ADHESION AND GROWTH OF ADIPOSE TISSUE-DERIVED STEM CELLS ON FIBRIN ASSEMBLIES WITH ATTACHED GROWTH FACTORS FOR TISSUE ENGINEERING OF HEART VALVES

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Abstract

Currently used xenogeneic biological heart valve prostheses are decellularized and crosslinked with glutaraldehyde. These grafts usually undergo degeneration and calcification. Pericardium-based heart valve prostheses, re-seeded with autologous cells, i.e. adipose tissue-derived cells (ASCs) and endothelial cells, could have longer durability and biocompatibility. In order to improve the adhesion of cells and their ingrowth into decellularized pericardium, various fibrin (Fb) layers were developed, i.e. Fb, Fb with covalently bound heparin (H), Fb with either vascular endothelial growth factor (VEGF) or fibroblast growth factor 2 (FGF) in various concentrations (1 ng/ml, 10 ng/ml, 100 ng/ml) or with both VEGF and FGF (100 ng/ml). Growth factors were attached onto Fb *via* heparin or were adsorbed. ASCs were seeded on theses layers in a DMEM medium supplemented with 2 % of fetal bovine serum, TGFβ1 and BMP-4 (both 2.5 ng/ml), and with ascorbic acid. Cell adhesion and growth/viability was assessed by counted cell number/MTS evaluation. ASCs were stained for differentiation markers of smooth muscle cells, such as alpha-actin, calponin, and myosin heavy chain. On day 7, ASCs on Fb_H_VEGF layers produced both calponin and alpha-actin. An increased FGF concentration caused reduced calponin staining of ASCs. Lack of heparin in fibrin assemblies with growth factors inhibited the production of both alpha-actin and calponin in ASCs. The highest ASCs density/viability was found on Fb_H_VEGF_FGF layer. The proper formulation of fibrin coatings could be favorable for ASCs growth and differentiation and could subsequently support endothelialization of cardiovascular prostheses with endothelial cells.

Keywords: Adipose tissue-derived stem cells, heart valves, fibrin assemblies, growth factors

1. INTRODUCTION

Currently used bovine pericardial prostheses or patches are glutaraldehyde-treated or decellularized. Glutaraldehyde (GA) treatment reduces host immune response, and improves the graft durability and mechanical stability. On the other hand, a long-term release of GA from the tissue results in the graft toxicity, macrophage activation, calcification, fibrotic deposition, chronic inflammation, and degeneration [1,2,3]. Decellularization improves pericardium biocompatibility by removing immunogenic and pro-calcific elements and, concurrently, it preserves extracellular matrix. Decellularized pericardial CardioCel patches (Admedus Regen Pty Ltd, Perth, Western Australia) [4], which were rapidly crosslinked with 0.6 % GA, allowed intima formation, neovascularization, and remodelling after their implantation in patients. Controlled recellullarization and endothelialization could improve biological properties of the grafts, reduce their degeneration, prolong their durability and could allow the graft growth in children. The choice of available cell type, optimizing cell culture conditions, composition of cell culture medium, and modifications of the scaffold structure are important factors

for rapid, homogeneous recellularization and directed cell differentiation. Valve interstitial cells, present in heart valves have a phenotype partially similar to fibroblasts and smooth muscle cells (SMCs). Adipose tissue-derived stem cells (ASCs) are able to differentiate into SMCs, which make them a suitable candidate for recellullarization of pericardium scaffolds [5]. The way of growth factor (GF) attachment onto the graft surface, their concentration or sustained release, or even their combination, are important factors for recellularization. In the present study, various fibrin layers with adsorbed/attached FGF and/or VEGF have been developed; heparin was used for the long-term GF binding. We evaluated modified fibrin layers in term of ASCs adhesion, proliferation, and their differentiation towards SMC phenotype.

