GROWTH OF HUMAN ADIPOSE-DERIVED STEM CELLS ON Ti-6Al-4V ALLOY WITH VARIOUS SURFACE MODIFICATIONS

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Abstract

Adipose-derived stem cells (ASCs) are of a great importance for bone tissue engineering. We compared ASCs obtained by liposuction under two pressures (-200 mmHg and -700 mmHg). The number and proliferation activity of cells isolated under -700 mmHg (ASC-H) were higher than in cells isolated under -200 mmHg (ASC-L). However, the ASC-L was more active in osteogenic differentiation, as manifested by a higher intensity of fluorescence of alkaline phosphatase and osteocalcin in these cells. The adhesion and growth of ASCs were then studied on Ti-6Al-4V samples either unmodified (K, roughness parameter R_a = 280 nm) or modified by shot blasting and tarnishing (A, R_a = 200 nm), vibratory finishing (B, R_a = 100 nm) and vibratory finishing, shot blasting and polishing (C, R_a = 80 nm). All modified samples were more wettable than the K samples. On day 1 after seeding, the size of cell spreading area on some modified samples was lower than on K samples, namely on B samples in ASC-L, on B and C in ASC-H and on A in control MG-63 cells. On day 3, the cell number on some modified samples became higher than on K samples, namely on C samples (ASC-L), on B (ASC-H) and on B and C s (MG-63 cells). On day 7, the numbers of ASC-H and MG-63 cells on all modified samples evened out and became significantly higher than on K samples. On ASC-L samples, the highest cell numbers were obtained on A samples. Thus, all studied modifications of Ti-6Al-4V enhanced growth of ASCs and human osteoblast-like MG-63 cells.

Keywords: Bone implants, bone tissue engineering, surface roughness, stem cells, liposuction, cell proliferation

1. INTRODUCTION

Stem cells in general are of a great perspective for tissue engineering and regenerative medicine. These cells are present not only in embryonic and extra-fetal tissues, but also in tissues and organs of an adult organism, e.g. in bone marrow, blood, skin, skeletal muscle, and particularly adipose tissue [1]. Adipose tissue is relatively abundant in many patients, and it is relatively easily accessible without considerable donor site morbidity due to its subcutaneous localization [2]. In comparison with the other sources of stem cells in the human body, e.g. bone marrow, ASCs in the adipose tissue are present in much larger quantities [3], and have a higher proliferation capacity [4] and delayed senescence [5]. ASCs can be induced *in vitro* to differentiate into other mesodermal cell types, such as osteoblasts, chondroblasts, skeletal myocytes, smooth muscle cells and endothelial cells, and they are also able to transdifferentiate into cells of ectodermal origin, e.g. neurons and epithelial cells, and cells of endodermal origin, such as hepatocytes and pancreatic islet cells [1, 5]. The quantity of harvested ASCs, their viability, growth and differentiation capacities can be influenced by the method of harvesting these cells. For example, it has been shown that the number of ASCs isolated from liposuction material is higher than the number of cells derived from excised adipose tissue [6]. Also the parameters of liposuction, such as the amount of negative pressure (vacuum) or the content of the solution injected into the fat, can have an important effect on the quantity and quality of the isolated ASCs. During *in vitro* cultivation, the adhesion, growth and differentiation of ASCs can be further modulated by the composition

of the culture medium and properties of the growth substrate, e.g. its chemical composition, wettability, roughness and topography.

In this study, we have focused (1) on the role of negative pressure during liposuction on the growth and osteogenic differentiation of ASCs and (2) on the influence of various surface treatments of Ti-Al-4V alloy, i.e. a material widely used for construction of bone implants, on the adhesion and growth of ASCs *in vitro*.

