Published in final edited form as:

Biomaterials. 2009 October ; 30(28): 5061–5067. doi:10.1016/j.biomaterials.2009.06.013.

Chondrogenic and osteogenic differentiations of human bone marrow-derived mesenchymal stem cells on a nanofibrous scaffold with designed pore network

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Abstract

The utilization of 3D scaffolds and stem cells is a promising approach to solve the problem of bone and cartilage tissue shortage and to construct osteochondral (cartilage/bone composite) tissues. In this study, 3D highly porous nanofibrous (NF) poly(L-lactic acid) (PLLA) scaffolds fabricated using a phase separation technique were seeded with multi-potent human bone marrow-derived mesenchymal stem cells (hMSCs) and the constructs were induced along osteogenic and chondrogenic development routes in vitro. Histological analysis and calcium content quantification showed that NF scaffolds supported in vitro bone differentiation. SEM observation showed an altered shape for cells cultured on a NF matrix compared with those on smooth films. Consistent with the morphological change, the gene expression of early chondrogenic commitment marker sox-9 was enhanced on the NF matrix. NF scaffolds were then used to support long term in vitro 3D cartilaginous development. It was found that in the presence of TGF- β 1, cartilage tissue developed on PLLA NF scaffolds, with the cartilage-specific gene expressed, glycosaminoglycan and type II collagen accumulated, and typical cartilage morphology formed. These findings suggest that NF scaffolds can support both bone and cartilage development and are excellent candidate scaffolds for osteochondral defect repair.

Keywords

mesenchymal stem cell; cartilage; bone; nanofiber; poly(L-lactic acid) scaffold; tissue engineering

Introduction

A current challenge in the field of tissue engineering is to construct composite tissues. Bone/cartilage composite (osteochondral) constructs are required to restore defective osteochondral

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tissues due to arthritis, trauma or tumor excision. Bone marrow-derived mesenchymal stem cells (MSCs) are multi-potential stem cells that can differentiate into bone, cartilage, fat, muscle, tendon and other tissue types when induced by the appropriate cues in vitro or in vivo [1,2], providing a suitable cell source for osteochondral tissue reconstruction. In addition to a suitable cell source, scaffolds play critical roles in accommodating cells, supporting their proliferation, differentiation, and directed 3D tissue formation [3]. Previously, the cartilage and bone have been separately generated on different scaffolds and then combined together using a glue to form an osteochondral construct [4]. A problem of this approach is the less-than-optimal integration of osteo and chondral tissues. A single scaffold may provide more continuous distribution of cells and integration of the two component tissue types [5]. However, the challenge is to find a scaffold that can meet the requirements of optimal differentiation for both bone and cartilage tissues. A collagen-glycosaminoglycan scaffold has been used to support rat mesenchymal stem cells differentiation along osteogenic and chondrogenic routes [6]. Although the scaffold supported bone differentiation well, the cartilage tissue was not fully developed on the scaffold. Electrospun nanofibers were also used to support multi-lineage differentiations of human mesenchymal stem cells (hMSCs) into bone, cartilage and fat tissues and showed promising results for multiple types of tissue formation [7]. However, without designed large pores for cell penetration, the size of fabricated tissues was limited. In a new approach, highly porous and interconnected nanofibrous (NF) scaffolds have been fabricated in our laboratory using a phase separation method combined with porogen leaching techniques [8,9]. We have previously shown that the NF scaffolds were characterized by high-porosity, NF matrix with a fiber diameter at the scale of natural collagen fibrils and high surface to volume ratios. These physical characteristics promoted protein adsorption, cell adhesion [10] and osteoblastic differentiation of primary osteoblasts and preosteoblast cell line cells [11, 12]. Another advantage of this kind of scaffold is that nanospheres containing different growth factors can be incorporated into the scaffolds. Controlled-releasing nanospheres containing BMP-7 have been incorporated into the scaffolds and implanted into rodents, showing significant ectopic bone formation in vivo [13]. Therefore, it is hypothesized that combined with chondrogenic and osteogenic factors, this system of an NF scaffold and nanospheres can be used to reconstruct osteochondral defect for the regeneration of bone and cartilage as well as their integrating interface simultaneously. To accomplish this goal, the first step is to test the possibility of NF scaffolds to support both bone and cartilage development under different inductive factors. In this study, hMSCs were seeded and cultured on NF scaffolds in vitro, with osteogenic or chondrogenic factors added respectively to induce bone or cartilage differentiation.

