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Brief communication

Donor age negatively affects the immunoregulatory properties of both adipose and bone marrow derived mesenchymal stem cells

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ABSTRACT

Purpose: Age negatively impacts the biologic features of mesenchymal stem cells (MSCs), including decreased expansion kinetics and differentiation potential. Clinically, donor-age may be within a wide spectrum; therefore, investigation of the role of donor's age on immunoregulatory potential is of critical importance to translate stem cell therapies from bench to bedside.
Methods: Adipose and bone marrow derived MSCs (ASCs and BMSCs) were isolated in parallel from Lewis and Brown Norway rats of young (less than 4-week old) and senior groups (older than 15-month). The presentation of cells and time required for growth to 90% confluence was recorded. FACS sorting based on the expression of CD90 and CD29 double positive and CD45 CD11 double negative quantified the proportions of MSCs. After expansion, ASCs and BMSCs from different age groups were co-cultured in mixed lymphocyte reaction (MLR; Lewis vs. Brown Norway) assays. The suppression of $CD3^+CD4^+$ and $CD3^+CD8^+$ T cell populations by different sources of MSCs were compared.
Results: The kinetics of cell growth was slower in old animals (17.3 ± 2 days) compared with young animals (8.8 ± 3 days), and cell morphology was irregular and enlarged in the senior groups. The yield of MSCs by FACS sorting was significantly higher in young groups compared to senior groups ($p < 0.02$). With regard to immunoregulatory potential, senior ASCs failed to induce any $CD3^+CD4^+$ T cell suppression ($p > 0.05$). In addition, young BMSCs-induced suppression was more prominent than seniors ($p < 0.05$).
Conclusions: Donor age should be taken into consideration when using recipient MSC of either bone marrow or adipose origin in clinical applications.

1. Introduction

The utilization of stem cell-based therapies, mesenchymal stem cells (MSCs)-based therapy in particular, has been proven to be a promising strategy to achieve transplant tolerance [1]. Despite their extensive proliferative and regenerative capacity, studies suggest that physical and biological properties of stem cells usually decline with advanced donor age [2]. This is primarily due to years of exposure to environmental damage and stress, which eventually leads to deterioration of tight control of multiple aspects of physiology, cell proliferation, and/or signal transduction [3].

Bone marrow derived mesenchymal stem cells (BMSCs) have shown great potential in regulating both alloreactive and non-specific immune responses when co-cultured *in vitro* through suppression of alloreactive T-cells [4,5]. However, accumulating evidence has shown that BMSCs exhibit both diminished differentiation and growth factor secretion with increasing donor age [6]. Yet, both transplant recipients and potential organ donors may be of any age. Thus, understanding the behavior and kinetics of these alterations in the quality and function of MSCs due to donor age is of great importance, as there is currently limited knowledge on how these potentially 'functionally compromised' MSCs would modulate transplantation outcomes.

Adipose-derived mesenchymal stem cells (ASCs) are robust, multipotent cells, with a protein expression phenotype comparable to that of BMSCs [7]. Several *in vitro* studies have established that ASCs share immunological qualities with BMSCs including low immunogenicity, immunoregulatory effects, and similar cytokine expression profile [8–10]. Furthermore, ASCs are comparable to BMSCs in treating severe graft-versus-host disease [11] and alleviating arthritis and colitis [12].

Abbreviations: MSCs, mesenchymal stem cells; ASCs, adipose derived stem cells; BMSCs, bone marrow derived stem cells; MLR, mixed lymphocyte reaction.

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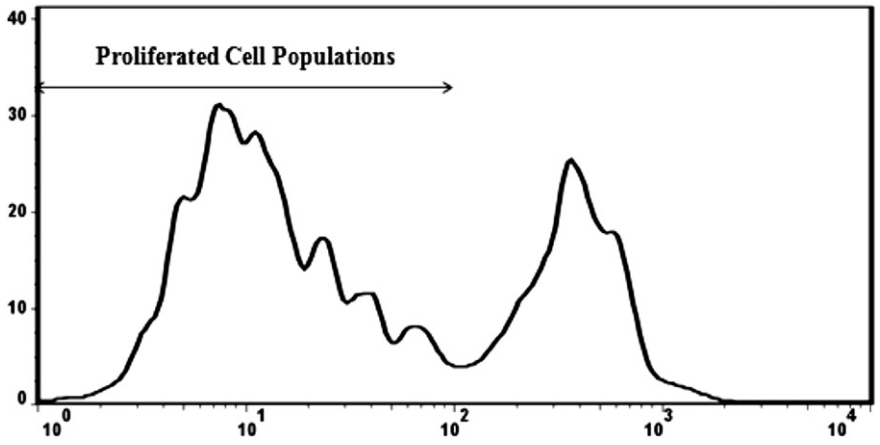

Fig. 1. The calculation of percentage of proliferated cell populations in a MLR. The percentage of all $CD3^+/CD4^+$ or $CD3^+/CD8^+$ cells was taken that no longer expressed CFSE as CD4 or CD8 T cells that had proliferated. Suppression was defined a lower percentage of cells proliferating. If the percentage of cells was the same with or without added MSC, this was defined as no suppression.