2. MATERIAL AND METHODS

2.1. Isolation and cultivation of ASCs

The adipose tissue was obtained from a patient (43 years old woman) by liposuction under a lower and higher negative pressure, i.e. -200 mmHg and -700 mmHg, respectively. For each pressure, 10 ml of lipoaspirate were taken, and the ASCs were isolated by a method described earlier [7]. Briefly, the lipoaspirate was repeatedly rinsed in the phosphate-buffered saline, digested by 0.1 % collagenase type I (37 °C, 1 hour), centrifuged, filtered through a cell filter (Cell Strainer, 100 µm, BD Falcon, USA) and seeded into polystyrene culture flasks (25 cm^2, TPP, Switzerland) in the amount of 0.16 ml of the original lipoaspirate per cm^2. The cells were cultured in Dulbecco's modified Eagle's Minimum Essential Medium (DMEM; Sigma, USA, Cat. N° D5648) with 10 % of fetal bovine serum (FBS; Sebak GmbH, Aidenbach, Germany), gentamicin (40 µg/ml, LEK, Ljubljana, Slovenia) and fibroblast growth factor-2 (FGF-2, 5 ng/ml) at 37° C in a humidified air atmosphere containing 5 % of CO_2. After reaching subconfluence, the cells were passaged using trypsin-EDTA solution (Sigma, U.S.A., Cat. No. T4174) in phosphate-buffered saline (PBS) for 5 min at 37 °C. Some cells were resuspended in DMEM medium with 20 % of FBS and 10 % of dimethylsulfoxide (10^6 cells/ml), frozen and stored at -80 °C.

2.2. Osteogenic differentiation of ASCs

The ASCs isolated at high pressure (ASC-H) and at low pressure (ASC-L) in passage 2 were seeded on microscopic glass coverslips (Menzel-Gläser, Germany, diameter 12 mm) and inserted into 24-well polystyrene plates (TPP, Trasadingen, Switzerland; well diameter 15 mm). As control cells, human osteoblast-like SaOs-2 and MG-63 cells (i.e. cells with high and low differentiation ability, respectively) were used. The cells were seeded in the density of 20 000 cells/well (about 11 400 cells/cm^2) and cultured for 5 days in the growth medium (DMEM with 10 % of FBS and gentamicin for MG-63 cells, the same medium with FGF-2 for ASCs, and McCoy`s medium with 15 % of FBS and gentamicin for SaOs-2 cells). In one-half of the samples, the growth medium was replaced with a differentiation medium, i.e. the growth medium with 10 mM β-glycerolphosphate, 2 mM L-glutamine, 50 µg/ml of ascorbic acid, 10^{-6} M dihydroxyvitamin D_3 and 10^{-8} M dexamethasone. In the second half of samples, the growth medium was replaced by the same growth medium. The cells then were cultured for additional 5 days, rinsed in PBS and fixed in 70% ethanol (-20 °C, 10 min). Collagen I, alkaline phosphatase (ALP) and osteocalcin, i.e., an early, middle and late marker of osteogenic cell differentiation, respectively, were then visualized in the cells by immunofluorescence [8]. The cells were photographed under the epifluorescence microscope (Olympus IX 51, objective 20x, DP 70 digital camera) at the same exposure time. For each experimental group, 20 micrographs were taken. The fluorescence intensity was measured with Fluorescent Image Analysis software (version 1.0) [9]. The intensity was subsequently normalized for the number of cells. The fluorescence intensity of control samples stained without primary antibodies was subtracted.

2.3. Surface modification and characterization of Ti-6Al-4V samples

Ti-6Al-4V samples in the form of discs (diameter 14 mm, thickness 2 mm) were modified by shot blasting and tarnishing (group A), vibratory finishing (group B) and vibratory finishing, shot blasting and polishing (group C). Unmodified Ti-6Al-4V discs were used as control samples (samples K). The surface roughness of the samples was evaluated by R_a parameter (i.e., departures of the roughness profile from the mean line), measured by

atomic force microscopy (AFM). The wettability and surface free energy of the samples was evaluated by contact angles of distilled water and ethylene glycol, measured using the Krüss DSA 100 reflection goniometer (Krüss GmbH, Germany). For each liquid and experimental group of samples, one sample was used, and the contact angle was measured in 7 drops (volume 3 µl) homogeneously distributed on the sample surface. Surface free energy was then calculated form the mean values of the contact angles using the Owens-Wendt-Rabel-Kaelble method.