Materials and methods

Reagents

PLLA with an inherent viscosity of approximately 1.6 was purchased from Boehringer Ingelheim (Ingelheim, Germany). Mesenchymal stem cell culture medium, trypsin-EDTA solution, osteogenic induction medium and chondrogenic induction medium were purchased from Lonza Walkersville, Inc. (Walkersville, MD, USA). TGF- β 1 was purchased from R&D systems, Inc. (Minneapolis, MN, USA). Dimethylmethylene blue, *N*-acetyl cysteine and shark chondroitin 4-sulfate were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA).

Fabrication of NF PLLA matrix and scaffolds

Fabrication of 3D NF scaffolds has been described in detail previously [14]. Briefly, 10% PLLA/tetrahydrofuran (THF) solution was cast into an assembled sugar template (formed from bound sugar spheres, 250–425 μ m in diameter) under mild vacuum. The polymer-sugar composite was phase separated at -20°C overnight and then immersed into cyclohexane to exchange THF for 2 days. The composite was then freeze-dried. The sugar spheres were

leached out in distilled water and the highly porous scaffold was freeze-dried again. Scaffolds were cut into circular disks with dimensions of 3.6 mm in diameter and 1 mm in thickness. Fabrication of thin NF matrix and smooth films has been described in details previously [12]. Briefly, 10% PLLA/THF solution was cast into a preheated glass mold. The mold was quickly sealed using a cover glass. The PLLA solution was phase separated at -20°C overnight and then immersed into ice/water mixture to exchange THF for 24 hours. Thin sheets of matrix were then vacuum-dried for 2 days. Smooth films were fabricated in a similar manner excluding the phase separation step. Instead, the solvent was evaporated at room temperature in a fume hood.

Cell culture and seeding

Human bone marrow-derived mesenchymal stem cells (hMSCs) were purchased from Lonza Walkersville, Inc. (Walkersville, MD, USA). The cells were cultured according to the manual provided by the supplier. Scaffolds were sterilized by soaking in 70% ethanol for 30min, washed three times with PBS for 30 min each, and twice in complete medium for 2 hours each on an orbital shaker at 75 rpm. 2.5×10^5 cells were seeded onto each scaffold (3.6 mm in diameter and 1 mm in thickness). After 2 hours of initial seeding, cell-seeded scaffolds were further cultured for 22 hours under static condition to promote cell spreading on scaffolds. To induce osteogenesis, cell-seeded scaffolds were transferred to 6-well plates on an orbital shaker, and maintained in 3mL osteogenic medium supplemented with 100 nM dexamethasone, 10 mM β -glycerolphosphate and 50 μ g/mL ascorbic acid. Controls were maintained in the same medium but without dexamethasone. The medium was changed twice a week. In order to induce chondrogenesis, cell-seeded scaffolds were transferred to 15 mL polypropylene culture tubes and maintained in 0.5 mL chondrogenic medium supplemented with 10 ng/mL TGF- β 1 under static condition. Controls were maintained in the same medium but without TGF- β 1. The medium was changed twice a week. Thin PLLA NF matrix and flat films were sterilized by soaking in 70% ethanol for 30 min, washed three times with PBS for 30 min each, and twice in complete medium for 1 hour each on an orbital shaker at 75 rpm. Cells were plated at a density of 10,000 cells/cm². After the initial 24 hours of seeding and culture, the cell culture medium was replaced by chondrogenic medium supplemented with 10 ng/mL TGF- β 1. Controls were maintained in the same medium but in the absence of TGF- β 1. The medium was changed twice a week.

