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Factors of Osteogenesis Influencing Various Human Stem Cells on Third-Generation Gelatin/β-Tricalcium Phosphate Scaffold Material

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Abstract

Human bone marrow-derived stem cells (hBMSCs) and adipose-derived stem cells (hASCs) have been used to regenerate bone. Both sources are claimed to have comparable osteogenic potential, but few comparative studies are available. Third-generation biomaterials have been developed to reduce steps in regenerating tissues. For osteogenesis gelatin/β-tricalcium phosphate (β-TCP) scaffolds with incorporated controlled-release bone morphogenetic protein-2 (BMP-2) as third-generation biomaterials were recently developed. So far, few studies on protein-induced osteogenesis versus chemical-induced osteogenesis have been performed. This study evaluates the osteogenic potential of hBMSCs versus hASCs derived on gelatin/β-TCP scaffolds *in vitro* under four different conditions. Gelatin/β-TCP scaffolds with and without incorporated controlled-release BMP-2 were seeded with hBMSCs or hASCs under oscillating fluid conditions in osteogenic (OS) medium or growth medium (GM). All were evaluated radiologically (computed tomography [CT] scan), histologically, biomechanically, and for gene expression at 1, 2, 4, and 6 weeks. The highest radiological densities were seen in specimens at 6 weeks with controlled-release BMP-2, close to native bone. HBMSCs, hASCs, OS, and GM conditions resulted in similar bone formation with gelatin/β-TCP scaffolds and incorporated controlled-release BMP-2. This was confirmed histologically by Toluidine Blue and van Kossa staining and biomechanically. Gene expression studies of these specimens showed the presence of preosteoblasts, transitory osteoblasts, and secretory osteoblasts. Specimens comprised of gelatin/β-TCP scaffolds without incorporated controlled release BMP-2 in OS medium showed lesser bone formation. hASCs and hBMSCs have similar osteogenic potential. hASCs are an attractive alternative to hBMSCs for bone regeneration using third-generation gelatin/β-TCP scaffolds with incorporated controlled-release BMP-2.

Introduction

Bone tissue usually has the ability to repair itself, but repair attempts may fail, especially in large bone defects such as after tumor resections. This results in the formation of a pseudarthrosis, nonunion of the fracture, and loss of function, requiring surgical intervention.[1] Autologous bone transplantation is the most effective method for bone restoration, because it provides three essential elements: Osteoconduction, osteoinduction, and osteogenic cells. However, the supply of these cells is very limited.[2,3] Allografts are believed to be osteoconductive, but they confer the risk of disease transmission and immune rejection.[4] Bone cement is readily available and does not cause either rejection or disease transmission issues. However, it does not allow bone formation and growth, because it is not biodegradable and may lower the threshold for infection at the surgical site.

Tissue engineering of bone is a new alternative that has the potential to overcome many of the drawbacks of autologous or allogenic bone transplantation. It is possible to create various tissues including bone using autologous cells on a variety of biomaterials.[1] The ideal material will be resorbed and replaced over time by the body's newly regenerated bone tissue.[5] Today several generations of biomaterials are available. First-generation biomaterials are considered materials already on the market for clinical use

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that could support cellular attachment and growth, such as poly(lactic acid) or fibrin glue. The second generation consists of mixtures of materials of the first generation and new materials to improve the formation of new tissue such as β-tricalcium phosphate (β-TCP)/poly(lactic-*co*-glycolic acid) (PLGA), β-TCP/hydroxyapatite (HA), HA/PLGA, etc. The third generation, also referred to as smart biomaterials, consists of first- or second-generation biomaterials with embedded slow-release factors to enhance cellular growth, tissue regeneration, and facilitate the use of undifferentiated stem cells. For mimicking the mineral phase of bone, a variety of bioactive inorganic materials are in the focus of clinical interest: TCP, HA, bioactive glasses, and their combinations.[6,7] To improve cellular attachment and delivery of nutrients to the cells, the scaffold material must be porous. Biomaterials that are porous and are osteoinductive are β-TCP or HA.[6] Another characteristic of biomaterial supporting bone growth is osteoconductivity. There are few porous materials that are osteoinductive and osteoconductive, such as β-TCP or hydroxyapatite.[6] However, the brittle nature of these inorganic materials makes them on their own an inappropriate choice for load-bearing applications.[1]

Poly(ε) caprolactone (PCL), poly(lactic acid) (PLA), or copolymers of PLA and polyglycolic acid (PGA) (i.e., PLGA) are synthetic polymers and offer versatile alternatives. They can be processed by porogen leaching,[8] gas foaming,[8] phase separation,[9] fiber meshing, and three-dimensional printing.[10] Biological polymers, such as hyaluronic acid or collagen, are interesting candidates for tissue engineering bone because they provide innate biological guidance to cells that favors attachment and promote chemotactic responses and the possibility to process them into porous scaffolds.[1,11] Gelatin is a denatured collagen and commercially available as a biodegradable polymer. It has been extensively used for pharmaceutical and medical purposes, and its biosafety has been proven through long-term clinical applications.[12] Some researchers have demonstrated that the surface coating of substrates with gelatin enhances the attachment and proliferation of cells thereon.[13–15]

These findings suggest that gelatin is one of the materials suitable to the substrate of cells. Other advantages of gelatin include the usability of materials with different charges and the easiness of chemical modification. β-TCP is advantageous from the viewpoint of biodegradability. The combination of both materials has shown that cellular attachment, proliferation, and osteogenic differentiation of mesenchymal stem cells (MSCs) were influenced by sponge composition of gelatin and β-TCP as the cell scaffold. Previous experiments have shown advantage in regenerating bone when using a combination of β-TCP and gelatin in a 50:50 proportion and providing additional biomechanical stability.[11] Recently gelatin microparticles as a controlled delivery system have been evaluated for bone morphogenetic protein (BMP-2) release pattern in gelatin/β-TCP scaffolds.[16]

Bone marrow-derived mesenchymal stem cells (BMSCs) and adipose-derived stem cells (ASCs) have high proliferation capacity and multilineage potential; they can be differentiated into osteoblasts and have been used to tissue engineer bone. Additional sources are human umbilical vein embryonic cells (HUVECs) and embryonal stem cells (ESCs). It has been demonstrated that mesenchymal- and adipose-derived stem cells have the capacity to differentiate into bone-like tissue. So far, there has not been a comparison of factors influencing the osteogenic proliferation potential of BMSCs versus ASCs. Therefore, the current study evaluates the influences of osteogenic factors on human BMSCs versus ASCs.

