolution Explore Locus scanner (GE Healthcare, Ontario, Canada) using a resolution of pixel size of 46 μm and an exposure time of 400 ms, along with an energy source of 80 kV and a 450 μA of current. Approximately 180 projections were acquired over a rotation range of 180°, with a rotation step of 1°. The bone was scanned at pixel size of 0.046 mm. The full length of each bone was scanned, and on average consisted of 850 slices. The three-dimensional bone was contoured and analyzed using a threshold of bone density 7000–8000. Three-dimensional virtual models of representative regions in the scaffolds were created and visualized using micro view image processing software (GEbHealthcare explore Microview V.2.2 software Guide, GE Healthcare, Ontario, Canada).

2.7. BrdU cell labeling and staining

The rBMSCs were labeled by incubation for 48 h at 37 °C using the bromodeoxyuridine (BrdU, Sigma, St Louis, MO, USA). The staining procedure of BrdU on in vivo implant was as follows. The deparaffinized slides were washed with PBS and incubated in 3% H_2O_2 (in PBS) for 10 min, followed by washing three times with PBS. The slides were incubated with 2 N HCl for 30 min at 37 °C and neutralized with boric acid (pH 8.5). Anti-BrdU antibody (DAKO, Calif, USA) was incubated for 40 min and washed then second antibody (Envision kit, DAKO, Calif, USA) was applied for 40 min. After being washed with PBS, the BrdU positive cells were detected with using 3,3′-diaminobenzidine-tetrahydrochloride (DAB; Envision kit, DAKO, Calif, USA) and counter stained with hematoxylin.

2.8. Histological analysis

At two and four weeks after implantation, the rats were sacrificed and the implants were individually dissected and removed from the cranial. The tissues were immediately fixed with 10% formalin and embedded in paraffin. The