DIFFERENTIATION OF MESENCHYMAL STEM CELLS ON FIBRIN ASSEMBLIES SUPPORTED BY IMMOBILIZED GROWTH FACTORS FGF2 AND VEGF

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Abstract

Bioartificial heart valves and vascular grafts prepared from decellularized tissues could be recellularized with bone marrow-derived mesenchymal stem cells (MSCs) that are able to differentiate into both smooth muscle cells and endothelial cells. MSCs differentiation is facilitated by sustained release of growth factors. In our study assemblies based on fibrin, fibrin with heparin, fibrin with adsorbed or covalently-immobilized vascular endothelial growth factor A165 (VEGF) or basic fibroblast growth factor (FGF-2) via binding to heparin attached to fibrin have been prepared and were evaluated for their stimulation of MSCs differentiation. We estimated the mRNA expression of endothelial marker CD31 (PECAM1), smooth muscle marker α-actin (ACTA2), osteoblast markers osteocalcin (BGLAP) and alkaline phosphatase (ALP). The gene expression was estimated using RT-PCR on days 1, 7 and 21 after seeding. The cell morphology and viability was evaluated by LIVE/DEAD staining. VEGF, both adsorbed and covalently bound, increased significantly the expression of smooth muscle marker α-actin. The mRNA expression of ACTA2 on day 7 and 21 raised more than 200 times in comparison to control samples (undifferentiated cells before seeding). The ACTA2 gene expression significantly exceeded the expression of all other evaluated genes at all time intervals. Moreover, on day 21, the late smooth muscle marker desmin (DES) was steeply rising in cells cultivated on assemblies containing heparin and covalently bound VEGF. The expression of osteocalcin was minimal. We conclude that fibrin assembly containing covalently bound VEGF is the most convenient for MSCs differentiation towards smooth muscle cells.

Keywords: Stem cells, differentiation, growth factors, smooth muscle cells, fibrin assemblies

1. INTRODUCTION

Biological xenogeneic heart valve prostheses and patches are made of decellularized porcine heart valve or from bovine pericardium. These replacements undergo degeneration after 15 years. There is an effort to prepare prostheses with autologous cellular component with regeneration potential and to reduce immune response. Bioartificial heart valves and vessels prepared from decellularized tissues could be recellularized with bone marrow-derived mesenchymal stem cells (MSCs). The ability to differentiate into smooth muscle cells makes the MSCs to be a suitable candidate for recellullarization of pericardium scaffolds [1]. MSCs differentiation toward smooth muscle cells, endothelial cells or valve interstitial cells could be facilitated by sustained release of growth factors (GF) [2]. The choice of GF, the way of GF attachment onto the graft surface, their concentration and even their combination or sustained release are important factors for recellularization process of biological scaffolds. Sustained delivery of GF prolongs their life time, and allows to use lower concentrations of GF necessary for cell differentiation [3]. In the present study, various fibrin layers with adsorbed/attached FGF or VEGF have been developed; heparin was used for the long-term GF binding. We have estimated the presence of smooth muscle differentiation markers after a long-term cultivation in the

presence of FGF2 and VEGF A165 immobilized on fibrin assemblies. Moreover we have tested the presence of endothelial or osteogenic markers to compare the prevailing type of differentiation and to make sure that the MSCs isolated from bone marrow do not differentiate towards osteoblast.

2. MATERIALS AND METHODS

2.1. Material preparation

The bottom of a 24- Well Glass Bottom Plate (Cellvis) was coated with a fibrin assemblies as described earlier [4] by a subsequent incubation of fibrinogen (5 µg/ml in Tris-HCl buffer, pH 7.4, TB, 4°C, overnight) (Sigma-Aldrich), thrombin (2 U/ml in TB, 30 min, room temperature) (Sigma-Aldrich), and a mixture of antithrombin III (0.5 U/ml in TB) (Chromogenix) and fibrinogen (200 µg/ml in TB) for 2 hours. Heparin sodium salt from porcine intestinal mucosa was activated using a procedure described previously [5], i.e. heparin (14 mg/ml in PBS) was oxidized by NaIO4 (2.4 mg/ml) (Sigma-Aldrich) in the darkness for 90 min and then periodate was removed by ultrafiltration. Activated heparin solution was diluted in the citrate buffer, and was incubated in the wells overnight. 100 ng/ml of human FGF2 (FGF-basic 154aa) and human VEGF A 165 (GenScript) in PBS were added to the wells coated with only Fb or Fb with attached heparin) for 2 hours. The prepared layers were washed three times with PBS and UV irradiated. Glass cover slips, pure Fb and Fb+heparin were used as control samples.

