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An allograft generated from adult stem cells and their secreted products efficiently fuses vertebrae in immunocompromised athymic rats and inhibits local immune responses

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Abstract

BACKGROUND CONTEXT—Spine pain and the disability associated with it are epidemic in the United States. According to the National Center for Health Statistics, more than 650,000 spinal fusion surgeries are performed annually in the United States, and yet there is a failure rate of 15%–40% when standard methods employing current commercial bone substitutes are used. Autologous bone graft is the gold standard in terms of fusion success, but the morbidity associated with the procedure and the limitations in the availability of sufficient material have limited its use in the majority of cases. A freely available and immunologically compatible bone mimetic with the properties of live tissue is likely to substantially improve the outcome of spine fusion procedures without the disadvantages of autologous bone graft.

PURPOSE—This study aimed to compare a live human bone tissue analog with autologous bone grafting in an immunocompromised rat model of posterolateral fusion.

DESIGN/SETTING—This is an in vitro and in vivo preclinical study of a novel human stem cell–derived construct for efficacy in posterolateral lumbar spine fusion.

METHODS—Osteogenically enhanced human mesenchymal stem cells (OEhMSCs) were generated by exposure to conditions that activate the early stages of osteogenesis. Immunologic characteristics of OEhMSCs were evaluated in vitro. The secreted extracellular matrix from

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OEhMSCs was deposited on a clinical-grade gelatin sponge, resulting in bioconditioned gelatin sponge (BGS). Bioconditioned gelatin sponge was used alone, with live OEhMSCs (BGS +OEhMSCs), or with whole human bone marrow (BGS+hBM). Efficacy for spine fusion was determined by an institutionally approved animal model using 53 nude rats.

RESULTS—Bioconditioned gelatin sponge with live OEhMSCs did not cause cytotoxicity when incubated with immunologically mismatched lymphocytes, and OEhMSCs inhibited lymphocyte expansion in mixed lymphocyte assays. Bioconditioned gelatin sponge with live OEhMSC and BGS+hBM constructs induced profound bone growth at fusion sites in vivo, with a comparable rate of fusion with syngeneic bone graft (negative [0 of 10], BGS alone [0 of 10], bone graft [7 of 10], BGS+OEhMSC [10 of 15], and BGS+hBM [8 of 8]).

CONCLUSIONS—Collectively, these studies demonstrate that BGS+OEhMSC constructs possess low immunogenicity and drive vertebral fusion with efficiency matching syngeneic bone graft in rodents. We also demonstrate that BGS serves as a promising scaffold for spine fusion when combined with hBM.

Keywords

Adult stem cells; Animal model; Scaffolds; Stem cells; Tissue engineering; Vertebral fusion

Introduction

Back pain caused by degenerative disc disease is the leading cause of disability in adults, with a rising cost of \$100 billion annually in the United States [1,2]. This is also a global problem, as demonstrated by the World Health Organization's declaration that back pain is an epidemic, affecting 80% of the population at some point in their lives [3]. Although the majority of back and neck issues can be treated conservatively, a significant proportion of patients go on to develop progressive issues including instability, deformity, neurologic deficit, and intractable pain [4,5]. The treatment in severe cases is arthrodesis (or fusion), where vertebrae are fixed together by an endoprosthesis, and an osteoconductive material is positioned between the vertebrae in an attempt to prompt bone growth. According to the National Center for Health Statistics, more than 650,000 spinal fusion surgeries are performed annually in the United States [6]. Posterolateral lumbar arthrodesis is the most common type fusion performed, and yet there is a failure rate of 15%–40% [7,8]. Failed procedures cause additional pain, damage, and often require complex and expensive corrective surgery [9–11].

One of the most common strategies to improve bone healing in spine surgery is to use supraphysiological doses of bone morphogenetic protein (BMP) to force the differentiation of osteoprogenitors into osteoblasts [12–14]. It is effective, but BMP can cause significant and even life-threatening complications such as airway edema, heterotopic bone formation in the spinal canal, osteolysis, seroma and hematoma formation, radiculopathy, and severe inflammatory responses [15–20]. Composites of bone marrow and bone fillers are safer, but achieving reproducibility has been a limiting factor because success is contingent on the quality of the bone marrow and the biocompatibility of the filler [21]. Indeed, bone substitutes such as synthetics or decellularized bone [22] are abundantly available, but they

frequently exhibit poor osteoconductivity, poor cell retention, and immunologic rejection [23–26]. Autologous bone graft from the iliac crest [24] is very effective, but the amount of available material is often insufficient and the additional surgery comes with significant morbidity. Allografts harvested from cadaveric bone tissue have been employed as an alternative to autograft [27], but the source of the material raises concerns regarding donor variation, time from death, unknown effects of freezing, and potential pathogen transmission. Sterility is also a major concern, with studies reporting that the rate of bacterial and fungal isolates from cadaveric allograft musculoskeletal tissue samples is higher than that from living donors, reaching 25% in some cases [28–32].