Sophisticated seeding methods for cells have been proven to support bone formation, especially dynamic fluid current aids in the structure and building of bone.[10,17] Previous experiments have shown superior bone formation using dynamic oscillating fluid movements compared to rotational fluid stress.[10] Thus, we decided to apply dynamic oscillating movements to seed two different sorts of stem cells onto porous gelatin-based scaffolds.

The hypotheses of this experiment are: (1) hASCs and hBMSCs have similar osteogenic potential *in vitro* when using the same factors influencing bone formation and (2) combining osteogenic (OS) medium and slow-release BMP-2 increases the bone formation of hASCs and hBMSCs *in vitro*.

Materials and Methods

For all tissue collections, consent from donors and approval of the Hospital Institutional Review Board (IRB) were obtained.

Mesenchymal stem cells

Bone marrow was aspirated from the sternum of 3 patients, ages 44–62, undergoing sternotomy for open heart surgery, and the cells obtained were pooled. Aspiration syringes contained heparin (100 U/mL bone marrow) to prevent clotting. The aspirate was suspended in growth medium (GM) (Dulbecco modified Eagle medium [DMEM] with 10% fetal bovine serum [FBS], 100 U/mL ampicillin, and 100 μg/mL streptomycin), washed three times with phosphate-buffered saline (PBS), and plated in 25-mL cell culture flasks (Corning, NY) for 3 days in GM. During this time, 3 mL of GM was added daily. Attached cells were then expanded using GM until 80% confluence was achieved. MSCs were passaged at a 2:3 ratio and, after a sufficient number was reached, 2×10^5 cells each were differentiated in OS medium for 14 days (GM supplemented with 100 nM dexamethasone, 50 μg/mL ascorbic acid, and 10 mM β-glycerolphosphate; all from Sigma–Aldrich Co., St. Louis, MO unless stated otherwise), chondrogenic medium for 21 days (GM supplemented with 10 ng/mL insulin-like growth factor-β1 [IGF-β1], 10^{-7} M dexamethasone, 50 μM ascorbate-2-phosphate, 40 μg/ml proline, 100 μg/mL pyruvate, and 50 mg/ml ITS + Premix; Becton, Dickinson and Company), and adipogenic medium for 3 days (GM supplemented with 0.5 mM isobutyl methylxanthine, 1 μM dexamethasone, 10 μM insulin, 200 μM indomethacin, and 100 U/mL ampicillin, 100 μg/mL streptomycin). MSCs not used for differentiation into bone, cartilage, and fat cultured in GM were used in the experiment.

Adipose-derived stem cells

Subcutaneous fat was harvested from 5 different burn patients, ages 42–52 years, and suspended in DMEM. The fat was minced, digested in 2% collagenase overnight, and plated in GM for 3 days. Adherent cells were expanded by changing GM every second day until 80% confluence was

achieved. Then cells were passaged in a 2:3 ratio. Subsequently 2×10^5 cells each were differentiated in OS, chondrogenic, and adipogenic medium. hASCs cultured in GM with a viability of more than 90% were used for the experiment.

Scaffolds, BMP-2, and cationized gelatin hydrogel microspheres

All gelatin/β-TCP scaffolds and gelatin hydrogel microspheres were provided by Kyoto University, Department of Biomaterials, Field of Tissue Engineering, Institute for Frontier Medical Sciences, Kyoto, Japan. The material was previously tested and was comprised of denatured collagen I, gelatin, and β-TCP in a ratio of 50:50 for improved biomechanical stability and to increase osteoinductivity.[11] The scaffold was a sponge with interconnected pore structure (200 μm average) to facilitate cellular attachment. Scaffolds were provided as 2-cm×2-cm×2-cm cubes and were cut to 1-cm^3 cubes for the experiment. The experimental group was comprised of additional cationized gelatin hydrogelmicrospheres of 10 μm incorporated in the scaffold (Table 1).

To measure the *in vitro* release of BMP-2, recombinant human BMP-2 (PeproTech Inc., Rocky Hill, NJ) was radioiodinated according to the chloramine-T method.[17a] (Greenwood FC, Hunter WM, Glover TC. The preparation of I-131 labeled growth hormone of high specific radiosensitivity. Biochem J 1963; 89: 114 – 123). Na ^{125}I solution (4 μg) was added to 40 μL of 1 mg/ml BMP-2 solution, containing 5 mM glutamic acid, 2.5 wt% glycine, 0.5 wt% sucrose, and 0.01 wt% Tween 80 (pH 4.5). Then, 0.2 mg/mL chloramine-T potassium phosphate-buffered solution (0.5 M, pH 7.5) containing 0.5 M sodium chloride (100 μL) was added to the solution. After agitation at room temperature for 2 min, 100 μL of PBS (pH 7.5) containing 0.4 mg of sodium metabisulfate was added to the reaction solution to stop the radioiodination. The reaction mixture was passed through an anionic-exchange column to remove uncoupled, free ^{125}I molecules. Recombinant human BMP-2 (PeproTech Inc., Rocky Hill, NJ) was incorporated into the microspheres at 0.25 μg/mL for slow release within the scaffold material over 6 weeks. Then treated microspheres were dispersed in PBS (Hyclone, Logan, UT) and injected into four scaffolds directly before use. Scaffolds injected with microspheres without BMP-2 and scaffolds not treated with microspheres served as controls.

Sixteen groups were formed (Table 1). Two scaffolds each were inserted into one 25-mL flask, then 2×10^6 cells/mL of suspension in OS medium or GM was added to cellular specimens and 5 mL of OS medium or 5 mL of GM without cells were added to acellular controls. All flasks were cultured under dynamic oscillating conditions on a titer plate shaker (Lab-Line Instruments, Melrose Park, IL) at 60 cycles/min. Scaffolds were cultured for 6 weeks and evaluated at 1-, 2-, 4-, and 6-week time points.

