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Endothelial Cell Lining of PET Vascular Prostheses: Modification with Degradable Polyester-based Copolymers and Adhesive Protein Multi-layers

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Abstract

Background: Bypass surgery for atherosclerosis is confronted with the absence of endothelial cells in the lumen of vascular prosthesis in humans. This imposes a risk of thrombosis. New biomaterials try to minimize surface thrombogenicity.

Methods: Knitted polyethylene terephthalate (PET) vascular graft patches were impregnated with degradable polyester polymers: poly (L-lactide-co-glycolide) (PLG) or poly (L-lactide-co-glycolide-co-ε-caprolactone) (PLGC). The luminal surface was coated with collagen type I (Co) to which extracellular matrix proteins laminin (LM), fibronectin (FN), or surface fibrin gel (Fb) were attached. Three types of prostheses (PET, PET–PLG and PET–PLGC) and 5 types of protein assemblies (+Co, +Co/LM, +Co/FN, +Co/Fb, +Co/Fb/FN) were fabricated. Scanning electron microscopy and measurements of the water contact angles were performed. The development of a bovine endothelial cell layer was studied in a static culture for 1 week.

Results: The cells reached confluence on all PET surfaces with the highest final density on +Co/FN. Impregnation of PET with polymers made it less adhesive for cells in the following order: PET > PET–PLG > PET–PLGC. However, additional coating with the protein assemblies enhanced the endothelial cell growth, especially on fibrin-containing surfaces.

Conclusion: Tri-component vascular grafts composed of PET, copolymers and cell-adhesive assemblies were fabricated. The endothelial lining on the polymer-coated grafts was promoted after modification with the protein multilayers.

Artificial vascular prostheses have been made of non-degradable, non-compliant and thrombogenic materials for more than 50 years. Thus, they resemble passive tubing. Potential bio-activation by degradable materials and by introduction of living endothelial cells may approximate these materials to native artery. This work provided a method to include bio-degradable polymers into vascular graft and to facilitate the growth of cell lining via adhesive protein multilayers.

Keywords: Blood vessel prosthesis; Polyethylene terephthalate; Poly (L-lactide-co-glycolide); Endothelial cells; Fibrin; Collagen; Laminin; Fibronectin

Abbreviations

AT III: Antithrombin III; Co: Collagen; deg: Degree; EC: Endothelial Cells; ePTFE: Expanded Polytetrafluoroethylene; ECM: Extracellular Matrix; FBS: Fetal Blood Serum; Fb: Fibrini, Fbg: Fibrinogen; FN: Fibronectin; LM: Laminin; PBS: Phosphate-Buffered Saline; PGA: Poly Glycolid Acid; PLA, PLLA: Poly L-Lactic Acid; PLG: Poly L-Lactide-Co-Glycolide; PLGC: Poly L-Lactide-Co-Glycolide-Co-E-Caprolactone; PLLACL: Poly L-Lactide-Co-E-Caprolactone; PCL: Poly E-Caprolacton; PET: Polyethylene Terephthalate; SMC: Smooth Muscle Cells; SEM: Standard Error of Mean; SFE: Surface Free Energy; Thr: Thrombin; TCP: Tissue Culture Polystyrene; TB: Tris Buffer

Introduction

The main cause of mortality in developed countries is atherosclerotic vascular disease of heart and peripheral arteries. Surgical treatment involves bypassing occluded vessels with autologous arteries or veins, which unfortunately are often unsuitable or completely absent [1]. Synthetic vascular prostheses are an artificial alternative; however, the long-term patency of small caliber (<6 mm) bypass materials is discouraging [2,3]. Currently implanted synthetic prostheses are represented by the two materials: polyethylene terephthalate (PET, Dacron) and expanded polytetrafluoroethylene(ePTFE, Teflon, Gore-Tex). Complete full-wall healing is defined as the incorporation of the entire graft within a fibrous tissue matrix with capillary in-growth and the flow surface covered with neo-intimal Endothelial Cells (EC) and/or modified Smooth Muscle Cells (SMC) [4]. For perhaps as long as 20 years,

spontaneous coverage of synthetic grafts with endothelium, as previously described in animals, was assumed to occur in humans. However, Berger et al. [5] clearly demonstrated the failure of synthetic grafts to develop any endothelial lining in man. Healing in humans consists in outer wall fibrous tissue and luminal coverage with more or less compacted fibrin only. Incomplete healing of synthetic nonresorbable vascular prostheses imposes a risk of thrombotic occlusion, particularly in medium-diameter or small-diameter blood vessels. There are several approaches to achieving a non-thrombogenic surface, one of the most effective of which is artificial endothelial cell seeding prior to implantation [6].

