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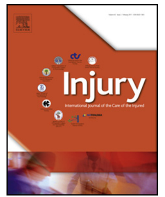

The regeneration and augmentation of bone with injectable osteogenic cell sheet in a rat critical fracture healing model

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ABSTRACT

Limitations in the current treatment strategies make cases with compromised bone healing challenging clinical problems. Osteogenic cell sheets (OCSs), fabricated from rat bone marrow stromal cells (BMSCs), contain enriched osteoblasts and extracellular matrix. Here, we evaluated whether the minimally invasive percutaneous injection of OCSs without a scaffold could be used as a treatment to increase bone regeneration in a critical fracture healing model. Critical fracture healing model was created in the femora of 60 male Fischer 344 inbred rats using marrow ablation and periosteal removal. The rats were then randomly divided into two groups. Six hours after fracture, one group received an injection of OCSs (OCS group), while the second group was injected with phosphate-buffered saline (PBS) (control group). Fracture healing was evaluated using radiological, histological, micro-computed tomography (CT) and biomechanical analyses. The radiological and histological evaluations demonstrated enhanced bone regeneration in the OCS group compared with that in the control group. By 12 weeks, the hard callus had been remodelled via recorticalization in the OCS group. By contrast, no fracture union was found in the rats in the control group. Biomechanical testing revealed a significantly higher maximum bending load in the OCS group compared with that in the control group. The results of the present study demonstrate that the injection of entire OCSs can enhance bone regeneration and lead to bony union in a critical fracture healing model. Therefore, this procedure offers a minimally invasive technique to promote hard tissue reconstruction and, in particular, bone repair strategies for cases with compromised bone healing.

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Introduction

Cases with compromised bone healing are a challenging clinical problem for orthopaedic surgeons. Although treatment options do exist for this challenging and recalcitrant clinical problem, they are complex and costly, and often multiple procedures are required for treatment. Fracture healing is normally a spontaneous sequence of events briefly summarised as the initial inflammation, then soft and hard callus formation, and finally bone remodelling [1], which involves the interactions between osteoprogenitor cells, growth factors and the extracellular matrix (ECM) [2]. When this process does not occur, as in cases with compromised fracture healing or segmental bone defect, surgical intervention is required, and it is commonly combined with autologous grafting, which improves the local repair environment by providing osteoprogenitor cells, structural substrates and bone-inducing proteins [1].

Autologous bone grafting remains the gold standard for the treatment of cases with compromised bone healing [3]. However, it is an invasive, open surgical procedure that requires autologous bone to be harvested from an alternative site within the patient. Furthermore, the primary concern is that an open surgery performed in nonunion patients may further reduce the osteogenic potential of the progenitor cells at the fracture site. As such, minimally invasive surgical options are desirable to avoid damaging the local environment and inducing a local

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inflammatory response in an already compromised bone healing site. The percutaneous injection of bone marrow has been suggested as one low-risk and inexpensive solution for the treatment of cases with compromised bone healing [4], and the success rate was reported to be relatively high in established nonunion cases [1]. However, the number and concentration of osteoprogenitor cells harvested using this method vary significantly between individuals [5,6], and fewer progenitor cells are present in marrow samples from older patients.

An alternative strategy to promote bony reconstruction is the implantation of tissue-engineered bone (TEB) developed from bone marrow stromal cells (BMSCs). A recently described injectable, cell-based TEB provides a noninvasive solution in addition to the current treatment options [7–9] that have attracted considerable attention within the field of orthopaedics. However, the use of foreign biomaterial compounds often stimulates an immune response, leading to granulation tissue formation at injury sites and ultimately fibrous tissue in the place of bone [10]. Reducing the immune response generated by these implanted biomaterials is therefore of critical importance, and it indicates the need for materials that are intrinsically nonimmunogenic or those with properties that can be modified to prevent recognition by the immune system. A scaffold-free material made from cells fits these requirements.

Interest in the concept of cell-sheet engineering for regenerative medicine applications has recently been increasing. This approach is gradually being established as a reliable alternative to traditional tissue engineering and regenerative medicine methods, namely biodegradable scaffolds used to create tissue substitutes and the injection of isolated cells [11]. When cultured cells are harvested as intact sheets including their deposited ECM, they can be easily attached to host tissues with minimal cell loss. This concept allows cells to be recovered within their own ECM as a sheet with cohesive cell–cell and cell–ECM interactions [12]. A custom-designed, temperature-responsive culture dish is required for successful harvest of the cell sheets. One advantage of using cultured cell sheets is that they eliminate the need for scaffolds, precluding the strong inflammatory responses that are typically induced when biodegradable scaffolds are broken down. Several studies have shown the potential of this technology for reconstitution of the cornea [13] and the myocardium [14], and in hepatocyte transplantation [15] and renal tube epithelial cell transfer [16].

