HISTOLOGY

Mesenchymal Stem Cell Aging, Their Environment and Methods to Restore Their Quality

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ABSTRACT

Objective: **This article aims to explore the aging of mesenchymal stem cells (MSCs) during cell expansion and available methods to restore their quality.**

Materials and methods: **we searched articles in PubMed/Medline and our library to collect relevant publications of MSC aging, their microenvironment (niche), and various approaches to prevent MSC aging.**

Results and Discussion: **MSC expansion during culture may cause cell aging, which is reflected by change in growth, genotype, and phenotype that can be seen in morphology and function. There are various approaches that can be used to prevent MSC aging. Approaches to prevent MSC aging in culture can be done by various methods, including by determining donor characteristics, genetic engineering approaches, regulating stem cells microenvironment *in vitro*, and *in vitro* physical modification**

Conclusion: **Prevention of MSC aging during culture can be done by selection of MSC sources, genetic modification, and microenvironment and physical adjustments.**

KEY WORDS

mesenchymal stem cells, aging, cell culture, propagation, micro environment

INTRODUCTION

Stem cells, in particular mesenchymal stem cells (MSCs) are promising therapeutic agent especially in degenerative diseases. In general, MSC therapy needs a large amount of cells for single or multiple administrations. High therapeutic MSC doses were used in MSC human trials, such as in multiple sclerosis and frailty due to aging[1,2]. In order to produce a high number of cells, culture expansion is needed. Providing cells with a same quality by long-term cell culture becomes a challenge. Donor's characteristics such as age may determine the MSC quality[3]. Cells may experience many changes in morphology, phenotype and genetic make-up during culture expansion[4]. These alterations might reflect natural stem cell conditions *in vivo*, which become exhausted due to aging[5].

One of the hallmarks of aging in an individual is stem cell depletion[6]. In aging, the cells enter their senescence, as well as their surroundings (micro-environment or niche) are also impaired. An *in vitro* study found that stem cell senescence affected their function and further their efficacy in stem cell treatment. Aged MSCs are less effective than the younger ones when they are used in an attempt to cure various disease models[7]. This fact may be important in autologous MSC administration in aging patients. Moreover, culture conditions such as cell seeding amount, passage number, culture medium, and other external environment may cause cell aging and differences in cell performance in cell therapy[8]. Therefore the aging of MSCs is important to observe, such the effects of aging on proliferation rate, genomic stability, differentiation potential, paracrine secretion, and immune modulatory properties. The aim of this mini-review was to explore the aging of MSCs during cell expansion and available methods to restore their quality, and therefore would highlight MSC aging, their microenvironment (niche), and various approaches to prevent MSC aging.

MATERIALS AND METHODS

We searched articles in PubMed/Medline within 10 years on June 24, 2020, using keywords "mesenchymal stem cell" and "aged" or "aging" or "senescence" and "methods" and "culture" We also added appropriate articles that were found in our library. All articles that con-

Received on January 19, 2021 and accepted on March 24, 2021

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tained information on MSC aging, characteristics of MSC aging, MSC microenvironment, and methods to restore aging MSC quality were included. Data were presented descriptively in the form of text and figures.

RESULTS AND DISCUSSION

Mesenchymal stem cell aging

MSCs are widely used because of their properties that underlie their potential therapeutic effects. Those properties are differentiation capacity, immunomodulatory and immunosuppressive properties, and the ability to support parenchymal cell and resident stem cell niche[9]. Mesenchymal stem cells, like any other cells may become senescent and show a functional decline. Therefore, MSC age-related changes might affect their therapeutic efficacy[10]. For example, MSCs become less immunosuppressive starting from passage-8[11]. The aging process of MSCs is influenced by the donor age, MSC tissue origin, and stress oxidative accumulation. In addition, sex may have influence to MSC properties, as bone marrow (BM)-MSCs that are derived from female donors tend to have better proliferation capacity[7]. Overall, senescent MSCs change in growth, genotype and phenotype, morphology and function.

