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Bone Marrow Stromal Cells Contribute to Bone Formation Following Infusion into Femoral Cavities of a Mouse Model of Osteogenesis Imperfecta

Feng Li¹, Xujun Wang¹, and Christopher Niyibizi^{1,*}

¹ Pennsylvania State University College of Medicine, Division of Musculoskeletal Sciences, Department of Orthopaedics and Rehabilitation, Hershey, PA 17033, USA

* Department of Biochemistry and Molecular Biology, Hershey, PA 17033, USA

Abstract

Currently, there are conflicting data in literature regarding contribution of **bone marrow stromal cells (BMSCs)** to bone formation when the cells are systemically delivered in recipient animals. To understand if **BMSCs** contribute to bone cell phenotype and bone formation in osteogenesis imperfecta bones (OI), MSCs marked with GFP were directly infused into the femurs of a mouse model of OI (oim). The contribution of the cells to the cell phenotype and bone formation was assessed by histology, immunohistochemistry and biomechanical loading of recipient bones. Two weeks following infusion of **BMSCs**, histological examination of the recipient femurs demonstrated presence of new bone when compared to femurs injected with saline which showed little or no bone formation. The new bone contained few donor cells as demonstrated by GFP fluorescence. At six weeks following cell injection, new bone was still detectable in the recipient femurs but was enhanced by injection of the cells suspended in pepsin solubilized type I collagen. Immunofluorescence and immunohistochemical staining showed that donor GFP positive cells in the new bone were localized with osteocalcin expressing cells suggesting that the cells differentiated into osteoblasts in vivo. Biomechanical loading to failure in three point bending, revealed that, femurs infused with **BMSCs** in PBS or in soluble type I collagen were biomechanically stronger than those injected with PBS or type I collagen alone. Taken together, the results indicate that transplanted cells differentiated into osteoblasts in vivo and contributed to bone formation in vivo; we also speculate that donor cells induced differentiation or recruitment of endogenous cells to initiate reparative process at early stages following transplantation.

Keywords

Osteogenesis imperfecta; Bone stromal marrow cells; Cell transplantation; Bone repair; Collagen

Correspondence to: Christopher Niyibizi, Ph.D., Department of Orthopaedics and Rehabilitation, Division of Musculoskeletal Sciences, Pennsylvania State University, College of Medicine, H089, 500 University Drive, Hershey, PA 17033, Tel: 717-531-5649, Cniyibizi@psu.edu.

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Introduction

Stromal cells are present in various tissues that include bone marrow, adipose tissue, muscle and other tissues [1–4]. Recent reports speculate that these cells may all derive from cells associated with blood vessel walls [5,6]. Potential of the cells to form bone in vivo [7] and to repair and regenerate various tissues in animal models is currently an area of active investigation [6,8–12]. Human **bone marrow stromal cells (BMSCs)** seeded onto scaffolds and implanted in bone defects created in animal models have been shown to augment repair and regeneration of the target tissues [9–14]. In most cases, **stromal cells** are supplemented with specific growth factors or seeded onto biomaterials to enhance and accelerate the reparative process [13–16].

Systemic transplantation of **BMSCs** has also been attempted and the initial results suggested that the cells have potential to migrate via the circulatory system and home to various tissues of the recipients [11,17–19]. Earlier studies in animal models of osteogenesis imperfecta (OI) suggested that the cells could prospectively be used to treat OI [17]. These studies lead to clinical trials in which **human BMSCs (hBMSCs)** were transplanted into patients with OI. The results from the clinical studies demonstrated that **hBMSCs** transplanted into OI patients led to reductions in bone fractures, increase in growth velocity and bone mineral density [20–22]. The number of donor cells that engrafted in the bones of the recipients was however low and it was not clear how such low numbers could lead to the positive effects seen in the recipient patients. Further studies in animal models have generated conflicting results regarding transplantability of **BMSCs** and contribution of the transplanted cells to the structural integrity of the recipients. Results from transplantation studies in animal models especially mice indicate that the level of **BMSCs** engraftment following systemic transplantation is very low and thus its therapeutic efficacy remains undefined [23–26]. Transplantation of the cells into developing animals or in utero has shown that the cells contribute to bone formation and structural integrity of the recipient animals [11,23,27]. Transplantation of the cells into adult animals has however generated mixed results [24,25].

