Osteogenic Differentiation of Mesenchymal Stem Cells in their Native Niche Like Environment

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Abstract

Niche is an important microenvironment regulating stem cell’s natural behavior and its fate. Mesenchymal Stem Cells (MSCs) are the most common cells used in bone tissue engineering. However, common culture procedures do not retain MSCs in their niche and apply in vitro expansion which can cause abnormal behavior. In this study, we propose that, by keeping MSCs in their native niche, natural behavior of these cells and their interactions are maintained and the quality of differentiation will enhance. In our previous studies, we isolated MSCs in their native niche like environment (native-MSCs) in the form of aggregates. Herein, in order to explore the osteogenic differentiation of these cells, they were isolated from bone marrow, and in vitro osteogenesis was assessed via alizarin red and real time PCR. It was observed that they differentiated after 6 days, which is remarkably faster than the conventional 21-day period. Higher expression of Runx2, Spp1 and alkaline phosphatase in native-MSCs also confirmed in vitro results. We also examine their attachment on poly (L-lactic acid) (PLLA) scaffold. In sum, native-MSCs exhibited faster differentiation than expanded MSCs which proves the importance of keeping stem cells in their niche.

Keywords: Niche; Mesenchymal Stem Cell; Osteogenic Differentiation; Bone Tissue Engineering.

List of Abbreviation: Native-MSCs: MSCs which are kept in their niche like environment; Ex-MSCs: Expanded MSCs are common MSCs which isolate and expand based on their adherent properties.

Introduction

Niche, as an important microenvironment first proposed by R. Schofield in 1978 [1], is responsible for regulating and preserving natural behavior of stem cells. Any stem cell’s niche substantially consists of the stem cell, mesenchymal or stromal cells, cell-cell, cell-extracellular matrix interactions, and external signals from other sources through blood vessels and neurons. These intrinsic and extrinsic factors influence the behavior and fate of stem cells as well as the shape, differentiation, and proliferation of them [2, 3]. The importance of niche is obvious as Leanne Jones (2008) has entitled it as a home for stem cells [4].

Mesenchymal Stem Cells (MSCs) have attracted great attention in regenerative medicine because of their multilineage potential and easy isolation from bone marrow, adipose tissue, and the umbilical cord [5, 6]. It is shown that bone marrow-derived MSCs (Bm-MSCs) are the most common cells used in bone tissue engineering [7, 8]. However, lack of identical markers for their selection and their low quantity leads to usage of cell culture procedures which are mainly based on plastic adhesion property and do not retain MSCs in their native niche. This can lead to detrimental consequences and affect MSCs natural behaviour, so that raise concerns about their safety for cell-based treatments [9-11]. In our previous studies, in accordance with other studies, we explain how in vitro culture of MSCs cause the chromosomal abnormality of these cells, which may influence vital properties of them such as homing ability [12, 13]. Due to unpredictable time of bone surgery and its urgency, using autologous MSCs seems to be far-fetched since for having a considerable amount of cells necessary for bone regeneration a minimum of a two-week culture is needed [14].

Some studies have attempted to use uncultured mononuclear cells (Bm-MNCs) as a source of MSCs in order to prevent the in vitro culture of MSCs and have used them more instantly [15, 16]. Although Bm-MNCs have recently gained a great interest for being applied in stem cell therapies, the quantity of MSCs in Bm-MNCs are very low [17]. For this aim, a suitable source of cells containin-
ing sufficient MSCs kept in their niche, without the need of in vitro expansion, is favored.

In our previous study, we suggested that MSCs have aggregate nature and ex vivo expansion of MSCs may become unnecessary because of having adequate multipotent cells [18]. By omitting in vitro expansion, intrinsic properties of MSCs will be sustained and can be used instantly for urgent situations. We also showed that these cell complexes had niche-like properties and, using this protocol, MSCs can be kept in their native niche-like environment [19]. In this study, we mainly focus on osteogenic differentiation of MSCs in their niche-like environment derived from BM, referred to as native-MSCs. We also investigate the feasibility of their attachment to scaffold for bone tissue engineering purposes.

