Influence of Collagen I Nanospheres on the Growth and Osteogenic Difference of Rat Bone Marrow Stromal Cells

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Abstract

Bone marrow stromal cells (BMSCs) are a promising cell source for bone tissue-engineering application due to their osteogenic differentiation potential. Effective growth and osteoblastic differentiation of BMSCs are required in promoting successful bone formation. In this study, the effect of collagen I nanospheres on the proliferation and osteogenic differentiation of rat BMSCs in vitro was investigated by the addition of collagen I nanospheres directly into the culture medium. The mitochondrial activity of rat BMSCs was measured by colorimetric assay: results showed that collagen I nanospheres promoted rat BMSC proliferation. Osteogenic differentiation of rat BMSCs was qualified via measurements of alkaline phosphatase (ALP) activity and matrix mineralization. Our results demonstrated that there was a striking difference in ALP staining and von Kossa staining. Collagen I nanospheres induced an increase in the number of ALP-positive cells. Bone nodule formation occurred under cells treated with or without collagen I nanospheres, but the extent of mineralization was increased when collagen I nanospheres were present in cultures. These data support that collagen I nanospheres might be suitable for the cultivation of BMSCs for clinical applications. Moreover, the observations suggest that collagen I nanospheres might have a great potential for bone tissue engineering.

Keywords: Bone marrow stromal cells (BMSCs), Osteogenic differentiation, Collagen I nanospheres

1. Introduction

Tissue engineering offers an emerging approach towards effective repair of damaged or diseased skeletal tissue. Successful bone tissue engineering requires a suitable cell source, appropriate culture conditions and a biocompatible scaffold as the basic elements. Recent advances in regenerative medicine have shown that stem cells are expected to play an important role in the repair of skeletal defects. Bone marrow stromal cells (BMSCs) have been demonstrated to be an attractive source of cells for bone tissue-engineering applications because of their self-replication and osteogenic differentiation capabilities [1]. The capability of BMSCs for self-renewing allows their extensive in vitro expansion to obtain an appropriate number of cells [2]. Moreover, BMSCs can differentiate along the osteogenic lineage and produce bone-like nodules with a mineralized extracellular matrix when cultured in medium supplemented with dexamethasone, β-glycerolphosphate and ascorbic acid [3]. Several studies have shown that BMSCs are able to repair bone defects in various animal models [4,5].

A number of physiological extracellular matrix proteins have been used as scaffolds for bone tissue engineering, such as collagen, hyaluronan and fibrin [6]. Type I collagen, one of the major structure proteins of bone, contains specific cell binding sites, particularly the arginine-glycine-aspartic acid (RGD) sequences, that promote cell adhesion and proliferation via direct interaction with integrin receptors on the cell surface [7]. Type I collagen-specific binding has also been found to mediate the osteogenic response of a bone cell line [8] and human BMSCs [9]. Various forms of collagen matrix, such as fibers, membranes, sponges and gels, have been developed for bone tissue reconstruction [10]. Moreover, many researchers have studied the influence of collagen on the growth and osteogenic differentiation of

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BMSCs [11-13]. Lund et al. prepared hydrogel microbeads consisting of collagen I and agarose in the presence of mesenchymal stem cells and found that the hydrogel bead format allows controlled cell differentiation [14]. Arpornmaeklong et al. reported that collagen sponges were able to support growth and oestoblastic differentiation of BMSCs to a greater extent than chitosan sponges [13]. Shih et al. prepared type I collagen nanofibers by electrospin technology and demonstrated that they supported the growth of mesenchymal stem cells without compromising their osteogenic differentiation capability [15].

Mammalian cells evolve in vivo in close contact with the extracellular matrix, which is a substratum with nanoscale features [16]. The interactions between cells and extracellular matrix affect the behaviors of the cells. A couple of investigations have characterized the growth and differentiation potential of BMSCs on collagen I nanofibers in vitro [11,15]. These studies demonstrated that collagen I electrospun nanofibers supported the growth and osteogenic differentiation of BMSCs to some extent.

To date, no investigation has employed nano-sized collagen particles to repair damaged bone. Moreover, no study has been focused on the use of culture medium that contains nano-sized collagen I particles to culture BMSCs. Culture medium composition is one of the key factors in optimization and control of the growth and differentiation of cultivated cells. Nano-particles show unique physical and chemical properties due to their ultra-fine size. Moreover, nanoparticles can provide huge surface areas for cell growth. Lan et al. studied the influences of nano-sized collagen I particles on hepatocyte cultures. The hepatocytes exhibited better viability and function after addition of high concentration of collagen I particles [17]. Our previous animal studies revealed that chitosan membrane containing collagen I nano-spheres promoted efficient re-epithelialization of the wound [18].