2.4. Adhesion and growth of ASCs on Ti-6Al-4V samples

The samples were cleansed in ethanol and distilled and deionized water and sterilized in an autoclave (120 °C, 1 atm., 30 min), inserted into 24-well polystyrene plates (TPP, Trasadingen, Switzerland, well diameter 15 mm) and seeded with ASC-H, ASC-L and MG-63 cells. Each well contained 10 000 cells (about 5 700 cells / cm^2) and 1.5 ml of DMEM medium with 10 % FBS and 40 µg/ml of gentamicin (in case of ASCs, also 5 ng/ml of FGF-2 was added). The cells were cultured for 1, 3 or 7 days, rinsed in PBS, fixed with ethanol (-20°C, 10 min) and stained with a combination of fluorescence dyes Texas Red C_2-Maleimide (stains the cell membrane and cytoplasm; 20 ng/ml in PBS), and Hoechst # 33258 (stains the cell nuclei; 5 µg/ml of PBS). For each experimental group, 2 samples were used, and on each sample, 10 pictures were taken under Olympus IX 51 microscope. On these pictures, the cells were counted, and the size of their spreading area was measured using the software Atlas (Tescan, Brno, and CR).

3. RESULTS AND DISCUSSION

3.1. Growth of ASCs obtained by liposuction at low and high pressure

At the higher negative pressure during liposuction (-700 mmHg), the cells were obtained in a larger quantity and proliferated faster. For example, five days after seeding, the ASC-H reached a population density of ~63 000 cells/cm^2, while ASC-L only ~40 000 cells/cm^2 (**Fig. 1**). These differences also persisted after passaging (2 - 3 passages) and cryopreservation of the cells. The final cell population densities were approx. by one third higher in ASC-H, even if both ASC-H and ASC-L were seeded in the same number. These results are rather surprising, because the majority of papers (e.g. [10]) reports that low negative pressure suction gives better results than high negative pressure for the number of viable stem cells.

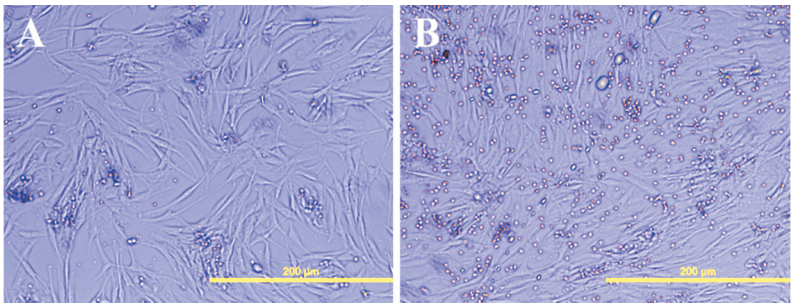

Fig. 1 Native ASC-L cells (**A**) and ASC-H cells (**B**) in primary culture on day 5 after isolation and seeding. Olympus IX 51 microscope, obj. 20x, DP 70 digital camera, bar = 200 µm

3.2. Osteogenic differentiation of ASCs

In contrast to the higher growth ability of ASC-H, the osteogenic differentiation was more pronounced in ASC-L cells. The intensity of fluorescence of collagen I was similar in ASC-L and ASC-H, but the intensity of fluorescence of ALP and osteocalcin was significantly higher in ASC-L. It has been often reported in various cell types, e.g. in osteogenic cells grown of rough substrates, that a lower proliferation capacity is associated with a higher degree of differentiation (for a review, see [11]). The differences in osteogenic differentiation of ASC-L and ASC-H were apparent in both, growth and differentiation media. However, in osteogenic media, the intensity of fluorescence of differentiation markers was generally higher than in growth media. In both

control cell lines of human osteoblast-like cells (SaOs-2, MG-63 cells), the intensity of fluorescence of all differentiation markers was relatively low and similar in both growth and differentiation media (**Fig. 2**).