Bone healing requires the presence of osteogenic cells, a scaffold that helps localize and maintain the cells, and appropriate stimuli that promote osteoblastic activity. Although all of these components are available in limited volumes in autograft, the need for large volumes of biologically active bone graft without increased patient morbidity makes a composite comprising osteogenic cells and an optimized scaffold an attractive method to mimic a natural bone graft. However, the generation of such a product has been hampered by reproducibility, biocompatibility of the scaffold, immune rejection, and poor preservation techniques. Mesenchymal stem cells (MSCs) can be readily cultured from human bone marrow (hBM) by their adherence to tissue culture plastic and they have the capacity to differentiate into osteoblasts when the tissue culture media is modified [33–37].

Traditionally, cytotherapy for bone tissue has been performed using osteoprogenitor cells that are derived from cultured human MSCs (hMSCs). However, hMSCs have a great degree of variation in osteogenic potential that can be exacerbated by variations in culture technique [38–41]. One method to improve osteogenic efficacy is to accelerate intracellular wingless (Wnt) signaling, which is a key regulator of osteogenic signal transduction in hMSCs [36,42–44]. This can be achieved by a number of means [45], but we have capitalized on the inhibitory cross talk that occurs between the adipogenic regulator peroxisome proliferator-activated receptor- γ (PPAR γ) and the Wnt signaling in hMSCs [46–48]. When PPAR γ is blocked by the inhibitor 2-chloro-5-nitrobenzanilide (GW9662) [49], the inhibitory cross talk on the Wnt pathway is released, thus enhancing Wnt signaling and osteogenesis in hMSCs [50]. Using this approach, we have generated a form of hMSC that has enhanced osteogenic potential (hereafter referred to as osteogenically enhanced hMSCs, or OEhMSCs) and the enhanced capacity to generate substantial amounts of extracellular matrix (ECM) containing a cocktail of collagen types found primarily in anabolic bone tissue. When the ECM is combined with the OEhMSCs, the material has excellent osteogenic capacity and thus could serve as an effective replacement for bone graft [51,52].

Over the last decade, MSC biology has been revolutionized by the discovery that like many primitive cell types, MSCs not only resist immunologic rejection but also have the capacity to inhibit a wide range of immune cells such as T and B lymphocytes, macrophages, neutrophils, and dendritic cells [53,54]. This suggests that a standardized allogeneic hMSC-derived implant would be feasible and if adequately cryopreserved, could be distributed to the point of care from a central manufacturing facility.

In this study, we describe a synthetic live bone tissue analog that could replace autologous iliac crest bone grafting. The analog consists of a non-mineralized scaffold composed of

OEhMSC-derived ECM proteins attached to a gelatin sponge (Gelfoam), thus creating a bioconditioned gelatin sponge (BGS). Two different sources of osteogenic cells were used with the BGS: live preparations of OEhMSCs and hBM aspirate.

Materials and methods

Detailed descriptions of methods can be found in the supplemental information.

Materials

Unless otherwise stated, reagents and materials were purchased from Sigma-Aldrich (St Louis, MO, USA).

Culture of MSCs

Bone marrow (2–5 mL) was collected with informed consent from human donors undergoing spine fusion procedures at Baylor Scott and White Hospital (Temple, TX, USA), in accordance with an institutionally approved human subjects protocol. Mesenchymal stem cells were generated from the nucleated fraction of bone marrow, as previously described [37].

Generation of OEhMSCs

Osteogenically enhanced human mesenchymal stem cells were generated by culture for 8 days in the presence of 10 μ M GW9662 and osteogenic supplementation, as previously described [50–52].

Generation of BGS

Bioconditioned gelatin sponge was generated by growth of OEhMSCs on clinical-grade Gelfoam (Pfizer, NY, USA or Ethicon, NJ, USA) followed by decellularization, as previously described [52].

OEhMSC viability studies

Briefly, 250,000 green fluorescent protein (GFP)-labeled MSCs were allowed to adhere to 0.25 cm³ BGS, and 10,000 or 50,000 peripheral blood mononuclear cells (PBMNCs) from an immunologically unrelated human donor were added to the cultures. After 3, 5, and 7 days, the BGS constructs were washed in PBS and total RNA was extracted using a commercially available kit (High Pure, Roche Diagnostics, Indianapolis, IN, USA). The number of surviving OEhMSCs was determined by real-time reverse transcriptase polymerase chain reaction for the human GFP transcript.