In this study, we compared proliferation and the osteogenic differentiation potential of BMSCs cultured in medium supplemented with and without nano-sized collagen I particles in order to assess whether nano-sized collagen I particles have a potential for bone tissue-engineering applications. MTT assay was used to investigate the effect of collagen I nanospheres on proliferation. The expression of osteogenic marker and the functional capacity to differentiate to form a mineralized bone nodule were assessed by ALP staining and von Kossa staining, respectively.

2. Materials and methods

Nano-sized collagen I particles with diameters of 20–30 nm, produced using a high-voltage electrostatic field system as described in detail elsewhere [17,19]. All chemicals used in this study were of reagent grade.

2.1 Isolation of BMSCs and cell culture

Rat BMSC cultures were prepared according to the procedure as described previously by van den Dolder et al., with only minor modification [20]. BMSCs utilized therein were obtained from the femur of 4–6 weeks old Sprague-Dawley rats (purchased from the National Laboratory Animal Center, Taiwan). Before the beginning of the study, the ethical committee for animal experiments at the Central Taiwan University of Science and Technology, Taichung, Taiwan approved the protocols. Rats were anaesthetized intramuscularly with Zoletil 50 (Virbac, France) and 2% Rompun solution (Bayer, Germany) (1:2 ratio, 1 mL/kg) in an aseptic animal operation room. The femurs were removed and dissected free of adhering soft tissues. The distal ends of the bones were then cut open with scissors, and the medullary cavities were flushed with low-glucose Dulbecco's modified Eagle medium (L-DMEM; Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Gibco) (standard growth medium), with a needle and syringe. A suspension of bone marrow cells was obtained by repeated aspiration of the cell preparation through a needle. Cells were plated in a 75-cm^2 cell culture flask (Costar, Cambridge, MA) in standard growth medium and incubated at 37°C under 5% CO_2. Culture medium was refreshed every 2 days. The adherent cells were allowed to reach ~80% confluence. Cells were passaged in culture and cells at their 2–3rd passage were used in all the experiments.

2.2 Analysis of cell proliferation

For the cell proliferation assay, a 0.1 μg/mL collagen solution that contained nano-sized collagen I particles was prepared in standard growth medium. 100 μL of 5×10^4 cells/mL of cultured BMSCs were seeded in individual wells of a 96-well tissue culture plate. Cell cultures were maintained in a humidified atmosphere with 5% CO_2 at 37°C. After culturing for 24 h, the culture medium was replaced with 100 μL of 0.1 μg/mL collagen solution. A culture without addition of collagen I particles acted as a control group. After culturing for 2 days, the proliferation of cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; USB, Amersham Life Science, Cleveland, OH) assay and was based on the mitochondrial conversion of tetrazolium salt. The medium was replaced with 10 μL/well of MTT solution (5 mg/mL) and 100 μL/well of culture medium and incubated at 37°C for 4 h to enable formation of insoluble dark-blue formazan crystals. The solution was then removed and 100 μL/well of acid isopropyl alcohol (0.04 M HCl in isopropyl alcohol) was added to all wells and mixed thoroughly to dissolve the crystals. After a few minutes at room temperature, the optical density was measured using an ELISA reader (uQuant; Bio-Tek Instruments, Inc., Sunnyvale, CA) at a wavelength of 570 nm and a reference wavelength of 650 nm. The number of viable cells in each well was calculated by converting the optical density values of the MTT assay into number of cells/well based on a standard curve.