This study is focused on endothelialization of knitted PET prostheses, which are currently produced by companies distributed over the whole world (e.g., VUP Joint-Stock Co., Brno, Czech Republic; Vascutek, a Terumo Company; LeMaitre Vascular, Inc.; Bio Nova International Pty Ltd, North Melbourne, Australia; Atrium, MaquetGetinge Group, Hudson, NH, U.S.A). However, the inner surface of knitted PET vascular graft is rough and irregular, with relatively large void spaces among the PET fibers, and thus not suitable for the adhesion, spreading and growth of endothelial cells, which prefer smoother surfaces. In addition, in its pristine unmodified state, PET is a highly hydrophobic polymer, having the sessile water drop contact angle more than 100°, which is not suitable for adhesion and growth of cells [7]. Therefore, for successful endothelialization, it is necessary to modify the knitted PET fabrics, e.g. by impregnation with other polymers and coating with bioactive molecules.

Impregnation of textile prostheses was proposed in 1960, the purpose being to make the prosthesis impervious and to avoid blood loss at the time of implantation. The most frequently used sealants were albumin, collagen and gelatin, and it was found that impregnation did not alter the infection, thrombosis or patency rates compared to the previous method of pre-clotting with patients' own blood, which was used at that time. However, after fulfilling the haemostatic role, the healing incorporation of the impregnated prosthesis was delayed for several weeks to months due to resorption of the matrix, depending on the type of protein and cross-linking agent. Moreover, the impregnated prostheses were not optimal substrata for direct endothelialization, because collagen impregnation is derived from fragmented biological tissue that is not created to optimize endothelial proliferation [8,9]. In addition, collagen as a component of bio-prostheses has often been cross-linked by glutaraldehyde and related agents, which are cytotoxic and may cause calcification of the grafts [10].

Therefore, in this study, we evaluated potential benefit of the impregnation of PET vascular grafts with synthetic polymers. This approach has been applied only rarely. For example, knitted Dacron vascular grafts were sealed with polyether-based urethane with carboxylic acid groups in order to reduce the blood permeability of the graft and to create "anchor" sites for protein attachment. However, these modified prostheses were not tested with cells; only the attachment of albumin was examined [11]. In another study, Dacron prostheses were impregnated with silicon. However, these grafts showed a higher permeability for blood and higher thrombogenicity compared to other clinically used vascular prostheses [12]. Impregnation of PET prostheses with a fluoropolymer solution (Fluoropassiv Graft, Vascutec Ltd.), and particularly polypyrrole with high electrical conductivity, even decreased the adhesion, growth, migration and viability of vascular endothelial cells [13,14].

Other promising candidates for impregnation of PET prostheses are absorbable aliphatic polyesters, namely poly (L-lactic acid) (PLA), poly (D,L-lactic acid) (PDLLA), poly (glycolic acid) (PGA), poly (εcaprolacton) (PCL) and their blends. Absorbable polymers act as temporary templates for the regeneration of vascular tissue. They elicit minimal chronic inflammatory foreign body reaction and they also meet two important but contradictory requirements: low bleeding permeability at the time of implantation and high cell-tissue permeability for better full wall healing. In bi-component vascular graft design, the non-absorbable fibers provide strength and the absorbable components are advantageous for tissue regeneration, mechanical properties and for reducing the foreign body reaction [15]. The first human use of a tissue-engineered vessel graft based on degradable polyesters was reported in 2001. The PCL-PLA copolymer reinforced with woven PGA and seeded with vascular cells was transplanted as a pulmonary artery in a 4-year-old girl with a cardiac defect [16].

In spite of these promising results, degradable polyesters have been still rarely used for improving endothelialization of PET vascular prostheses. Bicomponent vascular prostheses containing Dacron fabrics and PGA yarns showed good patency and no thrombus or aneurysma formation after implantation into dogs. Their luminal surface was lined with a layer of endothelial cells with myofibroblasts, fibroblasts and collagen underneath [15]. Similarly, when the inner surface of PET prostheses was coated with PLLA nanofibers, these grafts displayed a significantly better proliferation rate of endothelial cells and formation of endothelial cell monolayer than uncoated PET prostheses [9].

