Physiological Research Pre-Press Article

Attachment of Human Endothelial Cells to Polyester Vascular Grafts: Pre-coating with Adhesive Protein Assemblies and Resistance to Short-term Shear Stress

Jaroslav Chlupáč[1, 2], Elena Filová[1], Tomáš Riedel[3], Milan Houska[3], Eduard Brynda[3], Murielle Remy-Zolghadri[4], Reine Bareille[4], Philippe Fernandez[4], Richard Daculsi[4], Chantal Bourget[4], Laurence Bordenave[4] and Lucie Bacaková[1]

[1]Department of Biomaterials and Tissue Engineering, Institute of Physiology, Academy of Sciences of the Czech Republic, v.v.i., Prague, Czech Republic, [2]Department of Transplant Surgery, Institute for Clinical and Experimental Medicine, Prague, Czech Republic, [3]Institute of Macromolecular Chemistry, Academy of Sciences of the Czech Republic, v.v.i., Prague, Czech Republic, [4]Inserm U577, Université Victor Segalen, Bordeaux, France;

Corresponding author: Jaroslav Chlupáč**,** Department of Biomaterials and Tissue Engineering, Institute of Physiology, Academy of Sciences of the Czech Republic, v.v.i., Videnska 1083, 142 20 Prague 4-Krc, Czech Republic**,** +420 29 644 3743 jarach@centrum.cz

Short title: Endothelial Cells on Polyester Vascular Grafts

Summary

Cardiovascular prosthetic bypass grafts do not endothelialize spontaneously in humans, and so they pose a thrombotic risk. Seeding with cells improves their performance, particularly in small-caliber applications. Knitted tubular polyethylene-terephthalate (PET) vascular prostheses (6 mm) with commercial type I collagen (PET/Co) were modified in the lumen by the adsorption of laminin (LM), by coating with a fibrin network (Fb) or a combination of Fb and fibronectin (Fb/FN). Primary human saphenous vein endothelial cells were seeded (1.50×10^5 /cm^2), cultured for 72 h and exposed to laminar shear stress 15 dyn/cm^2 for 40 and 120 min. The control static grafts were excluded from shearing. The cell adherence after 4 h on PET/Co, PET/Co +LM, PET/Co +Fb and PET/Co +Fb/FN was 22%, 30%, 19% and 27% of seeding, respectively. Compared to the static grafts, the cell density on PET/Co and PET/Co +LM dropped to 61% and 50%, respectively, after 120 min of flow. The cells on PET/Co +Fb and PET/Co +Fb/FN did not show any detachment during 2 h of shear stress. Pre-coating the clinically-used PET/Co vascular prosthesis with LM or Fb/FN adhesive protein assemblies promotes the adherence of endothelium. Cell retention under flow is improved particularly on fibrin-containing (Fb and Fb/FN) surfaces.

Key words: blood vessel prosthesis, polyethylene terephthalate, endothelial cells, fibrin, shear stress

Introduction

Atherosclerosis is one of the most common diseases in the developed countries. Surgical intervention in the form of a bypass or blood vessel substitution is often required to restore the blood supply to the diseased heart, brain, extremities or inner organs (Balaz *et al.* 2009). Autologous (patient's own) artery or vein is an ideal natural material for bypass graft procedures, but it is often unavailable or unsuitable for reconstruction (Matia *et al.* 2007, Kachlík *et al.* 2008). Vascular prostheses made of non-degradable synthetic polymers are therefore widely used in cardiovascular surgery, namely expanded polytetrafluoroethylene (ePTFE, Teflon, Gore-Tex) or polyethylene terephthalate (PET, Dacron). Although they perform well as substitutes for large-caliber blood vessels, they fail in small-caliber applications (≤ 6 mm), *e.g.* in coronary, below-the-knee or micro-vascular regions (Kannan *et al.* 2005, Chlupac *et al.* 2009, Rémy *et al.* 2013).

The healing process of synthetic vascular prostheses is incomplete in humans. It comprises the formation of a fibrous capsule around the outer surface, minimal capillary in-growth into the fiber interstices, and no lining or sparse lining with endothelial cells (EC) in the lumen (Pasquinelli *et al.* 1990). Thus, the blood-contacting surface remains unhealed and covered with a layer of compacted plasma proteins only. Synthetic vascular grafts therefore fail, in part due to thrombosis resulting from the lack of endothelium (Zilla *et al.* 2007). Moreover, the mismatch in mechanical properties between the elastic native artery and the rigid prosthetic graft leads to proliferation (intimal hyperplasia - stenosis) of vascular smooth muscle cells (VSMC) which cannot be regulated by missing EC (Sarkar *et al.* 2006, Varga *et al.* 2013). Bio-functionalization of vascular prostheses and subsequent *in vitro* lining with EC was previously shown to reduce the risk of thrombosis and graft failure and to improve the clinical performance of vascular prostheses (Vara *et al.* 2005, Bordenave *et al.* 2008, Deutsch *et al.* 2009).

There is no significant difference in long-term patency between PET and ePTFE bypass grafts (Roll *et al.* 2008), and the choice between PET and ePTFE is usually left to the discretion of the implanting surgeon; however, some vascular specialists prefer PET over ePTFE because PET has better handling characteristics, and there is less bleeding from suture holes and a lower tendency to form peri-graft seroma (Jonas *et al.* 1987).

The objectives of our study were to modify the inner surface of clinically-used collagen type I-impregnated PET vascular prostheses with immobilization of the extracellular matrix (ECM) protein laminin (LM), coating them with a network of blood coagulation protein fibrin (Fb) or coating them with assemblies of fibrin and fibronectin (Fb/FN). In addition, we aimed to seed primary human patient-derived EC in the lumen of the grafts to approximate the synthetic prosthesis to the natural vessel. Initial adherence and subsequent retention of the cells under flow conditions up to 2 hours (h) representing a clinical small-diameter bypass graft were evaluated in a bioreactor with a view to tissue engineering of less thrombogenic bio-artificial vascular grafts.

Methods

Knitted crimped tubular PET vascular prostheses with bovine type I collagen impregnation were received from the manufacturer (6 mm inner diameter, courtesy of VÚP Joint-Stock Comp., Brno, Czech Republic) (Fig. 1).