Growth arrest in aging MSCs

As a cell, MSC may go into a replicative senescence, G1-phase growth arrest, and proliferation ability decline. *In vitro* lifespan of MSCs is 20 - 40 doublings, which depends on their telomere length. The average of the telomere length depends on the age of the donor. In fetal tissue, MSC telomere length is about 10-11 kb, while in postnatal tissue it is 7 kb. When the telomere length is 5.8 - 10.5 kb, the MSC stops to grow that is called telomere-dependent MSC senescence[9].

Genotype changes in aging MSCs

A review concluded that gene expression changed during MSC aging[9]. Those genes are cyclin-dependent kinase inhibitor 4/tumor suppressor (INK4a/ARF), high mobility group A2 (HMGA2), retinoblastoma (RB), and lamin A/C (LMNA). Gene INK4a/ARF is located in chromosome 9p21 and regulates two suppressor proteins, i.e. p16 and p14/p19. P16 is increased, while HMGA2 expression is decreased in aging. RB protein controls cell cycle and DNA methylation via DNA methyltransferase 1 (DNMT1) upregulation. Without RB gene, senescence occurs spontaneously. LMNA gene mutation produces progerin, and its accumulation induces premature senescence. As MSC aged, there is a loss of function mutation in ataxia telangiectasia mutated (ATM) gene, which encodes a serine threonine protein kinase, that regulates stress response against DNA damage. Moreover, master in response to oxidative stress, nuclear factor erythroid 2-related factor 2 (NRF2) also has an activity decline[9].

In addition, there is increased expression of senescence-associated β-galactosidase (SA-β-Gal) and senescence-associated lysosomal α-L-fucosidase (SA-α-Fuc). SA-β-Gal shows increased lysosomal activity, meanwhile SA-α-Fuc responds to DNA damage and oncogene-induced senescence. Further, lipofuscin aggregations cause an increase in auto fluorescence level of the aging MSCs[9].

Phenotype changes in aging MSCs

Senescent MSC phenotype changes morphologically as enlarged or flattened cell, with hypertrophic, granular cytoplasm, and constrained nucleus[9]. Actin fibers inside the cell are highly increased and the ability to attach on plastic surfaces is decreased. The MSC surface markers are also changed. High passages cause decrease in expression of Stro-1, CD106, and CD146. CD 106 is a surface marker that disappeared after the cells differentiate. CD295 is found increased in aging MSCs. Meanwhile, CD73, CD90, and CD105 are relatively stable during extended culture[7]. However, BM-MSCs show a decrease in CD90 expression along with passages. BM-MSCs, which are extensively studied, show that their phenotype is clearly changed in aging[11]. Surface marker alteration depends on donor, culture passage, cell seeding density, cell homing, and attachment properties[9].

Functional changes in aging MSCs

MSCs as multipotent cells show changes in differentiation capacity during expanded culture. As BM-MSC aged, the ability to differentiate is decreasing, and there is a loss of bone-forming efficiency and differentiation into adipocytes is more preferred[12].

MSC osteogenesis is regulated by leptin, glucagon-like peptide-1 (GLP-1), transforming growth factor-β (TGF-β)/SMAD3, and phosphatidylinositol 3-kinase-protein kinase B (PI3K-AKT) pathway. Meanwhile, the adipogenesis is controlled by LPL (Lipoprotein Lipase), CCAAT/enhancer binding protein (C/EBP), peroxisome proliferator-activated receptor γ (PPARγ), and insulin induced protein kinase B (AKT) phosphorylation[9]. Late passage MSCs show increased expression of RANKL (receptor activator of nuclear factor-kB ligand), which plays a role in osteoclasts differentiation and maintenance. Leptin is an important paracrine-signaling molecule. High level of leptin increases the proliferation and differentiation of the MSCs into osteoblasts and inhibits differentiation into adipocytes. GLP-1 up regulate the activity and expression of osteoblast mRNA, and calcium mineralization, and suppression of PPARγ, LPL, and adipocyte protein 2 (AP2). Likewise, LPL induces MSCs to differentiate into adipocyte[9]. Moreover, stem cell origin also dictates differentiation fate. Bone marrow MSCs are more prone to become bone cells, and adipose MSCs to become fat cells[13].