2. MATERIALS AND METHODS

2.1. Material preparation

Glass bottom wells in a cell culture plate (24- Well Glass Bottom Plate, Cellvis) were coated with a fibrin layer using technique described earlier [6]. The wells were incubated at 4°C with fibrinogen (5 µg/ml in Tris-HCl buffer, pH 7.4 (TB), (Sigma-Aldrich) overnight. Then, thrombin (2 U/ml in TB) (Sigma-Aldrich) was added for 30 min at room temperature. Subsequently, the wells were incubated with a mixture of antithrombin III (0.5 U/ml in TB) (Chromogenix) and fibrinogen (200 µg/ml in TB) for 2 hours. Heparin was activated by a procedure published earlier [7]. Heparin sodium salt from porcine intestinal mucosa (14 mg/ml in PBS) was oxidized by NaIO4 (2.4 mg/ml) (Sigma-Aldrich) in the dark for 90 minutes. The periodate was removed by ultrafiltration using Amicon Ultra Centrifugal filter unit with a molecular weight cut-off 3,000 Da. Activated heparin solution was diluted in the citrate buffer and immediately added to the wells and incubated overnight. In the last step, different concentration, i.e. 1 ng/ml, 10 ng/ml, and 100 ng/ml of human FGF (FGF-basic 154aa) and human VEGF 165 (GenScript) in PBS were added to the wells coated with only Fb or Fb with attached heparin) for 2 hours. Finally, the wells were three times washed with PBS, UV irradiated for 15 min, and seeded with cells. Glass cover slips, pure Fb and Fb_H were used as control samples.

2.2. Cell culture conditions

Human adipose tissue-derived stem cells (ASCs) were isolated from human lipoaspirates in compliance with the tenets of the Declaration of Helsinki for experiments involving human tissues and under ethical approval issued by the Ethics Committee in the Bulovka Hospital in Prague (August 28, 2014) [8,9]. Flow cytometry analysis revealed that the ASCs were positive for cell surface antigens CD73, CD90, CD105, and CD29, they were negative for CD31, CD34, and CD45, and about 3 % of the cells were positive for CD146.

The ASCs (passages 2-3) were seeded onto Fb layers at the density of 25,000 cells per well in DMEM supplemented with 2 % FBS, transforming growth factor β1 (ab50036, Abcam), and bone morphogenetic protein 4 (SRP6156-10UG, Sigma), both 2.5 ng/ml, 2-phospho - L - ascorbic acid trisodium salt (50 µg/ml 49752, Sigma), and cultured for 7 days.

2.3. Evaluation of cell adhesion, growth and differentiation, statistical evaluation

On day 1, the phase contrast images were taken using Olympus IX 51 microscope, equipped with DP70 digital camera from 30 microscopic fields (from 3 individual samples), and cells numbers were counted from the micrographs. On day 7, cell viability/metabolic activity was assessed by MTS assay (The CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega). 100 µl of MTS reagent was added into 500 µl of cell culture medium and cells were incubated for 45 min. Fluorescence intensity of 150 µl of the samples was measured at 490 nm in triplicates from 3 individual samples.

For evaluation cell number, ANOVA on ranks, Dunn's Method was used. Data were presented as median ± quartiles; p value ≤ 0.05 was considered significant. For MTS analysis, One way ANOVA, the Student-Newman Keuls method was used, data are expressed as mean + standard deviation.

Immunofluorescence staining of alpha-actin, calponin, and myosin heavy chain was performed. Briefly, the samples were incubated with solution containing 1 % bovine serum albumin (A9418, Sigma) and 0.1 % Triton X-100 (Sigma) for 20 min, washed with phosphate-buffered saline (PBS), and incubated with 1 % Tween 20 for 20 min. After washing with PBS, the primary antibodies (dilution 1:200) were applied overnight at 4°C, i.e. mouse monoclonal α-smooth muscle actin antibody (99A2547, Sigma), rabbit monoclonal anti-calponin antibody (EP798Y, ab46794, Abcam), mouse monoclonal anti-MYH11 (G-4) antibody (sc-6956, Santa Cruz Biotechnology, Inc.). After washing samples twice with PBS, secondary antibodies were applied for 1 hour at RT, i.e. Alexa Fluor 488 F(ab`)2 fragment of goat anti-rabbit IgG (H+L) (A11070), Alexa Fluor 546 goat anti-mouse IgG (H+L) (A11003), Alexa Fluor 488 F(ab`)2 fragment of goat anti-mouse IgG (H+L) (A11017) (dilution 1:400, Thermo Fisher Scientific). Cell nuclei were counterstained with Hoechst 33258 (Sigma). Finally, the samples were washed twice with PBS and the images were taken under IX71 Olympus epifluorescence microscope, equipped with DP71 digital camera.