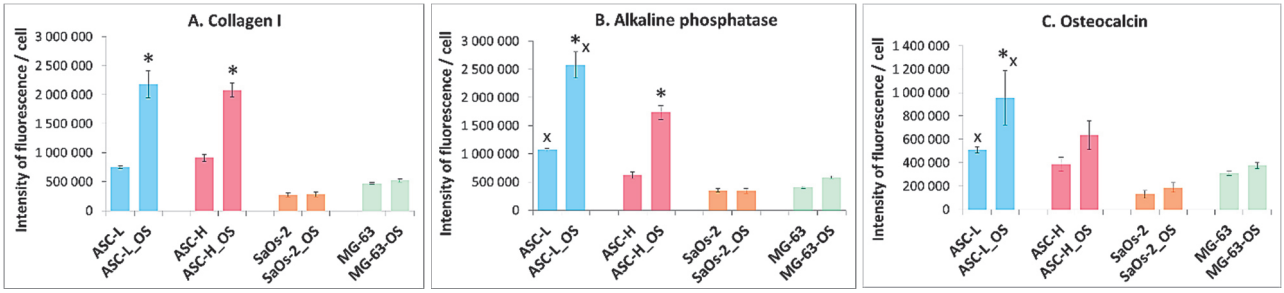

Fig. 2 Intensity of fluorescence of collagen I, alkaline phosphatase and osteocalcin in ASC-L, ASC-H, Saos-2 and MG-63 cells. The cells were cultured either in growth medium or in osteogenic medium (OS). Mean ± S.E.M. from 10 measurements for each experimental group. ANOVA, Student-Newman-Keuls method. Statistical significance: *: $p \leq 0.05$ compared to the corresponding sample in growth medium, x: $p \leq 0.05$ compared to the corresponding ASC-H

3.3. Physical and chemical properties of the material surface

The material surface roughness, measured by the R_a parameter, was lower in the modified samples than in the control sample, i.e. it was 280 nm, 200 nm, 100 nm and 80 nm in K, A, B and C samples, respectively. Similar trend was observed for the water drop contact angle, which was also lower in the modified than in control samples, i.e. the wettability of the modified samples was higher. The modified samples also showed a higher surface free energy. However, its polar component, which is important for the adsorption of cell adhesion-mediating molecules and the cell adhesion and growth (for a review, see [11]), was higher only in samples B and C, while in samples A, it was relatively low and similar as in control K samples (**Table 1**).

Table 1 Contact angle and surface free energy on Ti-6Al-4V samples

Sample	Contact angle [°]		Surface free energy [mN/m]		
	Water	Polyethylene glycol	Total	Disperse part	Polar part
K	85.6 ± 4.9	63.4 ± 4.1	25.4 ± 13.85	19.5 ± 8.47	6 ± 5.38
A	56.8 ± 2.1	12.4 ± 2.9	50.9 ± 2.72	45.3 ± 1.8	5.7 ± 0.9
B	52.7 ± 4.7	33.0 ± 0.7	47.0 ± 10.98	12.8 ± 3.42	34.2 ± 7.55
C	44.0 ± 2.3	30.4 ± 3.0	55.4 ± 5.97	8.7 ± 1.75	46.7 ± 4.22

Mean ± S.D. from 7 measurements

3.4. Adhesion and growth of ASCs on surface-modified Ti-6Al-4V samples

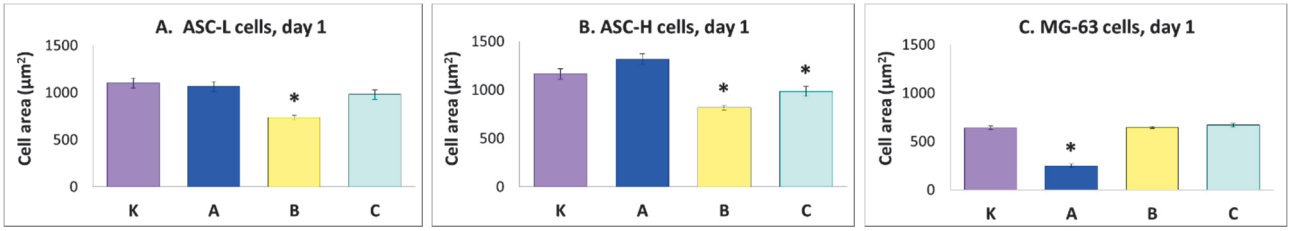

Fig. 3 The size of the cell spreading area of ASC-L, ASC-H and MG-63 cells on Ti-6Al-4V samples with various surface modifications (see Material and Methods). Mean ± S.E.M. from 141 to 289 cells for each experimental group. ANOVA, Student-Newman-Keuls method. Statistical significance: *: $p \leq 0.05$ compared to control K samples

On day 1 after seeding, ASC-L, ASC-H and MG-63 cells adhered in numbers from about 1 970 to 3 400 cells/cm^2 on all tested groups of samples, and the differences among these values were not significant. However, the size of the cell spreading area on the modified samples was often lower than on control samples, namely on B samples in ASC-L, on B and C samples in ASC-H and on A samples in MG-63 cells (**Fig. 3**). At the same time, the cell spreading areas were similar or slightly higher in ASC-H than in ASC-L cells. The cell spreading areas were generally higher in ASCs than in MG-63 cells.