2.3 Analysis of alkaline phosphatase and mineralized nodule formation

The effect of nano-sized collagen I particles on the osteoblastic differentiation in vitro was evaluated by ALP

staining and von Kossa staining over a period of 4 weeks. Briefly, 2 × 10^4 BMSCs/well were added to the osteogenic-differentiation medium containing 0.1 μg/mL of nano-sized collagen I particles in a 35-mm culture dish. Collagen I nanoparticle-free medium acted as a control group. The osteogenic-differentiation medium was standard growth medium supplemented with 50 μg/mL ascorbic acid (Sigma, St. Louis, MO), 10 mM β-glycerophosphate (Sigma) and 10^{-8} M dexamethasone (Sigma). Presence and activity of ALP in the cell cultures was shown through hydrolysis of naphthol AS-BI phosphate. At weeks 1, 2 and 4, the cell layers were rinsed twice with phosphate-buffered saline (PBS), fixed in 3.7% formaldehyde (Acros, Geel, Belgium) and stained using naphthol AS-BI alkaline solution (Sigma kit no. 86-R) according to the manufacturer's instructions. After washing three times with deionized water to remove the remaining stain, the cell layers were observed using an inverted optical microscope (Axiovert 25; Carl Zeiss, Inc., Göettingen, Germany).

Von Kossa staining was utilized to examine the formation of the mineralized matrix during osteogenic differentiation and performed as reported with some modification [21]. After 2, 3 and 4 weeks, cultures were washed with PBS twice. Fixation of the cells in 2% glutaraldehyde (Acros) for 20 mins was followed by three wash steps with deionized water. After incubation for 30 min with 5% silver nitrate (Union Chemical Works Ltd., Hsinchu, Taiwan) in darkness at room temperature, the cells were rinsed twice in deionized water. After being air-dried, cells were exposed to ultraviolet light for 1 h until color development was complete, and then the cells were immersed in 5% sodium thiosulfate (Union Chemical Works Ltd., Hsinchu, Taiwan) for 2 min. Finally, cellular nodular structures were visualized by counterstaining with 0.1% nuclear fast red (Sigma) dissolved in 5% aluminum sulfate (JT Baker, Phillipsburg, NJ) for 5 min. After washing twice with deionized water, the newly formed bone nodules were observed with an optical microscope. Calcium salts stain dark brown/black with this technique.

2.4 Statistical analysis

Statistical analysis was performed using a two-tailed, unpaired Student's *t*-test. Significant difference was set to $p < 0.05$.

3. Results

3.1 Cell proliferation in different culture conditions

After 2 days of culture, the MTT assay indicated that rat BMSCs proliferated typically in the medium with and without collagen I nanospheres (Fig. 1). Furthermore, the medium that contained nano-sized collagen I particles had significantly more cells than the control medium ($p < 0.05$). These results demonstrate that collage I nanospheres can increase the rate of BMSC proliferation.

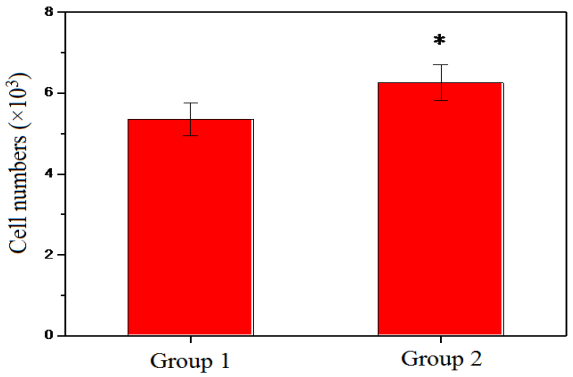

Figure 1. Effect of nano-sized collagen I particles on the cell number after culturing for 2 days, as determined by MTT assay. BMSCs were cultured in the medium without collagen (group 1) and in the medium with 0.1 μg/mL collagen (group 2). The results were obtained by three individual experiments and expressed as mean ± SD (n = 3). Statistical significance at the $p < 0.05$ level is shown by the asterisk.

3.2 Osteogenic differentiation of BMSCs

ALP is known to be localized on the cell membrane of osteogenic cells and well recognized as a marker for the differentiation of stromal cells to osteoblasts [22]. To elucidate the impact of collagen I nanospheres on osteogenic differentiation of rat BMSCs, ALP activity was measured by histochemical method after 1, 2 and 4 weeks of culture. As shown in Figure 2, ALP-positive cells were detected at 1 week of culturing in both samples in the presence or absence of collagen I nanospheres in culture, indicating induction toward the osteogenic phenotype. Subsequently, cells had relatively increased intensity of ALP stain during the following weeks of culture. However, the differences in the degree of ALP expression at all time points were apparent. A greater level of ALP positivity was produced by the addition of collagen I nanospheres. In addition, collagen I nanosphere-treated BMSCs showed significant changes in morphology, with cells clustering in discrete areas of the well after 4 weeks of culturing.