The prostheses were cut into pieces 20 cm in length and modified on the inner surface by immobilization of LM (Engelbreth-Holm-Swarm Murine sarcoma, Sigma) 40 μg/ml in phosphate-buffered saline (PBS) overnight or by a Fb network coating prepared by activation of human fibrinogen (Fbg) (Sigma) with surface-attached thrombin (Sigma) according to a previously-published method (Riedel *et al.* 2009). Briefly, Fbg (50 μg/ml in Tris buffer (TB) for 60 min) was adsorbed on the surface of the graft, rinsed with TB, followed by thrombin (2.5 NIH U/ml in TB for 10 min), TB, and Fbg (200 μg/ml in TB containing antithrombin III (Chromogenix) (0.5 U/ml) for 2 h. A surface fibrin network was formed by catalytic action of the surface-attached thrombin on the ambient fibrinogen solution. In the third sample, the fibrin network was further coated with FN (human plasma, Roche) 50 μg/ml in PBS overnight. An unmodified (commercial) PET/Co prosthesis served as a control graft. Thus, 4 graft types were prepared: PET/Co, PET/Co +LM, PET/Co +Fb and PET/Co +Fb/FN (Institute of Macromolecular Chemistry, Academy of Sciences of the Czech Republic, v.v.i.). They were all immersed for 12 h into M199 standard culture medium (Invitrogen).

Human saphenous vein endothelial cells (HSVEC) were enzymatically harvested from vein remnants (according to Fernandez *et al.* 2001) obtained from patients undergoing cardiac bypass

surgery (Université Victor Segalen, Bordeaux, France), cultured in polystyrene flasks (Falcon, 75 cm^2) and amplified to passage number P3-5. The procedure was approved by the local Ethics Committee. The culture medium consisted of M199, 20% of fetal calf serum (FCS) (PAA), heparin 50 IU/ml (Choay), basic fibroblast growth factor b-FGF 10 ng/ml (Promocell) and a mixture solution of penicillin (10×10^3 IU/ml), streptomycin (10 µg/ml) (Sigma) and amphotericin B (250 µg/ml) (Invitrogen). The cellular suspension was homogenized in a fresh medium and injected into the lumen of the prosthesis. Both ends were ligatured and the grafts were placed into a rotating device (Endostrabilisator, Biegler Co. Ltd., Austria) for 4 h (at 5 rpm) to achieve homogeneous cell seeding. The initial seeding density was about 1.50×10^5 cells/cm^2. The seeding was followed by 3 days of maturation in culture flasks with a fresh complete medium without rotation in a humid incubator at 37°C with 5% CO_2.

The flow experiment was performed in a custom-built hemodynamic chamber (Inserm U577, Université Victor Segalen, Bordeaux, France) (Fig. 2) consisting of a silicon tubing circuit (Tygon, Masterflex), a peristaltic pump (Cobe, Stöckert, Germany), a water container with a heater (Julabo, Labortechnik Ltd., Germany), a flowmeter (EMKA Technologies, Transsonic Systems Inc., USA), and a tensiometer (Sirecust 960, Siemens).

The prosthesis was mounted on a special metal connector creating laminar flow, and the dynamic conditions inside the bioreactor simulated human arterial circulation for 40 and 120 min (temperature 37°C, flow 500 ml/min, pulsatile pressure 120/60 mmHg, laminar shear stress (SS) 15 dynes/cm^2 and medium viscosity 0.04 Poiseuille - achieved by adding 8% Dextran (Sigma, D4751, MW 68 800). FCS (10%) was added to the medium in the 120-min experiment. A static control prosthesis was placed into a blind branch of the circuit (Fig. 2D) and was thus submitted to the same conditions of medium pressure and temperature but excluding flow and shear stress.

After each step of the experiment (seeding–maturation–flow) a prosthesis ring of defined surface area was cut open, washed with PBS and stained with the immunofluorescent Live/Dead (L/D) Viability/Cytotoxicity Kit (Molecular Probes) to visualize the homogeneity and the efficiency of the cell adhesion and retention (Olympus IX50 fluorescence microscope and Olympus DP70 digital camera, magnification 10 ×). The cells were then removed from the support using a PBS solution with

0.125% trypsin (Sigma) and 0.0625% EDTA (T/E, Sigma). M199 medium with 10% FCS was used to stop the action of the T/E solution. After centrifugation (5 min, 300g) and resuspension, the cells were counted in a 1 mm^3 Malassez chamber (8 counts) to obtain the cell number and to calculate the cell population density. The surface of the prosthesis was observed again in the fluorescence microscope to check that it was really devoid of cells after trypsinization. The cell densities on the flow-exposed prostheses were compared to the corresponding static grafts to evaluate the cell detachment during flow. Pieces of the seeded prostheses were also stained with mouse anti-human-β-actin IgG1 (1:100) monoclonal antibody (Sigma) or rabbit anti-human-von Willebrand Factor IgG (1:100) monoclonal antibody (Sigma), and the cell nuclei were counterstained with propidium iodide (5μg/ml) (Sigma). The morphology of the cells was then evaluated with confocal microscopy (Leica TCS SP2 AOBS) at magnification 10 ×.

Statistical analysis

The data is expressed as mean and standard deviation (SD) from 4 to 16 counts. The experiments on PET/Co and PET/Co +Fb (40 min) were run in duplicate and the data was pooled. One-way ANOVA multivariate analysis (Student-Newman-Keuls test) was applied for a statistical evaluation (SigmaStat 3.1 2004, Systat Software, Inc.). Statistical significance was considered when the probability of error was $p \leq 0.05$.

Results

The adherence of HSVEC to the PET vascular prostheses is shown in Figure 3A. The cell densities 4 h after rotational seeding amounted to 22% of the initial seeding density on PET/Co, 30% of the initial seeding density on PET/Co +LM, a value of 19% on PET/Co +Fb, and a value of 27% on PET/Co +Fb/FN. Thus, the cell adhesion and seeding efficiency was better on the vascular prostheses coated with LM ($p<0.001$) and Fb/FN ($p<0.05$) than on the control PET/Co prosthesis. Further differences were detected among the coated grafts – coating with LM resulted in significantly better adhesion than coating with Fb ($p<0.001$) or with Fb/FN ($p<0.05$). Deposition of Fb resulted in cell

density similar to the control graft, but adding FN to the Fb gel (PET/Co +Fb/FN) enhanced the cell adherence ($p<0.01$).

The morphology of the HSVEC is presented in Figure 4. Viable cells are green, dead cells would be visible in red. However, there are no dead cells on any of the grafts. The adhering cells are becoming spread on PET/Co and PET/Co +LM (Fig. 4A, B); however, they are slightly less numerous and still round in shape on PET/Co +Fb and PET/Co +Fb/FN (Fig. 4C, D). After 72 h of growth – before they are submitted to the shear stress – the EC form a confluent monolayer, which seems to be more compact in the valleys (Fig. 4F, H) than on the crests (Fig. 4E, G) of the crimped (*i.e.* waved structure) prosthesis.