In vitro *release evaluation*

Growth factor loading was achieved as described. Scaffolds with microspheres were incubated in buffer at 37°C and agitated at 70 rpm. The control was a Poly(propylene fumarate) (PPF) scaffold loaded with pluronic gel with an equivalent amount of BMP-2 that was injected into the scaffold according to the method of Patel.[16] At the time points of 0, 1, 2, 4, and 6 weeks, the buffer was removed and replaced by fresh buffer. Standards with known amounts of radiolabeled growth factor were used to account for radioactive decay. The release of growth factor was quantified by monitoring the radioactivity in the removed buffer using a gamma counter (ARC-301B, Aloka Co, Japan), and results were correlated to a standard curve. The percentage of cumulative release was determined by normalizing the total BMP-2 released by each time point with the total amount incorporated within the scaffolds. Release rates were calculated as slope of cumulative release over the stated period of time and are presented as percentage change of cumulated release.

Radiological analysis

Radiologically by computed tomography (CT) scanning, using the Lightspeed 16 (General Electrics, Piscataway, NJ) at settings: 120 KV, 60 mA and 10 mAs. CT images were taken every 0.625 mm. Radiological density of the samples was assessed by measuring Houndsfield density units (HU). The mean value for eight separate points in each sample was calculated, allowing for the comparison of the radiological density of different samples.

Histological analysis

For histological evaluation a 1-cm×1-cm×1-cm construct was harvested sterilely at 1, 2, 4, and 6 weeks. The samples were fixed in 10% phosphate-buffered formalin and embedded in paraffin. Sections of 4 μm were taken. The sections were stained with Hematoxylin & Eosin (H&E) to demonstrate cell morphology and evaluate newly formed tissue.

Table 1. Group of Cell Source Used and Scaffold Material Under Different Conditions

	hBMSC	*hBMSC*	*hASC*	*hASC*
Scaffold	Gelatin + BMP_2 + OS ($n=8$)	Gelatin + BMP_2 + GM ($n=8$)	Gelatin + BMP_2 + OS ($n=8$)	Gelatin + BMP_2 + GM ($n=8$)
Scaffold	Gelatin + OS ($n=8$)	Gelatin + GM ($n=8$)	Gelatin + OS ($n=8$)	Gelatin + GM ($n=8$)
Scaffold	Gelatin ($n=8$) w/o cells + OS	Gelatin ($n=8$) w/o cells + GM	Gelatin ($n=8$) w/o cells + OS	Gelatin ($n=8$) w/o cells + GM
Scaffold	Gelatin ($n=8$) w/o cells but with microspheres w/o BMP_2 + OS	Gelatin ($n=8$) w/o cells but with microspheres w/o BMP_2 + GM	Gelatin ($n=8$) w/o cells but with microspheres w/o BMP_2 + OS	Gelatin ($n=8$) w/o cells but with microspheres w/o BMP_2 + GM

OS, osteogenic media; GM, growth media.

Toluidine Blue staining for pericellular proteoglycan and van Kossa staining for calcium content in newly formed tissue were used to assess bone formation. In addition, staining for alkaline phosphatase to identify new bone like tissue formation was used.

Biomechanical compression testing

Biomechanical assessment for stiffness was performed on seven samples from each group at each time point. Compression testing of the specimens was performed using an Instron Analyzer (Instron Worldwide Headquarters, Norwood, MA). Samples were tested wet directly after harvest so as not to change any conditions *in vitro*. Unconstrained uniaxial compression was applied at a load of 5 kg, whereas compressive force and displacement were recorded after the probe tip contacted the sample at a rate of 1 mm/min. The load-deformation curve was obtained, and compression modulus of the samples was calculated from the initial slope of the load-deformation curve. Eight samples of each group were measured to calculate the average value and the standard deviation of the mean.

Gene transcription analysis

For transcription analysis, total RNA was purified from samples with RNA STAT-60™ according to the specifications of the manufacturer. RNA was quantified by spectrophotometrie techniques. All samples were normalized to 50 μg of RNA. One microgram of each sample was converted to cDNA in a 22-μL reaction containing 2×PCR buffer, 5 mM dNTPs, 0.5 μg random hexamer primers, and 200 units Moloney murine leukemia virus reverse transriptase (MMLV RT) (Applied Biosystems, Foster City, CA). The reaction was subsequently heat-inactivated and diluted to 100 μL with water. One-microliter aliquots were used in each 25-μL reverse transcriptase polymerase chain reaction (RT-PCR), using gene-specific primers and probes from Applied Biosystems. Quantitative RNA expression was measured after 44 cycles using the 7500 RNA Fast from Applied Biosystems. Tested RNA was: Osteonectin, osteopontin, osteocalcin, and Wnt. *BMP3* expression was tested as inhibitor of Wnt. Regular human bone served as a positive control, and RNase-free water as a negative control.

The primers used were:

Osteonectin (ON), TTCCCCTCCTCCTGTTCTC, ACCC ACCCGTCACTAAGACA; osteopontin (OPN), GGGCCTC ACAGTTGTTTGAT, CCGCAGGATTCACTATGGTT; osteocalcin (OC), CTGCATTCTGCCTCTCTGAC, CTATTCA CCACCTTACTGCCC; Wnt, CGTCTACTTCGAGAAATC GCCCAACTTC, TCACAGACACTCGAGCAGTACGCGC; BMP-3, TGTAGGGGTAATTTAGTAGGTAGG, AACCTCTA AAACTACAACTAACAAAA.

Statistical correlation analysis

Changes in the HU of each construct were correlated with biomechanical strength and RNA expression values. The Pearson, Kendall, and Spearman correlation coefficients were calculated, using MatLab 7.0 (The MathWorks, Inc., Natick, MA). Differences were calculated using the chi-sqaured test. A $p < 0.05$ was considered to be significant.

Results

Both stem cell sources were differentiated into osteogenic, chondrogenic, and adipogenic lineages, as was shown by Toluidine Blue, Alcian Blue, and Sudan Red staining (Figs. 1 and 2). No difference between hASCs and hBMSCs was observed histologically. Specimens from hASCs and hBMSCs with microspheres releasing BMP-2 showed a decrease of 30% in volume over time, as cellular number increased visibly on the surface. All other specimens did not reveal a decrease in size.