In the present report, to directly address whether **BMSCs** contribute to the bone cell phenotype and bone formation in the recipient's bones, murine **BMSCs** were infused directly into femurs of a mouse model of OI (oim). The oim mouse model used here for cell transplantation was described previously [18,28]. The mouse has defective synthesis of pro α 2(I) chains as a result of nucleotide deletion in the carboxy-terminal coding domain. This mutation leads to the synthesis of α 2(I) chains that cannot associate with pro α 1(I) chains resulting in the deposition of the α 1(I) homotrimers in tissues [28]. The mouse exhibits cortical thinning and very minimal trabecular bone [29]. Although the mutation in this mouse model does not represent the spectrum of most OI genotypes it serves as an excellent model for testing efficacy of cell therapy for the repair and regeneration of brittle bones. Owing to the fact that a large numbers of cells can be infused into femurs, this approach allows effective assessment of the efficacy of MSCs in regenerating OI bones.

Materials and methods

Cell isolation

Bone marrow stromal cells were isolated by flushing marrow from the femurs and tibia of normal mice syngeneic to oim mice; after marrow flush, the bones were split open and the cells associated with bone were scrapped and combined with the marrow harvested cells. Previous studies have reported presence of **BMSCs** in trabecular bone [30]. The **BMSCs** were cultured in T25 flasks in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin (P/S, v/v). After four days of culture, the medium was replaced with fresh medium and the cells were maintained in culture with passaging. At passage 3, the cells

were transduced with a retrovirus carrying the enhanced green fluorescent protein and Zeocin resistant genes.

Cell transduction

To aid in donor cell tracking in vivo, cell transduction was performed as described previously [11]. Briefly, the cells were plated in six-well plates in DMEM supplemented with 10% FBS and maintained in culture to 60% confluence. The cells were then treated with 1 ml of a high-titer DFG-retro virus carrying eGFP and Zeocin^r resistant genes in DMEM medium supplemented with 10% FBS [31,32]. **Bone marrow stromal cells** were incubated with the virus for 24 h. After 24 h, the medium was replaced with new medium supplemented with 10% FBS; the cells were then treated with the retroviral vector and incubated further for 24 h. After two more rounds of medium changes with addition of viral vector, new medium without the viral vector was added and the cells were incubated further for 24 h. To select a population of cells expressing eGFP, the transduced cells were maintained in DMEM supplemented with 10% FBS and 25 μ g/ml of Zeocin. The EGFP+ **BMSCs** were maintained in culture in presence of Zeocin until use.

Assessment of BMSCs differentiation

Alkaline phosphatase activity

Transduced **BMSCs** were treated with human recombinant bone morphogenetic protein-2 (rhBMP-2) at 100 ng/ml (RDI, Flanders, NJ) for 3 days. Alkaline phosphatase (ALP) activity was determined in cell lysates using an alkaline phosphatase assay kit (Sigma, St Louis, MO). Cell lysates were analyzed for protein content using a Bio-Rad protein assay kit, and the ALP activity was normalized for total protein concentration [11].

Osteogenic differentiation and Alizarin Red S staining

For osteogenic differentiation, **BMSCs** were maintained in DMEM supplemented with 10% FBS, 10^{-7} M dexamethasone, 10 mM glycerophosphate disodium, and 50 μ g/ml ascorbic Acid (all from Sigma). After 21 days, the cells were rinsed twice in PBS, fixed in 2% formaldehyde for 10 min, and washed in distilled water. The cells were covered with a 2% aqueous solution of Alizarin Red S, pH 4.2, for 10 min to stain for calcium deposits [11].

Adipogenic differentiation

Bone marrow stromal cells were plated onto 24-well plates in adipogenic medium, comprised of DMEM supplemented with 10% FBS, 1 μ M dexamethasone, 10 μ M insulin, 200 μ M indomethacin, and 0.5 mM isobutyl-methylxanthine (all from Sigma). The medium was replaced every 3 days. Adipogenic differentiation was assessed by Oil Red O staining at 3 weeks after initial adipogenic induction. Prior to staining, the cells were fixed in 2% formaldehyde for 10 min and washed in PBS. The cells were then incubated in 2% (w/v) Oil Red O reagent for 10 min at room temperature. The stained cells were observed under the light microscope and photographed [11,18].

Gene expression analysis

Gene expression analysis of osteogenic and adipogenic associated genes were performed as described previously [10]. Triplicate PCR reactions were carried out.