Mixed lymphocyte reactions

Mixed lymphocyte reactions were performed using human PBMNCs, as previously described [55]. Proliferation of lymphocytes was determined by the carboxyfluorescein succinimidyl ester dilution method.

Macrophage activation assays

Human MSCs or OEHMSCs were cocultured with GFP-labeled murine macrophages in the presence of $0.5 \mu\text{g mL}^{-1}$ lipopolysaccharide (LPS) for 15 hours. Interleukin 6 and tumor necrosis factor alpha levels in media were determined by enzyme-linked immunosorbent assay. Values were normalized to the number of macrophages by measuring the intensity of GFP and comparing the value with known standards.

Posterolateral fusion model

All animal procedures were performed in accordance with an approved animal use protocol from the Baylor Scott and White Animal Care and Use Committee. Fifty-three 6-week-old female athymic nude rats (Hsd:RH-Foxn^{nu}) were distributed between subgroups in accordance with previously described data [56] and our pilot studies. A bilateral inter-transverse process fusion at L5–L6 was performed on the rats as previously described [56], with some minor modifications.

Immunophenotyping

Mesenchymal stem cells were immunophenotyped as previously described [51] using a Cytomics FC500 flow cytometer (Beckman Coulter, Indianapolis, IN, USA) and data were processed using the manufacturer's software (CXP, Beckman Coulter).

Differentiation of MSCs

Differentiation of hMSCs from osteoblasts, adipocytes, and chondrocytes was performed using established techniques [37].

Manual palpation

The explanted spines were manually tested for intersegmental motion by the three blinded observers [56,57]. When dorsal-ventral or lateral motion was detected between the facets or between the transverse processes, this indicated failure of fusion. Interobserver variation was evaluated statistically by multi-rater free marginal kappa test [58–63]. Kappa value was 1.0 for all comparisons, except for the BCS+OEHMSC group with a score of 0.91.

Microcomputed tomography

Spines were then subjected to tomographic scanning using a Skyscan 1174 high-resolution specimen imager (Bruker, Kontich, Belgium). In method 1, volumetric measurements on the axial sections between the posterior region of the L6 vertebrae and the anterior region of the L4 vertebrae were generated and presented as a ratio of bone volume to total tissue volume. In method 2, the ratio between the lateral process volume and the core vertebral volume was measured.

Histology

After microcomputed tomography (μCT) scanning, spines were fixed, decalcified, embedded, and sectioned using standard techniques [52]. Masson trichrome staining was performed using a commercially acquired kit (American MasterTech Scientific Inc., Lodi, CA, USA). Micrographs were generated using an upright microscope (Nikon Eclipse 80i

fitted with a Retiga 2000 camera) running digital imaging software (Elements, Nikon, Melville, NY, USA).

Statistics

Parametric data were analyzed by one-way analysis of variance with Tukey or Dunnett posttest, where indicated. Binary (fusion) data were tested by two-tailed Fisher exact test using the method of summing small p-values. For analysis of variance and Fisher tests, a p-value of $<.05$ was considered statistically significant. Analysis of variance and Fisher exact tests were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA; www.graphpad.com), and assumptions of statistical tests were verified by the software. Testing of interobserver agreement was performed with the multi-rater free marginal kappa test [61–63]. A kappa coefficient of 0.9 was considered reliable.

Results

Immunologic properties of OEhMSCs and BGS

If BGS+OEhMSC constructs generated from universal donors are to be used as an “*off-the-shelf*” tool for fusion, immunologic rejection must not occur in allogeneic recipients. Human MSCs are excellent candidates for allogeneic tissue engineering because they are potent immunomodulators and resist immune rejection [64–66]. As such, we speculated that OEhMSCs may share the immunologic characteristics of hMSCs and performed a series of experiments to address this possibility.

Recently, we confirmed that OEhMSCs do not inherently activate proliferation of immunologically mismatched lymphocytes [52], but it was unclear whether lymphocyte exposure had a detrimental effect on OEhMSC viability. To address this question, GFP-labeled OEhMSCs were attached to BGS and exposed to immunologically mismatched PBMNCs. After 3, 5, and 7 days, the constructs were recovered, and real-time reverse transcriptase polymerase chain reaction for GFP transcript was employed to enumerate cells. We determined that over 7 days, immunologically mismatched PBMNCs did not affect the overall number of viable OEhMSCs attached to the BGS (Fig. 1A). Assays of apoptosis or necrosis demonstrated a marginal degree of cell death during the experiments that did not appear to be related to the addition of PBMNCs. Therefore, these data suggest that if OEhMSCs are implanted into allogeneic recipients, the likelihood of rapid immunologic rejection is low.