Over the course of 42 days, scaffolds with microspheres showed an initial small burst-like release and afterward a more linear and sustained cumulative release. The control scaffolds showed a significant higher burst release, followed by only minimal increase in release until day 42 (Fig. 3).

Histologically increasing tissue formation was observed in both groups, hBMSC and hASC. At 4 and 6 weeks, the majority of cells were small, round, and osteocytic in appearance within the scaffold at all time points, confirmed by H&E staining. The cells were distributed homogeneously within the scaffold. A progressing layer of dense tissue around the scaffold was observed. This tissue stained positively for calcium using alkaline phosphatase and for pericellular proteoglycan using Toluidine Blue (Figs. 4 and 5), beginning at week 2 and progressively over time until week 6. The newly formed tissue penetrated the entire scaffold material. Groups of hBMSCs and hASCs were comparable for cellular and histological appearance. The most bone-like tissue formation was observed at the 6-week time point within the group of hASCs on scaffold material releasing BMP-2. Lesser bone-like tissue was observed in the group of hBMSCs and hASCs on gelatin scaffolds in OS medium (Fig. 5). Specimens without microspheres showed lesser bone-like tissue at all time points. However, the amount of new tissue formed was

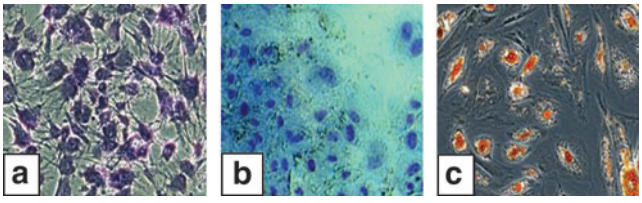

FIG. 1. Differentiated human bone marrow-derived stem cells (hBMSCs): (**a**) Osteogenic, (**b**) chondrogenic, and (**c**) adipogenic. Staining Toluidine Blue, Alcian Blue, and Sudan Red, respectively. Magnification, 200×. (Color image is available online at www.liebertpub.com/rej).

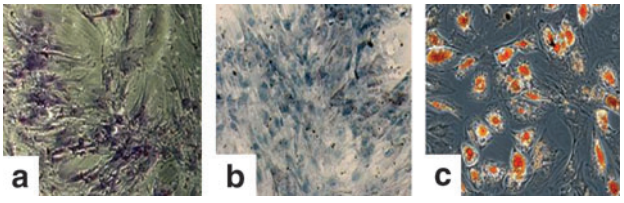

FIG. 2. Differentiated human adipose-derived stem cells (hASCs): (**a**) Osteogenic, (**b**) chondrogenic, and (**c**) adipogenic. Staining Toluidine Blue, Alcian Blue, and Sudan Red, respectively. Magnification, 200×. (Color image is available online at www.liebertpub.com/rej).

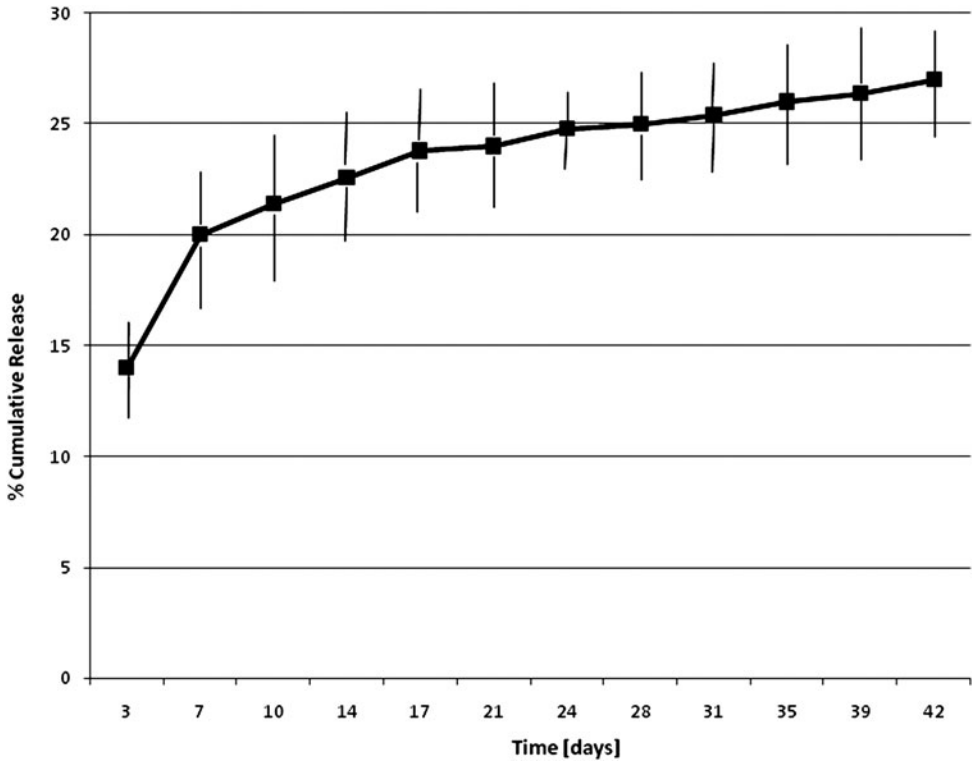

FIG. 3. Percent cumulative release profile of scaffolds with bone morphogenetic protein-2 (BMP-2). A poly(propylene fumarate) (PPF) scaffold injected with pluronic with an equivalent BMP-2 dose served as negative control.

similar in groups of hASCs and hBMSCs. Specimens without microspheres in OS medium showed more bone-like tissue formation than specimens in GM at all time points (results not shown). Controls without cells did not show any new tissue formation at any time point. A decrease in size of the incorporated microspheres was noted over the 6 weeks. At the 6-week time point, no further microspheres were present in the incorporated scaffold of specimens.