Material and Methods

Isolation of mesenchymal stem cells in their niche-like environment (native-MSCs) and expanded mesenchymal stem cells (ex-MSCs)

C57BL/6 mice were used as the source of native-MSCs and ex-MSCs. The mice euthanized morally and the ends of the tibia and femur of both legs were cut to extract the bone marrow in a centrifuge tube being centrifuged for 1 min at 800g. Bone marrow samples were diluted with phosphate-buffered saline plus the Ethylenediamine tetra acetate acid (PBS/EDTA; Gibco-BRL, GrandIslant, NY, USA) and filtered through a c. 20-μm strainer (a double-layered 40-μm nylon mesh; BD Biosciences, San Jose, CA, USA). Filter-retained fraction includes MSCs divided in two parts. One part was used as a source of native-MSCs without in vitro expansion, and another part was cultured with common culture conditions. This kind of scaffold was prepared by electrospinning technique; 0.45 g of poly (L-lactic acid) (PLLA) (sigma) was diluted in 9 ml chloroform (Merck, Germany) and 1 ml Dimethylformamide to reach 4.5 % (w/v) ratio. The solution was poured in a 10 ml syringe with a 21-gauge needle. A rotational collector, placed in a 15-cm distance from the needle, was used for collecting the electrospun fibers. Flow rate was set to 0.5 ml/h, and 10 KV voltage was used for transferring the solution droplet from the needle to the collector. After gaining 200 μm thickness, the mat was separated from the collector and the residual solvent was evaporated by vacuuming.

Osteogenic differentiation and alizarin red staining

In order to explore osteogenic differentiation, native-MSCs, instantly after isolation, and ex-MSCs were treated with Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS) (both from Gibco-BRL) plus 10 mM beta-glycerol phosphate (Merck, Darmstadt, Germany), 50 μg/mL ascorbic acid bi-phosphate (Sigma, St Louis, MO, USA), and 100 nM dexamethasone (Sigma). The medium was changed every 2 days. For confirming in vitro osteogenic differentiation of native-MSCs in comparison with ex-MSCs, alizarin red staining was performed. The cells were fixed with formaldehyde 4% for 10 min at room temperature, washed with PBS twice, and stained with 2% alizarin red solution (pH=4) for 15 min at room temperature. The results were assessed by alizarin red staining and compared on days 6, 11, and 21, respectively.

Table 1. Sequence of primers for quantitative real-time PCR.

| genes          | Forward primer          | Reverse primer          |
|---------------|------------------------|------------------------|
| Runx2         | 5′-AATGCTTCCCTGGGTGTATG-3′ | 5′-TCTGTCTGTTCGCCCTTCTTG-3′ |
| Spp1          | 5′-AACCAGCCAAGACTACTAC-3′ | 5′-CTTCAGAGGACACACACATT-3′ |
| Alkaline phosphatase | 5′-GGTAGATTTGCTCACAAACAC-3′ | 5′-CAGGCAAGTGTGTCAGG-3′ |
| Beta-actin    | 5′-CTCTTGGTGATGAATCTCTG-3′ | 5′-GTTGGCGCTAGAGGCAGG-3′ |

Real time RT-PCR

The expression of some osteogenic genes in native-MSCs and ex-MSCs was evaluated. Total RNA of cells on day 1, 3, and 6 was extracted and random hexamer primed cDNA synthesis was performed by Takara cDNA synthesis kit (Japan, clontec). Real-time PCR was done via Maxima™ SYBR Green/ROX qPCR Master Mix (Fermentas). 40 cycles PCR was performed in Applied Biosystems® 7500 Real-Time PCR Systems (life technologies, UK) and the melting curves were analyzed for assessing PCR specificity. All the reactions were repeated twice and the averages of threshold cycles were considered. REST software was used for data analysis. ΔΔCt method was used for comparing relative expression and Beta-actin was used as a housekeeping gene for normalization. Related primers are shown in Table 1.

Fabrication of poly (L-lactic acid) scaffolds

Native-MSCs were seeded on PLLA scaffolds and their attachment was examined for tissue engineering purposes. Before cell seeding, PLLA scaffolds were cut into a 2-cm diameter circular shape and both sides of them were sterilized with UV for 20 minutes. Due to the aggregate nature of our cell complexes, we were not able to count the cells. Therefore, 200 μl of filter retained fraction including native-MSCs was seeded on scaffolds, and basal medium was added to reach the final volume of 1 ml.

Scanning electron microscopy (SEM)

The surface morphology of scaffolds was assessed via a scanning electron microscope. The attachments of native-MSCs were also determined by scanning electron microscopy. Also, DAPI test was done in evaluating the presence of cells on the scaffolds before performing SEM.

Statistical analysis

All the experiments were repeated twice. The data are shown as mean ± standard deviation. The statistics were conducted via...
Results

Native-MSCs showed faster osteogenic differentiation than ex-MSCs

Ex-MSCs and native-MSCs were harvested and characterized as described before, and were treated with osteogenic medium [11]. However, native-MSCs were instantly treated by differentiation medium. Osteogenic outcomes of native-MSCs and ex-MSCs revealed a meaningful difference compared to their controls, indicating a proper differentiation induced by osteogenic medium. Native-MSCs showed osteogenesis on day 6, but no differentiation was screened for ex-MSCs on the respective day. Osteogenesis of native-MSCs increased on day 11 and ex-MSCs, still, had no osteogenic differentiation. By completing the 21-day interval, both native-MSCs and ex-MSCs had Osteogenesis. Control of native-MSCs showed no differentiation even after passing 21 days (Figure 1).