Several studies have demonstrated a potent immunomodulatory role for hMSCs. Indeed, hMSCs have been shown to actively inhibit lymphocyte expansion under immunostimulatory conditions [64]. To determine whether this is the case for OEhMSCs, we performed mixed lymphocyte reactions. After labeling of two PBMNC populations with CFSE and mixing, lymphocyte proliferation was detected by flow cytometry as a function of CFSE depletion. In these assays, the presence of hMSCs, OEhMSCs, and BGS+OEhMSC constructs inhibited expansion of mixed populations of MNCs (Fig. 1B) at a ratio of 1 MSC to 100 MNCs.

Human MSCs have also been shown to inhibit the secretion of inflammatory cytokines by macrophages under certain stimulatory conditions such as exposure to bacterial LPS. To determine whether OEhMSCs had the capacity to diminish the inflammatory capacity of macrophages, they were cultured in the presence of an LPS-activated murine macrophage cell line, and secretion of tumor necrosis factor alpha and interleukin 6 was measured. Osteogenically enhanced human mesenchymal stem cells inhibited secretion of both cytokines by macrophages, but to a lesser extent than conventional hMSCs (Fig. 1C,D).

Collectively, these data demonstrate that OEhMSCs are not destroyed by immunologically unrelated lymphocytes and may have the capacity to inhibit the cellular processes that contribute to inflammation and immune rejection.

Contribution of OEhMSCs and BGS to vertebral arthrodesis in an immunocompromised rat model

We successfully reproduced the spine fusion protocol of Grauer et al. [56] using athymic nude rats, with some minor modifications.

To generate clinically relevant positive control data, we implanted morselized iliac bone derived from syngeneic donor rats (approximately 500 mg per side) to the lateral processes of the L5 and the L6 vertebrae. Each graft was supplemented with 5 million syngeneic whole bone marrow cells delivered in 200 μ L of human plasma activated to clot with thromboplastin. We reasoned that this approach mimicked the use of autologous bone graft in humans. Under these conditions, we achieved 7 of 10 L5–L6 fusions as determined by manual palpation and μ CT scanning (Table, Fig. 2, Bottom). Controls that received decortication and plasma, but no graft or cells, did not fuse (Table, Fig. 2, Top).

The first series of experimental subjects (n=15) received BGS (approximately 1 cm³ per side, packed bilaterally along L5–L6 as $\approx$ 5 mm³ fragments) and 2 million (per side) OEhMSCs delivered in 200 μ L of activated human plasma. The BGS+OEhMSC constructs were well received by the immunocompromised rats and there were no signs of inflammation or immobility. After 8 weeks, 10 of 15 fusions were achieved accompanied by extensive bone growth along the lateral processes as determined by manual palpation and μ CT scanning (Table, Fig. 3, Bottom). Unlike the bone graft control group (Fig. 2, Bottom), it appeared that the fusion masses were entirely the result of de novo bone because these animals did not receive mineralized scaffold and the BGS is radiolucent on implantation. To determine whether BGS had inherent activity as an osteogenic agent, we repeated the experiments in the absence of OEhMSCs (n=10). After 8 weeks, we observed no sign of fusion in any of the subjects but there were signs of slight remodeling of the transverse processes in five cases (Fig. 3, Top). These data demonstrate that BGS+OEhMSCs constructs fuse the transverse processes vertebrae with efficiency equivalent to syngeneic bone graft and do not require the presence of a mineralized substrate.

Contribution of hBM and BGS to vertebral arthrodesis in an immunocompromised rat model

We reasoned that in some cases, autologous bone marrow would be the preferred source of progenitor cells and we questioned whether BGS could serve as an effective retention

scaffold for the cells in a manner similar to that observed for OEhMSCs. We therefore tested whether cryopreserved, concentrated hBM could be used with BGS to facilitate vertebral fusion. To perform the experiments, we prepared 200 million excess hBM cells from a single human donor. The hBM cells were purified by discontinuous density gradient centrifugation and cryopreserved at a concentration of 10 million cells per milliliter. When recovered from cryopreservation, viability was 80% and plastic-adherent hMSCs could be cultured from the hBM with the capacity for osteogenic, chondrogenic, and adipogenic differentiation (Fig. 4A–C), and the expected immunophenotype (Fig. 4D) [67].

We then performed the in vivo fusion assay with 5×10^6 hBM (per side, in 200 μ L activated plasma) and BGS in an identical manner to the OEhMSC experiments. After 8 weeks, fusion was evident in 8 of 8 rats accompanied by extensive de novo bone growth as determined by manual palpation and μ CT scanning (Fig. 4E, Table). Together, these data demonstrate that BGS has the capacity to support fusion by unfractionated hBM as well as OEhMSCs.