3. RESULTS AND DISCUSSION

The morphology of ASCs attached to Fb layers was polygonal with long and numerous protrusions - filopodia (**Figure 1**), which was probably caused by low stiffness of the fibrin layers. On hard glass surface, the cells seemed to be better spread with smaller filopodia. Similarly, in a study by Wong et al [10], performed on a micropatterned substrate composed of rigid micro-islets surrounded by a soft gel, fibroblasts attached to the stiff islets used filopodia to test rigidity of the surrounding gel. On the rigid islets, the filopodia were stable and allowed cell spreading. On the contrary, on soft surfaces, the filopodia were less stable and tended to retract. In another study, NIH 3T3 cells evolved higher traction forces, higher migration speed, and they had higher spreading area on stiffer surfaces; Young's modulus was 300 and 140 kdyn/cm^2 for stiff and soft surface, respectively [11].

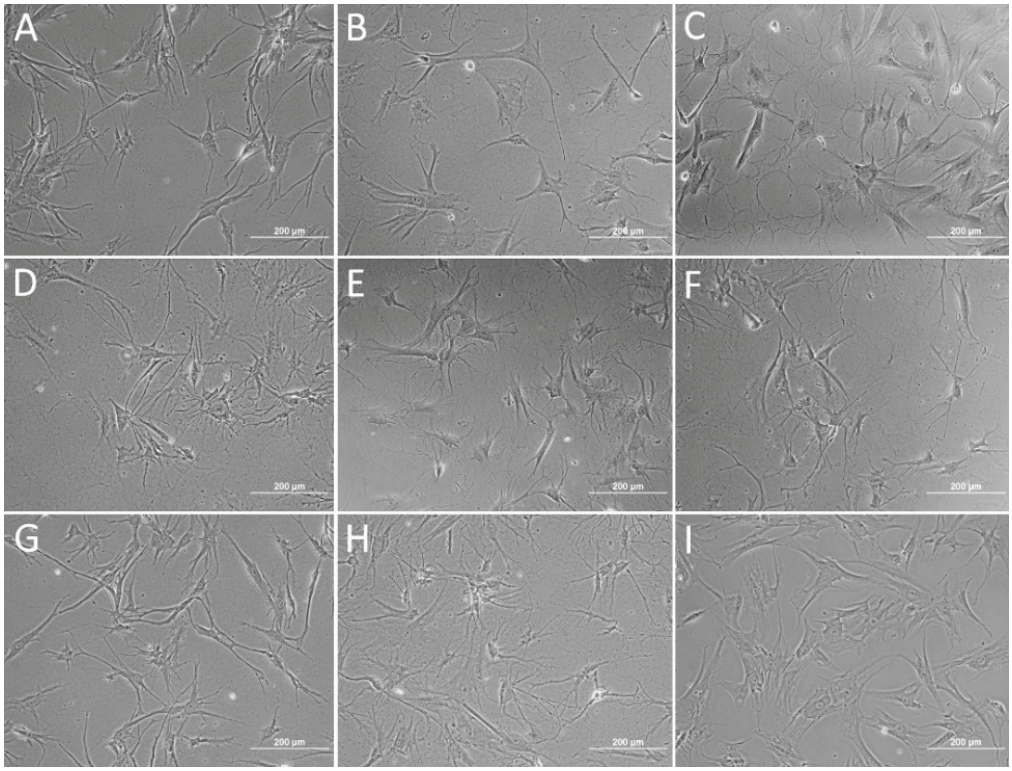

Figure 1 Phase contrast images of ASCs on Fb (A), Fb_VEGF (B), Fb_FGF (C), Fb_H (D), Fb_H_VEGF (E), Fb_H_FGF (F), Fb_VEGF_FGF (G), Fb_H_VEGF-FGF (H), and on glass coverslips (I) on day 1 after seeding. Olympus IX 51 microscope, DP70 digital camera, objective ×10

