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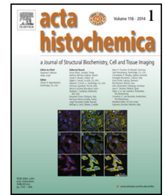

A 3-month age difference profoundly alters the primary rat stromal vascular fraction phenotype

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ABSTRACT

The stromal vascular fraction (SVF) is a heterogeneous population obtained from collagenase digestion of adipose tissue. When cultured the population becomes more homogeneous and the cells are then termed adipose stromal/stem cells (ASCs). Both the freshly isolated primary SVF population and the cultured ASC population possess regenerative abilities suggested to be mediated by paracrine mechanisms mainly. The use of ASCs and SVF cells, both in animal studies and human clinical studies, has dramatically increased during recent years. However, more knowledge regarding optimal donor characteristics such as age is demanded. Here we report that even a short age difference has an impact on the phenotype of primary SVF cells. We observed that a 3-month difference in relatively young adult rats affects the expression pattern of several mesenchymal stem cell markers in their primary SVF. The younger animals had significantly more CD90+/CD44+/CD29+/PDGFRα + primary cells, than the aged rats, suggesting an age dependent shift in the relative cell type distribution within the population. Taken together with recent studies of much more pronounced age differences, our data strongly suggest that donor age is a very critical parameter that should be taken into account in future stem cell studies, especially when using primary cells.
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1. Introduction

Within the last decade adipose tissue has been recognized as a rich source of stromal stem cells, which possess regenerative abilities. Numerous clinical and basic research studies are therefore performed using these cells in order to develop stem cell based therapies of different degenerative diseases.

Digesting and fractionating adipose tissue produces the population termed the stromal vascular fraction (SVF). This freshly isolated primary fraction is heterogeneous, containing lymphocytes, endothelial cells, fibroblasts, macrophages, pericytes, preadipocytes (Pettersson et al., 1984) and most importantly 1–2% stem cells (Strem, 2005). Culturing of SVF cells to obtain a larger cell number, produces a more homogeneous plastic adherent population, the adipose stromal/stem cells (ASCs). Interestingly, studies have shown that culturing the SVF to ASCs changes the phenotype, reduces the differentiation potential and may even reduce the regenerative abilities (Andersen et al., 2008; Boquest et al., 2005; Harada et al., 2013). Thus, the primary SVF cells may posses a larger regenerative potential than the cultured ASCs. Both primary SVF cells and ASCs exhibit multipotential characteristics; being able to differentiate into adipocytes, myocytes, epithelial cells, osteoblast and neuronal cells (Gimble and Guilak, 2003; Gimble et al., 2007). The regenerative abilities of SVFs and ASCs however seem mainly to be caused by their paracrine activities (Lin et al., 2012), including secretion of a wide range of growth factors, cytokines, chemokines and immune modulating factors (Kilroy et al., 2007; Rehman et al., 2004). The use of primary SVF cells and ASCs both in animal studies and human clinical studies has increased dramatically in recent years. As donor characteristics like anatomical site of fat deposits, gender and age (Aksu et al., 2008; Djian et al., 1983; Efimenko et al., 2014; Kaewkhaw et al., 2011; Schipper et al., 2008; Scruggs et al., 2013) have been suggested to influence the function of the SVF cells and ASCs, more knowledge regarding optimal donor charac-

Abbreviations: SVF, stromal vascular fraction; ASC, adipose stromal/stem cell; DMEM, dulbecco's modified eagle medium; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PDGFRα, platelet-derived growth factor alpha.

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teristics is warranted. Extensive age studies have been conducted in both cultured rodent and human ASCs, demonstrating evidence of biological aging of the cells, as well as age dependent changes in angiogenic- and differentiation potentials, cell yield and proliferation rate (Djian et al., 1983; Efimenko et al., 2011; Fei et al., 2013; Mantovani et al., 2012; Shi et al., 2005; Wu et al., 2014). Despite the extensive research concerning the effect of age on ASCs, few studies have investigated the SVF population. The literature on age studies and SVF cells evidence the use of a rather large age span of animals between otherwise comparable studies, which therefore may have a large impact on the results (Efimenko et al., 2011; Wu et al., 2014). In this study we hypothesized that the phenotype of primary SVF cells and cultured ASCs derived from younger rats with a relatively small age difference were distinct.

2. Materials and methods

2.1. Animals

Ten male Sprague Dawley rats (10 weeks of age) were purchased from Taconic Europe, and housed in plastic cages with a 12/12-h light/dark cycle, and fed *ad libitum* until experiments. All animal experiments were approved by the Danish Council for Supervision with Experimental Animals (#2013-15-2934-00877).