SEM observation

Constructs were rinsed in PBS, fixed in 2.5% glutaraldehyde, and post-fixed in 1% osmium tetroxide. Samples were dehydrated in increasing concentrations of ethanol and hexamethyldisilazane (HMDS). The samples were then sputter-coated with gold and observed under a SEM (Philips XL30 FEG) at 15 kV.

RNA analysis

Samples were homogenized with a Tissue-Tearor. Total RNA was isolated using RNeasy Mini Kit (Qiagen, Valencia, CA, USA), and DNA was digested by RNase-free DNase set (Qiagen) according to the manufacturer's protocol. The cDNA was reverse-transcribed with TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA, USA). Real time PCR was done using TaqMan Universal PCR Master Mix (Applied Biosystems) and specific primers for sox-9 (pre-designed, Assay ID Hs00165814_m1, Probe sequence CGAGCACTCGGGCAATCCCAGGGC) on an ABI Prism 7500 Real time PCR system (Applied Biosystems). The gene expression level was normalized against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression. RT-PCR was performed using specific primers for collagen type II (5'-CAGGTCAAGATGGTC 3'-and 5'-TTCAGCACCTGTCTCACCA-3'), aggrecan (5'-TGAGGAGGGCTGGAACAAGTACC-3'

and 5'-GGAGGTGGTAATTGCAGGGAACA-3') and GAPDH (5'-GGGCTGCTTTTAACTCTGGT-3' and 5'-GCAGGTTTTCTAGACGG-3') [7].

Histological and immunohistological analysis

Constructs were washed in PBS, fixed with 3.7% formaldehyde in PBS overnight, dehydrated through a graded series of ethanol, embedded in paraffin, and sectioned at a thickness of 5 μ m. For histological analysis, sections were deparaffinized, rehydrated, and stained with Alcian blue. For immunohistochemistry stain, rehydrated sections were pre-treated with papain solution for 15 min, incubated with collagen type II antibody (Thermo Fisher Scientific Inc., Fremont, CA, USA) at 1:100 dilutions for 1 hour and detected by a cell & tissue staining kit (R&D systems Inc., Minneapolis, MN, USA) according to the manual. All sections were counterstained with hematoxylin. For an immunofluorescence stain, rehydrated sections were pre-treated with papain solution for 15 min, incubated with collagen type II antibody at 1:100 dilutions for 1 hour, rinsed with PBS three times for 5 min each, then incubated with FITC-labeled secondary antibody at 1:100 dilutions for 1 hour. After rinsing three times with PBS, sections were mounted using mounting medium containing 4'-6-diamidino-2-phenylindole (DAPI) and observed under a fluorescence microscope.

Calcium content quantification

After 6 weeks of cell culture, constructs for mineralization quantification were washed three times for 5 min each in distilled water and then homogenized in 400 μ L 1N hydrochloric acid. The lysate was incubated overnight to extract calcium. A total calcium content of each construct was determined by o-cresolphalein-complexone method following the manufacturer's instructions (Calcium LiquiColor, Stanbio laboratory, Boerne, TX, USA).

Glycosaminoglycan (GAG) assay

Constructs were harvested after 3 and 6 weeks of culture, washed with PBS, and digested with 200 μ L papain solution (280 μ g/mL in 50 mM sodium phosphate, pH 6.5, containing 5 mM *N*-acetyl cystein and 50 mM EDTA) for 24 hours at 65°C. GAG content was measured by reaction with dimethylmethylene blue (DMMB) [15]. Optical density was determined at 525 nm and GAG content of each construct was calculated using shark chondroitin 4-sulfate as the standard.

