The International Journal of Biochemistry & Cell Biology 70 (2016) 1–12

Contents lists available at ScienceDirect

The International Journal of Biochemistry & Cell Biology

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

Aging induced loss of stemness with concomitant gain of myogenic properties of a pure population of $CD34^+/CD45^-$ muscle derived stem cells

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ARTICLE INFO

Article history:
Received 7 May 2015
Received in revised form
18 September 2015
Accepted 7 October 2015
Available online 13 October 2015

Keywords:
Muscle derived stem cells
Aging
Skeletal muscle
Myoblasts
Myogenesis

ABSTRACT

Aging is accompanied by the functional decline of cells, tissues, and organs, as well as, a striking increase in susceptibility to a wide range of diseases. Within a tissue, both differentiated cells and adult stem cells are susceptible to intrinsic and extrinsic changes while aging. Muscle derived stem cells (MDSCs) are tissue specific stem cells which have been studied well for their multipotential nature. Although there are reports relating to diminished function and regenerative capacity of aged MDSCs as compared to their young counterparts, not much has been reported relating to the concomitant gain in unipotent nature of aged MDSCs. In this study, we report an inverse correlation between aging and expression of adult/mesenchymal stem cell markers and a direct correlation between aging and myogenecity in MDSCs. Aged MDSCs were able to generate a greater number of dystrophin positive myofibres, as compared to, the young MDSCs when transplanted in muscle of dystrophic mice. Our data, therefore, suggests that aging stress adds to the decline in stem cell characteristics with a concomitant increase in unipotency, in terms of, myogenecity of MDSCs. This study, hence, also opens the possibilities of using unipotent aged MDSCs as potential candidates for transplantation in patients with muscular dystrophies.

1. Introduction

Advancement of time in the lifespan of a living organism is called aging. This universal phenomenon of aging is primarily associated with irreversible decline in tissue homeostasis and its regenerative capacity. At the organism level, the aging process is associated with senescence. There are numerous underlying molecular events for senescence. Such events include changes in gene expression consistent with a decrease in protein synthesis, oxidative defense, mitochondrial proteins, differential expression of genes involved in energy metabolism, DNA damage repair, stress response, immune/inflammatory response and RNA binding and splicing and proteosome degradation (Joseph et al., 2012; Lanza and Nair, 2010; Hiona and Leeuwenburgh, 2008; Short et al., 2005; Rooyackers et al., 1996; Hiona et al., 2010). However at a cellular level, most, if not all, human adult cells can undergo at most 50–60 divisions before they become senescent. This limited replicative potential is also known as the 'Hayflick limit,' named after its discoverer Leonard Hayflick (Hayflick, 1965; Shay et al., 2000; Olovnikov, 1996), and it is thought to be determined by chromosomal telomere shortening after each cell division. The way somatic cells escape this process of limited replicative potential is by acquiring mutations in specific genes in a process called transformation or in other words, cancer. In contrast, embryonic stem cells (ESCs) possess a very high degree of self-renewal capacity without requiring transformations to acquire these properties, at least in part because they express, in contrast to adult cells, high levels of telomerase (Zeng and Rao, 2007; Evans and Kaufman, 1981; Thomson et al., 1998; Carpenter et al., 2004; Rosler et al., 2004). Adult stem cells, on the other hand, are able to self-renew to some extent *in vivo* that is required for maintaining tissue homeostasis or serving as a reserve for cellular replacement or repair in case of minor or major injury, respectively (Simons and Clevers, 2011; Burkhalter et al., 2015; Weissman, 2000). In many mammalian tissues, there is a decline in the ability to replace mature cells with age. It is reasonable to hypothesize that some of this decline could be related to decreased stem cell functionality with

Abbreviations: Y-MDSCs, young muscle derived stem cells; A-MDSCs, aged muscle derived stem cells; TERT, telomerase reverse transcriptase; GAPDH, glyceraldehyde 3 phosphate dehydrogenase; MAPC, multipotent adult progenitor cells; VSELs, very small embryonic like stem cells; OCT, optimal cutting temperature.

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http://dx.doi.org/10.1016/j.biocel.2015.10.009
1357-2725/

age, resulting in diminished production of progenitors and differentiated cells that ultimately compromise the tissue or organ (Boehm et al., 2013; Liu and Rando, 2011; Fujita and Tsumaki, 2013; Drummond-Barbosa, 2008; Oh et al., 2014). It is still not clear whether this is due to the intrinsic aging of the cells, due to changes in the niche or to the external environment. The relative contribution of these factors might be different in different organs or tissues. For example-in mice greater than 2 years of age, there is a reduction in the number and proliferative potential of neural stem cells (Molofsky et al., 2006; Kuhn et al., 1996) and male germline stem cells (Ryu et al., 2006; Zhang et al., 2006). In contrast, with aging, hematopoietic stem cell (HSC) numbers are preserved but their function is lost (Morrison et al., 1996; Fuller, 2002). Bonafide muscle stem cells, also known as satellite cells, tend to lose stem cell properties with aging, but their abundance in old age remains controversial (Conboy and Rando, 2002; Conboy et al., 2003; Shefer et al., 2006; Collins et al., 2007). Huard et al. also reported that muscle derived stem and progenitor cells (MDSPC) from old, as well as progeroid mice, have diminished function and regenerative capacity as compared to their young counterparts (Lavasani et al., 2012).