2.2. Cell cultivation conditions

The MSCs (Lonza, passages 3) were seeded at the density of 5,000 cells/cm^2 onto six different types of fibrin layers: fibrin (Fb), fibrin+FGF2 adsorbed (FGF2), fibrin+VEGF adsorbed (VEGF), fibrin+heparin (H), fibrin+heparin+VEGF (H+VEGF), fibrin+heparin+FGF2 (H+FGF2) and glass control (glass). The cells were cultivated in α-MEM medium supplemented with 2.5% FBS and with aprotinin (3.73 mg/100 ml) for 21 days.

2.3. Evaluation of cell viability by LIVE/DEAD test

On day 1, MSCs viability on fibrin assemblies was assessed by LIVE/DEAD test. The fluorescent images were taken using Olympus IX 51 microscope, equipped with DP70 digital camera from 30 microscopic fields (from 3 individual samples), living cells are stained in green, the dead cells, in red.

2.4. Evaluation of cell differentiation by Real-time PCR (RT-PCR)

The mRNA expression of genes specific for smooth muscle cells ACTA2 (α-actin) and DES (desmin), endothelial marker PECAM1 (CD31), and osteogenic markers ALP (alkaline phosphatase) and BGLAP (osteocalcin) was tested to estimate the MSCs differentiation on fibrin assemblies. The total mRNA isolation was performed using Total RNA Purification Micro Kit (Norgen Biotek). The reverse transcription was performed using the Omniscript Reverse Transcription Kit (205113; Qiagen, Hilden, Germany) and random hexamers (New England Biolabs, Inc, Ipswich, MA, USA), and was carried out according to the manufacturer's protocol. The samples were stored at -20°C. The mRNA level was quantified using 5×HOT FIREPol Probe qPCR Mix Plus (ROX) (Cat. No. 08-36-00001; Solis BioDyne, Tartu, Estonia) and by TaqMan Gene Expression Assays (Cat. No. 4331182; Thermo Fisher Scientific for genes ACTA2 (Hs 05005341_m1), DES (Hs 00157258_m1), PECAM1 (Hs 00169777_m1), ALP (Hs 01129144_m1) and BGLAP (Hs 01587814_g1). The experiments were performed with GAPDH (Hs 02786624_g1) as a reference gene.

For RT-PCR analysis, One way ANOVA, the Student-Newman Keuls method was used. Data are calculated as $2^{-\Delta\Delta Ct}$ values and expressed as a multiple of the control and presented as mean ± standard deviation; p value ≤ 0.05 was considered significant. As the control sample, the mRNA isolated from cells before seeding was taken.

3. RESULTS AND DISCUSSION

3.1. LIVE/DEAD test

Cell viability on fibrin assemblies was assessed by LIVE/DEAD test (**Figure 1**) on day 1. MSCs adhered well and spread on all tested fibrin assemblies. The cells were alive (stained green); only some individual cells were dead (i.e. stained red). On the pure heparin assembly the cell settlement was sparse on day 1. We can summarize that all assemblies are convenient for MSCs growth. In our previous study [7] we tested the metabolic activity of the HUVECs, seeded on crosslinked decellularized pericardium coated with fibrin mesh or with a mesh containing attached heparin and/or fibronectin. The lowest degree of viability was attained for samples that were coated by pure heparin. In another study, heparin immobilized on chitosan films decreased smooth muscle cells proliferation, while heparin immobilized on chitosan-collagen films improved their proliferation [7].