Flow cytometry

Bone marrow stromal cells were harvested by trypsinization with 0.25% trypsin/EDTA and washed with cold PBS. Cell aliquots (1×10^6 cells) were incubated in a buffer containing monoclonal antibodies. Antibodies used for FACS analysis were phycoerythrin-conjugated

anti-CD11b, anti-CD13, anti-CD34, anti-CD44, anti-CD45, anti-CD73, anti-CD90, anti-CD117 (all from BD Biosciences, San Diego, CA), and unconjugated antibodies against CD105 (R&D, Minneapolis, MN), and anti-CD146 clone P1H12 (Chemicon, Temecula, CA).

Infusion of GFP marked cells in mice femurs

We used a total of 35 oim mice for the in vivo femur transplantation experiments. In all the following in vivo experiments, a volume of 20 μ l of PBS with cells (1×10^6) or with type I collagen (bovine pepsin solubilized type I collagen (0.1 mg/ml) or collagen alone or PBS was injected through the femoral condyles into the femoral cavities. Each group of mice was sublethally irradiated prior to treatment as described previously [11,18]. Four hours after irradiation, the mice were anesthetized by subcutaneous injection of 1ml of Ketamine and Xylazine (100 mg/ml, 2:1)/kg body weight. For **BMSCs** or any of the treatments of the femurs, a technique was developed to distribute cells within the entire bone cavity. A tunnel was created within the femur cavity using a 26 gauge needle (except untreated femurs in group 4). This was followed by insertion of a smaller gauge needle (30 gauge) attached to a syringe containing cells for injection. The cells were slowly introduced within the bone cavity by slowly retracting the needle while depositing BMSCs. Femurs that did not receive cells were treated similarly but no cells were injected.

The goal here was to determine the effect s of cells alone or cells suspended in collagen matrix for regenerating OI mice bones.

The following is a description of the experiments done with oim mice.

Right femurs of 5 oim femurs were injected with BMSCs suspended in PBS and left femurs were injected with PBS. These mice were sacrificed at 2 weeks following cell injection and were used for histology to assess bone formation and donor cell distribution. For six weeks analysis, two groups of 5 oim mice were used for histological analysis of recipient femurs; Right femurs of one group of mice were infused with GFP+ BMSCs suspended in collagen and the left femurs (contralateral) were infused with collagen in PBS. Femurs of the other group of 5 mice were infused with BMSCs suspended in PBS and the left femurs were infused with solubilized type I collagen. These mice were sacrificed at 6 weeks following cell injection and were processed for histology to assess bone formation.

A group of 20 oim mice divided into 4 groups of 5 mice were used for biomechanical testing and were treated as follows: The right femurs of group 1 mice were injected with GFP marked **BMSCs** suspended in solubilized collagen I. The left femurs were injected with **BMSCs** suspended in PBS. In group 2 mice, the right femurs received **BMSCs** suspended in solubilized type I collagen and the left femurs were injected with bovine solubilized type I collagen. In group 3 mice, the right femurs received **BMSCs** suspended in PBS and the left femurs received solubilized type I collagen. In group 4 oim mice; five femurs were infused with collagen I and left femurs were left untreated.

Histological analysis, frozen tissue sections

For histological analysis of the donor GFP+ cells in vivo, a method described previously was used [11,18]. Briefly, femurs harvested from the recipient mice were immediately fixed in freshly prepared 4% paraformaldehyde in PBS, containing 10% sucrose and kept at 4°C for 24 hours in the dark. After 24 hours, the bones were rinsed in PBS and then demineralized in 0.5M EDTA in PBS containing 10% sucrose at 4 °C for 48 hours in the dark. Two femurs from mice sacrificed at 2 weeks that were infused with BMSCs in PBS or PBS only after demineralization were slowly frozen in cold isopentane cooled on a dry ice bath. Frozen bones were embedded in Tissue-Tek optimal cutting temperature Compound

(OCT), and 10- μ m sections were cut and mounted on glass slides. The slides were directly observed under a fluorescent microscope without the cover slips, but the sections were kept hydrated in PBS to reduce autofluorescence. Images were acquired using the Spot RT SE digital camera (Diagnostics Instruments Inc., Sterling Heights, MI). Equivalent tissue sections were stained with hematoxylin and eosin.

Histological analysis, paraffin embedded tissues sections

Femurs harvested from recipient mice at 2 weeks post infusion with BMSCs in PBS or PBS only (6 femurs) were paraffin embedded. Femurs from mice that were infused with BMSCs or collagen at 6 weeks as described above were also paraffin embedded. Ten micron longitudinal tissue sections were cut, deparaffinized and stained in hematoxylin and Eosin (H and E). Some of the tissue sections were used for immunofluorescence and immunohistochemistry staining of GFP and osteoclastin.

