Biomaterials 35 (2014) 1176–1184

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Controlling stem cell-mediated bone regeneration through tailored mechanical properties of collagen scaffolds

Hongli Sun [a,b], Feng Zhu [c], Qingang Hu [c], Paul H. Krebsbach [a,b,*]

[a] Department of Biologic and Materials Sciences, University of Michigan, School of Dentistry, Ann Arbor, MI, USA
[b] Biointerfaces Institute, University of Michigan, Ann Arbor, MI, USA
[c] Department of Oral and Maxillofacial Surgery, School of Stomatology, Affiliated Stomatological Hospital, Nanjing University, Nanjing, Jiangsu, China

ARTICLE INFO

Article history:
Received 26 September 2013
Accepted 19 October 2013
Available online 7 November 2013

Keywords:
Mesenchymal stem cells
BMP2
Scaffold stiffness
Endochondral ossification
Bone regeneration

ABSTRACT

Mechanical properties of the extracellular matrix (ECM) play an essential role in cell fate determination. To study the role of mechanical properties of ECM in stem cell-mediated bone regeneration, we used a 3D *in vivo* ossicle model that recapitulates endochondral bone formation. Three-dimensional gelatin scaffolds with distinct stiffness were developed using 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) mediated zero-length crosslinking. The mechanical strength of the scaffolds was significantly increased by EDC treatment, while the microstructure of the scaffold was preserved. Cell behavior on the scaffolds with different mechanical properties was evaluated *in vitro* and *in vivo*. EDC-treated scaffolds promoted early chondrogenic differentiation, while it promoted both chondrogenic and osteogenic differentiation at later time points. Both micro-computed tomography and histologic data demonstrated that EDC-treatment significantly increased trabecular bone formation by transplanted cells transduced with AdBMP. Moreover, significantly increased chondrogenesis was observed in the EDC-treated scaffolds. Based on both *in vitro* and *in vivo* data, we conclude that the high mechanical strength of 3D scaffolds promoted stem cell mediated bone regeneration by promoting endochondral ossification. These data suggest a new method for harnessing stem cells for bone regeneration *in vivo* by tailoring the mechanical properties of 3D scaffolds.

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1. Introduction

At sites of tissue injury, both local and distant adult stem cells are recruited to the wound bed, subsequently differentiate to the specific progenitors, and when conditions are favorable, repair or regenerate the tissue. Stem cell fate in wound healing is determined by a combination of signals that includes growth factors, cytokines, hormones, biomechanical stress, interaction with cells at the site of injury, and the nature of the extracellular matrix (ECM) [1]. Recently, the mechanical properties of the ECM have attracted greater attention as the role of the ECM in stem cell differentiation has become better understood [2–5]. Previous studies indicated that soft matrices (~1 kPa) favored mesenchymal stem cell (MSC) differentiation into neuronal-like cells, while moderately stiff matrices (~10 kPa) promoted myogenic differentiation, and rigid matrices (~100 kPa) stimulated osteogenic differentiation [2,6,7]. These findings suggest that the mechanical properties of biomaterial scaffolds used for stem cell delivery in tissue engineering could modify cell behavior and that designing materials to mimic the injured tissue may be an advantage. These principles have already been exploited to some extent. Therefore, aligning the mechanical property with the tissue of interest is one of the reasons that hydrogels (soft, <1 kP) [8–11] and ceramics (stiff, >100 kP) [12–15] are the most widely used biomaterials for pre-clinical cartilage and bone tissue engineering, respectively. Another example supporting this concept is the use of decellularized tissues (native ECM) that have been successfully used in tissue engineering and pre-clinical applications [16,17].

Although decades of investigation have expanded our understanding of the ECM *in vivo*, much of our current knowledge regarding the mechanical properties of supporting matrices in stem cell fate determination is derived from *in vitro* cell culture models [2,3,5,18–23]. However, typical two dimensional cell culture systems are not able to fully mimic the complex microenvironment that naturally modulates stem cell behavior *in vivo* [24,25]. Therefore, *in vivo* studies will likely provide more instructive insights to

* Corresponding author. Department of Biologic and Materials Sciences, School of Dentistry, University of Michigan, 1011 North University Ave, Ann Arbor, MI 48109, USA. Tel.: +1 734 936 2600; fax: +1 734 647 2110.
E-mail address: paulk@umich.edu (P.H. Krebsbach).

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http://dx.doi.org/10.1016/j.biomaterials.2013.10.054

understand the role of mechanical properties of the ECM in stem cell-mediated tissue regeneration.