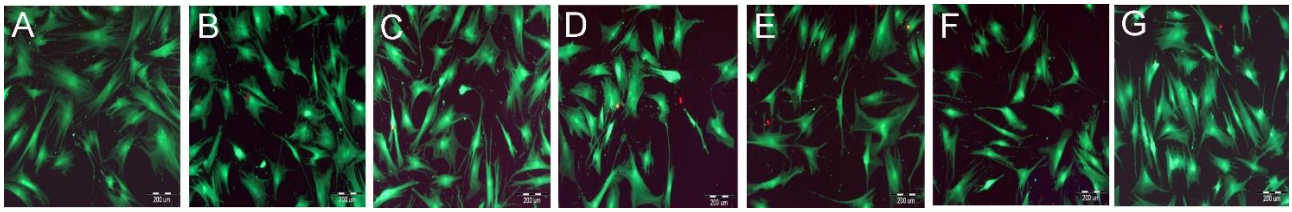
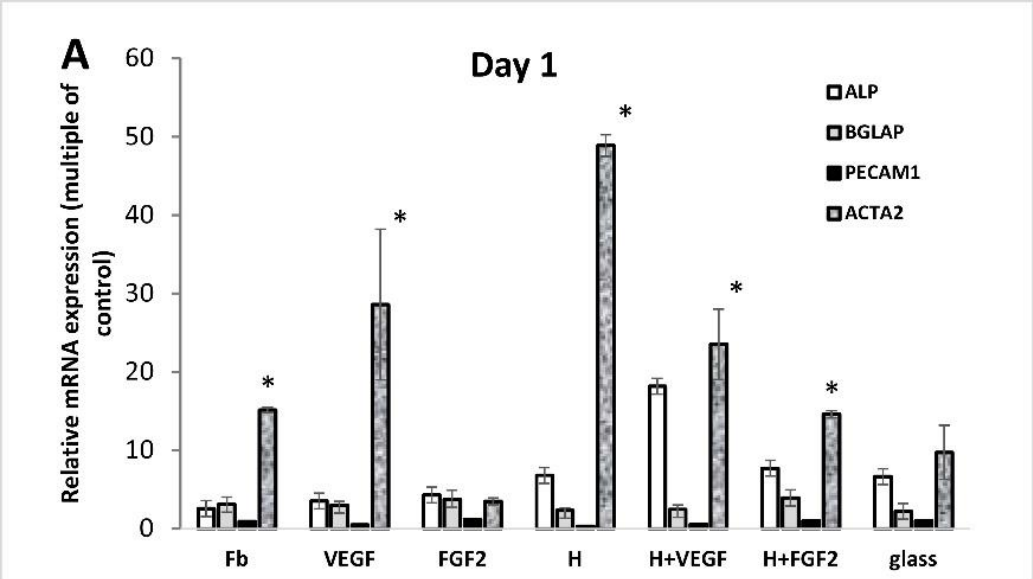


Figure 1 Viability of MSCs on day 1 after seeding on fibrin assemblies: fibrin (A), fibrin+FGF2 adsorbed (B), fibrin+VEGF adsorbed (C), fibrin+heparin (D), fibrin+heparin+VEGF(E), fibrin+heparin+FGF2 (F) and glass control (G). The cells were stained with a LIVE/DEAD Cell Viability Cytotoxicity kit (Life Technologies). Live cells are stained in green, and dead or dying cells are stained in red. Olympus IX 51 microscope, DP70 digital camera, obj. × 20, scale bar = 200 μm.

3.2. Real-time PCR

We evaluated the MSCs differentiation using Real-time PCR method. Differentiation towards smooth muscle cells and other cell types can be ascertained by the presence of early smooth muscle markers, i.e. smooth muscle alpha-actin, medium-term markers, such as calponin and H-caldesmon, and late markers, such as myosin heavy chain 11 and desmin [8,9].