On day 3 after seeding, the cell number on some modified samples became higher than on control samples, namely on C samples (ASC-L) and B samples (ASC-H) and B and C samples (MG-63 cells) (**Fig. 4**). These results could be attributed to the nanoscale surface roughness of B and C samples ($R_a \leq 100$ nm). The nanoscale surface roughness is considered as a factor promoting the adsorption of cell adhesion-mediating proteins (e.g., vitronectin, fibronectin) from the serum supplement of the culture medium in an appropriate geometrical conformation, which allows binding between the active sites in these molecules (e.g. amino acid sequences such as RGD) and the cell adhesion receptors (integrins). In addition, the samples B and C displayed relatively high wettability and high polar component of the surface free energy, i.e. other positive factors for the protein adsorption and the cell adhesion (for a review, see [11]).

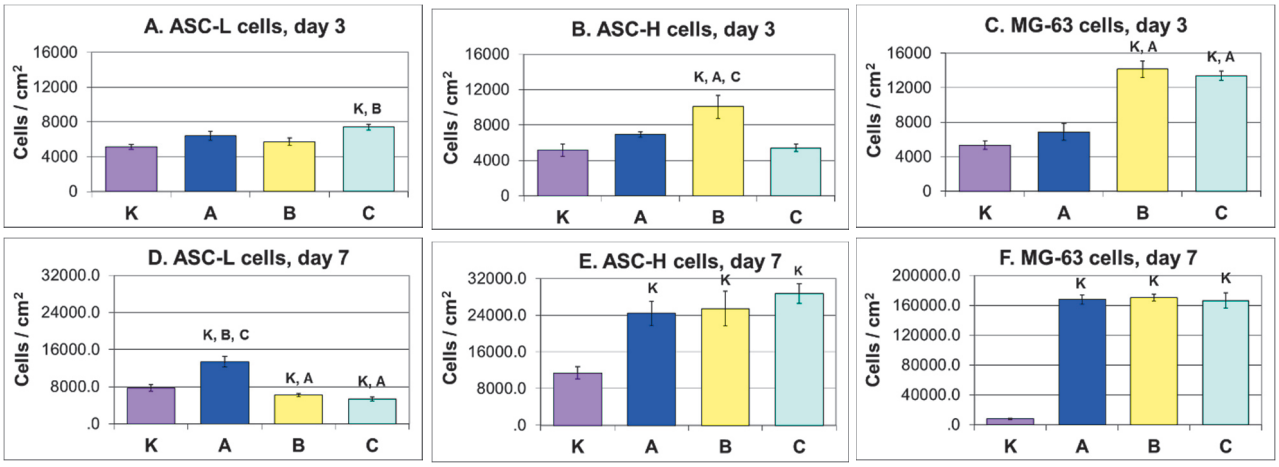

Fig. 4 The number of ASC-L, ASC-H and MG-63 cells on Ti-6Al-4V samples with various surface modifications (see Material and Methods) on days 3 and 7 after seeding. Mean ± S.E.M. from 20 measurements for each experimental group. ANOVA, Student-Newman-Keuls method. Statistical significance: *: $p \leq 0.05$ compared to the groups mentioned above the columns

Nevertheless, on day 7, the numbers of MG-63 cells and ASC-H cells on all modified samples evened out and became significantly higher than on control K samples. Only in ASC-L samples, the cell numbers on B and C samples were lower than on control K samples, and the highest cell numbers were obtained on A samples. The cell numbers were generally higher in ASC-H cells than in ASC-L cells, and in MG-63 cells than in both types of ASCs, which was clearly apparent particularly on day 7 (**Fig. 4**).

4. CONCLUSION

ASCs obtained by liposuction under high pressure (-700 mmHg) were more active in proliferation, while ASCs obtained under low pressure (-200 mmHg) were better in osteogenic differentiation. Both ASC-H and ASC-L are sensitive to physicochemical surface properties of Ti-6Al-4V alloy after various modifications. The modifications by vibratory finishing (B) and vibratory finishing, shot blasting and polishing (C) seem to be more suitable for induction of faster proliferation of cells than the modification by shot blasting and tarnishing (A). However, the final cell population densities were similar on all modified samples or even higher on A samples. Thus, all modifications can be considered as suitable for surface treatment of bone implants.

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