Mesenchymal stem cells niche

In aging pathophysiology, there are hormonal, inflammation, and metabolic changes[14]. All of these factors contribute to changes of the microenvironment of the stem cells. The interaction between stem cells and their surroundings might determine the efficacy of stem cell therapy[15].

Naturally, the stem cell niche consists of cells, extracellular molecules, and soluble factors (Figure 1). Mimicking *in vivo* microenvironment, *in vitro* culture medium may affect the cells and their secretions. Culture conditions including duration, oxygen level, and physical condition may play a role in MSC viability[16].

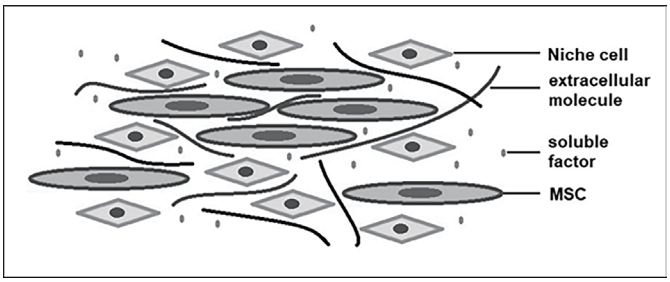

Figure 1: Mesenchymal stem cell niche

MSC microenvironment is essential to maintain the cell's capacity. The microenvironment affects the cells, and the cells themselves adjust to the microenvironment. MSC can alter their microenvironment by gene expression regulation and producing various factors. In culture condition, where there is an abundant amount of tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ), MSC through Toll-like receptor 4 (TLR4) becomes activated. It generates indoleamine 2,3-dioxygenase (IDO), prostaglandin E2 (PGE2), nitric oxide (NO), TGF-β, hepatocyte growth factor (HGF), and hemoxygenase (HO). Further, HO directs the MSC differentiation into the osteogenesis pathway[9].

Approaches to regulate MSC aging

In order to achieve plentiful amount of MSC while maintaining its therapeutic quality, researchers tried to manipulate the cells and also the surroundings. Approaches to regulate MSC aging can be done by various methods, including by genetic engineering approaches, regulating stem cells microenvironment *in vitro*, and *in vitro* physical modification (Figure 2).

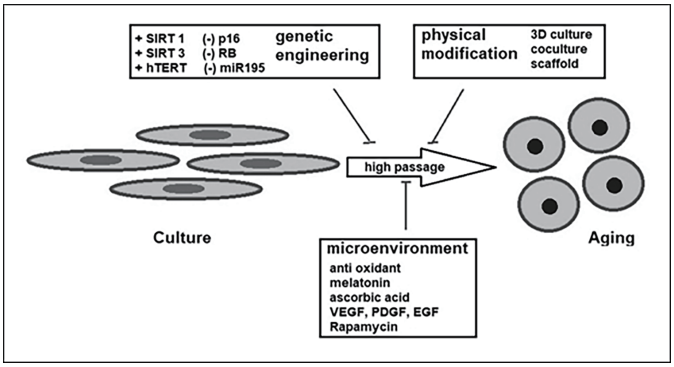

Figure 2: Approaches to regulate MSC aging

Genetic engineering to prevent MSC aging

Genetic engineering approaches have been tried to inhibit MSC *in vitro* aging. Studies explored gene knock out/silencing in isolated MSCs to improve the function of MSCs. Sirtuins (SIRT) is one of the targets in anti-aging therapy that is able to inhibit *in vitro* aging of MSCs. During expansion *in vitro*, SIRT3 overexpression and SIRT1 activation by miR-34 inhibition reduces MSC aging-related senescence, ameliorate oxidative stress and maintain MSC differentiation potentials[17,18].

