Adipose derived mesenchymal stem cells - A promising population of adult stem cells for regenerative therapy

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Abstract

The field of regenerative medicine and its applications in medical care has now been well accepted and novel improvisations have been implemented towards developing innovative therapies. This concept makes use of a combination of stem cells, biomaterial scaffolds and growth factors for developing next generation medical care. Stem cells being an inevitable part of tissue engineering strategy, focus on identifying autologous adult stem cell sources have always been of immense significance. Adipose tissue is a good source for obtaining mesenchymal stem cells (ASCs), a group of adult stem cells with a wide range of applications in the field of tissue engineering and regenerative therapy. This study emphasizes the isolation of rabbit adipose mesenchymal stem cells and its evaluation for the mesenchymal stem cell properties. Rabbit ASCs were isolated from the subcutaneous fat pad and were demonstrated for properties such as population doubling time and colony forming unit assay. Mesenchymal lineage demonstration was done by evaluating for MSC markers CD105 and CD90. The isolated cells were also demonstrated for their multi-lineage differentiation potential by differentiating them to osteo- and adipo- lineages.

Key Words: Adipose derived mesenchymal stem cells, Colony forming assay, Population doubling, Tisue engineering, Regenerative Medicne, Flow cytometry.

1. INTRODUCTION

Tissue engineering and regenerative medicine has developed as an impactful field focusing on addressing a lot of medical scenarios by development of bioengineered tissue constructs and novel therapies. To date, biomedical industry has attempted prototypes of various tissues and organs aiming at treating and addressing various disease conditions. The tissue engineering branch of regenerative medicine practices a triad combination to repair the failing organs. This uses a combination of cells, supported by biomaterials called scaffolds and growth factors to generate tissue constructs [1]. Identifying the suitable cell population and growing them on suitable scaffolds called biomaterials supported by accurate growth factor cues to direct the cell fate is the basic principle in tissue engineering.

To date a number of cells types have been identified and attempted in the field of tissue engineering of which adult stem cell types are prominently useful. Mesenchymal stem cells (MSCs), an adult stem cell group has gained attraction, as it can be isolated from various tissues such as, bone marrow, adipose, blood, umbilical cord, etc [2] and exhibits various advantages than other cell types used in this field [3]. MSCs exhibit potential to differentiate in to multiple lineages including osteo, adipo, chondro, neuro and even extra mesenchymal lineages. They also show the potential to self-renew and exhibit immune-modulatory properties upon implantation [4]. MSCs can secrete different growth factors and matrix proteins, also recruit host stem cells to injury sites promoting wound healing [5]. MSCs are a trustable source of autologous stem cells as they reside in various different tissues in the human body. All these properties make MSCs an ideal stem cell candidate for use in tissue engineering.

Adipose-derived stem cells (ASCs) are MSCs isolated from adipose tissue, with all the aforementioned properties. Also, adipose tissue is the most easily accessible source of MSCs. This stem cell population was identified within the adipose stroma and was termed as processed lipo aspirate (PLA) [6]. The term "adipose-derived stem cell" (ASC) refers the population of MSC within the stroma of adipose tissue [7]. ASCs were shown to be similar to bone marrow MSCs as they express the same set of CD markers but human adipose tissue is

considered as the easily assessable source of MSCs compared to bone marrow [8, 9]. Moreover, adipose tissue represents an abundant reservoir of ASCs with multi-lineage differentiation potential, immuno-modulation, anti-inflammatory properties and anti-angiogenic properties]for use in autologous or allogenic replacement therapies [10].

This work has demonstrated that ASC with specific stem cell properties can be isolated from subcutaneous fat pad of rabbits, which can considered as a source for autologous stem cells regenerative therapy.

2. Materials and Methods

2.1 Medium and other components for isolation and culture of ASCs

Dulbecco's Modified Eagles Medium (DMEM-HG) High Glucose supplemented with 10% Fetal bovine serum (FBS) and 1% antibiotics was used for isolation and expansion of ASCs in culture. This complete medium is referred to as growth medium for ease of usage. Cells were passaged or trypsinized using 0.5% trypsin. All the cell culture reagents were procured from Invitrogen – Thermo Scientific, unless otherwise mentioned.

2.2 Isolation, culture and expansion of ASCs

ASCs were isolated from the subcutaneous fat pad of rabbits. Tissue isolation and use of animals were approved by Institutional Animal Ethics Committee (IAEC) and were carried out as per the guidelines of CPCSEA. Subcutaneous fat was collected in sterile PBS (Phosphate Buffer saline) containing 1% antibiotics. The collected adipose tissue was chopped and minced well before digestion with type I collagenase for 1h at 37 ^{o}C. The digestion was stopped with serum containing media and then filtered with a 180-micron nylon mesh membrane filter (Millipore). The filtrate was centrifuged and plated in 25cm2 flask (Nunc) using growth medium. When the cells in culture attained 70 to 80 % confluence, they were passaged by trypsinization using trypsin-EDTA and plating them in new flasks.