The degradation rate and mechanical properties are also important parameters of aliphatic polyesters designed for tissue engineering. PGA lost its strength in vivo within 4 weeks and was completely degraded through hydrolysis of its ester bonds by 6 months to glycolic acid which was, in turn, metabolized and eliminated as water and carbon dioxide [17]. The poly (L-lactide-co-glycolide) (PLG) underwent pronounced degradation at 30 weeks in vivo after implantation as a porous scaffold in rat skeletal muscle [18]. PCL is a flexible semi-crystalline linear aliphatic polyester with total degradation time 2-3 years. This timescale is likely to be too slow for tissue engineering applications, and PCL-based materials have therefore typically been co-polymerized or blended with other poly-ahydroxy acids. PGA and PLA are rigid and this limits their use in soft tissue engineering. However, copolymers of PCL with PGA or PLA are elastomeric materials that can be elongated and recover from extension under cyclic loading. In addition, the degradation rate of the copolymers can be controlled by adjusting the ratio of two or three components [19]. Moreover, scaffolds composed of polymers of that kind are capable of transferring mechanical signals to the seeded cells [20].

Degradable polymers can be expected to improve the mechanical properties of PET vascular prostheses. It is generally known that PET prostheses exhibit relatively good mechanical properties in terms of their resistance to the cyclic stresses generated by pulsatile blood flow [21]. However, the PET prostheses are much stiffer and less compliant compared to the native blood vessels and related tissues [22]. Dacron grafts coated with a polymeric biodegradable sealant, i.e. polyethylene oxide-polylactic acid-segmented copolymers, exhibited a highly flexible elastomer-like mechanical response in vitro [23]. A hybrid material with excellent mechanical and biological properties has been produced by electrospinning a co-solution of PET and collagen [24].

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PLG and its copolymer with PCL also seem to be suitable candidates for combination with PET, because these polymers showed relatively good mechanical properties for construction of scaffolds applicable in soft tissue engineering [25-27].

Although the use of absorbable polyester-based materials is welldocumented in tissue engineering, their surface modifications must often be adopted due to the lack of natural recognition sites for the cells and poor hydrophilicity, which is particularly apparent in poly (ecaprolactone and its blends [28-30]. Combinations of synthetic and natural substrates, such as ECM-derivatives, have been used in order to benefit from the advantages of each of the materials, i.e. to provide cells with a more natural ECM-like environment and to create a dense, robust, leak-free synthetic substrate with desired mechanical properties [31,32].

Thus, the objectives of our study were to fabricate a hybrid tricomponent vascular graft consisting of (1) a semi-finished PET prosthesis without a commercial collagen sealant, (2) impregnation with synthetic degradable polyester-based copolymers, namely Poly (L-Lactide-Co-Glycolide) (PLG) or Poly (L-Lactide-Co-Glycolide-Co-E-Caprolactone) (PLGC) to prevent possible blood leaks and to provide tissue in-growth upon possible implantation, and (3) luminal coating with five different assemblies of ECM proteins (collagen, laminin and fibronectin) and fibrin gel to introduce cell-recognition sites and facilitate cell-biomaterial interactions. The proliferation rate of bovine pulmonary artery endothelial cells was explored to evaluate a potential applicability of those constructs for tissue engineering of vascular grafts.

Methods

Impregnation of vascular prostheses with copolymers

A copolymer Poly (L-Lactide-Co-Glycolide) (PLG), with a molar ratio of L-lactide to glycolide 85:15 (molecular masses: Mn=105 kDa, Mw=220 kDa), and a terpolymer poly (L-lactide-co-glycolide-co- ε -caprolactone) (PLGC), with a molar ratio of L-lactide to glycolide to ε -caprolactone 70:10:20 (molecular masses: Mn=91 kDa, Mw=173 kDa) were synthesized at the Centre of Polymer and Carbon Materials (Polish Academy of Sciences, Zabrze, Poland), according to a method described elsewhere [33,34]. PLG and PLGC were dissolved in methylene chloride (POCh, Gliwice, Poland) at a concentration of 5% (w/v). The detailed characteristics of PLG and PLGC processed in the form of foils by solvent casting can be found elsewhere [30,35]. These characteristics included: composition, contact angles, Surface Free Energies (SFEs), surface topography and average roughness (R_a) as well as mechanical properties in a tensile test.