The population densities of the HSVEC after exposure to the flow conditions in a bioreactor are given in Figure 3B. They are compared in relative numbers to the static control prosthesis (= 100%). The cell densities on PET/Co dropped to 78% (n.s.) after 40 min of shear stress and to 61% ($p<0.001$) after 120 min of shear stress, suggesting significant cell loss under flow of 2 h. The significant decrease in cell numbers was even more evident on the PET/Co +LM prostheses (55%, $p<0.001$ and 50%, $p<0.001$). However, no cell denudation was noted on the PET/Co +Fb vascular prostheses after both 40 min and 120 min of flow. Similarly, no cell loss was observed on PET/Co +Fb/FN during 120 min of shear stress application, though a significant decrease in cell density was noted after 40 min (68%, $p<0.05$).

The morphology of the HSVEC on PET/Co, PET/Co +LM and in PET/Co +Fb in static conditions and after subsequent application of flow in dynamic conditions, as seen by confocal microscopy, is shown in Figure 5. The EC form a confluent monolayer after 72 h of static growth (Fig. 5A-C) and they become sub-confluent due to certain cell denudation after delivery of 120-min-shear stress on PET/Co and on PET/Co +LM (Fig. 5D and E). However, the EC maintained their endothelial phenotype, as shown by immunofluorescence of the cytoskeletal β-actin (Fig. 5A and C, D-F) and of von Willebrand factor, which is stored in the secretion granulae (Weibel-Palade bodies) in the cytoplasm of EC (Fig. 5B). Cell detachment on the crimped prosthesis occurred mainly in the regions of the crests (middle third of Fig. 5D and the upper and lower third of Fig. 5E), whereas the cells in the valleys remained attached (upper and lower third of Fig. 5D and middle third of Fig. 5E). No flow-

dependent cell loss was observed when EC were seeded on PET/Co +Fb (Fig. 5F). In our study, we did not observe cell alignment with the direction of flow, since this physiological phenomenon occurs after longer application of shear stress, usually after 6-24 h (Punchard *et al.* 2007).

Discussion

Endothelial cell seeding on vascular implants was introduced in the 1980s, and it was proved to reduce the thrombogenicity of implanted prosthetic grafts (Ortenwall *et al.* 1990) and to enhance their patency (Meinhart *et al.* 2001). Impregnation of textile vascular prostheses with a collagen sealant was proposed in 1960 in order to make the graft impermeable to blood at the time of implantation; however, impregnated prostheses are not optimal substrata for direct endothelialization (Chakfé *et al.* 1999). To establish a cell monolayer on graft surfaces, optimal attachment and spreading of the cells is desired, together with the ability to withstand shear stress. Several research groups therefore investigated pre-coating vascular prostheses with cell-adhesive substrates and exposing the seeded cells to flow conditions (Rémy-Zolghadri *et al.* 2004, Bacakova *et al.* 2011).

EC seeding of rough-surfaced vascular prostheses with a tissue culture technique is problematic. Many authors have demonstrated the importance of providing a protein matrix to facilitate the adhesion, growth and retention of EC on prosthetic graft surfaces. Sentissi *et al.* (1986) cultured bovine aortic EC on ePTFE grafts pretreated with type I collagen and fibronectin. Cell detachment amounted to 7-11% after 1-hour of flow (Sentissi *et al.* 1986).

Gourevitch *et al.* (1988) seeded HSVEC and human omental EC on PET grafts treated with various protein coatings. The 100-min adhesion was as high as 86% on human cold insoluble globulin and 82% on 1% gelatin-coated grafts, and as low as 58% on alginate-treated PET and 48% on untreated PET. Compared to the initial cell density, they also reported EC retention of 73% after 102 min of flow in an *in vitro* circuit on 6 mm gelatin-coated Dacron tubular grafts when perfused with a culture medium, and 64% when perfused with heparinized human blood. This cell retention, however, was achieved after 30 min of maturation only (Gourevitch *et al.* 1988). Although the seeding efficacy in our study was much lower (Fig. 3A), the flow-dependent retention (40 and 120 min) of EC on our

commercial PET/Co was comparable (78% after 40 min and 61% after 120 min) with gelatin-coated Dacron grafts.

The advantage of providing a protein adhesive substrate to facilitate the endothelialization of vascular grafts was also demonstrated *in vivo*. Coating ePTFE grafts with fibronectin (FN) improved cell in-growth and surface endothelialization in a porcine interposition graft model; however, additional seeding of the FN-coated grafts with autologous EC (harvested from jugular vein) resulted in the most rapid EC coverage and excellent graft patency (Seeger and Klingman 1988).

Vohra *et al.* (1992) coated FN on ePTFE and gelatin on Dacron grafts. After seeding HSVEC (1.40×10^5 /cm^2) on both coated samples they reached 60% seeding efficiency on ePTFE and 75% seeding efficiency on Dacron, at 90 min after seeding (Vohra *et al.* 1992). The cell retention after 120 min of shear stress (1.7 and 2.6 dyn/cm^2) was 55% and 57% on FN-coated ePTFE and 69% and 67% on gelatin-impregnated Dacron grafts, respectively. In our study, the seeding density was similar (1.50×10^5 HSVEC/cm^2), but the seeding efficiency amounted to 22% only on PET/Co after 4 h of culture. Under 40-min flow and 120-min flow, the resulting cell retention was again comparable – 78% on FN-coated ePTFE grafts and 61%, on gelatin-impregnated Dacron grafts.

Pronk *et al.* (1994) investigated human peritoneal mesothelial cells (HMC) as an alternative source to EC for seeding Dacron vascular grafts. They found that the behavior of HMC was similar to that of human umbilical vein EC (HUVEC), and they reported 35% seeding efficiency after 60 min (2.50×10^5 HMC/cm^2) on plain Dacron and about 10% more on FN-coated (10 μg/ml) Dacron. Interestingly, a positive effect of FN pre-coating on cell adherence to the prosthesis was evident when the cells were seeded both in a serum-free medium (p=0.066, n.s.) and in a serum-containing medium (p=0.046, s.) (10% FCS) (Pronk *et al.* 1994). By analogy with this, we found 22% seeding efficiency of HSVEC on plain PET/Co, and this was further increased by surface pre-coating with LM up to 30% and with Fb/FN up to 27%. The slight discrepancy can be explained by the different cell type and by the lower seeding density in our study (1.50×10^5 HSVEC/cm^2). Similarly, we also seeded cells in a serum-containing medium (20% FCS).

However, Tunstall *et al.* (1995) found no enhanced adhesion of HUVEC (5.0×10^4 /0.3 cm^2) to Dacron prosthetic material pre-coated with 1% gelatin, with FN (15 μg/ml) or with LM (6 μg/ml)

compared to bare Dacron pre-incubated with the complete (*i.e.* 10% human serum-containing) medium. The authors reported the adherence of 50-70% of cells 2 h after seeding on 5 types of complete medium-pre-incubated Dacron grafts with different porosity (Tunstall *et al.* 1995). In our study, the variations found in cell adhesion to different substrata on PET/Co were statistically significant (Fig. 3A), and we also revealed differences by further testing the cell retention under flow conditions (Fig. 3B).

