Original Article

The CD34$^+$ Cell Number Alone Predicts Retention of the Human Fat-Graft Volume in a Nude Mouse Model

(fat grafting / resorption / mouse model / human / CD34$^+$)

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Abstract. Prediction of the final transferred fat volume is essential for the success of fat grafting, but remains elusive. Between 20 and 80 % of the initial transplanted volume can be reabsorbed. Although graft survival has many determinants, CD34$^+$ progenitor cells from the vascular stroma of adipose tissue play a central role by promoting growth of blood vessels and adipocytes. We aimed to verify the hypothesis that a higher proportion of total CD34$^+$ cells in the transplant is associated with better preservation of the graft volume. Human lipoaspirates from 16 patients were processed by centrifugation and two grafts per donor were subcutaneously injected into 32 nude mice in 1 ml volumes in the right upper flank area. The volume of each graft was measured using a preclinical MRI scanner immediately after grafting and at three months. The percentage of CD34$^+$ cells in the graft before implantation was determined by flow cytometry. The final graft volume at three months after implantation directly correlated with the percentage of CD34$^+$ cells in the grafted material (r = 0.637, P = 0.019). The minimum retention of the fat graft was 28 % and the maximum retention was 81 %, with an average of 54 %. Our study found that fat retention after fat transfer directly correlated with the fraction of CD34$^+$ cells in the graft. The simple and fast determination of the CD34$^+$ cell percentage on site can help predicting outcomes of fat transplantation.

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Abbreviations: ADSC(s) – adipose-derived stem cell(s), EC(s) – endothelial cell(s), EPC(s) – endothelial precursor cell(s), SVF – stromal vascular fraction.

Introduction

Autologous fat grafting has been performed for more than 100 years (Newman and Ftaiha, 1987). Fat constitutes a filler material with ideal characteristics because it is completely biocompatible and naturally integrates into the host tissue. Fat is abundantly available under the skin and is easily obtainable by liposuction. When used cosmetically in the facial area, fat grafts have the ability to rejuvenate aging skin by improving its elasticity, firmness and overall quality (Coleman, 2006). After breast reconstruction using a fat transplant, the areas of skin affected by radiation (from breast cancer radiotherapy) show softening of scars, resulting in visible improvement in appearance and reduction or elimination of local pain.

Since the 1990s, the fat grafting techniques have been improved and their effectiveness verified in both practice and experimental and clinical studies (Strem and Hedrick, 2005; Coleman and Saboeiro, 2007). The rejuvenating effects are due to the high concentration of mesenchymal stem cells in the fat tissue, which is even higher than that in the bone marrow. These cells not only have the potential to transform themselves and grow into various tissues, but also to stimulate growth of the adjacent tissues by secreting cytokines; this observation has been confirmed by both experimental and clinical studies (Gimble et al., 2007; Yoshimura et al., 2009; Zimmerlin et al., 2009).

Despite their proven advantages, postoperative retention of fat grafts remains difficult to predict because 25 to 80 % of the injected material may be reabsorbed, resulting in an insufficient filling effect (Bellini et al., 2017). Histological studies investigating evolution of the transplanted fat have revealed the importance of the growth of new blood vessels and transformation of pluripotent mesenchymal stem cells for survival of the graft. Only peripheral adipocytes in the graft to a depth of 2 mm from the margin can survive due to tissue ischaemia (Fu et al., 2013). The survival of cells situated deeper under this peripheral layer is dependent on centripetal new vascularization starting from the host tissues. These new blood vessels are evident on the fourth day after grafting (Pu, 2016). An important contribution to building new blood vessels is made by metaplasia of adipose-derived stem cells (ADSCs) and multiplication of endothelial cells from the vascular stromal component of the transplant (Dong et al., 2013; Philips et al., 2013; Zhu et al., 2015; Rinker and Vyas, 2016; Bellini et al., 2017; Choi et al., 2018). Cells located centrally in the graft will most often not survive and will be phagocytized by the host histiocytes. Their place will be taken by new adipose cells, probably grown from either premature adipose or stem cells located in the viable areas of the graft.