Histologic study

Specimens were sectioned and stained with Masson trichrome to evaluate histologic characteristics of the de novo bone within the fusion beds. As expected, specimens in the syngeneic bone graft group (Fig. 5, Left) consisted of large fragments of mature bone (blue) that were likely to originate from the donated tissue, with intermittent immature osteoid (red) that was generally contiguous with the mature bone. Fibrous tissue and cartilage was also evident in rare cases. In the BGS+OEhMSC group (Fig. 5, Middle), extensive areas of thick trabecular bone at various stages of maturity could be observed. Clusters of chondrocytes in cartilaginous lacunae were also evident, suggesting that bone had formed by endochondral ossification. Specimens that received BGS+hBM (Fig. 5, Right) were histologically distinct from both the bone graft controls and the BGS+OEhMSC groups. The fusion beds consisted of a bony exterior composed of mature and immature osteoid that enclosed diffuse and immature trabecular bone with an extensive marrow cavity. It is possible that the reduced frequency of osteoprogenitors contained in whole bone marrow aspirates as compared with the bone graft and the BGS+OEhMSC groups could account for the apparent delay in bone maturation.

Statistical analysis of fusion frequency

Frequency of fusion was determined by three individual observers, and interobserver agreement was confirmed by kappa testing. Fusion frequencies were then tested for statistical significance by Fisher exact test (Table). When all groups were tested against the no-intervention group (decortication and plasma only), frequency of fusion in the bone graft, BGS+OEhMSCs, and BGS+hBM groups was found to be significantly greater ($p=.0031$ – $.0001$). The BGS-only group was statistically equivalent to the no-treatment controls. When groups were tested against the bone graft control, frequency of fusion was statistically equivalent to bone graft for the BGS+OEhMSCs and BGS+hBM groups, but bone graft was found to be superior to both the BGS-only and the no-intervention groups. Of the groups that demonstrated fusion (bone graft, BGS+OEhMSC, and BGS+hBM groups), no significant differences between fusion frequencies were evident.

Quantification of bone growth

Several attempts were made to reproducibly distinguish the new bone from the mature transverse processes, but the outgrowths were completely contiguous with original bone in most specimens. Complex region of interest templates based on control specimens were initially designed, but slight variations in animal size introduced irregularity to measurements. Therefore, we employed methods that compared total volumes of fused vertebrae rather than subjectively determining the volume of de novo bone. This was achieved by μ CT scanning spine explants and performing volumetric measurements on the axial sections between the posterior edge of the L6 vertebrae and the anterior edge of the L4 vertebrae. To normalize for variations in specimen size and composition, we processed the data in two ways. In method 1, we employed a standard approach, calculating the ratio of bone volume to total tissue volume (Fig. 6A,C). We also developed method 2 in an attempt to internally standardize measurements (Fig. 6B,D). In this protocol, we measured the volume of the lateral processes (where the fusion masses are located) and the volume of the core region of the vertebrae (the body, facets, and spinous process) separately and calculated the ratio between the lateral process volume and the core vertebral volume (eg Fig. 6D). The method makes the assumption that in the model, the core vertebral structures are unaffected by the procedure, whereas the fusion masses are confined to lateral processes and adjacent regions. This assumption was supported by qualitative assessment of several axial cross sections. We found that method 2 was faster than classical methods, less subjective, and because it is internally controlled, it is independent of slight variations in radio-opacity that can occur between samples that differ in soft tissue and water content.

We found that the data were qualitatively equivalent for both methods (Fig. 6A,B). With the exception of the BGS-only group, we found that all experimental groups had significantly larger vertebral volumes when compared with the no-intervention controls. Furthermore, there was no significant difference between the bone graft controls and the experimental conditions that received cells and BGS, with one exception: the ratio of bone volume to tissue volume was significantly increased in the hBM group compared with the ratio in the autograft group ($p < .01$). It should be noted that in the case of the autograft control, it is not possible to distinguish between de novo bone and mature, donor-derived bone tissue, and as a result, the volume of new bone generated in the autograft control is likely to be overestimated. In contrast, no mineralized tissue was implanted in those animals that received BGS, and elevated values will reflect the presence of entirely new bone tissue. Overall, the results demonstrated that in the presence of a suitable progenitor cell type (OEhMSCs or hBM), BGS has the capacity to generate bony fusion masses with an efficiency equivalent or superior to syngeneic bone graft.

