

Original Article

The Frequency of Proliferative Stromal Cells in Adipose Tissue Varies Between Inbred Mouse Strains

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Abstract

Stromal cells derived from adipose tissue (ASCs) can proliferate as undifferentiated cells with a fibroblast-like morphology in cell culture, or can be induced to differentiate into a variety of cell types including, adipogenic, myogenic, neurogenic, osteogenic, chondrogenic and hepatic cells. There is increasing interest to understand the factors controlling the proliferation of ASCs since these cells might provide a readily available source of autologous stem/progenitor cells for cell therapy applications. To explore potential genetic factors that modify the properties of ASCs, we tried to identify relevant properties of ASCs that differ between inbred mouse strains. Plating cells in a modified colony forming assay indicates that the percentage of high proliferative cells among ASCs differs more than 2-fold between 129x1/svj and C57Bl/6J mice. The identification of genetic factors affecting the proliferative capacity of stem cell populations could improve the efficacy of cell therapy.

Key words; adipose stromal cell; stem cell, mouse, cell proliferation, strain difference

Introduction

Adipose stromal cells (ASCs) isolated from fat are a readily available source of autologous stem/progenitor cells. ASCs exhibit pluripotency having been shown to develop into myogenic, adipogenic, neurogenic, osteogenic, chondrogenic endothelial and hepatic cells^{3,6,9,12,14-17,19-23} *in vitro*. In addition, ASCs in culture include a significant percentage of highly proliferative cells enabling rapid expansion of pluripotent cells *in vitro*. Several recent publications suggest their potential value as a source of autologous, adult progenitors for cell therapy applications^{2,4,5,7,8,13,18,23,24}. To realize that potential, considerable effort has focused on identifying and understanding the factors that affect their proliferation and differentiation. These include the effects of media components and other cell culture parameters. In addition, there are likely unknown genetic factors influencing the behavior of ASCs.

It has long been recognized that individuals with the same genotype at particular genes of interest, often manifest variable phenotypes. While some of this variation reflects stochastic or environmental factors, part is attributable to the variation at other genetic loci that modify the biological process involved in the expression of the phenotype. The aim of this study was to determine whether properties related to the efficacy of stem/progenitor cells for cell therapy are affected by genetic background. If so, the identification of such genetic factors might suggest strategies to improve such protocols.

The role of modifier loci and genetic background is most pronounced in the phenotypic differences observed between animals of different inbred strains that have the same genotype at loci of interest. It is likely that these phenotypic strain differences extend to the properties of stem/progenitor cells. With the genomic tools that are now available, significant strain differences provide the opportunity to identify modifier loci affecting biomedically important biological

processes. Identifying genetic factors affecting the behavior of ASCs might identify new targets to improve the efficacy of ASCs for cell therapy applications as well as to control the generation of adipose tissue. The goal of the work described in this manuscript is to identify proliferative properties of ASCs that vary between inbred mouse strains.

Material and Methods

Animals C57Bl/6j and 129x1/svj mice were purchased from Harlan (Indianapolis, IN) and housed at the LARC animal facility at IUPUI with continuous access to conventional mouse chow. Before harvest of adipose tissue mice were anaesthetized and euthanized in accordance with practices approved by the Institutional Animal Care and Use Committee of IUPUI.

Cell Isolation ASCs were isolated from mouse inguinal and lateral abdominal fat of C57BL/6J and 129x1SVJ 6-week old male. Fat tissue was washed extensively with sterile phosphate-buffered saline (PBS) and cut into tiny pieces with scissors. Washed aspirates were treated with 0.075% type I collagenase (Sigma, St. Louis, MO) in DMEM for 25 min at 37°C with gentle agitation. After digestion, mixtures were centrifuged at 500 rpm for 5 minutes. The supernatant was discarded and the pellets were pipetted and incubated in red cell lysis buffer for 5 minutes at 37°C. After centrifugation at 500 rpm for 5 minutes, cell pellets were gently agitated in DMEM with 10% FBS and filtered through a 40-µm cell strainer (BD Labware) The cell population was transferred into T75 flasks. These cells were incubated in Myelocult (M5300, Stemcell Technologies, CA) for 12-24 hours. Unattached cells were discarded with the first change of media. The attached cell population was used for subsequent analysis. Mouse ASCs were cultured in MyeloCult medium at 37°C 5% CO₂. Cells were passaged when approximately 85% confluent and the media changed twice a week.