Sreerekha and Krishnan (2006) cultured ovine blood endothelial progenitor cells (EPC, 5.0×10^4 /cm^2) on Dacron graft patches pre-coated with fibrin composite matrix (Fb, FN, gelatin, factor XIII and vascular endothelial growth factor – VEGF). They reported 2-hour adherence of 60%, 70% and 80% on bare Dacron, low-density Fb (2 mg/ml) and high-density Fb (10 mg/ml)-coated Dacron, respectively. Moreover, Fb induced differentiation of EPC into EC, as shown by staining of von-Willebrand factor, uptake of acetylated low-density lipoproteins (LDL) and nitric oxide (NO) synthesis. The cell proliferation over a period of 72 h and the NO production was also highest on high-density-Fb Dacron when compared to Fb-coated ePTFE. Applying shear stress (20 dyn/cm^2) for 1 h resulted in no cell loss, and the NO production under dynamic conditions was again highest on high-density Fb-coated Dacron (Sreerekha and Krishnan 2006). In our study, the 4-hour adherence of HSVEC was only 22% on PET/Co, 19% on PET/Co +Fb, and 27% on PET/Co +Fb/FN. This discrepancy may be due to the different prosthesis manufacturer and the use of a different cell type. Nevertheless, no cell loss during 2-hour flow on PET/Co +Fb and PET/Co + Fb/FN in our experiment with tubular grafts is consistent with Sreerekha and Krishnan's study, confirming the concept that attached cells withstand the flow forces better on composite matrices resembling the natural ECM than on mono-protein layers.

The interaction of HUVEC with patches of commercial woven PET vascular prosthesis was extensively studied by Feugier *et al.* (2005). The cell adherence on plain grafts after 6-day culture was only 35% of the seeding density (1.05×10^5 /cm^2); however, the adhesion increased to 83% when the graft surface was experimentally bio-functionalized with a type I/III collagen coating. The cell retention under the action of 60-min shear stress of 10 and 20 dyn/cm^2, in turn, amounted to 66% and 49%, respectively. Although they reported enhanced cell spreading under flow, the cell loss was still

considerable on all of the grafts (Feugier *et al.* 2005). We studied HSVEC (1.50×10^5 /cm^2) on knitted PET with commercial type I collagen impregnation, and this data is in full accordance with our results – 78% of HSVEC retained after 40-min SS, and 61% retained after 120-min SS (12 dyn/cm^2). Interestingly, the number of cells in the study by Feugier *et al.* (2005) that remained adherent after shear stress was significantly higher on the smoother surface of ePTFE grafts than on the more irregularly shaped surface of the woven PET. The authors explain that EC adhered to the PET fibers in a more elongated fashion than to ePTFE, thus forming sub-confluence, and that this may have caused the difference. Moreover, local irregularities of PET may give rise to local variations of shear stress and flow disturbances.

Wong *et al.* (2006) seeded ovine carotid artery EC (4.00×10^5 /cm^2) on commercial knitted PET graft patches, and achieved coverage of about 30% after 3 d culture and 56% after 7 d culture. Cell retention under flow conditions decreased with increasing shearing and time of exposure values – 67% after SS = 0.018 N/m^2 (15 min) and 45% after SS = 0.037 N/m^2 (60 min) (Wong *et al.* 2006). In this experiment, both cell colonization in a static culture and cell resistance to flow were significantly better on PET grafts than on ePTFE grafts, in contrast to the findings of Feugier *et al.* (2005). Again, these results are comparable to our study with HSVEC on commercial knitted PET grafts.

Bérard *et al.* (2009) seeded human umbilical cord blood progenitor-derived EC (PDEC) on a commercial knitted PET prosthesis impregnated with bovine type I and III collagen in serum-free conditions. They found cell adherence of 20% at 1 h, 21% at 3h, and 29% (rounded values) at 24 h after seeding 2.00×10^5 PDEC/cm^2 on planar graft patches. Although the cells did not proliferate significantly until day 9 in static conditions, the application of flow for 6 h (after 3 static days) restored the endothelial monolayer in tubular grafts (Bérard *et al.* 2009). In our study, we seeded 1.50×10^5 HSVEC/cm^2 with very similar cell adhesion of 22% after 4 h. The flow experiment with tubular grafts was performed in the same bioreactor (Fig. 2.) with very similar dynamic conditions (6 mm prosthesis, 15 dyn/cm^2). By contrast, we observed significant cell denudation on the bare commercial PET/Co after 120 min of flow delivery. However, no cell loss was seen at 120 min when the cells were plated on PET/Co pretreated with either Fb or Fb/FN.

In our experiments, we chose crimped polyester vascular grafts (*i.e.* a wavy structure). These grafts have the clinical advantage of possible elongation (*e.g.* when the graft runs over a joint) and better resistance to bending (Hajjaji *et al.* 2012). The direction of the blood flow is perpendicular to the direction of crimping (Fig. 5D-F), and it runs over crests and valleys about 1 mm in length (Fig. 1A). Crimped vascular grafts are usually implanted with a certain degree of tension in an elongated fashion between the two sites of anastomoses to the native artery, thus partially removing the effect of crimping. However, in our study, the investigated prosthesis was mounted to a connector on one side only, thus retaining the crimped structure. The flow pattern had previously been investigated in a model of the crimped vascular grafts used in our study. Although a certain backflow was observed in the crimped tubes, no significant impact of crimping was found on the laminar flow pattern (Konfršt *et al.* 1994, 2005, Abdessalem *et al.* 1999). Moreover, no clinical difference between crimped and non-crimped grafts was found in terms of autologous cell seeding in a dog model (Baitella-Eberle *et al.* 1991). Interestingly, the HSVEC in our *in vitro* study were shed during flow more on the crests than in the valleys of the crimped grafts; however, this happened mainly on PET/Co and PET/Co +LM (Fig. 5D and E) – these are the grafts that sustained major cell detachment during flow.

To summarize current knowledge, it is evident that human EC of various origins can colonize commercial PET vascular grafts *in vitro*. However, substantial cellular detachment still occurs even after short-time action of flow conditions that represent the clinical situation. The contribution of our experiment consists in confirming these observations in clinically-used tubular 3D bypass grafts submitted to flow in a hemodynamic chamber.

Laminin coating has previously been shown to increase both cell attachment and cell retention, but no difference from other ECM molecules was demonstrated (Salacinski *et al.* 2001). Surface modification using LM was reported to accelerate endothelialization of porous vascular grafts in rats (Williams *et al.* 2011). However, all these tests were carried out on PTFE. The seeding efficiency of HSVEC on PET/Co +LM in our study was the highest – 30% of seeding density; however, the cell numbers significantly dropped to 55% after 40 min of flow, and to 50% after 120 min of flow, compared to static conditions.