2.3 Population Doubling Time

The population doubling time (PDT) was evaluated by seeding cells at passage #1 at a density of 2x105 ASCs per 60 mm standard tissue culture dishes (Nunc). The cells were trypsinized after 72h in culture with 0.25% trypsin and were counted using hemocytometer. 2x105 cells per 60 mm standard tissue culture dishes were again seeded in the next passage and the above protocol was followed till passage #10. PDT was calculated using the formula,

PDT = (T–T0) log2/(logNt–logN0), where T is 72h and T0 is 0h. N0 and Nt are the number of cells at the start and end of each passage (n=3).

2.4 Colony Forming Unit Assay

For Colony forming unit assay (CFU assay), 200 ASCs per 60 mm dish were seeded at P2, P4, P6, P8 and P10 passage. After 14 days culture in growth medium, the cells were fixed with paraformaldehyde; stained with DAPI for 10 min and imaged with a fluorescent microscope (Leica DMI 6000, Germany).

2.5 Characterization using Flow cytometry

ASCs at passage 4 were trypsinized with 0.25% trypsin and fixed with paraformaldehyde in suspension. The fixed cells were blocked with 1% BSA (Sigma) and were then divided in to four sets. One set was used as the unstained control. Other sets were stained with primary antibodies such as rat anti-CD105 (SC-71042, Santa Cruz Biotechnology), mouse anti CD90 (ab225, Abcam) and mouse anti CD34 (ab6330, Abcam) for 1h at room temperature. After primary staining, these sets were stained with corresponding secondary antibodies tagged with alexafluor®488 for 45min. Anti CD 105 was stained with anti-rat alexa flour 488. While anti CD 9 and anti CD 34 was stained with anti-mouse alexa flour 488. Later washed and resuspended in PBS. The fluorescence intensity was then recorded under flow cytometry (BD Biosciences FACS Aria II).

2.6 Differentiation of asc to Adipogenic and Osteogenic lineage

Rabbits ASC's were differentiated to adipo and osteo lineage to demonstrate stemness with multi lineage differentiation potential.

2.6.1 Induction of ASC to Adipogenic lineage

ASCs at passage 3 were induced to adipogenic lineage using Stempro adipogenic induction cocktail (Gibco, Thermo scientific) for a period of 21 days. The cells were then fixed with paraformaldehyde, washed with PBS and stained with Oil red O. Oil red O was prepared by making 0.3% solution with Oil Red O powder in isopropanol (Nice Chemicals). The working staining solution was then made by diluting 6 parts of 0.3% Oil Red O with 4 parts of distilled water. The cells were incubated with 60% isopropanol, stained with Oil Red O working solution for 10 min and the images were taken in Leica DM 6000.

2.6.2 Induction of asc to osteogenic lineage

Differentiation was achieved using osteogenic differentiation cocktail (Gibco). Induction period was for 28 days and the cells were then fixed with paraformaldehyde and washed with PBS. Osteogenic differentiation of ASCs was confirmed through presence of calcium and phosphate ions staining. The fixed cells were stained with 1% Alizarin red (Sigma Chemicals) to determine calcium deposition and was viewed under

Light Microscope (Leica DM 6000). For von Kossa staining, the fixed cells were incubated with 5% silver nitrate (Merck India) in distilled water and exposed to UV light for 5 minutes, washed; air dried and viewed under Light Microscope (Leica DM 6000).

3. Results and Discussion

3.1 Harvesting Rabbit Adipose tissue

As per the guidelines of the Institutional Animal Ethics Committee (ICAE) rabbit ASCs were isolated from the subcutaneous fat pad of rabbits. Mesenchymal stem cells (MSCs) represent a group of progenitor cells isolated from multiple tissue sites of which adipose and bone marrow offer the advantage of abundance and easy access. Adipose tissue derived MSCs were first harvested by Zuk et al., from the lipo aspirate and was reported to have multi lineage potential [11]. In the present study ASCs were isolated from the subcutaneous fat pad of rabbit models which is considered as a better candidate for tissue engineering related therapies [12]. The tissue was minced and digested with type I collagenase and the isolated cells were selected based on the property of plastic adherence [13]. Repeated washes and medium change facilitated removal of non-adherent cells after 10-15h. At passage 2 the cells seems to be more homogenous and exhibited spindle shaped fibroblastic morphology. When seeded in a T25 flask with optimal number of cells, they attained confluence in 3-4 days (Figure 1A).