Discussion

Our results suggest that we are close to solving some of the problems associated with donor variation and scaffold bio-compatibility that limit the current widespread use of hMSCs in bone tissue engineering. Specifically, our protocol for the generation of OEhMSCs reduces the effects of donor variation by maximizing the osteogenic potential of hMSCs, and the unique composition of BGS provides the necessary biological complexity that seems to be

necessary for maintenance of cell retention, functionality, and survival [51]. Furthermore, BGS is generated from OEhMSCs that mimic primitive osteoprogenitor-like cells, and prior in vitro assays suggest that it better resembles the composition of anabolic osteoid than bone fillers generated from cadaver tissue or synthetic substrates. Indeed, in related studies, we could demonstrate that the functional constituent of BGS could accommodate more hMSCs per unit volume than several synthetic bone mimics without subjecting cells to extremes of pH or scaffold dissolution that was observed with many of the currently used products [52,68].

Because OEhMSCs do not activate allogeneic lymphocytes, inhibit lymphocyte expansion, and also inhibit secretion of inflammatory cytokines by macrophages, the constructs are not likely to be rapidly rejected by the human immune system. These characteristics, albeit demonstrated in vitro, suggest that BGS+OEhMSCS may therefore serve as promising allogeneic constructs. A live allogeneic bone construct is likely to satisfy a great need in orthopedic medicine, particularly in those patients with limited ability for healing such as the elderly, patients with osteoporosis, or patients with diabetes. One drawback of this study is that we did not use immunocompetent animals to determine whether OEhMSCs indeed do have the capacity to evade immune rejection, but the goal of the current study was to initially assess efficacy of OEhMSCs and BGS. Nevertheless, although in vitro assays lack the complexity of in vivo studies, the data presented herein justify further investigation of the fate of OEhMSCs in immunocompetent systems. For example, it would be informative to examine how the putative anti-inflammatory effects of OEhMSCs influence the healing process. On one hand, OEhMSCs could attenuate serious inflammation occurring because of extreme trauma, but on the other hand there could be delays in healing, given the necessity for a transient inflammatory phase during fracture repair [69]. Given the extent of osteogenesis seen in the experimental groups, we predict that modest inhibitory effects on inflammation would have a negligible effect on healing, but it does raise the question whether a small reduction in the regenerative inflammatory response represents a reasonable compromise for allogeneic compatibility. We also acknowledge the need to confirm these findings in an immunocompetent model system that is less permissive to engraftment of foreign biomatter.

In previous studies, we determined that OEhMSCs alone or attached to collagen I-based carriers do not result in the levels of osteogenesis seen with OEhMSCs attached to scaffolds containing OEhMSC-derived ECM. These experiments were performed in a murine calvarial defect model [51] and a femoral segmental defect model [52], both of which permitted accurate volumetric measurement of de novo bone, a task that could not be accomplished in the spine fusion model. Given past observations in more quantitative experimental systems, and in the interests of animal preservation, we did not perform controls with Gelfoam scaffolds alone or with OEhMSCs alone in the spine fusion model.

Mesenchymal stem cells in various forms have been tested for efficacy in spine fusion with a range of reported outcomes. The majority of studies have employed rabbit or rat bone marrow-derived plastic-adherent cells that were cultured for a relatively short time before utilization in immunocompetent recipients [70–75]. Data from these studies strongly suggest that MSCs from bone marrow have the capacity to drive fusion of vertebrae, but outcome is

critically dependent on dosage and culture conditions. For example, Minamide et al. [70] demonstrated that high doses of plastic-adherent cells (1×10^8) were necessary to observe fusion with equivalent efficacy to autograft or BMP administration. Furthermore, they reported that lower doses of cells (1×10^6) were ineffective. Indeed, the majority of the literature strongly supports the need for preconditioning or enhancement of osteogenic capability to dismiss the requirement for excessive cell doses. To this end, genetic modification of osteoprogenitor cells to allow the cells to produce bioactive molecules such as BMP2 [71,76,77] or vascular endothelial growth factor [71] has been performed with some success. However, as with any form of genetic modification, there are serious safety concerns. Pretreatment of MSCs with classical osteogenic differentiation cocktails containing beta-glycerophosphate, ascorbate, and dexamethasone is a feasible approach, but results are variable [74,75], indicating that osteogenic pre-differentiation can improve or inhibit spine fusion in a manner that is dependent on the nature of the initial cell preparation and the culture conditions employed. Although supraphysiological doses of BMP2 are associated with safety concerns, it has been suggested that a combination of osteoprogenitors with very low doses of BMP2 could be effective. Indeed, when plastic-adherent MSC-enriched preparations are coadministered with low concentrations ($<5 \mu\text{g mL}^{-1}$) of BMP2, the rate of fusion can be comparable with autograft [72,75]. Bae et al. [78] also recently demonstrated that the osteogenic capability of unfractionated bone marrow can be profoundly enhanced with low-dose BMP2 (0.006 mg mL^{-1}), too. The doses of BMP2 required to enhance MSC activity in these reports are approximately one to two orders of magnitude lower than those typically employed in clinical procedures and therefore are likely to have a much improved safety profile. Nevertheless, transient preconditioning of MSC cultures with BMP2 and basic fibroblast growth factor is reported to improve MSC efficacy in experimental spine fusion [73], suggesting that direct administration of recombinant agents to the surgical site may be unnecessary if appropriate cells and biologically active scaffolds are employed.