Various studies thus suggest that stem cell plasticity may be higher when the organism is young and diminishes, or is lost, with age (Liang and Van Zant, 2003). However, there are no reports till date regarding the loss of stemness with a concomitant gain of unipotency of tissue specific stem cells. In our present investigation, we have isolated pure $CD34^+/CD45^-$ muscle derived stem cells (MDSCs) from young 5 week (Y-MDSCs), as well as, 2 year old (A-MDSCs) from C57BL6J mice. As the presence of CD34 in various tissues has been linked to progenitor and adult stem cell phenotypes (Sidney et al., 2014), we decided to isolate the MDSCs as pure population of $CD34^+$ cells from the muscle tissues. Moreover, MDSCs had earlier been reported to be $CD34^+$ (Lavasani et al., 2012). Secondly, during the isolation process using a single parameter sorting for CD34, there is a possibility of obtaining $CD34^+/CD45^+$ hematopoietic stem cells that are undesirable. Hence, we decided to obtain only the pure population of MDSCs by applying a double parametric sorting for $CD34^+/CD45^-$. We had further characterized the $CD34^+/CD45^-$ MDSCs *in vitro* at cellular and molecular level for myogenic and adult stem cell markers. Functional characterization of MDSCs was performed by quantifying their respective *in vitro* myogenic differentiation potentials. Also, we have studied the respective *in vivo* engraftment potentials of young and aged MDSC. Our findings suggest that A-MDSCs were more myogenic *in vitro* and *in vivo* when compared to Y-MDSCs. However, Y-MDSCs exhibited higher expression levels of mesenchymal stem cell (MSC) markers as compared to A-MDSCs, and hence we say that Y-MDSCs were more like stem cells.

2. Materials and methods

2.1. Ethics statement

All experiments involving animals were approved by the Nanyang Technological University Institutional Animal Care and Use Committee (IACUC), Singapore (Approval Number: ARF SBS/NIE-A 0057). Cell transplantations were performed under Ketamine/Xylazine anesthesia, and all efforts were made to minimize animal suffering.

2.2. MDSCs isolation and culture

MDSCs were isolated from the primary cultures of hind limb muscles of 5-week and 2-year-old WT male C57BL/6J mice respectively by "serial transfer and subsequent plating of the culture supernatant" with a few modifications. Each serial transfer/plating of supernatant is called a preplate/PP. This method of "serial transfer and subsequent plating of supernatant" is known as modified preplate technique (Gharaibeh et al., 2008; Li et al., 2010), which is commonly used by the muscle biologists. In the earlier reports (Gharaibeh et al., 2008; Li et al., 2010), the researchers have used the preplate 6 (PP6) cells directly and termed them as MDSCs. However, here we have incorporated a few modifications and sorted for pure population of $CD34^+/CD45^-$ cells from preplate 6 (PP6) populations (Fig. 1) and further cultured these $CD34^+/CD45^-$ cells on Matrigel (BD, NJ, USA) coated plates (Nunc, USA). Henceforth, we call these pure $CD34^+/CD45^-$ PP6 cells as MDSCs. The composition of MDSCs culture medium was Dulbecco's modified eagle's medium (DMEM high Glucose), 10% Horse serum, 1% penicillin and streptomycin (all of these components were from Invitrogen, Carlsbad, CA, USA), 10% fetal bovine serum (Hyclone, Logan, UT, USA), 0.5% chicken embryo extract (CEE) (US Biological, MA, USA) and 0.1 mM β-mercaptoethanol (Sigma–Aldrich, St. Louis, USA). All experiments were performed using early passage (1–5) MDSCs. Three independent isolations, each for young and aged MDSCs, were performed, on three different days. For each MDSC isolation, we had used six mice from that particular age group.

2.3. In vitro *myogenic differentiation*

MDSCs were plated at a density of 1000 cells/cm^2 and were cultured in MDSCs medium for 3 days as per established protocol for MDSCs myogenic differentiation (Deasy et al., 2009). After 3 days, the cells were transferred to induction medium containing Dulbecco's modified eagle's media (DMEM high Glucose) supplemented with 2% horse serum to induce myogenic differentiation for 72 h. WT primary myoblasts were also subjected to myogenic differentiation with 2% horse serum media in DMEM conditions for 72 h in parallel as control experiments. *In vitro* myogenicity was scored on the basis of the number of myotubes formed per unit area in 10 randomly chosen microscopic fields. The numbers of myonuclei were counted in at least 450 myotubes per culture. The results for myonuclei number were expressed as a percentage of myotubes with 3, 4, 5 and ≥10 myonuclei. Furthermore, Y-MDSCs, A-MDSCs and primary myoblasts derived myotubes areas were also assessed, using Image Pro software, in 100 myotubes per culture.

2.4. Quantitative real time-PCR (qRT-PCR)

Total RNA was extracted using the RNeasy mini kit (Qiagen, Germany, Catalog number-74104). cDNA was reverse transcribed using i-Script RT (Bio-Rad, USA, Catalog number-170-8891) and qRT-PCR was performed using Sso-Fast^{TM} Eva Green super mix (Bio-Rad, USA, Catalog number-172-5201), all as per manufacturer's instructions. Results were plotted as Relative gene expression of A-MDSCs expressed as fold change with respect to Y-MDSCs (Y-MDSCs value plotted as 1 in the Y-axis). Fold change was calculated by the $2^{(-\delta\delta Ct)}$ method. Expression of the housekeeping gene GAPDH in MDSCs was found to be consistent and high (Ct value ~17) throughout all the passages used for our experiments. The mRNA expressions of each of the cell types were first normalized to its respective expression of housekeeping gene GAPDH to obtain the δ Ct values. However, in case of pluripotency gene expression respective delta Ct values were plotted because of very little expression of pluripotency genes in MDSCs. The list of primers used is provided in Supplementary Table-S1.

2.5. Flow cytometry

Cells fixed with 4% paraformaldehyde in PBS were blocked using 5% normal donkey serum and stained with respective