2.2. Isolation and culture of SVF

All SVF isolations were performed independent of each other. Briefly, gonadal fat pads were excised from euthanized male Sprague Dawley rats (3, 6 or 12 months old), washed in Hank's Balanced Salt Solution (HBSS)/100U/mL penicillin-streptomycin (1% PS) (GIBCO, Invitrogen, Switzerland) and minced extensively. For tissue digestion the Collagenase NB 4 Standard Grade (Serva, Germany) was used. The enzyme is of high quality as it is equivalent to a GMP graded enzyme, the manufacturer guarantees batch-to-batch consistency and stable collagenase activity when stored correctly. All the manufacturer's recommendations were adhered to. The tissue was then digested with 0.86U/mL Collagenase NB 4 Standard Grade (Serva, Germany)/HBSS/2.265 mM Ca^{2+} at 37 °C for 60 min, and cells were released by gentle trituration at 37 °C in growth medium (DMEM/1.0 g/L glucose/25 mM HEPES supplemented with 4 mM Ultraglutamine/10% Fetal Bovine Serum (FBS) (all products from Lonza, UK)/1% PS). To remove undigested tissue, the cell suspension was filtered through a 100 μm cell strainer and centrifuged (400xg, 10 min). Red blood cells were lysed by 3 min incubation in 168 mM NH_4Cl/10 mM $NaHCO_3$/1.4 mM Na_4*EDTA, and the cell suspension was finally filtered through a 40 μm cell strainer. Cells were then harvested by centrifugation (400xg, 10 min), resuspended in growth medium, and now referred to as the stromal vascular fraction (SVF). Following hemocytometer cell counting, the SVF cells were either fixed immediately (Primary SVF) or plated at 55.000 or 16,000 cells/cm^2 (Cultured ASCs). Cells were then cultured at 37 °C in 5% CO_2 for 1 or 7 days, respectively. The medium was changed at day 1 and 4. On the day of harvest, ASCs were washed twice in PBS, detached by TrypLE Select (GIBCO, Invitrogen), and harvested in HBSS/5% FBS/1% PS. Following cell counting using hemocytometer the cells were fixed for flow cytometry.

2.3. Flow cytometry

SVF cells (primary) and ASCs (day 1 and day 7) were fixed (30 min on ice) in HBSS/5% FBS/1% PS/1% natural-buffered formalin (NBF), washed three times, and resuspended in HBSS/5% FBS/1% PS/0.05% azide for storage at 5 °C until analysis. Fixed cells were incubated with primary antibodies (60 min on ice), washed twice in HBSS/5% FBS, incubated with secondary antibodies (30 min on ice), washed twice, and finally suspended in HBSS/5% FBS. A FACSCalibur instrument (Becton Dickinson, CA, USA) and Flowjo 10.0.6 software (Tree Star Inc, OR, USA) were used for data acquisition and analysis, respectively. Primary antibodies were specific for rat CD45, CD90, CD44 (BD bioscience, 554875 (1:100); 554895 (1:50); and 554869 (1:100), respectively), CD29 (abcam, ab52971 (1:100)), CD34 (R&D, AF6518 (1:72)) and PDGFRα (cell signaling, 3164 (1:200)). As is normal procedure (Andersen et al., 2012) to obtain valid and robust flow cytometric results, we used isotype control antibodies, including sheep IgG (R&D systems, 5-001-A), rabbit polyclonal IgG, rabbit monoclonal IgG (Abcam, ab37415, ab125938), mouse IgG2a,k, and mouse IgG1,k (Sigma-Aldrich, M 5409, M 5284). Alexa 488 or 647 conjugated secondary donkey antibodies specific for rabbit IgG, mouse IgG, sheep IgG (all purchased at Invitrogen: 1:200) were used for visualization. The non-specific signal (%) was subtracted from the primary antibody signal, and depicted as such. Following flow cytometric analyses localization of the surface markers was confirmed by fluorescence microscopy.

2.4. Statistical analysis

Statistical analyses included, a nonparametric Kruskal-Wallis test and a two-way ANOVA test with a Bonferroni posttest on log-transformed data. The GraphPad Prism (5.0d Mac OS X, USA) software was used for all statistical calculations.

3. Results

Recent studies using large age differences indicate that donor age most likely affects the differentiation potential, phenotype and function of ASCs (Djian et al., 1983; Efimenko et al., 2014; Schipper et al., 2008; Scruggs et al., 2013). With the aim to further explore the role of age on SVF, we compared the rat SVF phenotype from three groups of different age. SVF cells were isolated from the gonadal fat pads of 3, 6 and 12 months old rats as in general (Lopez and Spencer 2011) and gave a cell yield of 1734 cells/mg tissue (SD, +/− 642,7, range 669–2546 cells/mg tissue). An overall yield difference was observed along age (P = 0.046 n = 3-4; Kruskal-Wallis test). This result is within the range of what other studies have found (Arana et al., 2013). While some primary cells were processed directly for flow cytometry, others were cultured for 1 and 7 days (ASCs). Many unattached cells, likely of hematopoietic origin, were removed at day 1 from these cultures, and remaining attached cells were either used as day 1 samples or further cultured until day 7. Comparable with similar studies, ASCs cultured for 7 days obtained the typical fibroblast-like morphology (Fig. 1) (Arana et al., 2013).

In order to determine whether age has an effect on the phenotype of primary SVF cells, we performed flow cytometry for mesenchymal stem cell markers on primary SVF cells from 3, 6 and 12 months old rats, in accordance with current recommendations (Bourin et al., 2013). Accordingly, we found that the number of primary cells positive for the mesenchymal stem cell markers CD90, CD29, CD44, the pericyt marker PDGFRα and the endothelial marker CD34 was significantly different between the three age groups (Fig. 2). Only the hematopoietic marker CD45 remained unchanged between groups. Thus, the primary phenotype is indeed affected by age.

In order to further investigate the role of age on the SVF phenotype we compared the SVF phenotype of groups (3 and 6 months) with a relatively short age difference of three months.

As such, we found that the number of primary cells, positive for CD90, CD29, CD44 and PDGFRα, was significantly higher in the young rats (1.48, 1.72, 1.14 and 4.35 fold, respectively) as compared to aged rats (Fig. 3). By contrast, no difference was observed for

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