Immunohistochemical staining for eGFP and osteoclastin

For immunohistochemical staining of GFP expression and osteocalcin synthesis by the donor cells in vivo, paraffin embedded tissue sections were prepared for antigen retrieval by enzymatic treatment. Following enzymatic antigen retrieval, the sections were treated with a monoclonal antibody against either eGFP (1:250, Abcam, Cambridge, MA) or mouse osteoclastin monoclonal antibody (1:100, BTI, Stoughton, MA). The sections were incubated with primary antibodies overnight at 4°C. For visualization, sections were stained with Vectastain Elite ABC kit (Vector Lab, Burlingame, CA). For GFP visualization, sections were stained using the Vector VIP substrate kit and for osteoclastin (OCN) staining, Vector VIP DAB substrate Kit was used. Glass cover-slips were mounted on 1 mm glass slides using a drop of Cytoseal 60 Mounting Medium (Richard-Allan Scientific, Kalamazoo, MI). Resulting immunostaining was evaluated using Spot RT SE digital camera (Diagnostics Instruments Inc., Sterling Heights, MI). For immunofluorescence staining of GFP and osteoclastin the same approaches as above were applied except for visualization; secondary antibodies conjugated to FITC or Cy3 were used.

Biomechanical testing

For biomechanical testing, six weeks following treatment, 5 oim mice (ten femurs) within each of the 4 groups were used for biomechanical testing. The femurs were loaded to failure in three point bending using a small animal biomechanical testing instrument (ElectroForce 3100 (Bose Corporation, Prairie Minnesota). Results from each group were collected and analyzed by assessing the differences in load to failure of the right and left femurs of the same mouse. The stiffness for each bone was also assessed. In all cases the right femur was set as experimental and the left femur served as a control. Data was expressed as percent of control; left femur served as a control regardless of treatment in all cases. The results were compared within groups but not across groups. These biomechanical approaches were previously used to assess mechanical strengths of bone in rat models of osteoporosis [33].

Statistics

Assessment of differences in loads between left and right femurs within groups was determined and the results were assessed for significance using paired samples t-test. Alkaline phosphatase activity between BMP-2 treated and untreated groups were compared and assessed and the results were analyzed for significance using one way analysis of variance (ANOVA). Significance was set at $p < 0.05$.

Results

Cell isolation

The method for isolating BMSCs was described previously [11]. We previously showed that the cells associated with bone are highly osteogenic in vitro and in vivo [11]. Flow activated cell sorting analysis (FACS) showed that the cells expressed surface markers attributed to MSCs (Fig 1A). The cells expressed putative markers of MSCs CD13, CD73 and CD105 and did not express markers associated with hematopoietic cells (CD45) and macrophages (CD11b) (Fig. 1A). The cells did not express CD146; a marker expressed by human BMSCs [34]. CD146 is expressed by pericytes; it is likely that the present marrow preparation did not contain pericytes. We previously demonstrated that cells expressing this antigen increase in culture with passaging in murine adipose derived MSCs [35] suggesting that the present bone marrow stromal cell population exhibits differences with the adipose derived preparation. Morphological appearance of the cells is shown (Fig. 1A). The cells exhibited small spindle shaped morphology and upon transduction with the retrovirus, they maintained a similar morphology (Fig. 1B).

Next, the cells were assessed for differentiation toward osteogenic and adipogenic cell lineages in vitro. The results indicated that the cells exhibited robust differentiation toward osteogenic and adipogenic cell lineages upon culturing in osteogenic and or adipogenic medium. Differentiation toward these cell lineages was demonstrated by Alizarin Red and oil Red O staining respectively (Fig. 2A, B). When the cells were treated with BMP-2, they expressed high levels of alkaline phosphatase activity (Fig. 2C). Expression of the osteoblasts and adipocytes associated genes confirmed osteogenic and adipogenic differentiation of the cells toward these cell lineages respectively (Fig. 2D). These data demonstrate that the cells have potential to at least differentiate toward osteogenic and adipogenic cell lineages.

BMSCs induce bone formation in recipient OI mice femurs

Initially, femurs were infused with BMSCs suspended in PBS; histological analysis of the recipient femurs at 2 weeks following cell injection, showed new bone formation in metaphyseal regions of the recipient femurs (Fig. 3A,B). Examination of the serial tissues sections for the donor cells by fluorescence microscopy, revealed that a high number of GFP + donor cells were concentrated at the injected site with few donor cells within the newly made bone (Fig 3C, D). These data suggest that the newly deposited bone in recipient femurs may not have been entirely due to the donor cells alone but by the endogenous cells as well. Tissue sections from oim femurs injected with PBS showed minimal or absence of trabecular bone; extensive trabecular was present in femur tissue sections of wildtype mice as expected (Fig 3E, F). Taken together, the results clearly establish that the donor cells contribute to bone formation in vivo either by depositing new bone or inducing endogenous cell differentiation.