Clinically, ASCs are more widely available, easier to harvest and expand in cell culture, and cause less donor-site morbidity than MSCs from other sources. Therefore, ASCs present a promising alternative to BMSCs in many clinical applications.

To our knowledge, MSCs derived from either bone marrow or adipose tissue have not been directly compared between young and aged donors for immunoregulatory capacities. Many different groups and investigators are now advocating ASCs as a potential alternative to the traditional BMSCs. This leads to the second questions we aimed to address in this study: does donor's age affect in the same way both BMSCs and ASCs?

2. Materials & methods

2.1. Animals

Lewis and *Brown Norway* rats (Harlan, Indianapolis, IN) were divided into young (age under 4-week old) and senior (older than 15-month) groups, with $n = 3$ in both age groups from each strain. All animal procedures were approved by and performed in accordance with the Johns Hopkins University Animal Care and Use Committee.

2.2. Isolation of Rat BMSCs and ASCs

Animals were euthanized by carbon dioxide suffocation and sterilized by iodine and ethanol solution. Soft tissue was stripped from femur & tibia, and the femur was disarticulated from the tibia. Long bone ends are cut and bone marrow flushed from the bones into a petri dish. The suspension was centrifuged at 1380 rpm for 5 min. Supernatant was aspirated, the pellet dislodged, and 20 cc of complete media consisting of DMEM/F12 with 10% heat-inactivated fetal bovine serum, 1% penicillin/streptomycin and 1% Fungizone added, then suspensions of 50,000 cells were plated into T-50 flasks. All reagents were purchased from Invitrogen, CA unless otherwise noted.

Inguinal fat pads were removed and minced with scissors in collagenase solution consisting of Hanks' balanced salt solution, bovine serum albumin and 1% type II collagenase (3.0 mg/g of fat) (Worthington Biochemical Corporation, Lakewood, NJ). Centrifuge tubes were then shaken at 100 rpm for 50 min at 37 °C. Following digestion, the content of each tube was filtered through double-layered sterile gauze. The filtrates were then centrifuged at 1000 rpm for 10 min at 37 °C. The pellets were suspended in the aforementioned plating medium and then preparations of 50,000 cells were added in 50 cm^2 flasks and placed in 20% carbon dioxide incubator. Adherent ASCs were expanded for a period of 3 days at 37 °C, and the medium changed every 2 days until the cells achieved confluence.

2.3. Cell sorting, re-expansion and certification

After the MSCs reached confluence, they were detached by a treatment with 0.25% trypsin and ethylenediaminetetraacetic acid (EDTA) and then approximately 10^5 cells for each of the senior groups, and 2.5×10^5 cells for the young groups were stained with Sytox-Blue (Invitrogen, Life Science, CA), CD29-FITC, CD90-PE, CD45-PerCP, and CD11b/c-AlexaFluor647 (BioLegend, US). Labeled (single) cells were sorted by gating on the CD29-FITC and CD90-PE double positive population, and excluding the Sytox-Blue, CD45-PerCP, and CD11-APC positive populations. We set 2×10^4 cells as target number of sorted cells to collect (when possible). Post-sorting cells were then expanded in culture for an additional 5 or 12 days and then used for purity analysis by flow cytometry, staining with anti-MHC class II-Alexa 647 (BD Biosciences, San Jose, CA), anti-CD31-eFluor 660 (eBioscience, San Diego,

Table 1
The biological difference in isolation of ASC.

	Brown Norway		Lewis	
	Senior ($n = 3$)	Young ($n = 3$)	Senior ($n = 3$)	Young ($n = 3$)
Body weight (g)	497 ± 7.81	41.67 ± 1.27	497.33 ± 7.77	44.33 ± 1.37
Adipose tissue harvested (g)	4.63 ± 0.54	0.37 ± 0.04	5.24 ± 0.69	0.48 ± 0.06
Cell yield from adipose tissue($\times 10^4$)	13.58 ± 1.23	5.42 ± 0.52	12.00 ± 1.15	5.67 ± 0.29
Cell/g Body weight	273 ± 29.26	1303 ± 161.98	241 ± 20.08	1279 ± 79.54
Cell/g Adipose tissue($\times 10^3$)	29.3 ± 4.3	146.5 ± 21.2	22.9 ± 3.7	118.1 ± 16
Days to reach confluence	15.0 ± 0	7.3 ± 0.58	17.6 ± 1.15	8.0 ± 0