Fluorescent Activated Cell Sorting of adipose stromal cells Fat tissue was isolated and treated with collagenase as above. After centrifugation, the cell pellet was incubated in red cell lysis buffer for 5 minutes and the cell suspension centrifuged at 500 rpm for 5 minutes to pellet a stromal cell fraction. The cell pellet was resuspended and stained simultaneously with phycoerythrin- (PE)-conjugated monoclonal anti-mouse Sca-1 (e13-161.7; BD Pharmingen), allophycocyanin- (APC)-conjugated monoclonal anti-mouse CD45 and c-kit (30-F11 and 2B8, respectively; BD Pharmingen), and biotin-conjugated anti-mouse Thy-1 (53-2.1; BD Pharmingen). Secondary staining was performed using PE-Cy7-conjugated streptavidin (Caltag Laboratories, Burlingame, CA). All stainings were performed at 4°C for 20 minutes, and cells were washed with PBS + 1% FBS after staining. Flow cytometry was performed on a FACS Vantage SE (Becton Dickinson Immunocytometry System, San Jose, CA).

Determining frequency of high proliferative cells

To determine the frequency of high proliferative cells, primary ASCs that attached to the surface of the T75 flask after 12 hour incubation were trypsinized with 0.05% Trypsin-EDTA (Gibco, 25300) and plated directly onto 48-well plates at a density of 10 cells/well. Cells were cultured for 9-11 days and media changed twice a week. The number of wells containing less than 100 cells were counted and used to determine the frequency of wells with no high proliferative cells. Using a Poisson distribution, one could calculate the average number of high proliferative cells per well.

Results

Percentage of Proliferative cells. An important property of stem/progenitor cells is their ability for self-renewal which requires the maintenance of proliferative capacity. Therefore, we determined the percentage of the ASC cell population that maintained the ability to form colonies and retain the potential

for significant proliferation. A modified colony formation assay was used in which wells of a 48 well plate were seeded with 10 cells per well. After 11 days of culture, the plates were examined and the percentage of wells for which there was no significant growth (less than 100 cells in a well) was determined. Assuming the distribution of stem/proliferative cells in a well follows a Poisson distribution, then the percentage of wells with no significant growth represents the '0 cell' class which occurs with an expected frequency of $P(0) = e^{-N} N^0 / 0! = e^{-N}$ where N is the average number of high proliferative cells per well.

Thus N, the number of proliferative cells (among the 10 cells plated per well) is $N = -\ln(P(0))$ and the frequency of proliferative cells, $f(pc)$ is the number of proliferative cells among the 10 cells in a well ($f(pc) = N/10$).

Using this assay, the frequency of high proliferative cells was determined for ASCs isolated from adipose tissue from two strains of mice. The frequency of high proliferative cells among ASCs isolated by attachment to the plastic culture dish in the 129x1/svj strain was significantly greater than that of ASCs isolated from C57Bl/6 (19.1% vs 6.9%, $p = 0.01$). (Table 1., Fig. 1).

Figure 1

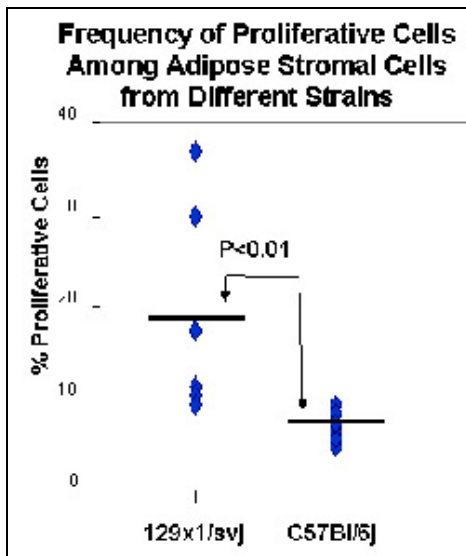


Figure 1. Percentage of Proliferative Cells Among ASCs in 129x1SVJ and C57Bl/6J. ASCs were isolated from 6 129x1svj and 9 C57Bl/6J males and plated at 10 cells per well in a total of 40 wells. The percentage of high proliferative cells among the ASCs is more than 2 fold greater in 129x1/svj than C57Bl/6J mice (19% vs 6.9%; $p<0.01$) (See Table 1 and text for details).

Figure 2

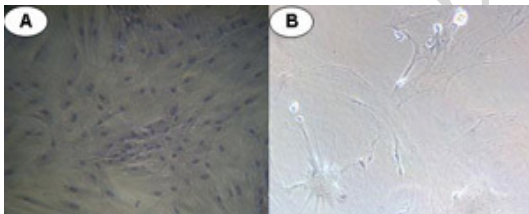


Figure 2. Limited Proliferative Potential of Murine ASCs. Photomicrographs of C57Bl/6J ASCs growing in Myleocult (Stem Cell Technology, CA) after passage 2 (frame A) and passage 6 (frame B). Early passage cells maintain fibroblast-like morphology; later passage cells are senescent and stop proliferating. Similar results are obtained for ASCs from 129x1/svj mice.