BMSCs suspended in collagen matrix leads to enhanced bone deposition in the femurs of the recipient mice at 6 weeks

Initial experiments at 6 weeks showed that BMSCs suspended in PBS made less bone based on histological analysis. Based on published reports indicating that collagen immobilizes transplanted cells in recipient bones [36], we assessed whether supplementation of BMSCs with pepsin solubilized type I collagen enhanced bone deposition in the recipient mice femurs. Bone marrow stromal cells were suspended in PBS containing pepsin solubilized type I collagen and were then injected into the femurs of the oim mice. Contralateral femurs were either injected with PBS alone, type I collagen or BMSCs suspended in PBS. The recipient femurs were harvested at 6 weeks post cell infusion and subjected to histological

assessment. The results showed that **oim** femurs injected with BMSCs suspended in PBS-collagen contained more bone at 6 weeks when compared to femurs injected with BMSCs suspended in PBS only as revealed by histological analysis (Fig. 4A). The femurs injected with cells in PBS showed less bone deposition when compared to cells suspended in type I collagen and injected in the femurs at 6 weeks (Fig. 4A, B). The contralateral femurs injected with PBS-Collagen I or PBS alone revealed no bone formation as shown (Fig. 4C). In all cases, five femurs were assessed for each treatment.

Next, we assessed if the donor cells differentiated into osteoblasts in vivo and participated in bone formation. Paraffin embedded sections harvested from the recipient mice were prepared for immunofluorescence staining for osteocalcin synthesis and GFP expression (Fig. 5). The results showed that GFP positive donor cells detected by immunofluorescence staining were present in bone and marrow (Fig. 5A phase, B green arrows, metaphyseal region of recipient femur). Immunofluorescence staining for osteocalcin in the same tissue section revealed that osteocalcin expression was restricted to cells present in bone not the marrow (Fig. 5C, red arrows). Overlay image of B and C showed that GFP positive donor cells colocalized with cells expressing osteocalcin (Fig. 5D, yellow arrows, endogenous osteocalcin positive cells, red arrows). The data clearly demonstrated that donor cells associated with bone differentiated into osteoblasts while the cells in the marrow probably remained as progenitors. These data are consistent with our previously published observations[18,37]. Overlay of image A (image observed in phase of a serial section) with image D clearly demonstrated that donor cells were associated with bone as well as the marrow (Fig. 5E). Figure 5F shows a serial section stained with H and E; the boxed area is equivalent to the areas shown in A–E tissue sections. We speculate that new bone deposited in recipient femurs must have been generated by a combination of donor and endogenous cells. Figure 5G, H show serial control tissue sections in which primary antibodies to GFP and osteocalcin were omitted respectively.

The above results were further confirmed by immunohistochemical staining using specific antibodies to GFP and osteocalcin in paraffin embedded tissue sections. Figure 6A shows a hematoxylin and eosin (H and E) stained tissue section of oim femur that was infused with MSCs suspended in collagen. The boxed area in A is shown at a higher magnification in figure 6B of a serial tissue section. Immunohistochemical staining for osteocalcin in serial section revealed by ABC staining protocol identified cells expressing osteocalcin (Fig. 6C, red arrows representative cells). Immunohistochemical staining for GFP expressing cells in the equivalent serial section is shown in figure 6D. Close examination of the osteocalcin staining in figure 6C and GFP staining in figure 6D, clearly revealed that there were cells expressing both GFP and osteocalcin (Fig. 6C red arrows and D green arrows). Taken together, the data shows that donor cells differentiated into osteoblasts in vivo as indicated by osteocalcin expression and were co- distributed with endogenous cells expressing osteocalcin shown by red arrows (Fig. 6C, red arrows). The data clearly showed that in specific areas within the recipient femur, donor cells positive for GFP were also expressing osteocalcin. The data imply that donor cells differentiated into osteoblasts and osteocytes and participated in bone formation in vivo. Since the femurs injected with PBS or collagen did not show any new bone formation, the excess bone made in recipient femurs must result from a combination of exogenous and endogenous cells. This conclusion is based on osteocalcin staining results and a previous report indicating that osteocytes also express osteocalcin [38]. BMSCs containing empty vector were not prepared because GFP has never been demonstrated to induce bone formation.