Statistical analysis

Values were reported as mean $\pm$ S.D. based on triplicate cell cultures (n=3). The experiments were performed twice to ensure reproducibility. To test the significance of observed differences between the study groups, the Student's t-test was applied. A value of $p < 0.05$ was considered to be statistically significant.

Results

Osteogenesis of hMSCs on NF scaffolds

hMSCs were seeded on NF scaffolds and cultured in the presence or absence of dexamethasone (Dex) for 6 weeks. After 6 weeks of cell culture in osteogenic medium, the constructs were highly mineralized throughout, shown by von Kossa stain (Fig. 1A). For control culture without Dex, there was no mineralization (Fig. 1B). In conjunction with histological data, the calcium quantification assay showed that Dex treatment greatly induced cells to deposit calcium in constructs (Fig. 1C). This data showed that NF scaffolds support in vitro differentiation of hMSCs into osteoblasts, resulting in mineralized bone formation.

Morphology and sox-9 gene expression of hMSCs on thin NF matrix and smooth films

To investigate the effect of NF matrix on chondrogenic differentiation of hMSCs, the cells were seeded and cultured on thin NF matrix and smooth films. After the initial 24 hours of culture, the cells on smooth films were flat with larger spreading areas (Fig. 2A and C, low and high magnifications). In contrast, the cells on NF matrix showed a more rounded morphology with smaller spreading areas (Fig. 2B and D, low and high magnifications). After 24 hours of culture on NF matrix or smooth films, culture medium was replaced by chondrogenic medium with or without TGF- β 1 supplement. It was shown, after 7 days of induction in the presence of TGF- β 1, sox-9 gene expression was significantly increased only on NF matrix (Fig. 2E).

Gene expression of cartilage differentiation markers on NF scaffolds

After 24 hours of initial seeding and culture, cells aggregated inside the pores of the NF scaffolds (Fig. 3A and B, low and high magnifications), mimicking the condensation process of MSCs during early in vivo chondrogenic development. The constructs were cultured in the presence or absence of TGF- β 1 for 3 weeks. The expression of aggrecan gene was greatly increased and the expression of collagen type II gene was specifically induced for cultures in the presence of 10 ng/mL TGF- β 1 (Fig. 3 C).

Histological and immunohistological analysis of cells cultured on NF scaffolds

Cells were seeded on NF scaffolds and cultured in the presence or absence of TGF- β 1 for 3 and 6 weeks. Sections were first stained with Alcian blue. It was found that for all the samples, after 3 weeks of culture, the cells were distributed throughout the entire scaffolds and ECM was secreted and deposited in the scaffolds. In the absence of TGF- β 1, Alcian blue stain was negative both for 3-week (Fig. 4A) and 6-week (Fig. 4B) cultures. With the supplement of TGF- β 1, 3-week cell culture showed weak stain (Fig. 4C), and 6-week culture showed very strong Alcian blue stain (Fig. 4D). Higher magnification view showed that the cells were fibroblast-like in the absence of TGF- β 1 after 6 weeks of cell culture (Fig. 4E); but in the presence of TGF- β 1, round chondrocyte-like cells were embedded in lacunae (Fig. 4F), resembling the typical cartilage tissue structure. After the constructs were cultured for 6 weeks, collagen type II protein was not detectable by immunohistological method for constructs in the absence of TGF- β 1 (Fig. 4G), but strongly detected for cultures in the presence of TGF- β 1 (Fig. 4H). Since the pore walls were also stained positive for TGF- β 1 treatment samples, to exclude the possibility of adsorption of developed color substances by the NF matrix walls during the immunohistochemistry procedure, samples were also processed by immunofluorescence method. It was shown that collagen type II stain was negative for constructs cultured in the absence of TGF- β 1 (Fig. 4I), but strongly detected for cultures in the presence of TGF- β 1 (Fig. 4H). Collagen type II protein was mainly distributed in the extracellular matrix (ECM) and on the surface of pore walls of scaffolds. Meanwhile, it was found that there were more cells for the cultures with TGF- β 1, showing enhanced proliferation in the presence of TGF- β 1.