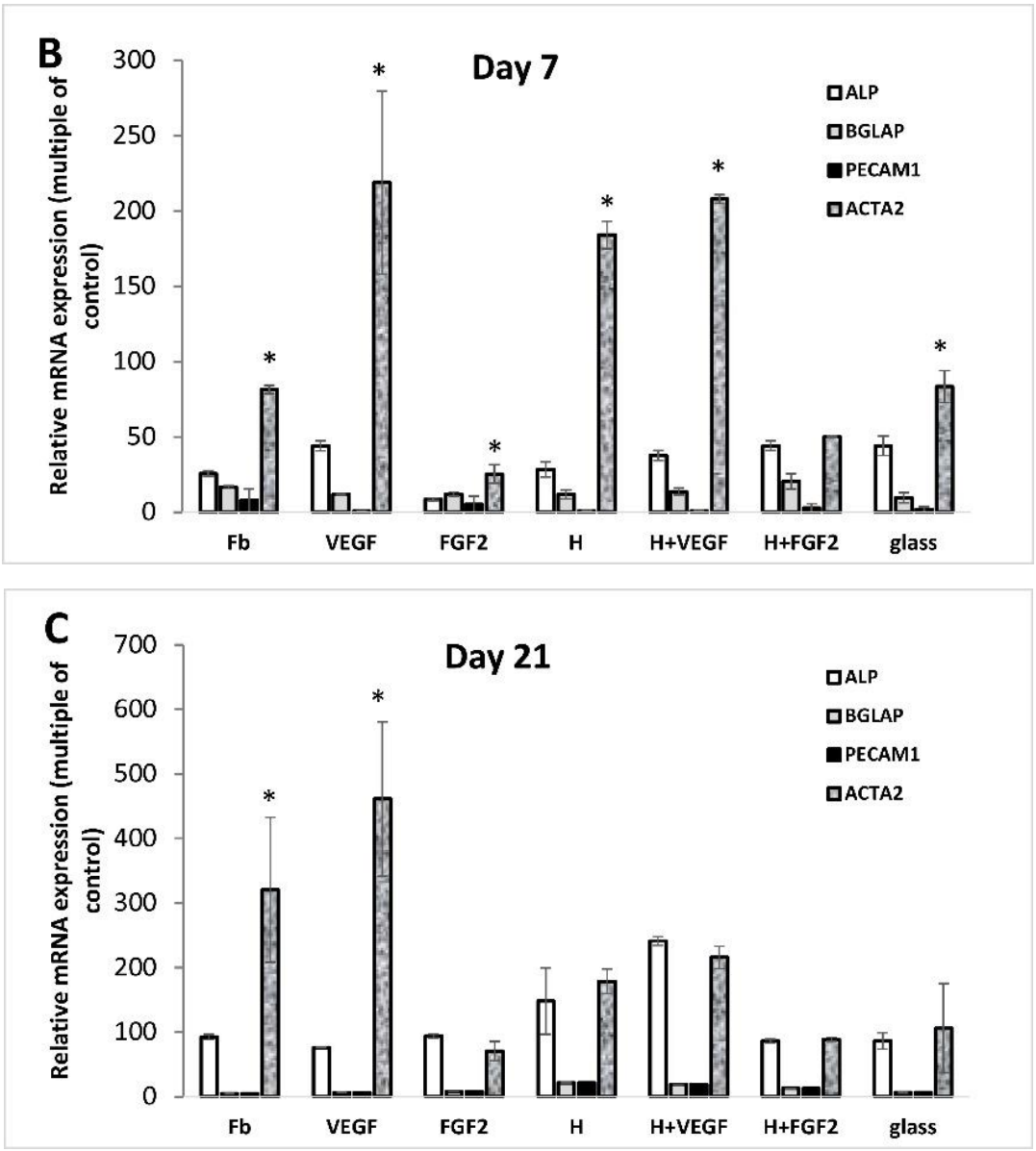


Figure 2 Relative mRNA expression of ALP (alkaline phosphatase), BGLAP (osteocalcin), PECAM1 (CD 31) and ACTA2 (smooth muscle α-actin) on fibrin assemblies in MSCs on day 1 (A), on day 7 (B), and on day 21 (C) after seeding. Fibrin assemblies: fibrin (Fb), fibrin+FGF2 adsorbed (FGF2), fibrin+VEGF adsorbed (VEGF), fibrin+heparin (H), fibrin+heparin+VEGF(H+VEGF), fibrin+heparin+FGF2 (H+FGF2) and glass control (glass). Statistical significance ($p \leq 0.05$) among genes on the same assembly type on the same day is marked by (*) above the columns.