Given the evidence of the important role of cells from the vascular stroma that surrounds the adipocytes in survival of the fat graft, studies focus on enriching the stem and endothelial cell content of the transplant by collecting the stromal vascular fraction (SVF). This fraction contains a mix of ADSCs, endothelial precursor cells (EPCs), endothelial cells (ECs), macrophages, smooth muscle cells, lymphocytes, pericytes, and pre-adipocytes and other cells (Bora and Majumdar, 2017).

The preclinical and clinical results published to date are not uniform, although most have shown better survival of grafts enriched with ADSCs. Survival is measured in terms of residual volume, weight, or histological appearance (Toyserkani et al., 2016). In particular, two studies comparing cohorts of patients with breast augmentation by fat grafting using either stem cell-enriched grafts or regular fat transplants observed no significant difference, which raised questions about the benefits of this approach (Peltoniemi et al., 2013).

Despite the fact that mesenchymal stem cells are considered CD34 negative (Kobolak et al., 2016; Zołocińska, 2018), the ADSCs in SVF express CD34 (Smith and Reid, 2018). Moreover, endothelial progenitor cells, which are very important for the transplant survival, are also CD34 positive (reviewed in Bora and Majumdar, 2017). The role of $CD34^+$ ADSCs for the graft survival has been directly demonstrated by Philips et al. (2013). The exact cellular mechanisms remain poorly understood; however, it is believed that differentiation of these $CD34^+$ adipose-derived and endothelial progenitor cells contribute to better retention of fat graft results (Matsumoto et al., 2006). However, multicolour flow cytometry for exact evaluation of the graft cellular composition is not available in many hospitals. The aim of our study was to evaluate whether only $CD34^+$ cell assessment in the graft can predict graft retention in an immunodeficient mouse fat transplantation model. We hypothesize that a higher percentage of $CD34^+$ cells would be associated with a higher retained graft volume.

Material and Methods

Human Subjects

Adipose tissues were harvested from 16 women with an average age of 47 years (32 to 66) and an average BMI of 24.7 (22.5 to 26.0).

The study protocol was approved by the Ethics Review Board of Na Bulovce Hospital, Prague, and all patients signed an informed consent form before participating in the study. The studied group was recruited from patients undergoing elective liposuction.

Lipoaspirate Harvesting and Processing

All procedures were performed under general anaesthesia. Before liposuction, we infiltrated the subcutaneous tissue with a tumescent solution containing 1 ml of adrenaline per 1 l of saline. Fat was harvested from the abdominal wall, flanks, or lateral thighs using a 60 ml Toomey syringe (handheld) and a 3 mm Mercedes cannula (Mentor, Santa Barbara, CA) and was processed by centrifugation at 1,200 × *g* for 3 min in 10 ml syringes (Medilite; Mentor) at standard room temperature. Luer-Lock connectors were used to transfer the lower one-third of the middle layer of the purified lipoaspirate to 1 ml syringes. The lower and upper layers (blood with infiltration liquids and oil, respectively) were removed. The animals were injected subcutaneously with the lipoaspirate (1 ml per mouse); then, 1 ml of the lipoaspirate was further processed to determine the $CD34^+$ cell content.

Preparation of Fat Tissue for Analysis by Flow Cytometry

The adipose tissue was digested in a buffer solution containing 20 mg of type I collagenase (Sigma Aldrich, St. Louis, MO, Lot #SLBQ1885V) in 5 ml of buffer solution per 5 ml of adipose tissue for approximately 30 min in a 37 °C water bath while being gently shaken. Thereafter, the adipose tissue was centrifuged (10 min at 180 × *g*) and subsequently filtered through a 100-µm mesh cell strainer to obtain a debris-free cell suspension.