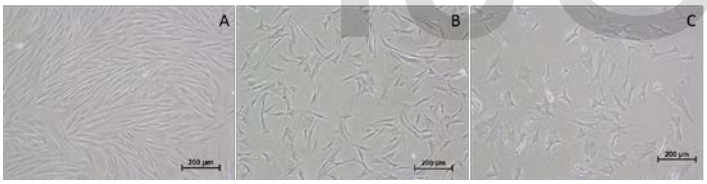

Fig 1: phase contrast image of adipose derived MSCs (ASCs) (A) a confluent monolayer at passage 3 (B) ASCs at passage 8 (C) ASCs at passage 13 with morphological changes and vacuolization.

3.2 Properties of ASCs

ASC alter their properties upon passaging and their proliferation rate decreases and cells approach senescence with increase in passage number [14]. Healthy cells were observed until 6th passage. After that the cells started showing traits of morphological changes (Figure 1B). At passage 10, narrow spindle shaped cells were observed and in later passages the cells showed vacuolization, failed to proliferate thereafter (Figure 1C).

Population doubling time (PDT) analysis showed a consistent doubling period for ASCs up to Passage 7 and thereafter the cells showed longer doubling times to proliferate (Figure 2A). The graph was plotted with PDT against passage number and it depicted an increase in proliferation time over passages. According to this analysis, rabbit ASCs in normal growth conditions maintain their properties till passage 7 and then showed changes in their morphological pattern and growth behavior. ASCs lose their potential to proliferate over time with more passages and approach senescence [15] and are reported to have decreased growth rate [16].

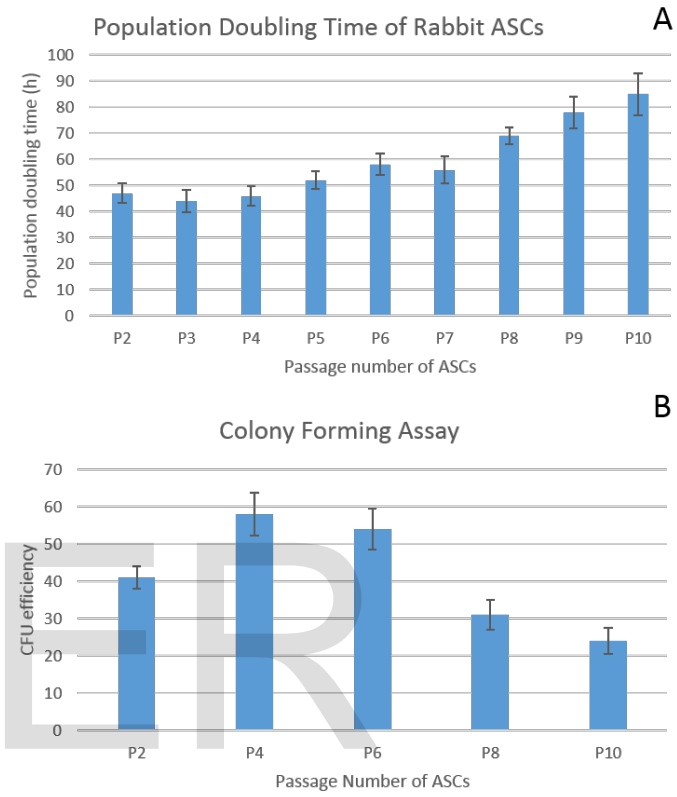

Fig 2: Properties of ASCS (A) population doubling time of ASCs from passage 2 to passage 10. (B) Colony forming efficiency of ASCs.

The reduction in proliferation rate and growth potential was also observed in colony forming assay (Figure 2B). The number of colonies with more than 100 cells were counted at different passages. It was observed that the number of colonies that ASCs could form in the later passages were very less as compared to the colony forming efficiency of ASCs at early passages. The growth rate had declined over passaging and was reflected in the colony forming unit assay. Passage 2 showed less number of colonies compared to Passage 4 and Passage 6 which can be attributed to the initial adaptation of the cells to the surroundings. Later in Passage 8 and Passage 10, ASCs showed decline in their potential to form large colonies, indicating reduction in growth curve and replicative senescence in vitro with increase in passage number [17]. For further experiments cells from P3 to P5 passage were used.

3.3 Characterization of rabbit adipose mesenchymal stem cells

Flow cytometry was used to confirm the mesenchymal lineage origin of the isolated cell population. The isolated cells were confirmed positive for mesenchymal stem cell markers CD 105, CD 90 and were negative for CD 34, a hematopoietic marker (Figure 3). Since there is no published consensus on a single marker for identifying ASCs, a set of positive and negative markers were used to identify the mesenchymal origin of ASCs [18]. ASCs at Passage 4 and Passage 5 were evaluated by FACS, and more than 85% positivity was observed for CD 105 and CD 90 markers, while CD 34 was found to be least expressed on ASC surface.

CD105 and CD 90 have previously been reported as positive markers for MSC identification. The obtained data was also in line with the requirements set by ISCT (International Society for Cellular Therapy) regarding identifying a group of cells as MSCs [19].