Table 1

Mouse	Number Of Wells With 0 Proliferating Cells	Number Of Proliferative Cells/10cells/Well	Mouse	Number Of Wells With 0 Proliferating Cells	Number Of Proliferative Cells/10cells/Well
129-1	13	1.1	C57-1	25	0.47
129-2	2	3.0	C57-2	20	0.69
129-3	14	1.1	C57-3	22	0.60
129-4	16	0.9	C57-4	19	0.74
129-5	7	1.7	C57-5	16	0.92
129-6	1	3.7	C57-6	21	0.64
			C57-7	17	0.86
			C57-8	23	0.55
			C57-9	19	0.74
	Mean Number Of Proliferative Cells Per 10 Cells In Well	1.9±1.1		Mean Number Of Proliferative Cells Per 10 Cells In Well	0.69±0.14

Table 1. Percentage of Proliferative Cells Among ASCs in 129x1SVJ and C57Bl/6J. ASCs were isolated from 6 129x1svj and 9 C57Bl/6J males and plated at 10 cells per well in a total of 40 wells. The number of wells in which there was no appreciable growth (<100 cells) were recorded. The number of proliferative cells in each well (N) was calculated using the Poisson distribution where $N=-\ln(P(0))$ where $P(0)$ is the fraction of wells with no significant cell growth (see text). Results are plotted in Figure 1.

Case et al. (2005) described the isolation of common pluripotent stem cells (coPSC) from muscle and adipose tissue. These cells formed colonies derived from a single cell and maintained pluripotency. Descendants of a significant number of colony forming cells maintained the ability to develop into multiple cell types including, adipocytes, neural cells and myocytes. These cells were identified out of a population of CD45-Sca-1+c-kit-Thy-1+ isolated from either skeletal muscle or adipose tissue. Since the percentage of high proliferative cells in fat varied with strain, we wished to determine if the frequency of CD45-Sca-1+c-kit-Thy-1+ cells also differed between strains. After the digestion of fat tissue, the stromal cell population was isolated and the percentage of CD45-Sca-1+c-kit-Thy-1+ cells determined by flow cytometry. Consistent with the data on proliferative cells, 129x1/svj mice contained a higher percentage of CD45-Sca-1+c-kit-Thy-1+ than C57Bl/6J mice (12.2% vs 3.4%; $p<0.05$).

Proliferative potential

In addition to the percentage of proliferative cells, the proliferative potential of ASC colonies isolated from both strains were compared. The wells containing proliferating

colonies were grown to approximately 85% confluence and passaged. Even though the 129x1/svj generated a higher percentage of proliferating cells, the proliferative potential of the cells were similar in the two strains. The mouse ASCs could only be passaged 6-7 times before a significant number of senescent cells appeared and the cells ceased proliferating (Figure 2).

Discussion

By understanding the factors that control the properties and behavior of stem/progenitor cells, it might be possible to improve their efficacy for cell therapy applications. Most attention focuses on environmental factors such as media components, growth factors, matrix composition and oxygen tension. However, genetic factors likely play an important role. Mesenchymal stromal cells isolated from bone marrow of different mouse strains have been shown to have different developmental potential and respond differently to various media formulations^{10, 11}.

In this report, we show that the frequency of high proliferative cells within ASC populations isolated by two different methods is higher in the 129x1/svj strain than in C57Bl/6J mice. These differences are statistically significant as the variation among mice in each strain is less than the difference between strains.

While the frequency of proliferative cells is different, there does not appear to be a difference in the proliferative potential among individual clones from each strain in ex vivo cell culture. The proliferation of these murine cells is quite limited; the clonal lines can only be passaged approximately 7 times before the cells become senescent. This limited propagation of murine ASCs is unlike the proliferative potential of ASCs isolated from other species including humans and rats. Conceivably, since the lifespan of mice is quite short, there is no selection to maintain high proliferative capacity of a progenitor cell population. Similar observations have been

observed with endothelial progenitor cells where it is much more difficult to isolate highly proliferative endothelial progenitors in mice than many other species (M. Yoder, personal communication).

The strain differences in the frequency of proliferative cells among ASCs suggest that genetic modifier loci influence the frequency of stem progenitor cells. Although the identity of these loci is unknown, it is possible that selective backcrossing of 129x1/svj:C57Bl/6J hybrids that have a high frequency of proliferative ASCs, into a C57Bl/6J background could enrich for 129x1/svj derived loci that effect the percentage of proliferative progenitor cells. The identification of such loci could provide important therapeutic targets to increase the regenerative potential of damaged tissue.

Conclusion and/or Summary

We have compared the percentage of adipose stromal cells isolated from two different inbred mouse strains that are capable of significant proliferation (forming initial colonies of greater than 100 cells). The frequency of high proliferative cells is greater than 2-fold higher in 129x1/svj than C57Bl/6J mice, indicating there are allelic differences at modifier loci that affect adipose progenitor cells. The identification of putative modifier loci could provide targets to improve the efficacy of ASCs for cell therapy applications.

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