Flow Cytometry

The cell fraction was kept chilled and prepared for analytical flow cytometry by immunostaining. The cell suspensions were pelleted (200 × *g* for 7 min) and stained with 2 µl of anti-human CD34-PE (Clone 531, BioLegend, San Diego, CA) for 20 min at 4 °C in the

dark. Then, the cells were incubated for 7 min at 37 °C with 0.5 ml of a phosphate-buffered solution containing 0.5 µg of Bisbenzimide H33342 (B2261, Sigma-Aldrich) for nuclear object discrimination. Only nuclear objects were analysed in a Canto II flow cytometer (Becton Dickinson, San Jose, CA). Immediately before the flow cytometric measurement, propidium iodide was added to a final concentration of 4 µg/ml of sample to discriminate dead cells.

Animals

A total of 32 eight-week-old female athymic nude mice (strain Crl:CD-1-Foxn1^{nu}, origin Charles River, Erkrath, Germany) were bred in the animal specific pathogen-free facility of the First Faculty of Medicine, Charles University (Prague, Czech Republic) and maintained in individually ventilated cages (IVC) (12 : 12 h light-dark cycle, 22 ± 1 °C, 60 ± 5 % humidity) during the experiments. The mice had access to standard laboratory chow and sterilized water ad libitum. The experiments were performed in accordance with national and international guidelines for laboratory animal care and approved by the Laboratory Animal Care and Use Committee of the First Faculty of Medicine, Charles University, and the Ministry of Education, Youth and Sports of the Czech Republic (MSMT-46654/2015-10).

Xenograft Implantation

The sample of fat from each patient (1 ml/mouse) was administered subcutaneously into two experimental animals under ether anaesthesia using a 25-gauge blunt-tip infiltration cannula. Each animal received 1 ml fat injection into the upper right flank region.

The collection and processing of the fat tissue took place on the same day in as short a time span (2–3 h) as possible to maintain maximum viability of the transplanted cells.

Magnetic Resonance Imaging

An experimental rodent 1T magnetic resonance imaging scanner (ICON, BrukerBioSpin, Ettlingen, Germany) allowed long-term observation of changes in the volume and structure of the transplanted adipose tissue.

Standard animal handling before measurement covered applied anaesthesia (1.5% isoflurane mixed with air), continual external heating of animals under anaesthesia to prevent hypothermia, and application of eye gel to prevent dry eyes during the anaesthesia. The MRI measurement protocol covered positioning of the animal inside the MRI scanner, selected acquisition conditions (T2-weighted sequence), and final aftercare (recovery wake-up time after each measurement during continued external heating).

The T2-weighted sequence was performed with the following parameters: TE/TR = 50/2555 ms, 20–25 slices, 1 mm slice thickness, number of averages = 10×, field size (FOV) = 8 × 8 cm, image matrix size = 256 × 256 px, and a total length of 9 min. This protocol, which had a total length of approximately 15 minutes (including anaesthesia and placement of the animal in the MRI), was selected to ensure a sufficient image quality and a short length of anaesthesia to minimize strain on the animal.

Each animal was imaged three times – shortly after the graft transplantation (in order of days after the fat application) and one and three months after the application. The adipose tissue volume was determined using own MATLAB programmed calculation at each of the three time points (after application, one month after application and three months after application) and any structural changes or other application site-specific changes were recorded.

Statistical Analysis

Dependencies between the observed variables were assessed using Pearson's correlation coefficients arranged in a correlation matrix.

Results

In total, 32 immunodeficient mice were transplanted with fat tissue (fat tissue from the same patient was administered into two mice). Nine mice were excluded from the study for the following reasons: one mouse missed a CD34 and fat cell count (due to the lack of material for flow cytometry testing), five mice spontaneously died during the anaesthesia, and three mice were terminated due to cachexia.