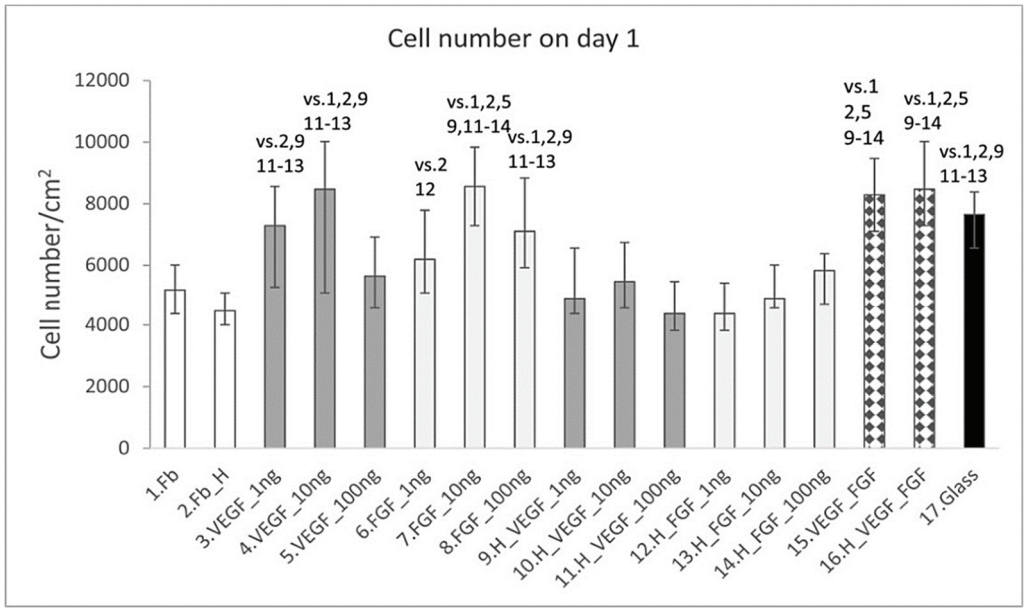

Figure 2 Number of ASCs on Fb layers modified by the attachment of H, VEGF, and FGF on day 1, measured from 30-31 microscopic fields. Data expressed as median ± quartiles. Statistical significance compared to the samples of the same number is displayed above the columns. P value ≤ 0.05 is considered significant.

High densities of ASCs were observed after one day on Fb modified with adsorbed VEGF and FGF (both 10 ng/ml) without heparin and on Fb modified with a combination of both growth factors regardless heparin presence (**Figure 2**). Heparin generally decreased cell adhesion, except for Fb_H_VEGF_FGF layer.