Biomechanical testing results

We determined whether femurs that received BMSCs were biomechanically stronger than femurs that were injected with PBS or type I collagen by loading the bones to failure.

Because we did not perform micro CT following cell injections, we only examined load to failure in 3 point bending as well as stiffness. Recipient mice were divided into 4 groups; for each mouse within a group, one femur (left) served as a control and the contralateral (right) femur served as experimental. For data analysis, the left and the right femurs of the same mouse within each group were compared in terms of the load required to failure. Results for mice in group 1: comparison of right femurs that received the cells plus collagen to the left femurs that received cells plus PBS showed that the right femurs were mechanically stronger (Fig. 7 and table 1). In group 2 mice, the right femurs that received BMSCs supplemented with collagen were biomechanically stronger than femurs receiving collagen matrix alone. These data suggest that transplantation of collagen alone had no effect on mechanical properties of the recipient bones; presence of exogenous BMSCs was necessary for the improvement in mechanical strengths of the recipient femurs (Fig. 7). In group 3, femurs that received cells plus PBS were stronger than the femurs that received collagen alone. In group 4; femurs that were infused with collagen I and contralateral femurs that were not treated demonstrated no difference in mechanical properties thus emphasizing the fact that presence of cells is the determining factor in improving mechanical properties of the oim femurs. Biomechanical comparison of the data is summarized in table 1. Stiffness results followed the trend of mechanical loads (Table I). In summary, the biomechanical testing results showed that infusion of BMSCs into OI femurs contributed to the improvement in the strength of the OI femurs but inclusion of collagen with cells potentiated the effects of the cells on the structural integrity of the recipient femurs. Figure 1 supplied in supplemental data showed that donor cells deposited bone in diaphyseal regions of bone and appeared to increase cortical thickness as revealed by histological analysis.

Discussion

Stem cell transplantation for treating OI raised high expectations originally but contribution of the transplanted cells to the structural integrity of OI bones has not been clearly established. Results of MSCs transplantation in patients with OI suggested that marrow derived BMSCs transplantation resulted in the reduction in fractures and increase in growth velocity of OI patients [20–22]. The number of donor cells that were present in the bones of OI patients following transplantation was however small. It was not clear how such small number of cells could have contributed to the positive effects observed on the structural properties of the patients' bones. In the present report we have shown that by 2 weeks following infusion of BMSCs into the femurs of oim mice, extensive new bone deposition was present as revealed by histological analysis; the new bone however appeared to contain few donor cells based on GFP positive cells within the recipient femur. These data suggested that not only did the donor cells contribute to bone formation in vivo but the endogenous cells participated in this process as well. It is likely that the presence of donor cells led to the recruitment of endogenous cells which initiated the reparative process. The femurs injected with PBS or collagen alone showed absence or minimal bone deposition. These observations suggest that donor cells as they differentiate induce or recruit endogenous cells to initiate reparative process. The present findings are consistent with current concepts that stromal cells secrete factors that modulate microenvironments for tissue repair [6,39–42].

Supplementation of the cells with collagen enhanced bone deposition and biomechanical properties of recipient femurs; the mechanism by which collagen participates in this process is not clear but speculation is that, collagen may provide an osteoconductive milieu for the cells to undergo accelerated differentiation and matrix production. The most likely explanation is that collagen enhances retention of transplanted cells in recipient femurs. This assumption is supported by a published report [36]. Application of BMSCs for tissue engineering of musculoskeletal tissues like bone and cartilage requires the use of scaffolding material for cell attachment and matrix deposition. Some scaffolding materials are

osteoinductive by enhancing cell differentiation while others are osteoconductive by providing an environment for cell attachment conducive for cell differentiation [43,44]. A recent report showed that intrafemoral injection of marrow derived cells suspended in rat tail tendon collagen aided in the retention of the cells in the recipient femurs [36]. It is likely that injection of BMSCs suspended in pepsin solubilized type I collagen helped in retaining cells in the recipient femurs and hence enhancement of bone formation in vivo.