When the PET/Co prostheses in our study were coated with Fb or Fb/FN assemblies, there was much better cell retention during the flow experiment, and no significant cell denudation was noted under shear stress up to 2 h (Fig. 5F). The EC adhere to collagen through integrin receptors $\alpha_1\beta_1$ and $\alpha_2\beta_1$, and to laminin through $\alpha_3\beta_1$, $\alpha_6\beta_1$ and $\alpha_6\beta_4$. Binding of fibronectin requires $\alpha v\beta_1$, $\alpha v\beta_3$ and $\alpha_5\beta_1$, and binding of fibrin also requires $\alpha v\beta_3$ and $\alpha_5\beta_1$ (Hynes *et al.* 2007, Takada *et al.* 2007). Integrin receptors $\alpha v\beta_3$ and $\alpha_5\beta_1$ play a crucial role in the adaptation of EC to hemodynamic force in terms of integrin activation, intracellular signaling and change in gene expression (Jalali *et al.* 2001). The fact that these integrins do not interact either with Co or with LM can explain the delamination of EC on PET/Co and PET/Co +LM under flow in this study. However, better cell coverage was observed on PET/Co +Fb and PET/Co +Fb/FN. Thus lining the vascular prosthesis with Fb or Fb/FN can provide the seeded EC with more matrix-specific signals (mediated by integrins β_1 and β_3) (Katsumi *et al.* 2004) that elicit desired cellular responses under shear stress.

The advantages of using fibrin as a substrate for cell seeding are that it can be isolated from autologous blood, it contains recognition sites for growth factors and cell adhesion receptors, and it induces matrix synthesis and differentiation of EC (Filova *et al.* 2008, Riedel *et al.* 2009). Moreover, a combination of fibrin gel with additional ECM components (*e.g.* FN) preserves EC adhesion and proliferation, and enables the formation of more mature contacts of focal adhesion (Filová *et al.* 2013). This was demonstrated by the higher cell density on PET/Co +Fb/FN than on PET/Co +Fb (Fig. 3A).

Kjaergard and Weis-Fogh (1994) described a technique for sealing a high-porosity polyester prosthesis with autologous fibrin obtained from patients' own blood. The grafts could withstand pressure of 300 mmHg, possessed good handling characteristics, and had no risk of any disease transmission (Kjaergard and Weis-Fogh 1994). Similarly, the commercial sealant of knitted PET vascular prostheses was experimentally replaced by autologous fibrin matrix, and these grafts were seeded with bone marrow cells in a porcine model. These constructs showed lowered risk of short-term (2 weeks) neo-intimal formation and thrombosis than grafts pre-clotted with whole blood; however, 3-month implantation resulted in substantial intimal hyperplasia, possibly due to the multi-potency of bone marrow cells (Cardon *et al.* 2000). Hasegava *et al.* (2007) implanted 2 mm thrombin-free fibrin-coated knitted polyester vascular grafts in carotid arteries in a rabbit model. Reduced

platelet deposition and infiltration of immune cells, decreased expression of inflammatory markers, and improved fibrinolytic balance were observed when autologous fibrin was used rather than when fibrin of xenologous origin was used. In addition, spontaneous endothelialization was promoted (Hasegawa *et al.* 2007)

Fibrin glue has previously been used as a support for endothelialization in arterial prosthetic grafts, both experimentally (Fernandez *et al.* 2007) and in human trials, and it has been clearly shown that these conduits have better clinical performance (Meinhart *et al.* 2001, Deutsch *et al.* 2009); however, the underlying bypass graft material was ePTFE in most cases. A further exceptional contribution of our study is that we have adopted the approach of coating fibrin gel or fibrin/fibronectin assemblies onto clinically-used PET/Co bypass grafts. These types of vascular prostheses have been clinically implanted for decades; however, the lack of an endothelial lining in human often results in thrombotic events with serious health complications. Our results are clinically relevant with potential direct clinical application, because we used endothelial cells derived from patients with cardiovascular disease, and our experimental coating of the prosthesis resulted in excellent shear stress resistance of the seeded cells.

Conclusion

Human primary endothelial cells can sufficiently colonize a bare commercial knitted PET/Co vascular prosthesis in a serum-containing medium; however, significant cell detachment occurs after flow is applied. Coating the inner surface with laminin improves the cellular adhesion, but the shear stress resistance remains poor. Conversely, the resistance of the adhering cells to dynamic flow conditions mimicking the blood flow is significantly enhanced when the cells are plated on prostheses modified by a fibrin network or by fibrin/fibronectin assemblies. These types of modifications may be suitable for the purposes of tissue engineering of vascular grafts.

Conflicts of interest

We declare no conflicts of interest.

Acknowledgements

Supported by the Ministry of Education, Youth and Sports of the Czech Republic (Barrande 2005-06-036-1 and grant No. EE2.3.30.0029), by the Grant Agency of the Czech Republic (grant No. P108/11/1857), and by the Grant Agency of the Ministry of Health of the Czech Republic (project No NT11270-4/2010). We also thank Dr. Vera Lisá and Mrs. Ivana Zajanová for their excellent technical assistance in cell culturing and immunocytochemical staining, and Dr. Lucie Kubinová for helping with the confocal microscopy. We thank Dr. Elzbieta Pamula, D.Sc. (AGH University of Science and Technology, Krakow, Poland) for providing the SEM picture of the vascular prosthesis. Mr. Robin Healey is gratefully acknowledged for the language revision of the manuscript.

References

ABDESSALEM SB, CHAKFE N, LE MAGNEN JF, BEAUFIGEAU M, ADOLPHE D, GENY B, AKESBI S, RIEPE G, KRETZ JG, DURAND B: Influence of crimping textile polyester vascular prostheses on the fluid flow kinetics. Eur J Vasc Endovasc Surg 18(5): 375-80, 1999.

BACAKOVA L., FILOVA E., PARIZEK M., RUML T., SVORCIK V: Modulation of cell adhesion, proliferation and differentiation on materials designed for body implants. Biotechnol Adv 29(6): 739-67, 2011.

BAITELLA-EBERLE G, LACHAT M, MÜLLER-GLAUSER W, NEUDECKER A, VON SEGESSER LK, TURINA M: Effect of hemodynamic conditions and prosthesis structure on endothelialization of clinical and experimental small lumen vascular prostheses. Helv Chir Acta 57(4): 659-63, (Article in German), 1991.

BALAZ P, ROKOSNY S, KOZNAR B, ADAMEC M: Combined infrainguinal reconstruction and infrapopliteal intraluminal angioplasty for limb salvage in critical limb ischemia. Interact Cardiovasc Thorac Surg 9(2): 191-4, 2009.