3.3 Effect of collagen I nanospheres on mineralized nodule formation by BMSCs

The final stage of progenitor cell development into the osteogenic lineage is matrix mineralization. Therein, the influence of collagen I nanospheres on the ability of rat BMSCs to mineralize the extracellular matrix that they produce was evaluated by von Kossa staining over a 4-week period. Von Kossa staining is specific for calcified extracellular matrix [23]. Dark staining of the nodules demonstrates deposition of mineralized matrix characteristic of bone formation. BMSCs cultured in the control medium were not able to form the mineralized nodules until 3-week culture, while mineralization of collagen I nanospheres-treated BMSCs was observed after 2 weeks of culturing (Fig. 3). Mineralization within the cultures increased over time for both groups. However, deposition of calcified extracellular

matrix was greater with collagen I nanosphere-treated BMSCs compared with the control for each time point. Moreover, cultures in the absence of collagen I nanospheres formed isolated mineralized nodules widely dispersed on the culture dish surface at 3 and 4 weeks. In contrast, the mineralized nodules clustered in the well were observed at 3 and 4 weeks for collagen I nanosphere-treated BMSCs. These results indicate that collagen I nanospheres can enhance the secretion of extracellular matrix of BMSCs to form the mineralized nodules.

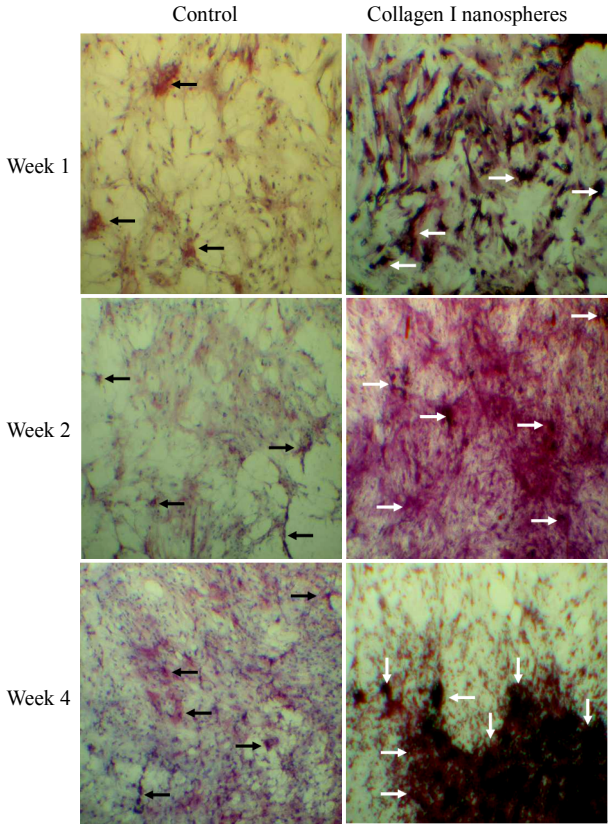

Figure 2. ALP reaction appeared as staining of osteoblasts differentiated from BMSCs cultured in the presence or absence of collagen I nanospheres after 1, 2 and 4 weeks' culturing (original magnification, × 40). Positive reaction (arrows) was observed as red staining in the cells.

4. Discussion

Bone formation involves a complex sequence of stages including (1) proliferation of osteoprogenitors from mesenchymal stem cells; (2) the differentiation phase, which goes along with the increased activity of ALP; and (3) the maturation stage, which is characterized by induction of mineralized nodule formation [21,24]. In this study, we focused on the application of collagen I nanospheres in bone regeneration. Collagen I was chosen due to the fact that it is the major organic macromolecule in bone matrix. The influence of collagen I nanospheres on the proliferation, differentiation and maturation stage of rat BMSCs was assessed by MTT assay, ALP staining and von Kossa staining, respectively.