Cell therapy protocols involve direct delivery of cells into target tissues and the hope is that the cells will differentiate into cells of the target tissues and repair or regenerate host tissues. The time course for the donor cells to differentiate into cell phenotype of the target tissues is not known. At 2 weeks following cell infusion into femurs, the cells appeared to remain aggregated at the injection site in the recipient femurs with few cells scattered in bone. Examination of the femurs that received the cells at 6 weeks following cell infusion, showed distribution of the cells on bone surfaces as well as within the newly made trabecular bone. The results indicate that the cells undergo a gradual differentiation in vivo. Previously, we showed that adipose derived stem cells infused into femurs of normal mice undergo differentiation and become partitioned into bone and marrow compartments at 2 weeks following cell infusion [37]. The cells that became associated with bone exhibited distinct morphologies and expressed genes associated with osteoblast differentiation while the cells that remained in bone marrow appeared to be progenitors [37]. These previous observations support our conclusion that the cells undergo gradual differentiation toward osteoblastic lineage in vivo. The differentiating donor **BMSCs** continued to have an effect on endogenous cells perhaps by generating factors that influence differentiation of the endogenous cells. It is likely that some of the factors produced by the engrafted MSCs include members of TGF- β family of proteins; BMPs and VEGF. Several reports have now clearly shown that **BMSCs** produce factors that modulate the host microenvironment for tissue repair [45].

Studies involving intrauterine transplantation of **BMSCs** into the fetuses of the oim mouse showed that at 12 weeks following cell transplantation, new bone was evident in recipient animals. The new bone contributed to the improvement of mechanical properties of the recipient bones [23]. Another report using a similar approach but in a different mouse model of OI has generated similar results[27]. The mechanisms by which donor cells contributed to the structural integrity of the recipients' bones were not investigated. In a related work, we reported that MSCs systemically transplanted into developing oim mice led to the engraftment, differentiation, matrix synthesis and deposition of new bone in the recipient mice at 4 weeks following cell transplantation [11]. Results from the present studies are supported by these previous reports which showed that transplanted BMSCs contribute to bone formation in recipients' bones. The sustainability of the newly made bone remains to be determined; our analysis was done at 6 weeks following cell infusion. Because at 2 weeks following cell infusion donor cells begin to differentiate into osteoblasts, biomechanical testing was not done at this time point.

In summary, we have demonstrated that BMSCs infused into femurs of the mouse model of osteogenesis imperfecta contribute to bone formation in vivo. Improvement in mechanical properties of the recipient bones may be the result of bone deposition by both endogenous and donor cells or paracrine actions of donor cells. We hypothesize that, initially, the donor cells exert paracrine actions in vivo by perhaps recruiting or inducing endogenous cell differentiation to initiate reparative process and at later time points the donor cells undergo gradual differentiation and participate in bone formation. We followed the recipient mice for 6 weeks following cell infusion into femurs, whether the positive affects seen last beyond this time period remains to be investigated. The speculation that donor cells induce or recruit

endogenous cells to initiate reparative process remains to be confirmed as well as the mechanisms involved in this process.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Surface mark expression and morphology of the BMSCs isolated from murine bones
 The cells in bone marrow and endosteal surfaces were combined and expanded in culture. FACS analysis demonstrated expression of markers attributed to BMSCs. The cells were devoid of hematopoietic cells and macrophages as indicated by lack of CD45 and CD 11b expression respectively (A). The cells comprised of small spindle shaped morphology; they were transduced with a retrovirus carrying eGFP and the Zeocin resistant genes at passage 3 (B). Transduction did not affect their morphological appearance or differentiation potential. (Magnification 100X).

Figure 2. BMSCs differentiated toward osteogenic and adipogenic cell lineages

BMSCs cultured in osteogenic medium for 21 days deposited calcium as demonstrated by Alizarin red staining (**A**), BMSCs maintained in adipogenic medium displayed adipogenic differentiation (**B**). BMSCs treated with BMP-2 for 3 days expressed high levels of alkaline phosphatase activity (ALP) (**C**). Expression of genes associated with osteoblasts and adipogenic differentiation are shown (**D**).

Figure 3. Representative tissue sections made from oim mouse femurs either infused or not infused with BMSCs

(A) Histological section from **oim** femur transplanted with BMSCs and stained with H and E to show the newly made bone. (B) A higher magnification of the H and E stained serial section showing extensive newly made bone. (C) GFP fluorescence of the recipient femur showing location of the donor cells. (D) Overlay image of GFP positive donor cells and the H and E stained tissue section indicating the exact location of the donor cells in the recipient bone. (E) Tissue section from oim mouse femur that was injected with PBS indicating minimal or absence of trabecular bone. (F)

Tissue section from the wild type femur showing extensive trabecular bone. Recipient femurs were harvested at 2 weeks following BMSCs injection. All sections are from the metaphyseal regions. Tissue sections A, C, D, E, F original magnification 40X. B original magnification 100X.