Glycosaminoglycan (GAG) accumulation of cells cultured on NF scaffolds

The GAG content was quantified for constructs cultured for 3 weeks and 6 weeks. In the presence of TGF- β 1, GAG was induced to secrete after 3 weeks and 6 weeks of culture, and increased with culture time (Fig. 5).

Discussion

In this study, we tested the possibility of NF scaffolds to support both bone and cartilage development. We have demonstrated that PLLA NF scaffolds effectively supported both osteogenesis and chondrogenesis of hMSCs under specific inductive factors.

PLLA NF scaffolds incorporating BMP-7 containing nanospheres have been implanted subcutaneously in rats in a previous study [13]. Significant ectopic bone formation was induced by this controlled-releasing system, showing NF scaffolds supporting bone formation with proper inductive factors provided. Consistent with in vivo observations, the present study further demonstrated that the NF scaffolds can also support hMSCs along osteogenic development route in vitro.

The chondrogenesis of hMSCs on the same scaffold was then investigated. Chondrogenesis is a tightly controlled development event, involving multiple steps including condensation of mesenchymal stem cells, chondrogenic commitment, and differentiation into chondrocytes [16], which is controlled by both extracellular and intracellular cues. The extracellular factors, including ECM and certain growth factors, elicit the activation of intracellular events, leading to the cartilage specific gene expression under the control of certain cartilage-specific transcription factors, such as Sox-9 [17]. Members of the TGF- β family of growth factors play critical roles in chondrogenesis during embryonic skeletal development. It has been shown that several TGF- β s, including TGF- β 1, 2, 3 stimulated in vitro chondrogenesis [18-20]. In our study, we used TGF- β 1 to induce the cartilage development on both 2D thin matrix and 3D scaffolds. Since 3D culture itself may also induce chondrogenesis [21], to separate the 3D culture effect from the matrix structure effect, hMSCs were first seeded on thin NF matrix and smooth films. It was found that after 7 days of culture, the expression of sox-9 gene was increased only on NF matrix, showing the synergistic effect of NF matrix with TGF- β 1 to the early chondrogenic commitment of hMSCs. It has been reported that as mesenchymal stem cells become chondrogenic, the cells acquire a spherical morphology and express the essential chondrogenic transcription factor Sox-9 [22]. Disruption of stress fibers and alteration of cell shape induced the chondrocyte phenotype [23,24] and promoted MSCs to commit to the chondrogenic lineage [25]. The cells showed a round shape on the NF matrix and this was consistent with the effect of NF matrix on hMSCs commitment. This data showed promising effects of NF matrix on chondrogenic differentiation of hMSCs. Therefore, porous NF scaffolds were used to support long-term 3D cartilaginous development. After 3 weeks of culture with TGF- β 1, cartilage specific collagen type II gene was induced to express, as well as the aggrecan gene expression was further enhanced, showing the chondrogenic development was induced. The morphology of cartilage tissue was observed by histological and immunohistological analysis. After 6 weeks of culture, a cartilage-like tissue formed on 3D NF scaffolds, composed of round, chondrocyte-like cells surrounded by ECM rich in GAG (as shown by Alcian blue stain) and collagen type II (as shown by immunostain). Quantitative biochemical assay also confirmed the accumulation of GAG into the constructs during 6 weeks of culture. Taken together, these observations strongly suggest that the NF scaffolds resembling a native collagen fibrillar matrix, with appropriate pore sizes, and a high porosity, effectively support chondrogenesis of hMSCs in the presence of TGF- β 1.