Flow cytometry

Flow cytometry measurements were performed for all obtained human adipose tissue samples. Fig. 1 shows the gating strategies used for flow cytometry to obtain quantities of CD34$^+$ cells for the statistical analysis. The minimal percentage of CD34$^+$ cells out of all living cells was 3.48 % and the maximal percentage was 28.10 %, with an average of 18.85 % (Table 1).

MRI results

Long-term MRI observation provided large data sets for each animal to search for possible structural changes inside the fat draft, calculation of volume changes (Fig. 2) and observation of the health status of laboratory animals.

Automatic evaluation of MRI data was carried out in MATLAB and correlated with the percentage of CD34$^+$ cells in the fat graft (results from flow cytometry). The ratio of retention was calculated as a comparison of results from the last measurement (three months after the graft transplantation) to the first measurement (around two days after the transplantation). This approach was chosen to minimize the inaccuracies in volume calculation (due to the slice thickness of MRI images) and retention ratio calculation (because the graft volume could differ from the originally applied fat volume).

The results show a wide spread of the retention ratio of the applied fat graft: minimal/maximal retention = 28.0 % / 81.2 % (volume change between the first and

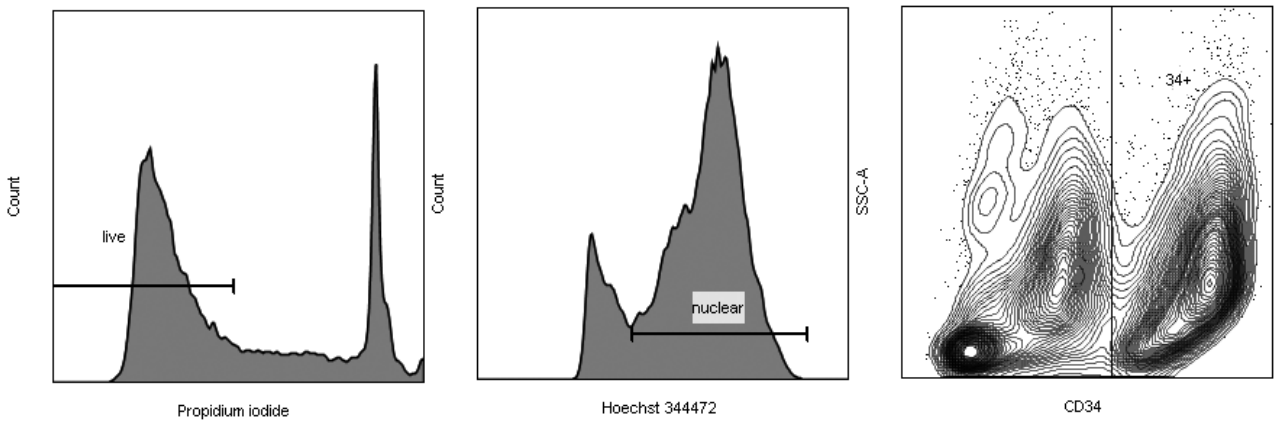

Fig. 1. Flow cytometry gating strategy for fat adipose tissue samples applied to laboratory mice. (**a**) Determination of live cells, (**b**) determination of nucleated cells by Hoechst 33342 staining, and (**c**) CD34+ cell gating.

Table 1. The percentage of CD34+ cells for each patient sample and the fat retention volumes (%)

Patient's ID	Mouse's ID	% CD34+ cells	% retention of fat
P02	M06	24.00 %	70.2
P02	M07		72.8
P03	M010	27.20 %	81.2
P04	M12	11.80 %	51.5
P04	M13		66.6
P05	M14	17.60 %	54.0
P05	M15		37.4
P06	M16	24.30 %	55.5
P06	M17		75.9
P07	M18	3.48 %	45.0
P07	M19		30.3
P08	M20	8.52 %	40.2
P08	M21		28.0
P09	M22	28.10 %	37.6
P11	M26	26.00 %	52.5
P11	M27		70.1
P12	M28	18.20 %	44.3
P12	M29		66.1
P14	M32	22.90 %	61.5
P14	M33		58.2
P15	M21	23.80 %	61.8
P16	M36	8.66 %	47.7
P16	M37		35.0

the third measurements) with average retention of 54.04 % over all measurements. The correlation between the retention rate and percentage of CD34+ cells in the graft indicate that the higher count of CD34+ cells could contribute to the higher retention of the transplanted adipose tissue.