Planar patches 1.6 cm in diameter were punched from a tubular non-crimped PET vascular prosthesis (VÚP, Joint–Stock Comp., Brno, Czech Republic). This prosthesis was a semi-finished product without commercial collagen impregnation. A PLG or PLGC solution 150 μ l in volume was poured on to the luminal surface of each sample, and the materials were air-dried overnight, followed by vacuum drying for the next 72 h. The microstructure of the prosthesis was studied with a scanning electron microscope (Nova NanoSEM 200, FEI, USA), accelerating voltage 5 or 10 kV, magnifications: 400× and 2000×, vacuum 60 Pa. On these samples, there was no coating with a conductive layer (Figure 1).

Modification of vascular prostheses with protein assemblies

The control PET samples and the PLG- or PLGC-impregnated samples were coated with 300 µl of collagen type I (rat tail, BIOSCIENCE) solution in 0.02 M acetic acid (75 µg/ml), exposed to ammonia vapors for 5 min, rinsed with water and then with Phosphate Buffered Saline (PBS). Fibronectin (human plasma, ROCHE) and laminin (Engelbreth-Holm-Swarm murine sarcoma basement membrane, SIGMA) were attached to the collagen-coated surface by 3-hour incubation with FN and LM solutions in PBS at concentrations of 50 µg/ml and 40 µg/ml, respectively. The surface-attached fibrin gel was prepared by rinsing the collagen-coated samples with Tris buffer (TB) followed by rinsing successively with solutions of fibrinogen (Fbg, human plasma, SIGMA, 50 µg/ml TB) for 60 min, TB, Thrombin (Thr, human plasma, SIGMA, 2.5 NIH U/ml) for 10 min, TB, Fbg (200 µg/ml) containing antithrombin III (AT III 0.5 U/ml) for 2 h, TB and PBS. A fibrin network was formed on the surface by catalytic action of the surface-attached thrombin on the ambient Fbg solution. FN was attached to the Fb network by incubating the sample with FN solution (50 µg/ml) in PBS overnight. The composition of the samples is summarized in Table 1.

1.	2.	3.	4.	5.	6.
PET	+Co	+Co/LM	+Co/FN	+Co/Fb	+Co/Fb/FN
PET-PLG	+Co	+Co/LM	+Co/FN	+Co/Fb	+Co/Fb/FN
PET-PLGC	+Co	+Co/LM	+Co/FN	+Co/Fb	+Co/Fb/FN

Table 1: Samples of vascular prostheses coated with extracellular matrix proteins and fibrin. Polyethylene Terephthalate (PET), Poly (L-Lactide-Co-Glycolide) (PLG), Poly (L-Lactide-Co-Glycolide-Co-E-Caprolactone) (PLGC), Collagen Type I (Co), Laminin (LM), Fibronectin (FN) and Fibrin Gel (Fb).

Measuring the contact angles

The surface wettability, i.e. an important parameter influencing the cell adhesion and growth, was characterized by measuring the sessile drop contact angles using the Laplace-Young method (OCA 20 Dataphysics, Germany). Millipore Q water droplets 2 μ l in volume were put on the surface, and the average contact angle values were obtained by 3× measuring the contact angles of individual droplets.

Endothelial cell culture

The constructs were UV sterilized for 30 min, inserted into tissue culture 24-well-plates (TPP, Switzerland) and seeded with EC of passage 20-22, derived from bovine pulmonary artery (line CPAE ATCC CCL-209, Rockville, MA, USA) at a density of 5×10⁴ cells/cm². The cells were cultured at 37°C and 5% CO₂ for 7 days, and the culture media, as recommended by the supplier of the CPAE line, consisted of 1.5 ml of Minimum Essential Eagle Medium supplemented with 2 mM L-glutamin, Earle's BSS with 1.5 g/l sodium bicarbonate, 0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate (all chemicals from SIGMA, USA) and 20% of fetal bovine serum (FBS). Aprotinin (200 IU/ml, CARL ROTH, Germany) was added to the medium on Fbcontaining assemblies to inhibit the fibrinolytic activity of EC. On culture days 1, 4 and 7, the samples were washed with PBS and the cells were detached from the support using 4 µl of trypsin-EDTA solution (SIGMA, USA) working for 15 min at 37°C. After neutralization with 800 µl of MEM + 20% FBS, the suspended cells were counted using an automatic cell counter (Vi-CELL XR 2.03, BECKMAN COULTER, Inc., Miami, FL, USA), and the corresponding cell densities were plotted against the culture time. The automatic counting of cells in the Vi-CELL analyser includes trypanblue exclusion test. The principle of this test is staining cells with trypan blue, which penetrates the membrane of damages or dead cells only. In our experiments, the cell viability was very high, reaching 95-99%, and was similar for all tested samples. The percentage of dead cells is in the range of physiological cell loss observed in cell populations [36]. Only viable cells were used for the evaluation of the cell adhesion and growth on the tested samples.