Radiological evaluation showed increasing density within the scaffolds over the 6-week period in the cellular samples of groups of hBMSCs and hASCs. Highest values were found at the 6-week end point in specimens of hASC and hBMSC on gelatin scaffolds with microspheres containing slow-release BMP-2 in OS medium and GM (Figs. 6 and 7). These values reached HU of 300, coming close to native bone in hASC and hBMSC groups with microspheres in OS medium and cells on gelatin scaffolds in OS medium. There was no statistically significant difference between hASC and hBMSC specimens with BMP-2. The densities were not statistically different in hASCs and hBMSCs specimens at time points of 1, 2, 4, and 6 weeks. Lower densities of 100 HU up to 200 HU were observed in the samples of hASCs and hBMSCs seeded on scaffolds without microspheres and scaffold with microspheres but not releasing BMP-2. The densities were higher for all time points in groups of hASC and hBMSC and for OS medium conditions. Lowest HU were in controls scaffolds only at −900 HU. Over time, only an increase of the initial scaffold density in the scaffold was measured, peaking at the 4-week time point and lesser at the 6-week end point. This was taken into account when measuring the densities of the cellular specimens.

Biomechanically, specimens with microspheres on gelatin scaffolds from hASCs and hBMSCs groups had the highest stiffness at the 6 weeks time point. An increasing stiffness was noted in these groups over the four time points, from 100 g at 1 week to 3.2 kg at 6 weeks. Values were not statistically different in hASC and hBMSC specimens under OS medium and GM conditions. A different result was found for hASCs and hBMSCs on gelatin scaffolds. Stiffness increased over time in both groups, with higher values in specimens in OS medium than in GM. However, stiffness was significantly lower than specimens without slow-release BMP-2. Lowest stiffness was found in the controls (Figs. 8 and 9). Values were also significantly lower than in microsphere/gelatin groups.

At the 4-week time point, bone marker RNA expression was detected in samples from BMP-2/gelatin samples with hASCs and hBMSCs under OS medium conditions. At 6 weeks, samples comprised of BMP-2/gelatin with hASCs or hBMSCs showed expression of bone-specific RNA under OS medium and GM conditions. No statistically significant difference was found between BMP-2/gelatin hASCs and hBMSCs under OS medium conditions, or for BMP-2/gelatin samples with hASCs or hBMSCs under GM conditions. Osteopontin and osteocalcin were more highly expressed than osteonectin. There was a statistically significant difference in bone-specific RNA expression between BMP-2/gelatin specimens under OS medium and under GM (Table 2). This was consistent with histological, biomechanical, and radiological findings.

Higher HU corresponded to the increased biomechanical strength of the scaffolds (Spearman correlation = 0.72, Pearson = 0.63, Kendall = 0.58). This association was robust

Table 2. Relative Quantitative Expression of Bone Specific Proteins

hBMSC	*Osteonectin*	*Osteocalcin*	*Osteopontin*	*Wnt*	*BMP3*
6Wks CellGelatinBMP2OS	5.31E+14	3.69E+16	3.72E+23	5.73E+15	Not detected
6 Wks CellGelatinBMP2GM	3.62E+14	2.25E+15	3.53E+22	8.95E+16	Not detected
4Wks CellGelatinOS	2.40E+14	3.00E+17	9.44E+23	1.51E+16	Not detected
6Wks CellGelatinOS	6.34E+14	6.12E+17	3.84E+24	7.29E+16	Not detected
6Wks CellGelatinGM	2.87E+14	4.98E+16	1.73E+23	3.54E+15	Not detected
hASC	*Osteonectin*	*Osteocalcin*	*Osteopontin*	*Wnt*	*BMP3*
6Wks CellGelatinBMP2OS	5.01E+14	3.37E+16	3.41E+23	5.49E+16	Not detected
6 Wks CellGelatinBMP2GM	3.41E+14	2.25E+15	3.60E+22	8.87E+16	Not detected
4Wks CellGelatinOS	2.28E+14	2.26E+17	9.14E+23	1.22E+16	Not detected
6Wks CellGelatinOS	6.18E+14	6.41E+17	3.65E+24	6.99E+16	Not detected
6Wks CellGelatinGM	2.57E+14	4.59E+16	1.74E+23	3.40E+15	Not detected

BMP_3 as Wnt antagonist is not detected at any time point. BMP_2/gelatin constructs have the highest expression of proteins at 6 weeks. Regular human bone served as positive control, RNase free water as negative control.

across the different correlation coefficients. A high correlation between HU and gene transcription values was found (Spearman correlation = 0.92, Pearson = 0.92, Kendall = 0.88). Also robust associations were found between biomechanical stiffness values and gene transcription (Spearman correlation = 0.71, Pearson = 0.66, Kendall = 0.60). Bivariate correlations were tested nonparametrically using Spearman rank correlation and Kendall tau b statistics. Spearman rank correlations showed: Biomechanical stiffness − HU = 0.64 ($p < 0.04$), biomechanical stiffness − gene transcription values = 0.57 ($p < 0.11$), HU − gene transcription values = 0.96 ($p < 0.002$). Kendall tau b statistics revealed: Biomechanical stiffness − HU = 0.62 ($p < 0.04$), biomechanical stiffness − gene transcription values = 0.44 ($p < 0.20$), HU − gene transcription values = 0.88 ($p < 0.004$).

Discussion

Adipose-derived stem cells are an attractive alternative as a source of stem cells to BMSCs, because obtaining autologous hBMSCs is combined with a higher morbidity.[18–21] Human ASCs and BMSCs are known to have osteogenic potential.[18–20] Although most authors in the literature agree on a similar osteogenic potential of human adipose-derived and bone marrow-derived stem cells, this idea is still controversial.[20] There are few comparisons of the influence of osteogenic factors on different sources of stem cells on third-generation biomaterials. In our study, we examined the conditions influencing the osteogenic potential of adipogenic and marrow-derived stem cell sources on a third-generation biomaterial.

Gelatin has been commonly used as a gelling agent in food, pharmaceuticals, photography, and cosmetic manufacturing. Gelatin is a protein produced by partial hydrolysis of collagen extracted from the bones, connective tissues, organs, and some intestines of animals such as domesticated cattle and horses. The natural molecular bonds between individual collagen strands are broken down into a form that rearranges more easily. Gelatin melts when heated and solidifies when cooled again. Together with water, it forms a semisolid colloid gel. Gelatin forms a solution of high viscosity in water, which sets to a gel on cooling, and its chemical composition is, in many respects, closely similar to that of its parent collagen. In clinical applications it is often used as coating material, in cosmetics, or as carrier material.[12] Gelatin scaffolds preserve MSC differentiation potential.[21] Other advantages of gelatin are easy chemical modification and the commercial availability of samples with different physicochemical properties.