In the current study, we used a PPAR γ inhibitor to upregulate Wnt signaling in hMSCs, thereby activating a primitive osteogenic phenotype. This does not initiate terminal, mineralizing osteogenesis seen with dexamethasone-mediated protocols, but rather promotes secretion of bioactive molecules such as ECM components and osteogenic or angiogenic protein ligands [50–52]. Our data suggest that the resultant OEhMSCs are partially differentiated, retaining the beneficial engraftment and survival characteristics of MSCs while possessing osteogenic properties. When coadministered with a scaffold composed of their own ECM components (in this study, BGS), OEhMSCs remain attached to the BGS and secrete factors into the surrounding injury, supporting osteogenesis under appropriate homeostatic control. Osteogenically enhanced human mesenchymal stem cells probably enhance osteogenesis by the secretion of several factors, but in particular, we have detected enhanced secretion of BMP2 by OEhMSCs when attached to BGS [52]. For example, in a previous in vitro study, we detected approximately $5.6 \text{ fg cell}^{-1} \text{ day}^{-1}$ output from OEhMSCs when attached to OEhMSC-derived ECM. If we predict that of the 2×10^6 OEhMSCs that were administered to each site in the fusion model 50% survive for 24 hours, this could result in secretion of $5 \mu\text{g}$ of BMP2 in addition to other beneficial factors. Whole bone marrow contains a minute proportion of MSC-like osteoprogenitors, but when

administered with BGS, a potent osteogenic response was observed. It is noteworthy to add that BMP2 attached to OEhMSC-derived ECM can be detected when analyzed by immunoblotting, and a few MSCs could be recovered from the hBM specimen used in the study. It is possible that BMP2 and other factors covalently attached to BGS could elicit a potent stimulus to the osteoprogenitor and angiogenic cells in bone marrow, suggesting that BGS could represent a powerful and biocompatible scaffold for bone regeneration in autologous settings, too.

It is important to note that BGS is entirely radiolucent on administration. Therefore, all bone generated in the experiments should be regarded as *de novo*. This is in contrast with the corticocancellous iliac grafts used in positive controls. Because the initial graft was detectable radiographically in these specimens, whereas BGS composites are radiolucent on implantation, it is probable that volumetric measurements significantly underestimate the regenerative potential of OEhMSCs and BGS. We therefore propose that although BGS may lack the initial physical properties of bone, it has potential advantages over hard scaffolds owing to its superior bioactivity and its ability to clearly define new bone growth radiographically. If initial strength and rigidity is absolutely required, OEhMSC-derived ECM could be attached to existing orthopedic materials to promote healing via enhanced surface interactions with osteoprogenitors and angiogenic cells.

When the mass of the rats was accounted for as compared with human recipients, the amounts of material deposited in each fusion bed were significantly larger as compared with what would be used in the clinical setting. This was done to maximize the effectiveness of the syngeneic graft in an attempt to reflect success rates of autograft typically observed with human patients. Larger doses of fusion material are necessary to account for the contrasting anatomy of rat as compared with humans, and the lack of accessories that confine and localize the osteogenic fusion materials that are commonly used in human procedures. In our opinion, it is quite possible that far less material would be required in the clinical setting with appropriate technology for fixation and biomaterial delivery.

In conclusion, we demonstrate that both OEhMSCs and unfractionated bone marrow have excellent osteogenic capacity when placed on a biologically active scaffold coated with ECM generated by the OEhMSCs themselves. The OEhMSCs and their ECM materials did not elicit significant immune responses and can even inhibit certain immune reactions, suggesting that they could be used as a virtually limitless allogeneic cell source for spine fusion procedures. To our knowledge, this is the first study demonstrating an approach of this kind.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix: Supplementary material

Supplementary material related to this article can be found at <http://dx.doi.org/10.1016/j.spinee.2016.10.009>.

Fig. 1.