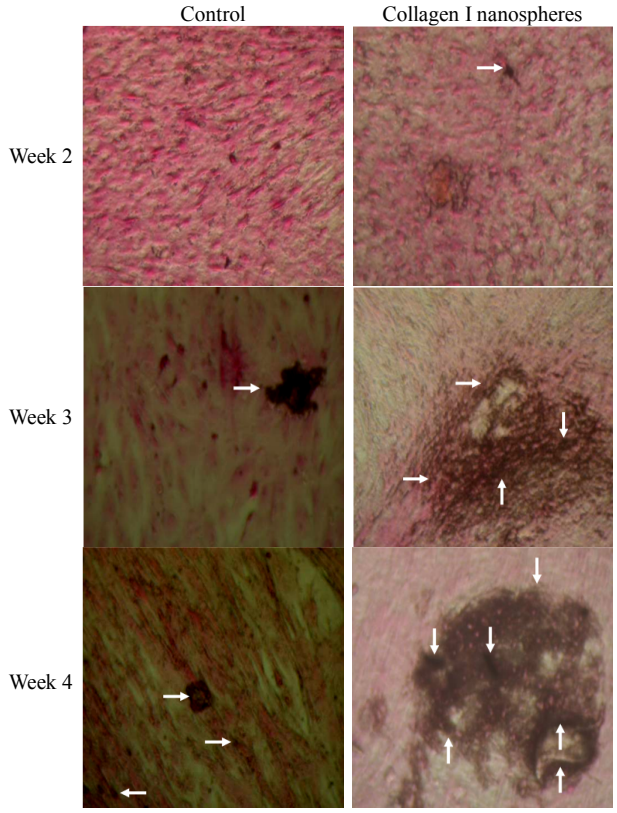

Figure 3. Von Kossa staining of rat BMSCs treated with collagen I nanospheres or in control conditions for 2, 3 and 4 weeks (original magnification, × 100). Dark staining of the nodules (arrows) demonstrates deposition of mineralized matrix characteristic of osteogenesis. Cell nuclei have been counterstained in red.

It is important to possess a high proliferative capacity to use BMSCs in tissue engineering applications such as for the reconstruction of massive bone defects. Previous studies have shown that type I collagen can be successful used as a scaffold material for 3D culture of BMSCs [13,25]. The current result indicates that the addition of collagen I nanospheres significantly increases the number of cells. Nano-sized particles or fiber could provide huge surface areas for cell growth. Shih et al. reported higher cell viability of mesenchymal stem cells on electrospun type I collagen nanofibers compared to smooth surfaces [15].

Osteogenic differentiation from BMSCs is an important step of bone formation. ALP, a membrane-bound enzyme, is a differentiation marker of early osteoblasts. There was a tendency for better differentiation of BMSCs towards osteoblasts after addition of 0.1 μg/mL of nanoscale collagen I particles into the culture medium, as demonstrated by ALP staining. Several studies have demonstrated that type I collagen increased the expression of ALP and other markers of the osteoblastic phenotype [13,26,27]. Shih et al. also found that type I collagen nanofibers supported osteogenic differentiation in which cellular ALP production was apparent [15].

The initiation of mineralization was delayed in the BMSC cultures in the absence of collagen I nanospheres, while the addition of 0.1 μg/mL of nano-sized collagen I particles to the

cell cultures promoted the formation of nodules and increased the rate of mineralization of nodules. The enhancement of osteogenic differentiation and mineralization of bone matrix through type I collagen is well documented. Mizuno et al. reported that BMSCs showed high ALP activity and collagen synthesis and formed mineralized tissues when they were cultured with type I collagen matrix gel [9]. They also found that collagen-α2β1 integrin interaction plays a crucial role in the osteoblastic differentiation of bone marrow cells. In addition, several studies showed that collagen contains specific cell binding sites, particularly the RGD amino acid sequences, which induce cell adhesion and promote differentiation on the surface of biomaterials [28].

In summary, our data indicated the potential of proliferation and osteogenic differentiation of BMSCs cultured in medium containing collagen I nanospheres and the possibility of using collagen I nanospheres for bone regeneration.

5. Conclusions

In the present study, we investigated the effect of nano-sized collagen I particles on the growth and proliferation of BMSCs, with respect to their suitability in bone tissue engineering. As a result, we found that collagen I nanospheres had better enhancing effect on cell proliferation as compared to those BMSCs cultured in the absence of collagen I nanospheres. Furthermore, ALP increased in the medium with nano-sized collagen I particles from BMSCs, suggesting that nano-sized collagen I particles induced a greater osteoblastic differentiation in these cells. Finally, the matrix deposition and mineralization were enhanced by addition of collagen I particles, as determined by von Kossa staining. These observations are favorable for the nano-sized collagen I particle-containing scaffold to be used in bone tissue engineering. Further study is currently being conducted to examine the proliferation and osteogenic differentiation of BMSCs in three-dimensional scaffolds composed of collagen I nanospheres. The feasibility of using the three dimensional (3D) scaffolds seeded with BMSCs for repairing damaged bone in vivo will be also evaluated.

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