Bone-related genes expressed higher in native-MSCs on early days

As osteogenesis of native-MSCs was seen on day 6, we proposed that alteration in the expression pattern of osteogenic genes may initiate before the 6th day; hence, time points prior to this time, including day 6, was explored. Three osteogenic genes were compared on day 1, 3, and 6 in ex-MSCs and native-MSCs after differentiation. The expressions of Runx2 and Spp1 in native-MSCs and ex-MSCs had an ascendant trend. Their expressions were significantly higher in native-MSCs compared to ex-MSCs. However, native-MSCs showed a higher level of Spp1 than ex-MSCs on the first day. Although the difference between the expression of Spp1 in native-MSCs and ex-MSCS was decreased on day 3 and 6, Spp1 was expressed higher in native-MSCs. While an increase in alkaline phosphatase expression level was detected on the first day of osteogenic differentiation of native-MSCs, a decrease was observed in the succeeding days. By the 6th day, the expression of alkaline phosphatase was higher in ex-MSCS. Runx2 was expressed more in native-MSCs than ex-MSCs on all respective days and the level of this transcript was remarkably higher in native-MSCs compared to ex-MSCs on day 6 (Figure 2).

Discussion

In this study, the osteogenesis of MSCs kept in their native niche-like environment (native-MSCs) was investigated. These cells were derived from bone marrow and showed efficient and faster osteogenic differentiation.

Based on SEM photos, PLLA scaffold benefitted appropriate fibrous structure and the native-MSCs were attached and distributed on the scaffold properly. SEM photos also showed the aggregate nature of our derivative cell complexes (Figure 3).

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On days 1, 3, and 6, Runx2 was expressed higher in native-MSCs than ex-MSCs. High levels of this gene, as an essential transcription factor in osteogenesis causing osteoblast differentiation, resulted in osteogenesis [22]. Our results confirmed the correlation whereby high level of Runx2 leads to increase in Spp1 [23]. Spp1 is the murine ortholog of Osteopontin, and is one of the extracellular matrix proteins that play a crucial role in osteogenesis and bone remodeling. The increased expression of Spp1 in native-MSCs compared to ex-MSCs suggests a higher osteogenic potential in native-MSCs. This could be attributed to the native environment in which the MSCs were cultured, which allowed for a more efficient induction of bone differentiation.

The results of this study indicate that native-MSCs have a faster and more efficient osteogenic differentiation compared to ex-MSCs. This is likely due to the native environmental cues that are present in the native niche, which are lost during the ex vivo expansion process. The use of native-MSCs could potentially lead to more effective bone regeneration in clinical applications, as they maintain their osteogenic potential even after a short period of culture.
lar bone proteins which mainly induces natural mineralization and bone formation and is expressed by a variety of cells, including osteoblasts and osteocytes [24, 25]. Higher expression of alkaline phosphatase on early days in native-MSCs is another clue for fast bone regeneration. Previously, in accordance with our findings, McNamara LM. et al (2012) illustrated that culture of MSCs with conditioned medium derived from osteocytes caused an increase in alkaline phosphatase level on the early days and a decrease as the cells matured and mineral deposition happened. While in their study attempts were made to mimic niche-like environment for faster differentiation of MSCs [26], we introduced a cell source in which MSCs are kept in their natural niche like environment without any manipulation.

As our technique used native-MSCs instantly, it can be a suitable source for bone tissue engineering. Therefore, we examined the attachment of these cells on PLLA scaffolds. This scaffold is a promising candidate for tissue engineering purposes because of its integrity and supporting cell proliferation [27]. It was observed that native-MSCs were attached and distributed properly on this scaffold.

Mimicking and manipulating native niche has been explored recently in order to improve bone tissue engineering and also studying the niche mediated regulation of MSCs [28-30].

Using MSCs in their own niche derived from bone marrow can be worthwhile because they are kept in a native microenvironment with all intrinsic cellular interactions and signaling. By not separating MSCs from their native niche, lots of cell-cell, ECM and cytoskeleton interactions, and cell signaling which are pivotal for the natural behavior of MSCs are maintained [31, 32]. Our results found this protocol promising due to the fact that MSCs were not expanded in vitro and were kept in their niche-like environment so that the intrinsic behaviors of stem cells were retained. This study indicates notable osteogenic properties of native-MSCs and, for the first time, makes it possible to differentiate MSCs in their niche-like environment. Because of their independence from in vitro expansion, they can be also suggested as a beneficial source for instant cell therapy. While further studies are needed to identify the distinct component of these niche-like environments, it is also worthwhile to examine the multilineage differentiation of native-MSCs and their usage in bone tissue engineering and other subclasses of regenerative medicine.

Acknowledgments

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