Immunologic characterization of OEhMSCs and BGS. (A) Viability of BGS+OEhMSC constructs after incubation with 0, 10,000, and 50,000 MNCs for 3, 5, and 7 days. Measurements are mean cell numbers with standard deviations. Statistics are ANOVA with Tukey posttest (data not significantly different). (B) Mixed lymphocyte reaction (MLR) assays in the presence of conventional hMSCs, OEhMSCs, or BGS+OEhMSCs at a ratio of 1 MSC to 100 MNCs. In the absence of MSCs, the mixed lymphocytes proliferate, causing dilution of CFSE and multiple distinct populations of progeny. Mesenchymal stem cells inhibit proliferation, resulting in a single population of fully labeled MNCs that have not divided. (C, D) Enzyme-linked immunosorbent assay (ELISA) to detect inflammatory mediators tumor necrosis factor alpha (TNF α) and interleukin (IL6) secreted by murine macrophages. The presence of cocultured hMSCs or OEhMSCs reduce TNF α and IL6 output. Measurements are mean TNF α or IL6 output normalized to macrophage number and culture duration with standard deviations. Statistics are ANOVA with Dunnett posttest (* p < 0.05, ** p < 0.01).

Fig. 2.

Vertebral fusion in athymic nude rats with syngeneic iliac bone graft and bone marrow. Representative μ CT scans of the lumbar region of rat spines. The axial views (left in each panel) refer to the intervertebral region between L4 and L5 (above, left) and between L5 and L6 (below, left) in each case. X-ray scans (center and right in each panel) represent coronal plane and sagittal plane views respectively. (Top) Images represent a negative control rat that received decortication and activated plasma only (no intervention). (Bottom) Images represent a positive control rat that received syngeneic iliac bone graft and syngeneic bone marrow (bone graft).

Fig. 3.

Vertebral fusion in athymic nude rats with BGS and OEhMSCs. Representative μ CT scans arranged as in Fig. 2. (Top) Images represent a rat that received decortication, activated plasma, and BGS only (BGS only). (Bottom) Images represent a rat that received decortication, BGS, and 1×10^6 OEhMSCs per side in activated plasma (BGS+OEhMSC). Note the large fusion masses in those rats that received OEhMSC and BGS (Bottom) consisting entirely of de novo bone, whereas bone fragments were added to the syngeneic bone graft controls (Fig. 2, Bottom).

Fig. 4.

Vertebral fusion in athymic nude rats with BGS and hBM. (A–C) Differentiation of hMSCs recovered from cryopreserved bone marrow samples. Cryopreserved vials of 10 million hBM cells were recovered in monolayer culture, and hMSC colonies were expanded from them. The hMSCs were then subjected to standard osteogenic (A) and adipogenic (B) differentiation assays on confluent monolayers or micro-mass chondrogenic assays (C). Mineralized nodules were stained with alizarin red S, lipid-filled vacuoles were stained with oil red O, and proteoglycans were stained with toluidine blue borate. Original magnification: 10× (A, B) and 4× (C). (D) Immunophenotyping of the hBM-derived MSCs by flow cytometry confirms expression or absence of currently accepted surface markers for hMSCs as defined by Dominici et al. [67]. Gates represent signal intensity above isotype controls. (E) Representative μ CT scans were arranged as in Fig. 2. Images represent a rat that received decortication, BGS, and 1×10^7 hBM cells per side in clotted plasma (BGS+hBM). Note large fusion masses consisting entirely of de novo bone.

Fig. 5.

Histologic analysis of fusion sites. Specimens were decalcified, embedded in paraffin, and sectioned in the transverse plane. Sections represent a region of the L5 vertebrae adjacent to the L5–L6 intervertebral space. SC represents the location of the spinal canal.

Fig. 6.

Quantitative comparisons of vertebral bone formation. (A) Ratio of bone volume to total tissue volume between the L4 and the L6 vertebrae (method 1 in text). (B) Ratio of lateral vertebral volume to core vertebral volume (method 2 in text). (C) Depiction of the ROI used to calculate measurements in method 1. (D) Depiction of the lateral ROI and core ROI used to determine measurements for method 2. (A, B) Box and whisker plots, where the boxes represent 25%–75% of the range of values, the bars represent the full range, and the internal lines represent the mean. Statistics, one-sided ANOVA with Tukey multi-comparison posttest. * $p<.05$, ** $p<.01$, *** $p<.005$.

Table

Frequency of fusion determined by manual palpation and statistical analysis

Group	Fusion frequency	p-Value versus no intervention*	p-Value versus bone graft*
Bone graft	7/10	.0031	n/a
No intervention	0/10	n/a	.0031
OEhMSC+BGS	10/15	.001	n/a
hBM+BGS	8/8	.0001	0.2157
BGS only	0/10	n/a	.0031

OEhMSC, osteogenically enhanced human mesenchymal stem cell; BGS, bioconditioned gelatin sponge; hBM, human bone marrow.

* Two-sided Fisher exact calculations performed using GraphPad Prism by using the method of summing small p-values. Significantly different data are indicated in bold.