One common strategy to improve the bone forming capacity of biomaterials is to add hydroxyapatite (HA) to polymer-based scaffolds because HA is not only able to increase the mechanical strength, but may also mimic the composition and structure of natural bone mineral [26–29]. However, it is often difficult to distinguish the contribution of mechanical properties from other modifications (e.g., chemical composition and structure). Previous observations suggest that very different mechanical strengths are required to support stem cells to differentiate to chondrocytes versus osteoblasts. However, many bone regeneration strategies, especially those induced by bone morphogenetic proteins (BMPs), are typically directed through an endochondral ossification process; that is, progenitor cells first differentiate to chondrocytes that subsequently undergo hypertrophy, are invaded by blood vessels and are subsequently replaced by osteoblasts [30,31]. To mimic endochondral bone formation, a strategy was developed in which stem cells were induced to chondrogenic differentiation *in vitro* prior to being transplanted *in vivo* [30,32,33]. Although chondrogenesis is a prerequisite for endochondral bone formation, osteogenesis and chondrogenesis may impede each other during bone development and regeneration [34,35]. It is therefore essential to recognize that endochondral bone formation is a dynamic process that cannot be recapitulated in *in vitro* cell culture models. We hypothesized that the mechanical microenvironment required for differentiation by stem cells *in vivo* was different from that functioning in *in vitro* systems. Therefore, to study the role of mechanical properties of the ECM in stem cell-mediated bone regeneration, we used a BMP-induced, 3D *in vivo* ossicle model that represents an endochondral ossification process [29,36]. Three-dimensional gelatin scaffolds with distinct elastic moduli were generated by crosslinking the material with 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC). EDC has been widely used in polymeric scaffold fabrication because it is a zero-length, non-toxic crosslinker that conjugates carboxylates (−COOH) to primary amines (−NH2) without the addition of linking molecules [37–39]. Moreover, we developed a technique to maintain the microstructure of gelatin scaffolds to prevent swelling during chemical crosslinking [40]. Therefore, the *in vivo* ossicle provided us a new and contrasting *in vivo* model to investigate the role of mechanical properties of matrices in stem cell-mediated bone regeneration.

2. Materials and methods

2.1. Chemical crosslinking of scaffolds

Three-dimensional porous gelatin scaffolds (Pharmacia and Upjohn, Kalamazoo, MI), were crosslinked as previously described [40]. Briefly, the scaffolds were incubated in 50 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl (EDC) (Thermo Scientific, Rockford, IL) and 50 mM N-hydroxy-succinimide (NHS) (Sigma, St Louis, MO) and 50 mM (2-(N-morpholino) ethanesulfonic acid) hydrate (MES) buffer (pH 5.3) (Sigma, St Louis, MO) at 4 °C for 24 h. To maintain the microstructure of gelatin matrices, a 90/10 (v/v) acetone/water solvent mixture was used instead of water. Scaffolds treated with MES buffer/acetone/water served as the control groups. All scaffolds were then washed with distilled water 5 × 30 min and frozen at −80 °C for at least 12 h. The scaffolds were subsequently freeze-dried and stored in a desiccator.

2.2. Scaffold characterization

The surface morphology of the scaffolds was observed by scanning electron microscopy (SEM, Philips XL30 FEG) as previously described [40]. Briefly, the scaffolds were coated with gold particles using a sputter coater (DeskII, Denton vacuum Inc.) with gas pressure of 50 mtorr and 40 mA current for 200 s. Samples were analyzed at 30 kV. The elastic moduli of the 3D gelatin scaffolds were determined with an AR-G2 rheometer (TA Instruments, New Castle, DE) in the oscillatory mode with a fixed frequency of 1 Hz and an applied strain of 1% [41]. The scaffolds were soaked overnight in distilled H_2O before undergoing mechanical testing. Four scaffolds were tested for each group ($n = 4$).

2.3. Cell culture

Bone marrow mesenchymal stem cells (BMSCs) were harvested and cultured as described previously [15,29]. The marrow content of 4–6 bones was plated into a T75 culture flask in BMSC growth medium comprised of α-MEM containing 10% fetal bovine serum (FBS) and 100 U/ml penicillin, 100 mg/ml streptomycin sulfate (Gibco, Grand Island, NY). Non-adherent cells were removed and adherent BMSCs were cultured and expanded for further experiments. Primary cells prior to passage 4 were used in the experiments. The mouse mesenchymal stem cell line, C3H10T1/2, and pre-osteoblastic MC3T3-E1 cells were cultured in α-MEM containing 10% FBS, 100 U/ml penicillin, 100 mg/ml streptomycin sulfate (Gibco, Grand Island, NY). C3H10T1/2 cells were transfected with adenovirus encoding BMP-2 (Ad-BMP2, University of Michigan Vector Core Laboratory, Ann Arbor, MI) at 500 MOI for cell differentiation *in vitro*. For osteoblastic differentiation *in vitro*, MC3T3-E1 cells were cultured in osteogenic medium containing α-MEM containing 10% FBS, 10 mM β-glycerophosphate and 50 mg/mL ascorbic acid-2-phosphate (Sigma–Aldrich, St. Louis, MO). Chondrocytes were isolated from fresh articular cartilage of adult bovine knee joints attained from a local slaughterhouse. Minced cartilage pieces ($\sim 1\ mm^3$) were digested overnight at 37 °C in a 5% CO_2 incubator with 0.25% collagenase (Sigma; St. Louis, MO) in high-glucose DMEM. The chondrocytes were collected by centrifugation at 500 g for 5 min after filtering the suspension with a 70 μm strainer. The cells were expanded in high-glucose DMEM containing 10% FBS, 100 U/mL penicillin and 100 mg/L streptomycin (Invitrogen; Carlsbad, CA).