Some of the samples were fixed with 80% methanol for 10 min and washed in PBS. The cells on PET samples were stained with mouse monoclonal anti-βactin primary antibody (clone AC-15, SIGMA, Cat. No. A5441; incubation overnight at 4°C). After rinsing in PBS, the samples were incubated with goat anti-mouse IgG (H+L) secondary antibody, F(ab')2 fragment, conjugated with Alexa Fluor 488 (2 mg/ml, INVITROGEN, Cat. No. A11017; incubation for 45 min at room temperature). Both antibodies were diluted in PBS at a ratio of 1:400. The cell nuclei were counterstained with propidium iodide (5 µg/ml, SIGMA, Cat. No. P4170). The cells were evaluated in a Leica TCS SP2 AOBS confocal microscope, z-series of 50 images with a zstep of 1 μ m, at magnification 40× and zoom 2×. In addition, the cells on the PET-PLG and PET-PLGC samples were stained with Texas Red C2-maleimide (1:500, 10 ng/ml, INVITROGEN) and Hoechst 33342 (5 µg/ml, SIGMA) fluorescence dyes to visualize the membrane and cytoplasmic proteins and the cell nuclei, respectively. Images were captured with a Leica SPE DM 2500 confocal microscope with a z-

series of 30 images and a z-step of 1 μm at magnification 40× and zoom 2×.

Statistical analysis

For each analysis, three independent experiments have been performed, and within each experiment, 3 samples were used for each experimental group and time interval. On each sample, 3-6 measurements were performed. The quantitative data was then expressed as mean and standard error of mean (SEM). The data was compared using one way ANOVA analysis (Holm-Sidak test) for multiple comparison (SigmaStat 3.1 2004, SYSTAT Software, Inc.). Statistical significance was considered at $p \le 0.05$.

Results

Scanning electron microscopy

The scanning electron microscopy images of various types of dried vascular prostheses are shown in Figure 1. Niches could be seen between the individual fibers in the knitted textile structure of the PET (Figure 1A and F); however, commercial impregnation with type I collagen (Figure 1B and G) had a tendency to span these interstices (this type of prosthesis was not subject to cell culture). A similar effect was achieved by impregnation of PET with PLG (Figure 1C and H) or PLGC (Figure 1D and I). Coating PET with Co/Fb gave an even greater impression of surface-smoothing (Figure 1E and J).



Figure 1: Scanning electron microscopy of modified vascular prostheses. Bare polyethylene terephthalate prosthesis (PET) (A and F); commercial collagen-impregnated PET prosthesis (B and G); PET impregnated with poly (L-lactide-co-glycolide) (PLG) (C and H); PET impregnated with poly (L-lactide-co-glycolide-co- ϵ -caprolactone) (PLGC) (D and I); PET coated with collagen type I (Co) and fibrin gel (Fb) (E and J). NOVA nanoSEM 200, FEI, USA, magnification 400× and bar=300 µm (A-E), 2000× and 50 µm (F-J).

Measuring the contact angles

The contact angles values (θ) on PET and PET–PLG vascular prostheses are presented in Table 2. Modification of hydrophobic PET (θ ~126 deg) with protein assemblies led to a mild decrease in contact angles i.e. to increased wettability, significantly on PET+Co/FN (θ ~106 deg, p<0.001). The cell population, in turn, reached the highest density on that surface on day 7, as shown further.