Conclusions

Multi-potent human bone marrow-derived mesenchymal stem cells (hMSCs) are seeded on highly porous nanofibrous (NF) PLLA scaffolds fabricated using a phase separation technique, and the constructs are induced for osteogenesis and chondrogenesis. Under osteogenic conditions, the NF scaffolds support osteoblastic differentiation of hMSCs, resulting in enhanced mineralized bone formation. Under chondrogenic conditions (with TGF- β 1), hMSCs

cultured on an NF matrix assume a chondrocyte-like morphology and express early chondrogenic commitment marker gene *sox-9* at significantly high level over that on the control (smooth-walled) scaffold. In the presence of TGF- β 1, cartilage tissue is developed on PLLA NF scaffolds, with the cartilage-specific gene expressed, glycosaminoglycan and type II collagen accumulated, and typical cartilage morphology formed. These findings suggest that NF scaffolds can support both bone and cartilage development and are excellent candidate scaffolds for osteochondral defect regeneration.

Acknowledgments

The authors would like to acknowledge the financial support from the National Institutes of Health (Research Grants DE015384, GM075840 and DE017689: PXM).

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Figure 1.

Osteogenesis of hMSCs on NF scaffolds. After 6 weeks of culture in the presence of dexamethasone (Dex), constructs were mineralized throughout as shown by von Kossa staining (A). There was no significant mineralization for control constructs cultured in the absence of Dex (B). Scale bar: 200 μm . The total calcium content was quantified for the constructs cultured with or without Dex (C) (** $p < 0.01$, compared to cultures without Dex supplement).

Figure 2.

Morphology and sox-9 gene expression of cells cultured on PLLA NF thin matrix and smooth films. SEM view of cells cultured on smooth films (A, C) and NF matrix (B, D) for 24 hours. Cells were more spreading on flat films and rounded on NF in matrix. Sox-9 gene expression was increased only for cells cultured on NF matrix with TGF-β1 supplement after 7 days of culture (E) (** $p < 0.01$, compared to smooth film culture with TGF-β1 or NF matrix culture without TGF-β1).

Figure 3.

Morphology and chondrogenic marker genes expression of cells cultured on 3D NF scaffolds. (A, B) SEM view of hMSCs after 24 hours of culture on PLLA NF scaffolds. Aggregates of cells formed inside pores of NF scaffolds. (C) After 3 weeks of culture with or without TGF- β 1 supplement, total RNA was analyzed for aggrecan and collagen type II gene expression. TGF- β 1 treatment strongly increased aggrecan gene expression and induced cartilage-specific collagen type II gene expression on NF scaffolds.

Figure 4.

Histological analysis of hMSCs cultured on NF scaffolds. Cultures were maintained in the presence or absence of TGF- β 1 for 3 or 6 weeks. Sections were stained with Alcian blue or processed for immunohistological analysis. In the absence of TGF- β 1, Alcian blue staining was negative both for 3-week (A) and 6-week (B) cultures. With the supplement of TGF- β 1, 3-week culture showed weak stain (C); 6-week culture was strongly stained by Alcian blue (D). Higher magnification view showed the cells were fibroblast-like in the absence of TGF- β 1 after 6 weeks of culture (E); while round chondrocyte-like cells were embedded in lacunae in the presence of TGF- β 1 (F). Immunohistological staining of collagen type II was done for constructs cultured for 6 weeks. Collagen type II protein was not detectable in the absence of TGF- β 1 culture (G) though strongly detected in the presence of TGF- β 1 (H).

Immunofluorescence staining of collagen type II was weak for the constructs cultured without TGF- β 1 supplement (I), but strong staining was observed for constructs cultured in the presence of TGF- β 1 (J). Collagen type II was mainly distributed in the newly synthesized ECM and pore wall surface. Scale bar: 100 μ m

Figure 5.

GAG accumulation during chondrogenesis on NF scaffolds. GAG content was quantified for constructs cultured for 3 and 6 weeks. In the presence of TGF-β1, GAG secretion was induced in the 3-week and 6-week cultures (** $p < 0.01$, compared to cultures without TGF-β1 supplement), and the GAG content increased with culture time (## $p < 0.01$, compared between 3-week and 6-week cultures).