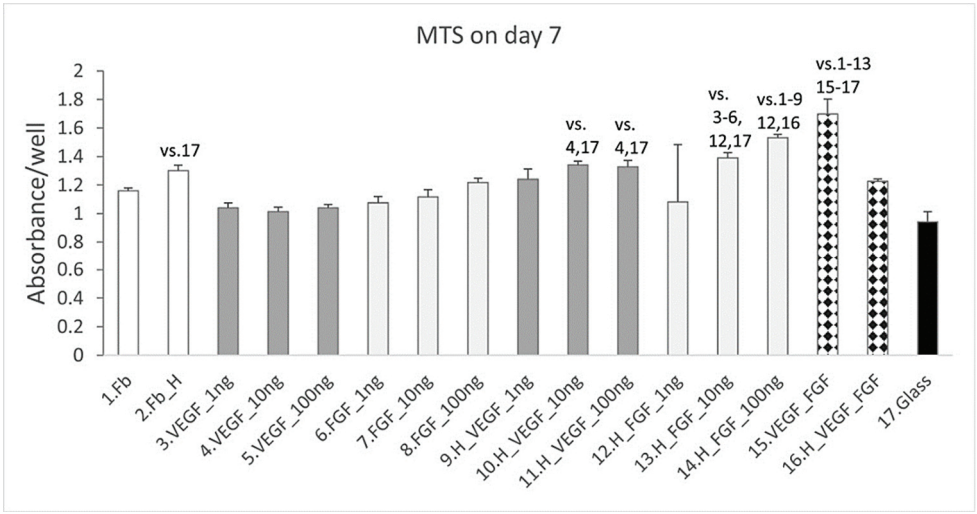

Figure 3 Viability/metabolic activity of ASCs on fibrin Fb layers modified by the attachment of H, VEGF, and FGF on day 7, measured from 3 samples. Data expressed as mean + standard deviation. Statistical significance compared to the samples of the same number is displayed above the columns.

Cell viability/proliferation was assessed by MTS assay (**Figure 3**) on day 7. The highest values were found on Fb_VEGF_FGF, slightly lower on Fb with either heparin-bound FGF or heparin-bound VEGF at the concentration of 100 ng/ml and 10 ng/ml. On glass and on Fb with adsorbed growth factors, ASCs proliferation was significantly lower. On Fb_H, cell viability was higher than on glass. On another study, heparin immobilized on chitosan films decreased smooth muscle cells proliferation, while heparin immobilized on chitosan-collagen

films improved their proliferation increased [12]. We suppose that attachment of growth factors via heparin decreased their degradation and prolonged their activity compared to the Fb samples with just adsorbed GFs.

ASCs differentiation towards smooth muscle cells (SMCs) can be evaluated by the presence of early SMC markers, i.e. SM alpha-actin, myocardin, and SM22-α, medium-term markers, such as calponin and H-caldesmon, and late markers, such as meta-vinculin, smoothelin-B, myosin heavy chain 11, and desmin [5,8,9]. We assessed the presence of SM alpha-actin, calponin (**Figure 4**) and myosin heavy chain 11. The cells were apparently positively stained for SM alpha actin on all H-containing samples, on Fb, Fb_VEGF_FGF, and on glass. The presence of adsorbed growth factors reduced the production of alpha-actin in ASCs. This might be caused by different kinetics of GF release and degradation. Similarly, staining of calponin showed its higher production in ASCs growing on Fb with covalently-bound heparin, and among them on Fb_H_VEGF_10ng. The Fb_H_FGF surfaces, especially those with the highest concentration of FGF, are less convenient for stimulation of ASCs differentiation towards SMCs. The staining for myosin heavy chain 11 was negative for cells on all samples.