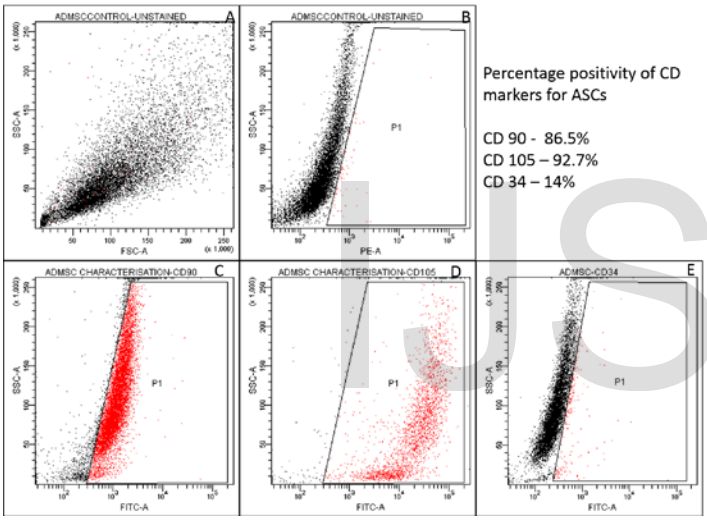

Fig 3: Characterization of ASCs (A) dot plot (B) Gated population of secondary stained cells (C) Gated positive cells for CD 90 cell surface marker(D) Gated positive cells for CD 105 cell surface marker (E) Gated positive cells for CD 34 cell surface marker

3.4 Differentiation to Multiple lineages

Further to demonstrate ASCs as an effective source of adult stem cells for regenerative medicine, multi-lineage differentiation potential of ASCs were evaluated. MSCs in general are considered tissues of mesodermal origin, with ability to differentiate in to adipogenic, osteogeneic and chondrogenic lineages. They were also reported to differentiate in to tissues of ectodermal origin and endodermal origin [20]. Adipocyte induction was confirmed through triglyceride specific staining dye Oil Red O [21]. It is a fat soluble dye and gave deep red color when stained for oil globule or fat accumulation within differentiated ASCs (Figure 4A). The accumulation of fat globules inside ASCs were considered an indication of adipogenic lineage differentiation [22].

Collagen and mineralized matrix deposition were considered as an indication of osteogenesis. Alizarin Red was used in demonstrating the calcified deposition by cells when they differentiate in to osteogenic lineage (Figure 4B). Calcium mineralization is considered an early marker and a crucial step towards bone formation [23]. Alizarin red reaction is not specific for calcium but it reacts with any divalent cation. With calcium, alizarin red forms a dark red color [24]. Another indication towards osteogenesis is determined by von kossa staining of the mineralized matrix. Von kossa stain indicates the presence of calcium phosphate deposition by forming brownish black precipitates. This is formed when silver ion reacts with phosphate ions and the photochemical degradation of silver phosphate appears as dark brown precipitates [25] ASCs differentiated to osteogenic lineage formed dark brown precipitates with von kossa indicating lineage differentiation (Figure 4C). Altogether, this indicates the multi lineage differentiation potential of ASCs and their relevance as one of the most suitable cell source for tissue engineering applications.

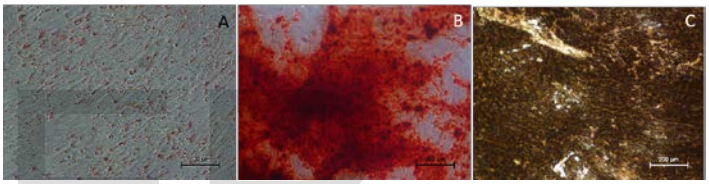

Fig 4: Multi lineage differentiation of ASCs: (A) Oil red O staining indicating adipogenic differentiation. (B) Alizarin red staining and (C) von Kossa staining– demonstrating mineral deposition, an Indication towards osteogenisis.

4. Summary and Conclusion

Tissue engineering (TE) has gained its prominence as a novel clinical approach to repair and reconstruct human tissue defects. Cell sources being an inevitable part of tissue engineering therapeutic interventions, a search of novel cell populations are of prior importance at any time line of tissue engineering research. Mesenchymal stem cells represent the most potential adult stem cell group with numerous properties supporting their use in regenerative medicine therapies. Adipose tissue is a good source of abundant stem cell population and is obtained by minimal invasive procedures. ASCs have also been shown to have multi-lineage and extra mesenchymal differentiation potential with immuno-modulatory and anti-inflammatory properties. ASCs have also been used safely and effectively in autologous and allogenic therapies. This makes it important to learn ASCs and their properties in depth to exploit this group of stem cells for the benefit of mankind.

5. ACKNOWLEDGMENTS

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