Figure 4. Histological tissue sections of oim mice femurs infused with BMSCs -PBS collagen or BMSCs-PBS or PBS- collagen without BMSCs

(A) The oim femur infused with BMSCs-PBS collagen I; (B) The femur injected with BMSCs-PBS; (C) The femur injected with PBS-collagen. The experimental femurs were injected with 1×10^6 cells suspended in PBS and collagen I or in PBS; the contralateral femurs were injected with PBS or collagen I in PBS. The femurs injected with cells suspended in collagen showed more bone by histology than the femur injected with BMSCs suspended in PBS. The femur injected with collagen I showed minimal or absence of trabecular bone. Histological sections original magnifications 40X. The sites of needle tracks re indicated.

Figure 5. Immunofluorescence staining for GFP and osteocalcin in tissue sections of a femur at 6 weeks following infusion of B MSCs

A) Section observed under phase f oim mouse femur infused with BMSCs indicating location of marrow and bone. B) Immunofluorescence staining for GFP in the same tissue section shown in A indicating GFP positive cells in bone, bone surfaces and marrow (green arrows). C) Immunofluorescence staining for osteocalcin in the same tissue section showing osteocalcin positive cells in bone, bone surfaces but absent from marrow (red arrows). **D)** Overlay image of B and C images showing cells positive for GFP and osteocalcin (yellow arrows) endogenous cells are indicated by red arrows. **E)** Overlay image of A and D images showing location of osteocalcin and GFP positive cells which represent the donor cells

(yellow arrows); Endogenous cells are indicated by red arrows, donor cells in marrow are indicated by green arrows. **F)** H&E stained section from oim recipient mouse showing sampled area shown in A at higher magnification. **G, H)** Serial sections in which primary antibodies for GFP and osteoclastin were omitted.

BMSCs suspended in collagen I were infused in oim mouse femurs and the recipient mice were sacrificed at 6 weeks. The donor cells are present in both bone and marrow. Only osteoclastin positive cells are present in bone. Original magnifications (A, B, D, E, G, H) 400X, F 40X.

Figure 6. Immunohistochemical staining of osteocalcin and GFP in tissue sections made from oim mouse femur transplanted with BMSCs in collagen I and harvested at 6 weeks
 Tissue section of a femur infused with BMSCs suspended in type I collagen indicating newly made bone (A); A higher magnification of the boxed area in A is shown (B); Staining for osteocalcin (C); GFP staining in the area(D). Representative cells that are expressing osteocalcin and GFP are indicated by red arrows (C) and green arrows (D) respectively. The data show that the GFP+ donor cells differentiated into osteoblasts and became associated with newly made bone thus indicating that they participated in bone formation. Paraffin embedded tissue sections were prepared for immunolocalization of osteocalcin and GFP using specific antibodies and were visualized by ABC staining kit. Original magnifications in A 40X; B, C, D 200X.

Figure 7. Biomechanical testing results of the oim femurs that received BMSCs, collagen I or PBS or untreated

In group 1 mice, the femurs receiving BMSCs required 24 % more load to failure. In group 2 mice, the femurs that received cells plus collagen I required 33% more load to failure. In group 3 mice, the femurs that received the cells in PBS required 15% more load to failure. In each group, 5 mice were sacrificed; left and right femurs were harvested and subjected to biomechanical testing. In each group, 10 femurs were tested, 5 femurs were experimental and 5 contralateral femurs served as controls. The values for each group were averaged and standard deviations were determined. Significance was determined using two – tailed paired-samples T-test and the significance was set at $P<0.05$. The significance is expressed as $\text{mean} \pm \text{SD}$. There was no difference between femurs injected with collagen or untreated.

Table 1

Comparison of loads and stiffness on left and right femurs within each group

	Group 1(mean±SD)		Group 2(mean±SD)		Group 3(mean±SD)		Group 4(mean±SD)	
	Cells+PBS	Cells+Matrix	Matrix	Cells+Matrix	Matrix	Cells+PBS	Untreated	Matrix
Loading(N)	9.126±0.693	11.316±1.468 *	9.006±2.418	12.152±1.505 *	7.572±1.357	8.762±1.686 *	7.925±1.941	7.660±1.884
Stiffness(N/mm)	63.810±6.35	73.803±13.41 *	48.771±8.43	69.135±11.67 *	48.376±7.07	59.135±3.47 *	50.580±21.22	49.843±20.08

* P<0.05 compared in each group