2.4. Cell seeding on scaffolds

The EDC-treated and control gelatin scaffolds were prepared for cell seeding as previously described [42] with modifications. Briefly, the scaffolds were cut into defined dimensions (3.5 × 3.5 × 3.5 mm) and soaked in 70% ethanol for 30 min and then exchanged with phosphate-buffered saline (PBS) for three times (30 min each). Subsequently, the scaffolds were dried to remove residual medium and air by compressing them between sterile gauze with moderate pressure. One million cells (C3H10T1/2/Ad-BMP2 or MC3T3-E1) were subsequently seeded into the scaffolds by capillary action [43] in a ultra-low attachment 96-well plate (Corning, Lowell, MA). All cell/scaffold constructs were transferred to a 24-well plate with 1 mL α-MEM containing 10% FBS in each well after a 2 h-attachment period and were cultured at 37 °C, 5% CO_2. Cells on EDC-treated and non-treated control scaffolds were cultured for 1, 4 and 7 days and harvested for analysis. To estimate the number of the cells within each scaffold, total DNA was quantified using a DNeasy Blood & Tissue Kit (QIAGEN, Germantown, MD). The recombinant BMP2 concentration in the supernatant produced by C3H10T1/2 was quantified using an enzyme-linked immunosorbent assay (ELISA) kit following the manufacturer's protocol (R&D Systems, Minneapolis, MN).

2.5. Gene expression analysis

Total RNA was isolated using the RNeasy®Micro Kit (Qiagen, Germantow, MD). RNA concentration was determined at 260 nm and an equivalent amount of RNA (0.5 μg) was processed to generate cDNA using the High Capacity cDNA Reverse Transcript kit (Applied Biosystems, Forster City, CA). Quantitative PCR was performed with Taqman gene expression assays (Applied Biosystems, Forster City, CA) using the ABI PRISM 7500 sequence detection system (Applied Biosystems, Carlsbad, CA, USA). The primers/probes: GAPDH (Mm99999915), Runx2 (Mm00501584), OCN (Mm03413826), PPARγ (Mm01184322), FABP4 (Mm00445878), Sox9 (Mm00448840), COL2 (Mm01309555) and Aggrecan (Mm00545809) were all purchased from Applied Biosystems (Forster City, CA).

2.6. In vivo transplantation and analysis

For *in vivo* bone regeneration, mouse BMSCs were transduced with Ad-BMP2 at 500 MOI one day before implantation. One million BMSCs/Ad-BMP2 were then seeded on the gelatin scaffolds as described above. Inbred C57BJL/6 mice with an age range of 5–6 weeks (Charles River Laboratories) were used in the study. Animal surgeries were performed according to the guidelines approved by the University of Michigan Committee of Use and Care of Laboratory Animals. The dorsum of the animals was shaved and disinfected with povidone-iodine. One midsagittal incision was made on the dorsa and two subcutaneous pockets were created using blunt dissection. One scaffold was implanted subcutaneously into each pocket. Each animal received 2 cell-seeded constructs. After placement of scaffolds, the incisions were closed with staples. Five mice ($n = 5$) were euthanized at 1 day, 4 days and 7 days after surgery, respectively. Each sample was split into two; one half was used for gene expression and one half for protein analysis. Five ($n = 5$) and ten mice ($n = 10$) were euthanized at 14 days and 42 days after surgery, respectively. Three and one transplants failed to form typical ossicles at 42 days after surgery in control and EDC treated group, respectively. The ossicles were fixed in Z-fix (Anatech Ltd.) for 2 days and then transferred to 70% ethanol until analyzed.

The ossicles were scanned at a voxel size of 18 μm using a microcomputed tomography (μCT) scanner (GE Healthcare Pre-Clinical Imaging). Micro View software (GE Healthcare Pre-Clinical Imaging) was used to generate a three-dimensional (3D) reconstruction. A fixed threshold (1000) was used to extract the mineralized bone phase and the bone morphometry was calculated as previously described [31].

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