Impregnation of PET with PLG resulted in a significant drop (p<0.001) in the contact angle (θ ~96 deg); however, the endothelial cell lining was somewhat inferior. In contrast to PET, the modification of PET–PLG with the protein assemblies was followed by a significant rise (p<0.001) in the contact angles on all multi-component protein assemblies, with the exception of mono-component PET–PLG+Co. Interestingly, this change was in turn followed by moderate promotion of cell growth, as shown below. These measurements were not

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	1. PET	2. +Co	3. +Co/LM	4. +Co/FN	5. +Co/Fb	6. +Co/Fb/FN
Contact angle (deg)	126 ± 4	123 ± 2	114 ± 0	106 ± 5	112 ± 3	117 ± 11
ANOVA, p ≤ 0.05	vs. 4, 7	vs. 4, 8		vs. 1, 2, 10		
	7. PET-	8. +Co	9.	10. Co/FN	11.	12.
	PLG		+Co/LM		+Co/Fb	+Co/Fb/FN
Contact angle (deg)	PLG 98 ± 8	90 ± 9	+Co/LM	140 ± 9	+Co/Fb 116 ±5	+Co/Fb/FN 125 ± 10

performed on PET-PLGC; however, we assume that the surface wettability would have a pattern similar to PET-PLG [29].

Table 2: Water contact angles of the PET and the PET-PLG vascularprosthesesmodifiedwithcell-adhesiveproteinsassemblies.Polyethyleneterephthalate (PET), poly (L-lactide-co-glycolide) (PLG),collagentype I (Co), laminin (LM), fibronectin (FN), fibrin gel (Fb),versus(vs.).Mean ±SEM from 3 independent experiments, eachperformed in triplicates.ANOVA

Endothelial cell culture - comparison of coatings with protein assemblies

The cell population densities on different vascular grafts coated with protein multilayers including statistical significances are shown in Figure 2. Interestingly, no significant differences were detected among the cell densities on either type of prosthesis with various protein assemblies on day 1 and on PET–PLG and PET–PLGC on day 4 (Figure 2A-2C).

The highest density on PET was on +Co and the lowest density was on +Co/Fb on day 4. The highest final population was reached on +Co/FN on day 7. Interestingly, none of the remaining protein multilayers was better for cell growth compared to pure PET (Figure 2A). On the other hand, the cell populations on protein-modified PET-PLG and PET-PLGC on day 7 tended to be higher than on bare polymer surfaces, however, this was significant again only on +Co/FN (Figure 2B and 2C).

For acceleration of growth significant increment in cell number was observed on all of the PET surfaces between days 1 and 4 and between days 4 and 7 (Figure 2A). The cell behavior on PET–PLG showed significant increase in the population density on +Co/LM and Co/FN between days 1 and 4 and on all of the protein-coated surfaces between days 4 and 7 except for +Co/Fb where cells increased their number between days 1 and 7 (Figure 2B). In the case of PET–PLGC, the cell growth was significantly improved on the +Co and +Co/LM surfaces between days 1 and 4 and on the +Co/FN and Co/Fb between days 4 and 7. No increase in cell number was detected on pure PET–PLGC (Figure 2C).



Figure 2: Population densities of bovine endothelial cells of the CPAE line on PET (A), PET–PLG (B) and PET–PLGC (C) vascular prostheses on Day 1 (D1), Day 3 (D3) and Day 7 (D7) after seeding. The seeding density was 5×10^4 cells/cm². Mean \pm SEM from 3 independent experiments. Within each experiment, 3 samples for each experimental group and time interval were used. ANOVA, Holm-Sidak test. Statistical significance (*p \leq 0.05) is indicated by numbers of experimental groups above the columns. Polyethylene Terephthalate (PET), Poly (L-Lactide-Co-Glycolide) (PLG), Poly (L-Lactide-Co-Glycolide) (PLG), Collagen type I (Co), Day (D), Laminin (LM), Fibronectin (FN), Fibrin gel (Fb), versus (vs.), Non-Siginificant (n.s.).

Endothelial cell culture - comparison of polymer impregnations

The growth curves of bovine EC on the tested surfaces including statistical significances are illustrated in Figure 3. The cell densities were compared on three vascular graft types (PET, PET–PLG and PET–PLGC) modified with the same protein assemblies and on identical culture days.