Other measures to prevent MSC senescence to maintain their therapeutic effect are overexpression of human telomerase reverse transcriptase (hTERT), p16 knockdown, RB silencing, and miR-195 silencing, but all of these measures are believed to increase carcinogenesis risk[19-21].

Regulating stem cell microenvironment in vitro

Regulating the culture medium and additives during MSC expansion *in vitro* may maintain MSC quality during passages. In adipose-derived MSC culture, human serum is more superior to fetal bovine serum (FBS) in maintaining genomic stability and DNA methylation profiles[22]. As MSC culture medium, alpha modified minimal Eagle essential medium (αMEM) is better than Dulbeco modified MEM (DMEM) for osteogenic differentiation purpose[4].

Adding exogenous substances also can maintain MSC characteristics during culture expansion. A study showed that antioxidants, such as reduced glutathione and melatonin, were able to preserve adipose derived mesenchymal stem cell stemness after long term passaging[23]. Another study showed that ascorbic acid could inhibit reactive oxygen species (ROS) production and activate protein kinase B/mammalian target of rapamycin (Akt/mTOR) signaling in order to prevent MSC senescence[24]. Rodrigues *et al* showed that various growth factors, such as VEGF, PDGF, and EGF increased survival of MSCs[25]. Inhibitors such as Rapamycin, which is an mTOR inhibitor, and histon acetyltransferase inhibitor were used in several studies to prevent MSC senescence through p16 regulation[26,27]. Further, a study showed that lysophosphatidic acid stimulated MSC proliferation during culture, exerted anti apoptotic effect, and did not interfere with cell differentiation capacity[28].

Moreover, controlling gas composition during MSC culture expansion may influence MSC characteristics. Adding 3% of hydrogen gas in culture may extend replicative life span of BM-MSCs[29], and keeping oxygen at low level (1%) may improve biological characteristics of MSCs[30].

In Vitro Physical Modification

Physical conditions during *in vitro* expansion may influence stem cell properties. Initiating the culture at low cellular density may result in consistent morphology and preservation of differentiation potential of MSCs[28]. A study showed that three-dimensional (3D) co-culture had beneficial impact on stem cell phenotype[31]. Another study showed that spheroid culture delayed adipose-derived mesenchymal stem cell senescence and also increased the therapeutic potential for tissue regeneration[32]. Further, biomaterial micro engineering of MSC niche may affect MSC signaling and secretion. As a scaffold, biomaterial properties such as swelling, porosity, and degradation may contribute to stem cell behavior. Degradation of the biomaterial releases bioactive substances that take part in stem cell differentiation fate, as well as affecting stem cell bioactive secretion[33].

In natural condition, stem cells exist together with other cells. Therefore, culturing stem cells together with other cells may have a mutual interaction. A study showed that 3D co-culture of adipose-derived stem cells and endothelial colony-forming cells reversed the stem cell senescence and displayed superior therapeutic potential[34].

Finally, various approaches have been proposed and show improvement in senescent inhibition, but still, expansion of MSCs is limited due to senescence. Therefore more studies are needed to provide an easy and effective high quality MSC expansion method for therapeutic purposes.

CONCLUSION

Expanding mesenchymal stem cells *in vitro* should consider their quality to optimize the cell therapeutic potentials. Therefore, maintaining the cells and microenvironment during culture expansion is highly important. Methods to maintain cell qualities are available ranging from determination of donor age, tissue origin of the cells, genetic modification, microenvironment regulation, and adjusting physical culture condition. Further researches are still needed to optimize MSC capacities in order to achieve optimal therapeutic effect.

ACKNOWLEDGEMENT

This work was supported by a research grant from the Ministry of Research, Technology and Higher Education of the Republic of Indonesia, Hibah Penelitian Pengembangan 2019, contract no.NKB-1804/UN2.R3.1/HKP.05.00/2019

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