BÉRARD X., REMY-ZOLGHADRI M., BOURGET C., TURNER N., BAREILLE R., DACULSI R., BORDENAVE L: Capability of human umbilical cord blood progenitor-derived endothelial cells to form an efficient lining on a polyester vascular graft in vitro. Acta Biomater 5(4): 1147-57, 2009.

BORDENAVE L, MENU P, BAQUEY C: Developments towards tissue-engineered, small-diameter arterial substitutes. Expert Rev Med Devices 5(3): 337-47, 2008.

CARDON A, CHAKFÉ N, THAVEAU F, GAGNON E, HARTUNG O, AILLET S, KERDILES Y, DION YM, KRETZ JG, DOILLON CJ: Sealing of polyester prostheses with autologous fibrin glue and bone marrow. Ann Vasc Surg 14(6): 543-52, 2000.

CHAKFÉ N, BIZONNE SC, BEAUFIGEAU M, URBAN E, CARDON A, DOILLON C, LE MAGNEN JF, DURAND B, KRETZ JG: Impregnated polyester arterial prostheses: performance and prospects. Ann. Vasc. Surg 13(5): 509-23, 1999.

CHLUPÁC J, FILOVÁ E, BACÁKOVÁ L: Blood vessel replacement: 50 years of development and tissue engineering paradigms in vascular surgery. Physiol Res 58 Suppl 2: 119-39, 2009

DEUTSCH M, MEINHART J, ZILLA P, HOWANIETZ N, GORLITZER M, FROESCHL A, STUEMPFLEN A, BEZUIDENHOUT D, GRABENWOEGER M: Long-term experience in autologous in vitro endothelialization of infrainguinal ePTFE grafts. J Vasc Surg 49(2): 352-62, 2009.

FERNANDEZ P, BAREILLE R, CONRAD V, MIDY D, BORDENAVE L: Evaluation of an in vitro endothelialized vascular graft under pulsatile shear stress with a novel radiolabeling procedure. Biomaterials 22(7): 649-58, 2001.

FERNANDEZ P, BOURGET C, BAREILLE R, DACULSI R, BORDENAVE L: Gene response in endothelial cells cultured on engineered surfaces is regulated by shear stress. Tissue Eng 13(7): 1607-14, 2007.

FEUGIER P, BLACK RA, HUNT JA, HOW TV: Attachment, morphology and adherence of human endothelial cells to vascular prosthesis materials under the action of shear stress. Biomaterials 26(13): 1457-66, 2005.

FILOVÁ E, BRYNDA E, RIEDEL T, BACÁKOVÁ L, CHLUPÁC J, LISÁ V, HOUSKA M, DYR JE. Vascular endothelial cells on two-and three-dimensional fibrin assemblies for biomaterial coatings. J Biomed Mater Res A 90(1): 55-69, 2009

FILOVÁ E, BRYNDA E, RIEDEL T, CHLUPÁČ J, LISÁ V, HOUSKA M, PIRK J, BAČÁKOVÁ L: Endothelial Cells on Surface Attached Fibrin Structures Containing Extracellular Matrix Proteins. J Biomed Mater Res A (accepted, in press), 2013.

GOUREVITCH D, JONES CE, CROCKER J, GOLDMAN M: Endothelial cell adhesion to vascular prosthetic surfaces. Biomaterials 9(1): 97-100, 1988.

HAJJAJI R, ABDESSALEM SB, GANGHOFFER JF: The influence of textile vascular prosthesis crimping on graft longitudinal elasticity and flexibility. J Mech Behav Biomed Mater 16: 73-80, 2012.

HASEGAWA T, OKADA K, TAKANO Y, HIRAISHI Y, OKITA Y: Autologous fibrin-coated small-caliber vascular prostheses improve antithrombogenicity by reducing immunologic response. J Thorac Cardiovasc Surg 133(5): 1268-76, 2007.

HYNES RO: Cell-matrix adhesion in vascular development. J Thromb Haemost 5(Suppl 1): 32-40, 2007.

JALALI S, DEL POZO MA, CHEN K, MIAO H, LI Y, SCHWARTZ MA, SHYY JY, CHIEN S: Integrin-mediated mechanotransduction requires its dynamic interaction with specific extracellular matrix (ECM) ligands. Proc Natl Acad Sci U S A: 98(3): 1042-46, 2001.

JONAS RA, SCHOEN FJ, ZIEMER G, BRITTON L, CASTANEDA AR: Biological sealants and knitted Dacron conduits: comparison of collagen and fibrin glue pretreatments in circulatory models. Ann Thorac Surg 44(3): 283-90, 1987.

KACHLÍK D, STINGL J, SOSNA B, STRAKA Z, LAMETSCHWANDTNER A, MINNICH B, FÁRA P: Morphological features of vasa vasorum in pathologically changed human great saphenous vein and its tributaries. Vasa 37(2): 127-36, 2008.

KANNAN RY, SALACINSKI HJ, BUTLER PE, HAMILTON G, SEIFALIAN AM: Current status of prosthetic bypass grafts: a review. J Biomed Mater Res B Appl Biomater 74(1): 570-81, 2005.

KATSUMI A, ORR AW, TZIMA E, SCHWARTZ MA: Integrins in mechanotransduction. J Biol Chem 279(13): 12001-04, 2004.

KJAERGARD HK, WEIS-FOGH US: Autologous fibrin glue for sealing vascular prostheses of high porosity. Cardiovasc Surg 2(1): 45-47, 1994.

KONFRŠT J., LIEPSCH D, WEIGAND C: Experimental study of pulsatile flow in elastic model of PETP vascular grafts. Proceedings of the 3rd International Symposium of Biofluid Mechanics, Munich, Germany, Abstract book: 582, 1994.

KONFRŠT J: Mechanical and hydrodynamical properties of arterial grafts. PhD Thesis, Czech Technical University, Faculty of Mechanical Engineering, Prague, Czech Republic, 2005.

MATIA I, JANOUSEK L, MARADA T, ADAMEC M: Cold-stored venous allografts in the treatment of critical limb ischaemia. Eur J Vasc Endovasc Surg 34(4): 424-31, 2007.

MEINHART JG, DEUTSCH M, FISCHLEIN T, HOWANIETZ N, FRÖSCHL A, ZILLA P: Clinical autologous in vitro endothelialization of 153 infrainguinal ePTFE grafts. Ann Thorac Surg 71(5) Suppl: 327-31, 2001.

ORTENWALL P, WADENVIK H, KUTTI J, RISBERG B: Endothelial cell seeding reduces thrombogenicity of Dacron grafts in humans. J Vasc Surg 11(3): 403-10, 1990.

PASQUINELLI, G., FREYRIE, A., PREDA, P., CURTI, T., D'ADDATO, M., LASCHI, R: Healing of prosthetic arterial grafts. Scanning Microsc 4(2): 351-62, 1990.