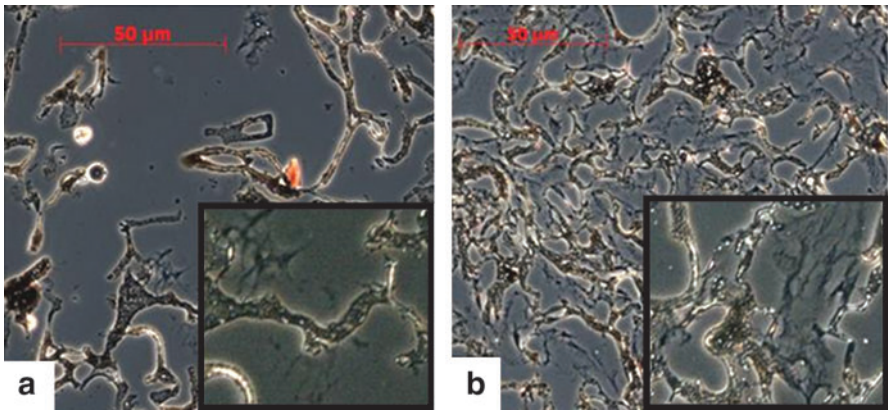

FIG. 4. Two-week (**a**) and 4-week (**b**) specimens of human bone marrow-derived stem cells (hBMSCs) with bone morphogenetic protein-2 (BMP-2)/gelatin scaffolds in osteogenic (OS) medium. Increasing new tissue formation is visible, penetrating the entire scaffold and staining positively for calcium. Staining for alkaline phosphatase. Bar, 50 μm. Magnifications, 100× and 200×. (Color image is available online at www.liebertpub.com/rej).

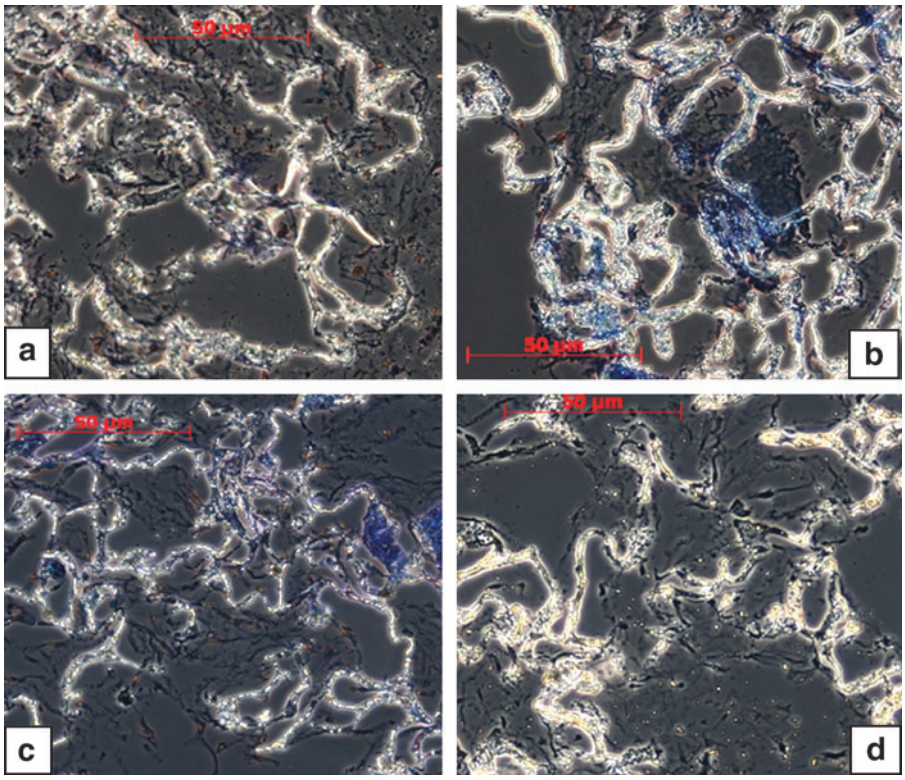

FIG. 5. Histologically **s**imilar bone-like tissue formation of specimens at 6 weeks: Human bone marrow-derived stem cells (hBMSCs)/ bone morphogenetic protein-2 (BMP-2)/gelatin in osteogenic (OS) medium (**a**), human adipose-derived stem cells (hASCs)/BMP-2/gelatin in OS (**b**), and hBMSC/BMP-2/gelatin in growth medium (GM) (**c**). Histologically lesser bone-like tissue formation of hBMSC/gelatin specimens in OS media (**d**). Staining Toluidine Blue. Bar, 50 μm. (Color image is available online at www.liebertpub.com/rej).

β-TCP has been successfully tested for bone regeneration and can be chemically linked to gelatin in different concentrations. In experimental and clinical cases, porous β-TCP has been widely used because of its inherent osteoconductivity.[6–8,10,22] However, it has slow biodegradability and is brittle in nature. In addition, it is difficult to freely change the shape of bioceramics upon applying them to the body site of different shapes during the operation. Because spontaneous gelation of gelatin solution homogeneously dispersing various amounts of β-TCP takes place immediately after leaving them in at 4°C, various amounts of β-TCP were tested before. These tests revealed increased mechanical resistance of sponges against compression in a dry state increased with the increased β-TCP content. In addition, this gelatin/β-TCP scaffold was recently successfully tested for supporting bone formation using MSCs.[11] In our experiment using OS medium or GM, no difference was noted for hBMSCs or hASCs. Similar results were found in studies by Guilak et al., Mosna et al., Rider et al., and Zannettino et al.[23–26] Niemeyer et al. and Liu et al., however found in their studies inferior capability of hASCs versus hBMSCs.[27,28] While certain characteristics of hASCs seem to be similar to BMSCs, such as surface markers,[26] differences seem to occur in their differentiation into the three lineages of cartilage, bone, and fat.[28]

Even more radiological densities were similar for hASC and hBMSC samples in both groups, similar to the findings in the literature.[23,24] These results were also found for biomechanical stiffness and were concordant with histological findings and gene expression. Although an increase in HU of the scaffold material itself was noted, peaking at 4 weeks, HU values were less at the 6-week end point. This was taken into account when specimen densities were evaluated radiologically. The scaffold seemed to degrade slowly, providing additional mechanical stability for the newly formed tissue. This slow degradation might have contributed to the 30% volume loss at the 6-week end point in all cellular samples. Previous experiments have shown a comparable loss in volume.[16,30,31] Different from our results, Patel et al. have observed a continuous decrease in the density/stability of the gelatin scaffolds.[16]