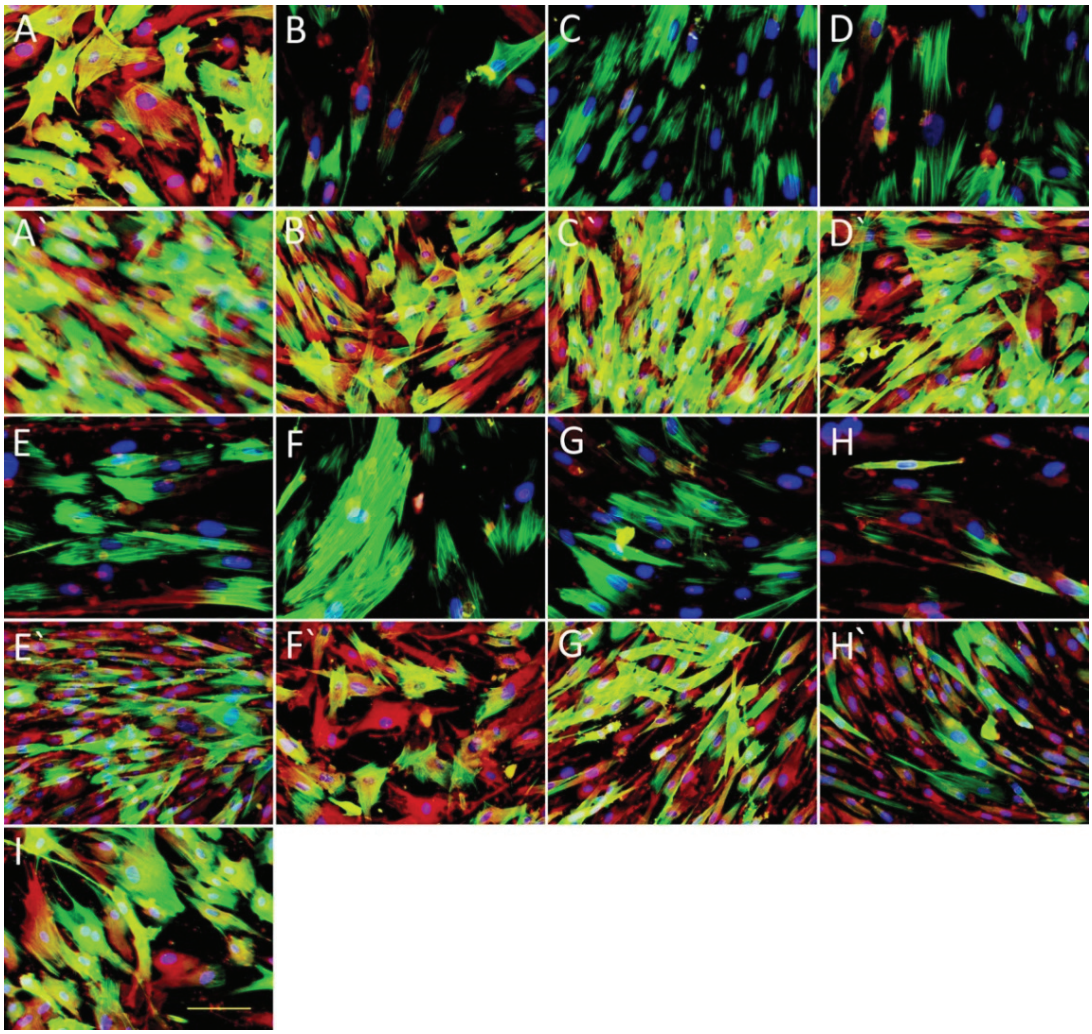

Figure 4 Differentiation markers of smooth muscle alpha actin (red) and calponin (green) in ASCs on various modified Fb layers as follows: Fb (A), Fb_H (A`), Fb_VEGF_1ng (B), Fb_H_VEGF_1ng (`B), Fb_VEGF_10ng (C), Fb_H_VEGF_10ng (C`), Fb_VEGF_100ng (D), Fb_H_VEGF_100ng (`D), Fb_VEGF_FGF (E), Fb_H_VEGF_FGF (E`), Fb_FGF_1ng (F), Fb_H_VEGF_FGF_1ng (F`), Fb_FGF_10ng (G), Fb_H_FGF_10ng (G`), Fb_FGF_100ng (H), Fb_H_FGF_100ng (H`), and glass (I), obj. × 20, scale bar = 100 μm. Cell nuclei counterstained with Hoechst 33258 (blue).

4. CONCLUSIONS

Various fibrin layers with either adsorbed or heparin-bound VEGF and/or FGF were prepared and their effect on ASCs adhesion, proliferation, and differentiation was assessed. We have observed that Fb samples with attached growth factors *via* heparin mostly decreased initial cell adhesion of ASCs. However, these samples supported cell viability/metabolic activity on day 7, and early differentiation towards smooth muscle cells. Heparin-bound VEGF or combination of both VEGF and FGF seems to be more effective than heparin-bound FGF for improving cell differentiation towards SMC phenotype.

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