PRONK A, HOYNCK VAN PAPENDRECHT AA, LEGUIT P, VERBRUGH HA, VERKOOYEN RP, VAN VROONHOVEN TJ: Mesothelial cell adherence to vascular prostheses and their subsequent growth in vitro. Cell Transplant 3(1): 41-48, 1994.

PUNCHARD MA, STENSON-COX C, O'CEARBHAILL ED, LYONS E, GUNDY S, MURPHY L, PANDIT A, McHUGH PE, BARRON V: Endothelial cell response to biomechanical forces under simulated vascular loading conditions. J Biomech 40(14): 3146-54, 2007.

RÉMY M, BAREILLE R, RERAT V, BOURGET C, MARCHAND-BRYNAERT J, BORDENAVE L: Polyethylene terephthalate membrane grafted with peptidomimetics: endothelial cell compatibility and retention under shear stress. J Biomater Sci Polym Ed 24(3): 269-86, 2013.

RÉMY-ZOLGHADRI M, LAGANIÈRE J, OLIGNY JF, GERMAIN L, AUGER FA.: Endothelium properties of a tissue-engineered blood vessel for small-diameter vascular reconstruction. J Vasc Surg 39: 613-20, 2004.

RIEDEL T, BRYNDA E, DYR JE, HOUSKA M: Controlled preparation of thin fibrin films immobilized at solid surfaces. J Biomed Mater Res A 88(2): 437-47, 2009.

ROLL S, MÜLLER-NORDHORN J, KEIL T, SCHOLZ H, EIDT D, GREINER W, WILLICH SN: Dacron vs. PTFE as bypass materials in peripheral vascular surgery - systematic review and meta-analysis. BMC Surg 19: 8-22, 2008.

SALACINSKI HJ, TIWARI A, HAMILTON G, SEIFALIAN AM. Cellular engineering of vascular bypass grafts: role of chemical coatings for enhancing endothelial cell attachment. Med Biol Eng Comput 39(6): 609-18, 2001.

SARKAR S, SALACINSKI HJ, HAMILTON G, SEIFALIAN AM: The mechanical properties of infrainguinal vascular bypass grafts: their role in influencing patency. Eur J Vasc Endovasc Surg 31(6): 627-36, 2006.

SEEGER JM, KLINGMAN N: Improved in vivo endothelialization of prosthetic grafts by surface modification with fibronectin. J Vasc Surg 8(4): 476-82, 1988.

SENTISSI JM, RAMBERG K, O'DONNELL TF JR, CONNOLLY RJ, CALLOW AD: The effect of flow on vascular endothelial cells grown in tissue culture on polytetrafluoroethylene grafts. Surgery 99(3): 337-43, 1986.

SREEREKHA PR, KRISHNAN LK: Cultivation of endothelial progenitor cells on fibrin matrix and layering on dacron/polytetrafluoroethylene vascular grafts. Artif Organs 30(4): 242-49, 2006.

TAKADA Y, YE X, SIMON S: The integrins. Genome Biol 8(5): 215.1-9, 2007.

TUNSTALL A, EBERHART RC, PRAGER MD: Endothelial cells on Dacron vascular prostheses: adherence, growth, and susceptibility to neutrophils. J Biomed Mater Res 29(10): 1193-9, 1995.

VARA DS, SALACINSKI HJ, KANNAN RY, BORDENAVE L, HAMILTON G, SEIFALIAN AM: Cardiovascular tissue engineering: state of the art. Pathol Biol (Paris). 53(10): 599-612, 2005.

VARGA M, MATIA I, LODEREROVA A, ADAMEC M: The minimal dose of FK506 is sufficient to facilitate the arterialisation of venous allografts in rats. European Surgery 45(1): 8-14, 2013.

VOHRA R, THOMSON GJ, CARR HM, SHARMA H, WALKER MG: The response of rapidly formed adult human endothelial-cell monolayers to shear stress of flow: a comparison of fibronectin-coated Teflon and gelatin-impregnated Dacron grafts. Surgery 111(2): 210-20, 1992.

WILLIAMS SK, KLEINERT LB, PATULA-STEINBRENNER V: Accelerated neovascularization and endothelialization of vascular grafts promoted by covalently bound laminin type 1. J Biomed Mater Res A 99(1): 67-73, 2011.

WONG CS, SGARIOTO M, OWIDA AA, YANG W, ROSENFELDT FL, MORSI YS: Polyethyleneterephthalate provides superior retention of endothelial cells during shear stress compared to polytetrafluoroethylene and pericardium. Heart Lung Circ 15(6): 371-7, 2006.

ZILLA P, BEZUIDENHOUT D, HUMAN P: Prosthetic vascular grafts: wrong models, wrong questions and no healing. Biomaterials 28(34): 5009-27, 2007.

Figures legends

Fig. 1A. Knitted crimped polyethylene-terephthalate (PET) vascular prostheses (6 mm) with commercial bovine type I collagen impregnation (VÚP Joint-Stock Comp., Brno, Czech Republic), bar = 1 cm. **B.** Ultrastructure of the textile knitted fashion of the prosthesis, scanning electron microscopy (NOVA nanoSEM 200, FEI, USA), magnification 400 × and bar = 300 μm (B).

Fig. 2. Hemodynamic bioreactor consisting of a circuit of silicon tubing (A), a peristaltic pump (B), a water container with a heater (C), a static control prosthesis that is located in a blind branch (D), a dynamic tested prosthesis that is exposed to flow (E) and a flow-meter (F) (Inserm U577, Université Victor Segalen, Bordeaux, France).

Fig. 3A. Adherence of human saphenous vein endothelial cells to PET vascular prostheses 4 h after seeding 1.50×10^5 cells/cm^2. The percentage of initial seeding density is shown at the bottom of each column. **B.** Flow-resistance of human saphenous vein endothelial cells on PET vascular prostheses. The laminar shear stress was 15 dyn/cm^2. The statistical differences are given versus the corresponding static control (= 100%), which was left without shearing (white columns). Data are presented as mean ± standard deviation (SD), number of measurements n = 4-16. The experiments on PET/Co and PET/Co +Fb (40 min) were run in duplicate. Polyethylene terephthalate (PET), collagen type I impregnation (Co), laminin (LM), fibrin gel (Fb), fibronectin (FN), standard deviation (SD), non-significant (n.s.).

Fig. 4. Morphology of adhering human saphenous vein endothelial cells on PET vascular prostheses 4 h after seeding 1.50×10^5 cells/cm^2 (A-D) and 72 h after seeding (E-H). Staining with the Live/Dead Viability/Cytotoxicity Kit (Molecular probes), viable cells are in green. Olympus IX50 fluorescence microscope, Olympus DP70 digital camera, magnification 10 ×, scale bar 200 μm. Polyethylene terephthalate (PET), collagen type I impregnation (Co), laminin (LM), fibrin gel (Fb), fibronectin (FN).