The growth factor VEGF A 165 supported the differentiation of MSCs preferentially into smooth muscle cells (**Figures 2A-C**) on all fibrin assemblies. The presence of FGF2 reduced the expression of gens specific for smooth muscle cells (**Figure 2, Figure 3**). The expression of endothelial marker PECAM 1 is moderately rising on assemblies with VEGF (**Figure 2**). The differentiation into osteoblasts was very poor (**Figures 2A-C**). After a long-time cultivation on fibrin assemblies containing heparin or covalently bound VEGF A 165 the MSCs differentiation towards smooth muscle subtype was supported. There was extremely high expression of ACTA2 compared to all other genes at all time intervals. On day 21 the late smooth muscle marker DES expression increased markedly in MSCs cultivated on assemblies containing heparin and covalently bound VEGF. These results indicates that the MSCs differentiation was developing towards smooth muscle cells [9].

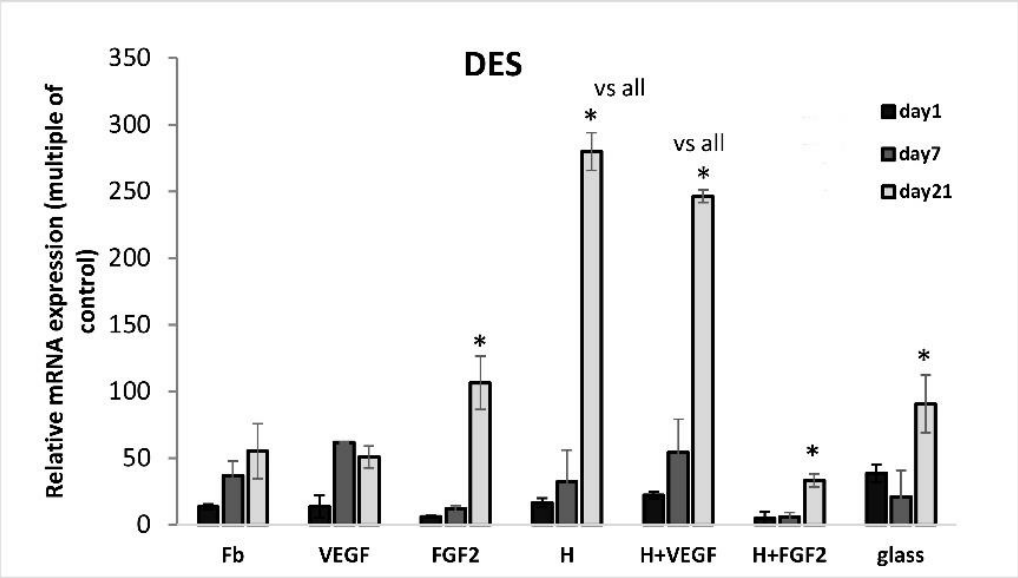


Figure 3 Relative mRNA expression of DES (desmin) on fibrin assemblies on day 1, 7 and 21 after seeding. Fibrin assemblies: fibrin (Fb), fibrin+FGF2 adsorbed (FGF2), fibrin+VEGF adsorbed (VEGF), fibrin+heparin (H), fibrin+heparin+VEGF(H+VEGF), fibrin+heparin+FGF2 (H+FGF2) and glass control (glass). Statistical significance ($p \leq 0.05$) of DES expression among different time intervals of the same assembly is marked by (*). The highest DES expression among all experimental groups (vs all) is depicted above the columns.

4. CONCLUSIONS

We have prepared various fibrin assemblies with adsorbed or covalently bound growth factors FGF2 and VEGF and we have evaluated differentiation of human MSCs on these surfaces in regards to their potential use in cardiovascular tissue engineering. MSCs differentiated mainly into the smooth muscle cells on fibrin assemblies containing VEGF. Moreover, the late smooth muscle marker DES was steeply rising in the cells cultivated on the assembly containing heparin and covalently bound VEGF. The MSCs differentiation into osteoblasts or into endothelial cells was negligible. We conclude that fibrin assembly containing covalently bound VEGF is the most convenient for MSCs differentiation towards smooth muscle cells and for the preparation of bioartificial vascular or heart valve replacements.

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