Recent developments in tissue regeneration have provided biomaterials for the slow release of proteins over a period of time, thus giving cells continuous stimuli to differentiate into desired cells.[1] We have used cationized gelatin hydrogel microspheres releasing BMP-2 over a 6-week period within microporous gelatin scaffolds. BMP-2 plasmids were crosslinked to the gelatin hydrogel and released as the hydrogel microspheres were degraded enzymatically by the cells.[29] Previous studies showed sustained release of plasmids via gelatin hydrogel microspheres in a maintained concentration over 3 weeks.[30,31] In our experiment, we still detected microspheres histologically at 4 weeks. Gelatin hydrogel microspheres are advantageous for controlled drug delivery, because continuous enzymatic degradation delivers the desired plasmid in maintained concentration at the desired

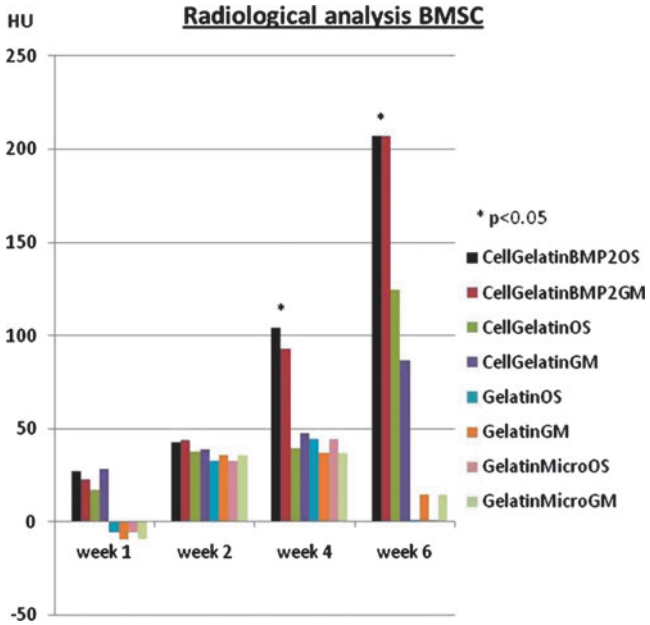

FIG. 6. Radiological densities of human bone marrow-derived stem cell (hBMSC) samples at 1, 2, 4, and 6 weeks. Bone morphogenetic protein-2 (BMP-2)/gelatin samples have the highest density, close to native bone. GelatinMicroOS or GelatinMicroGM = samples with microspheres but not releasing BMP-2; GelatinOS and GelatinGM = acel-acellular controls without microspheres. HU, Houndsfield density units; OS, osteogenic medium; GM, growth medium. (Color image is available online at www.liebertpub.com/rej).

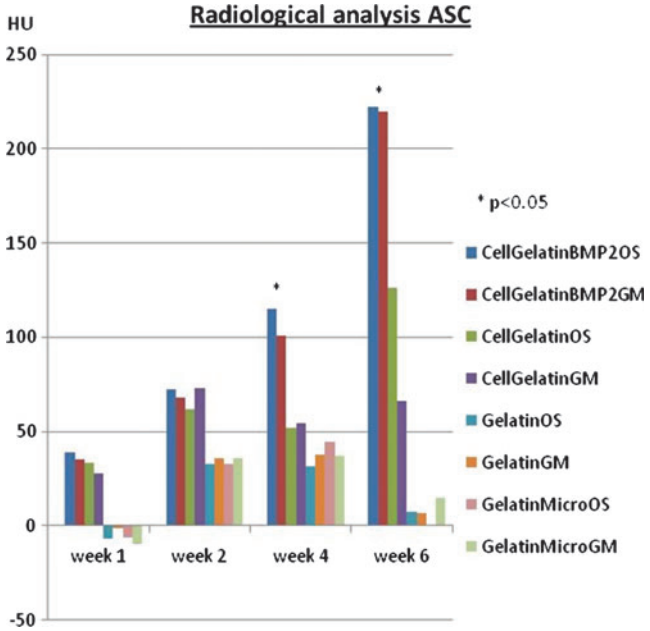

FIG. 7. Radiological densities of human adipose-derived stem cell (hASC) samples at 1, 2, 4, and 6 weeks. Bone morphogenetic protein-2 (BMP-2)/gelatin samples have the highest density, close to native bone. GelatinMicroOS or GelatinMicroGM = acellular controls with microspheres but not releasing BMP-2; GelatinOS and GelatinGM = acellular controls without microspheres. HU, Houndsfield density units; OS, osteogenic medium; GM, growth medium. (Color image is available online at www.liebertpub.com/rej).

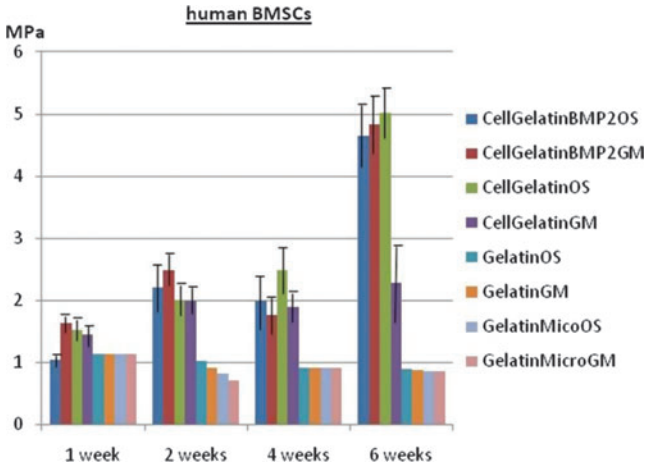

FIG. 8. Relative stiffness of human bone marrow-derived stem cell (hBMSC) specimens over 6 weeks' time course. Bone morphogenetic protein-2 (BMP-2)/gelatin samples have the highest stiffness at the 6-week end point. GelatinMicroOS or GelatinMicroGM = acellular controls with microspheres but not releasing BMP-2; GelatinOS and GelatinGM = acellular controls without microspheres. mPa, megapascals; OS, osteogenic medium; GM, growth medium. (Color image is available online at www.liebertpub.com/rej).

