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Double intratibial injection of human tonsil-derived mesenchymal stromal cells recovers postmenopausal osteoporotic bone mass

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Abstract

Background and aims: Osteoporosis, which is a disease characterized by weakening of the bone, affects a large portion of the senior population. The current therapeutic options for osteoporosis have side effects, and there is no effective treatment for severe osteoporosis. Thus, we urgently need new treatment strategies, such as topical therapies and/or safe and effective stem cell therapies. *Methods:* We investigated the therapeutic potential of directly injecting human tonsil-derived mesenchymal stem cells (TMSC) into the right proximal tibias of ovariectomized postmenopausal osteoporosis model mice. Injections were given once (1×) or twice (2×) during the 3-month experimental period. At the end of the experiment, micro-computed tomographic images revealed some improvement in the proximal tibias and more significant improvement in the femoral heads of treated mice. *Results:* Osteogenic effect was qualitatively and quantitatively more pronounced in TMSC/2×-treated mice. Furthermore, TMSC/2× mice exhibited significant recovery of the serum osteocalcin level, which is pathologically elevated in osteoporosis, and increased serum alkaline phosphatase, which indicates bone formation. TMSC therapy was generally well tolerated and caused no apparent toxicity in the experimental mice. Moreover, TMSC therapy reduced visceral fat. *Conclusion:* Our results demonstrate that double injection of TMSC directly into the proximal tibia triggers recovery of osteoporosis, and thus could be a potential therapeutic approach for severe bone loss.

Key Words: *femoral head, intratibial injection, osteoporosis, ovariectomized, tonsil-derived mesenchymal stem cells, visceral fat*

Introduction

Osteoporosis, which is a prevalent disease of the elderly, is characterized by thinner and weaker bones and an increased likelihood of bone fracture (1). The current osteoporosis medications (*e.g.*, bisphosphonates and estrogen) act as antiresorptives to slow down the natural breakdown of the bone (resorption), but they have side effects. Daily oral bisphosphonate intake is inconvenient, and a large fraction of patients fail to maintain proper treatment (2). Hormone replacement therapy (HRT) and the use of selective estrogen receptor modulators (SERM) may have thromboembolic complications (1). Osteoanabolics trigger bone formation, but the only Food and Drug Administration (FDA)-approved regimen is parathyroid hormone (PTH), which carries the inconvenience of daily subcutaneous injection and the risk of adverse effects. Therefore, we urgently need to develop new therapeutic strategies capable of stimulating bone formation.

For patients with severe osteoporosis or osteoporotic fractures, there are few available options for

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treatment. Various materials are researched for bone replacement (3–6), but these are for fractures of healthy bones. Although PTH is prescribed for severe osteoporosis, the anabolic window limits its use to only 2 years (7). Some researchers have sought to reduce possible unwanted systemic effects by delivering drugs directly to the site of bone loss, such as by topical injection. Various therapeutic agents have been administered directly to the bone, including bisphosphonates, PTH and bone morphogenetic proteins (BMP) (8). These therapies have proven effective for healing and prevention of osteoporotic fractures (9). Because of the high degree of bone resorption in severe osteoporosis, the stimulation of bone formation is the last hope. Hence, we need to develop additional bone formation–stimulating agents, such as stem cells, for the local treatment of severe osteoporosis.

Mesenchymal stem cells (MSC) have great regenerative potential due to their differentiation capacity and immune-modulating effects. They act both directly and indirectly for tissue regeneration, and are frequently used for stem cell therapy because they are safe in terms of cancer formation and immune rejection (10–12). Human tonsil-derived MSC (TMSC) were isolated from discarded children's tonsillar tissues after tonsillectomy, and shown to be a useful source of MSC for therapeutic purposes (13). These cells show an attractive regenerative potential and have proven capable of differentiating into PTH-releasing cells for hypoparathyroidism treatment (14,15), Schwann cell–like cells for peripheral nerve regeneration (16) and myogenic cells for skeletal muscle restoration (17). TMSC therapy has been shown to improve liver fibrosis (18,19), allergic rhinitis (20) and senile osteoporosis (21) without any preconditioning differentiation of the cells. Moreover, TMSC have demonstrated robust osteogenic potential (13,22) .

To examine their potential for bone regeneration in a mouse model of severe osteoporosis, we injected TMSC locally into the proximal tibias of ovariectomized mice, and later examined the injected proximal tibias and the distant femoral heads. To gain more comprehensive insights, we also explored changes in serum bone-related markers and the visceral fat mass.

Materials and methods

TMSC isolation

Tonsillar tissues were obtained from the medical waste generated from tonsillectomies performed at the Department of Otorhinolaryngology–Head and Neck Surgery, Ewha Womans University Medical Center (EWUMC, Seoul, Korea), with informed written consent. This study protocol was approved by the institutional review board of Ewha Womans University Medical Center (EWUMC, institutional review board number ECT-11-53-02). TMSC were isolated from the obtained tissues as previously described (14,23). Briefly, tonsillar tissues were acquired from five donors who had undergone tonsillectomy (three boys and two girls). Isolated tonsils were mechanically digested by cutting, mincing and grinding, and then were enzymatically digested with collagenase type I (Thermo Fisher Scientific) and DNase (Sigma-Aldrich) at 37°C for 30 min. The obtained cell suspension was filtered through wire mesh, and unwanted cells were removed using Ficoll-Paque (GE Healthcare) density gradient centrifugation. The obtained mononuclear cells were cultured in high-glucose (4500 mg/L) Dulbecco's modified Eagle's medium (DMEM-HG; Welgene Inc.) supplemented with 10% fetal bovine serum (FBS), penicillin-streptomycin and antibiotic-antimycotic (all from Thermo Fisher Scientific), and allowed to adhere to the culture plates. The resulting adherent mononuclear cells, which were taken as TMSC, were expanded and banked according to need. TMSC of passages five to seven were used for experiments.

Flow cytometry analysis

Flow cytometry measurements were performed to examine the cell surface immunophenotypes using a fluorescence-activated cell sorting (FACS) (FACS Calibur flow cytometer, BD Biosciences). At least 1×10^5 cells (in 100 μL phosphate-buffered saline [PBS]/0.5% bovine serum albumin [BSA]/0.01% NaN_3) were incubated with fluorescein isothiocyanate (FITC)–labeled antibodies against human CD34, CD45, CD90 and CD105 (BD Biosciences), phycoerythrin (PE)-labeled antibodies against human CD14 and CD73 (BD Biosciences) or the respective isotype controls (BD Biosciences) for 30 min at 4°C. After incubation, cells were washed, and then the labeled cells were used for FACS analysis.

Adipogenic, chondrogenic and osteogenic differentiation

The mesodermal differentiation capacity of TMSC was examined as previously reported (13,22) with slight modifications. Briefly, the TMSC were cultured with commercially available adipogenic media (Invitrogen) for adipogenic differentiation for 3 weeks. Differentiated TMSC were washed twice with PBS, fixed in 4% paraformaldehyde for 15 min at room temperature and then washed with PBS and stained with 2%