Zhou et al. reported a method to harvest intact cell sheets from standard culture dishes using a cell scraper, which made the use of cell-sheet technology more convenient and widely applicable [17]. Our group demonstrated that osteogenic cell sheets (OCSs) fabricated from BMSCs with dexamethasone (Dex) and ascorbic acid phosphate (AscP) can form bone tissue without the need for a scaffold after transplantation [18]. OCSs derived from different cell sources have been used in the production of vascularised tissue-engineering constructs [9,19], for the regeneration of periodontitis defects [20] and to enhance tendon–bone healing [21]. In a recent study from our group, we demonstrated that an OCS can enhance bone formation in a rat nonunion model [22]. Further, the percutaneous injection of an entire OCS was previously reported by our group, and the OCS, injected into subcutaneous sites on the dorsal surface of rats, formed the bone in the absence of a scaffold [23]. However, whether this minimally invasive approach encourages hard tissue reconstruction at bony sites and induces fracture repair has not yet been tested. To this end, the aim of the present study was to determine whether the percutaneous injection of entire OCSs enhances bone regeneration in order to demonstrate a novel method for the minimally invasive treatment of cases with compromised bone healing.

Materials and methods

Study design

Sixty male Fischer 344 inbred rats were divided into two groups: OCS and control groups ($n = 30$ each). A femoral critical fracture healing model was created in each rat, and the rats were sutured. After 6 h, two sheets of OCS in 1-ml phosphate-buffered saline (PBS) (Gibco, Invitrogen, Carlsbad, CA, USA) were injected into the osteotomy site in rats in the OCS group. Rats in the control group received an injection of PBS only. Femora were harvested from each group at 4, 8 and 12 weeks after the injection. Fracture healing was evaluated with radiological and histological analyses at 4, 8 and 12 weeks ($n = 4$). Micro-computed tomography (CT) and biomechanical analyses were also performed at 4, 8 and 12 weeks ($n = 6$). Ten additional rats were used to prepare sufficient quantities for injection. This research protocol was approved by the Institutional Animal Care and Use Committee of Nara Medical University, following all appropriate guidelines.

Critical fracture healing model of the femur

We modified a rat femur critical fracture healing model described previously [22]. Briefly, 12-week-old male Fischer 344 inbred rats (approximately 280 g) were anaesthetised with 2% isoflurane. The operative site was shaved and prepared with ethanol. A 5-cm lateral incision was made on the hind limb parallel to the femoral shaft, extending from the femoral condyle to the proximal part of the femur. The midshaft of the femur was exposed by dividing the vastus lateralis and the biceps femoris muscles, and a fracture was created by transverse osteotomy with an oscillating mini saw. The femoral canal was reamed with an 18-G needle (Terumo, Tokyo, Japan), and it was irrigated with 20 ml of sterile saline so that the bone marrow was completely ablated. The periosteum was removed as much as possible from the proximal to the distal ends of the femur, and the muscle attached to the periosteum was also removed. A K-wire (0.8 mm in diameter) was then inserted into the medullary canal in a retrograde fashion with the use of a motor-driven drill. The K-wire was positioned within the proximal part of the femur, and the distal end was then cut close to the articular surface of the knee. The wound was then irrigated with 10 ml of sterile saline, and it was closed with a 4/0 nylon suture. Unprotected weight bearing was allowed immediately after the operation. Postoperative pain was managed by the subcutaneous administration of buprenorphine hydrochloride. Postoperative antibiotics were administrated by intramuscular injection of penicillin prophylactically. The rats were fed a standard maintenance diet, and they were provided water ad libitum.

BMSCs preparation from bone marrow aspirates

The method for BMSCs preparation has been reported previously [18,22,23]. Briefly, bone marrow was obtained from the femoral shafts of 7-week-old male Fischer 344 inbred rats. Both ends of the femur were cut at the epiphysis and the bone marrow was aspirated from the marrow cavity using 10 ml of standard culture medium expelled from a syringe using a 21-G needle. Standard culture medium consisted of minimal essential medium (MEM) (Nacalai Tesque Inc., Kyoto, Japan) containing 15% fetal bovine serum (FBS) (Gibco, Invitrogen, Carlsbad, CA, USA) and 1% antibiotics (10,000 U/ml penicillin and 10,000 μg/ml streptomycin, Nacalai Tesque Inc.). Harvested cells were then transferred into two T-75 flasks (Falcon, BD, NJ, USA) containing 15 ml of standard culture medium. Cell culture was maintained in 95% humidified atmosphere with 5% CO_2 at 37 °C. After reaching confluence,

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