A statistically significant correlation between the graft retention and CD34+ cell content in the graft has been found (Fig. 3). Based on the correlation coefficient r = 0.637, P = 0.019, we evaluated the dependence as moderate.

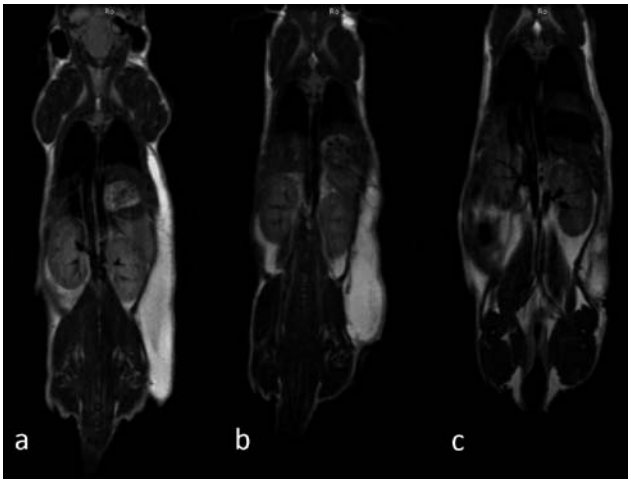

Fig. 2. Representative MRI images of a single animal (mouse M32 with a graft from patient P16) showing changes in the volume of the transplanted graft three days after the graft application (**a**); one month after the graft application (**b**); three months after the graft application (**c**). The graft of patient P16 included 22.9 % stem cells, and the total retention ratio (comparison of the first and third measurements) is 61.5 %.

Discussion

An accurate prediction of the final volume of a fat transplant for reconstructive or cosmetic purposes is essential for the success of the procedure. These predictions remain elusive; although most results are satisfactory, neither the patient nor the surgeon wants to be confronted by the need to perform subsequent surgery to correct unacceptable results.

To obtain better control of fat graft survival, numerous studies have explored the possibility of enriching

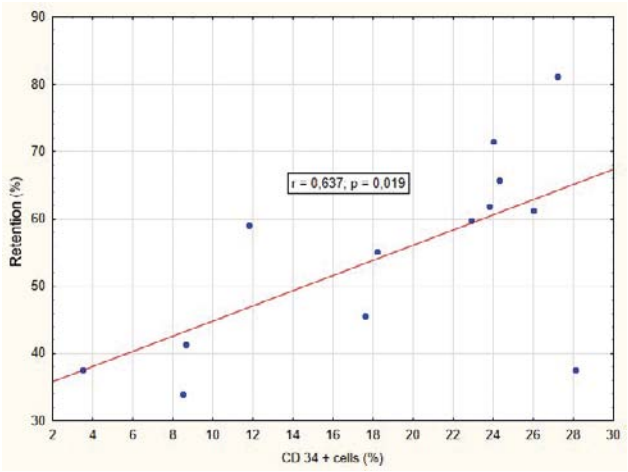

Fig. 3. Graph showing correlation between the fat retention and CD34$^+$ cell content in the graft.

the stem cell content of the transplanted material. Such attempts were based on the potential of these cells to generate new blood vessels inside the graft, maintain the trophicity of the surrounding tissues, and produce new fat cells to replace those lost due to the initial hypoxia in the transplant. Although numerous studies have reported better results using ADSC-enriched fat transplants (Toyserkani et al., 2016), other studies have found no benefits (Laloze et al., 2018).