Fig. 5. The morphology of human saphenous vein endothelial cells on PET/Co (A and D), PET/Co +LM (B and E) and PET/Co +Fb (C and F) vascular prostheses 72 h after seeding in static conditions (A-C) and after subsequent application of 120 min of flow (D-F). The flow direction is indicated by the arrow (D-F). Immunofluorescence of β-actin (green) (A and C, D-F) and of von Willebrand factor (green) (B) with cell nuclei counterstained with propidium iodide (red) (A-F). Leica TCS SP2 AOBS confocal microscope, magnification 10 ×, zoom 2 ×, scale bar 300 μm (A and D), magnification 10 ×, scale bar 200 μm (B and C, E and F). Polyethylene terephthalate (PET), collagen type I impregnation (Co), laminin (LM), fibrin gel (Fb).

Fig. 1

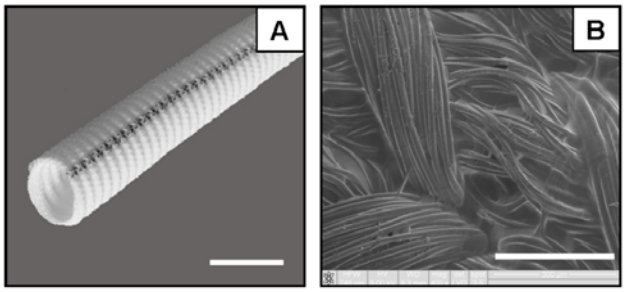

Fig. 2

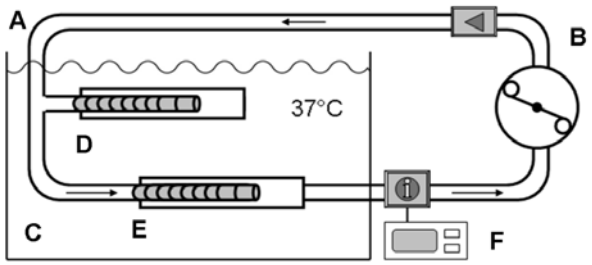

Fig. 3

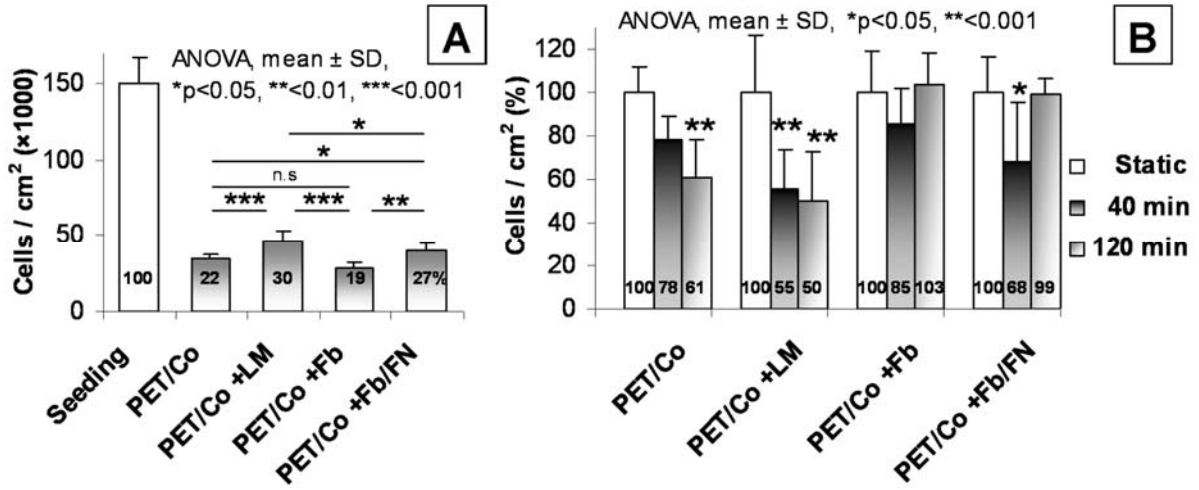
A
ANOVA, mean ± SD,
*p<0.05, **<0.01, ***<0.001
Cells / cm² (×1000)
150
100
50
0
n.s
*
*
***
***
**
100
22
30
19
27%
Seeding
PET/Co
PET/Co +LM
PET/Co +Fb
PET/Co +Fb/FN
B
ANOVA, mean ± SD, *p<0.05, **<0.001
Cells / cm² (%)
120
100
80
60
40
20
0
**
**
**
*
100 78 61
100 55 50
100 85 103
100 68 99
Static
40 min
120 min
PET/Co
PET/Co +LM
PET/Co +Fb
PET/Co +Fb/FN

Fig. 4

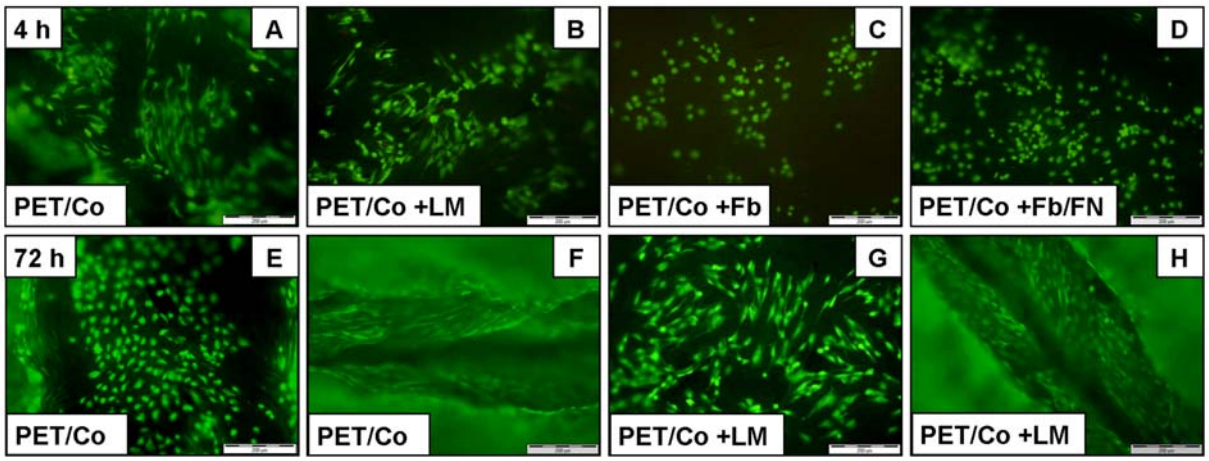
4 h
A
PET/Co
B
PET/Co +LM
C
PET/Co +Fb
D
PET/Co +Fb/FN
72 h
E
PET/Co
F
PET/Co
G
PET/Co +LM
H
PET/Co +LM

Fig. 5