tissue site. The shape of the hydrogel does not influence the release profile of the plasmid, because the release is governed by the degradation of the hydrogel carrier.[30–33] The level of gene expression in the surrounding tissue can be enhanced by delivery of plasmid DNA using hydrogel microspheres, more than plasmid DNA solution. In our experiment BMP-2 was crosslinked to cationized gelatin microspheres and incorporated into gelatin/β-TCP scaffolds. This led to signifi-

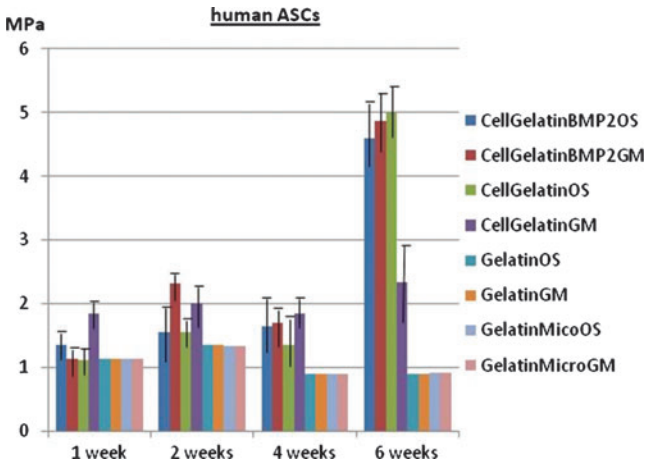

FIG. 9. Relative stiffness of human adipose-derived stem cell (hASC) specimens over the 6-week time course. Bone morphogenetic protein-2 (BMP-2)/gelatin specimens have also the highest stiffness. GelatinMicroOS orGelatinMicroGM = acellular controls with microspheres but not releasing BMP-2; GelatinOS and GelatinGM = acellular controls without microspheres. mPa, megapascals; OS, osteogenic medium; GM, growth medium. (Color image is available online at www.liebertpub.com/rej).

cantly increased osteogenesis compared to the chemically induced osteogenesis in specimens. At the 6-week end point, no microspheres were observed histologically; it appeared that microspheres distributing BMP-2 were absorbed. In previous studies, Patel et al. showed a continuous release of BMP-2 *in vitro* and *in vivo*.[16] However, no study on the longevity of the microspheres was included.

In our study, the 6-week end point was chosen for radiological evaluation and gene expression of bone proteins because bone fracture healing is normally completed at this time point and the newly formed matrix bone is visible radiologically. Our CT imaging study revealed highest densities up to 300 HU in both sample groups, BMP-2/gelatin with hASCs and hBMSCs, and that bone formation was present in both groups. Highest levels of RNA expression and biomechanical stiffness correlated with radiological results. No densities within the range of bone were found in gelatin hASC or hBMSC specimens in GM. It seems that chemical stimuli such as β-glycerolphosphate in the OS medium and biological stimuli from BMP-2 provided in slow release by the microspheres led to equal results for bone regeneration using bone marrow-derived or adipose tissue-derived stem cells. In an *in vivo* study, Hayashi et al. showed that radiologically specimens of ASCs and BMSCs seeded on hydroxyapatite ceramic had similar densities at 6 weeks.[20]

We tested the hypothesis that combining OS medium and slow release would enhance osteogenic differentiation of the seeded cells. HBMSCs and hASCs did not show increased bone formation radiologically, histologically, biomechanically, or by gene expression over the course of 6 weeks, when combining slow-release BMP-2 and OS medium. Both groups showed a decrease in biomechanical stiffness at 4 weeks, but a sharp increase at 6 weeks. However, these results were not the same as in radiological density. This might be due to an increase of bone-like tissue on the outside of the specimens, reflected by radiological and histological findings, but not in the same amount within the scaffold, seen in stiffness. Gene expression studies showed the presence of preosteoblasts, transitory osteoblasts, and secretory osteoblasts, mainly at 6 weeks.[34] Interestingly, histological, radiological, biomechanical, and gene expression studies were comparable to specimens using only slow-release BMP-2 in GM. However, specimens comprised of cells under BMP-2 slow-release conditions had significantly higher radiological densities, biomechanical stiffness, and expression of bone-specific proteins. This was concordant with histological findings.

When comparing both stem cell sources, no difference in osteogenic potential was observed in histology, radiological, biomechanical, and gene expression. Osteogenic differentiation of BMSCs and ASCs was seen as described in the literature with maturing bone-like tissue from MSCs.[34] Previously, similar capacity of osteogenic and chondrogenic differentiation of ASCs and BMSCs in quantitatively gene expression of collagen II and alkalic phosphatase were observed.[35] Liu et al. observed similarities in the osteogenic and chondrogenic pathways of ASCs and BMSCs, suggesting that MSCs from different sources are derived from a similar cell source.[19] These results confirm our findings that hASCs and hBMSCs did not differ in bone regeneration. The reason might be that the source of hASCs and hBMSCs could be the same.[19] Although the authors described similar osteogenic potential in the early differentiation, late differentiation shows lesser osteogenic potential for ASCs than BMSCs. Because our study used cells in the early differentiation, our results are concordant with these studies. Surprisingly, we saw a relatively low expression of osteocalcin; however, we would expect an increase of these values over time. However, it has been shown that repaired bone does not express the same high amount of osteocalcin as native bone,[3] which might explain the lower level of osteocalcin.

In conclusion, we have demonstrated that hASCs and hBMSCs have similar bone-regenerating potential *in vitro* on gelatin scaffolds. This makes hASCs an attractive alternative to hBMSCs for bone regeneration. Comparable results in bone formation were found for combined chemical and proteinous stimuli and proteinous stimulus alone. These stimuli led to better bone formation compared to OS medium only. It seems that BMP-2 as an osteogenic differentiating stimulus is sufficient for hASCs and hBMSCs, resulting in good bone formation. Combining third-generation smart biomaterials with integrated BMP-2 slow release with easy accessible adipose-derived stem cells offers attractive new pathways for regenerating bone.

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