The simple method of partially enriching the fat graft by mesenchymal stromal stem cells and vascular progenitors by centrifugation and immediate transplantation of the centrifuged fraction containing the maximum number of these cells has substantial advantages over the use of cultured stem cells due to its one-step execution. Nevertheless, some researchers compared fat grafting alone with cultured stem cell-enriched fat grafts in nude mice and found significantly greater final graft volumes 15 weeks after transplantation, when stem cells were added to the transplant (Choi et al., 2018). Alternatively, if the volumes were not significantly different, a better histological appearance denoting higher viability was observed in the progenitor cell-treated grafts than in simple fat transplants (Ko et al., 2011).

In this study of preservation of the human fat graft volume over three months in immune-deficient mice, the residual graft volume was correlated to the percentage of CD34$^+$ cells out of the total viable nucleated cells in the fat at the time of grafting. We found a significant positive correlation ($P = 0.019$) between the two variables, showing that higher percentage of CD34$^+$ cells in the transplanted material was associated with larger graft residual volumes after three months.

We elaborated on the results reported by Philips et al. (2013), who found a strong direct correlation ($r = 0.78$, $P < 0.05$) between the percentage of CD34$^+$ ADSCs in the stromal vascular fraction of the centrifuged fat and graft retention in nude mice. The authors used sophisticated multicolour flow cytometry to predict the graft retention. This method would not be available to many hospitals. Therefore, we focused on the possibility that the simple determination of CD34$^+$ cells in centrifugation-enriched adipose tissue could predict the fat graft survival. This simple test could be done using any flow cytometer, or even the cheap cassette-based portable flow cytometer (e.g., Moxi GO II, ORFLO, Ketchum, ID) that does not need extensive cytometry skills and can be installed in any laboratory. Such setting could thus be used anywhere for simple and fast prediction of the fat volume needed for transplantation.

Another finding of our study was that the percentage of CD34$^+$ cells in the transplanted tissue varied largely among the donors. A similar observation was reported by Philips et al. (2013). This finding suggests significant individual variations in the fat tissue cellular composition and the content of CD34$^+$ cells. Therefore, to improve the success rate of a fat graft, determination of the CD34+ cell content in a sample of fat from the intended harvesting area by flow cytometry may be useful. Should the content of such cells be low, the patient may be advised to have stem cells augmented in culture before undergoing grafting.

The wide variations in the residual graft volumes between mice injected with fat from the same donor also raised questions. Another study found inter-mouse variations for small grafts (0.3 ml) of approximately 8.44 % (Kokai et al., 2017), whereas our results attained an average of 13.8 % variation. A possible explanation for the increased variation may be that the larger size of the graft may result in variable vascularization rate in different animals, leading to uneven inner fat cell survival due to different oxygen and nutrient availability.

In our study, we used an experimental MRI scanner specifically designed for small laboratory animals; this equipment has the advantage of not producing any radiation while offering high measurement precision. In human studies of fat grafting, the use of MRI volume measurement has been reported several times; however, to the best of our knowledge, this study is the first report of the use of an experimental MRI scanner for the study of fat grafting in mice.

This study provides evidence for a beneficial effect of the higher CD34$^+$ cell concentrations in grafted fat for the graft survival and preservation of its volume. It also suggests that testing the CD34$^+$ cell content in the fat tissue before its grafting may predict the graft retention. Further research to pursue this avenue may contribute to a better outcome of the fat grafting.

Conclusion

This study found a moderate direct correlation between the volume retention of human fat xenografts in nude mice at three months and the percentage of CD34$^+$ cells out of the total viable cells in the adipose tissue at the time of transplant. The data support the hypothesis that CD34$^+$ cells contribute to the viability of the fat graft. We suggest that the simple determination of the CD34$^+$ cell content in the stromal vascular fraction can be used for prediction of the fat grafting outcome.

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