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Figure 3: Growth curves of bovine endothelial cells of the CPAE line on PET, PET–PLG and PET–PLGC vascular prostheses. Cell densities were compared on various vascular grafts with the same assembly of proteins and on identical culture days. Seeding density was 5×10^4 cells/cm². Mean \pm SEM from 3 independent experiments. Within each experiment, 3 samples for each experimental group and time interval were used. ANOVA, Holm-Sidak test. Statistical significances (i.e., $p \le 0.05$) highlighted with *asterisks were positive versus PET, #PET–PLG versus PET–PLGC. Polyethylene Terephthalate (PET), Poly (L-Lactide-Co-Glycolide) (PLG), Poly (L-Lactide-Co-Glycolide-Co-E-Caprolactone) (PLGC), Collagen type I (Co), Laminin (LM), Fibronectin (FN) and Fibrin gel (Fb).

On grafts without proteins, the cell densities were similar on all three graft types on day 1; however, significantly lower cell population density was observed on PET-PLG and PET-PLGC on days 4 and 7 (Figure 3A). Similarly, PET-PLGC+Co was significantly less populated on day 1 than PET+Co (Figure 3B). Moreover, PET-PLG and PET-PLGC +Co and +Co/FN showed significantly lower cell densities on day 4 and +Co/LM on day 7 compared to corresponding protein-coated PET (Figure 3B-D). Comparing PET-PLG to PET-PLGC, lower cell number was observed on PET-PLGC+Co and +Co/Fb on day 1 (Figure 3B and E). No more significant differences were found in the cell densities among the constructs coated with +Co and Co/FN on day 7. This fact suggests delayed acceleration in the cell growth on PET-PLG and PET-PLGC modified with +Co or Co/FN, which compensated for a lower initial cell density on day 1 and 4. However, this was not the case for the fibrin-containing surfaces (PET-PLG+Co/Fb, +Co/Fb/FN and PET-PLGC+Co/Fb, +Co/Fb/FN), where no significant variances in cell populations were observed on any of the prosthesis types and on any of the culture days when compared to corresponding PET surfaces (Figure 3E and 3F). This observation indicated a unique role of including fibrin in the design of cell-adhesive protein assemblies.

The morphology of bovine endothelial cells cultured on different vascular prostheses on day 7 is depicted in Figure 4. Cells seeded on all of the PET samples almost reached confluence (Figure 4A-F). The cells on unmodified PET-PLG and PET-PLGC were distributed more sparsely and were spindle-shaped, oriented along the fibers in the PET fabrics (Figure 4G and M). However, the cells cultured on PET-PLG and PET-PLGC modified with protein assemblies showed a more favorable cobblestone-like appearance and grew to sub-confluence

(Figure 4H-L, 4N-R), especially on fibrin-containing surfaces (Figure 4K, L, Q, R). Moreover, the cells displayed a well-developed β -actin cytoskeleton (Figure 4A-F) (data for PET–PLG and PET–PLGC coated with the protein assemblies not shown). Thick actin cables have been considered as a marker of endothelial cell phenotypic maturation [37].



Figure 4: Morphology of bovine endothelial cells of the CPAE line seeded on PET vascular prostheses, PLG– and PLGC–impregnated PET vascular prostheses and further modified with cell-adhesive protein assemblies. Day 7 after seeding 5×10^4 cells/cm². A-F: immunofluorescence of β -actin (green), cell nuclei counterstained with propidium iodide (red). G-R: staining of the cell membrane and cytoplasm with Texas Red C2-maleimide (red), cell nuclei counterstained with Hoechst #33342 (blue). Leica TCS SP2 AOBS confocal microscope (A-F), z-series of 50 images with a z-step of 1 µm, magnification 40×, zoom 2×, bar=20 µm. Polyethylene Terephthalate (PET), Poly (L-Lactide-Co-Glycolide) (PLG), Poly (L-Lactide-Co-Glycolide-Co-E-Caprolactone) (PLGC), Collagen type I (Co), Laminin (LM), Fibronectin (FN) and Fibrin gel (Fb).

Discussion

We have found that the impregnation with PLG or PLGC did not improve significantly the adhesion and subsequent growth of endothelial cells, measured by the cell population densities on days 1, 4 and 7. The endothelial cell lining was improved only after coating the pure and impregnated prostheses with protein assemblies and after 7day-cultivation. On day 7 after seeding, the highest cell number was achieved on the assemblies containing fibronectin (Co/FN). The most favorable cell morphology, i.e. the most pronounced cobblestone-like shape of endothelial cells, was achieved on fibrin-containing coating (Co/Fb, Co/Fb/FN) on PLGC-impregnated prostheses.

However, some other authors have found a positive effect of degradable synthetic polymers on endothelialization of PET prostheses. A prototype of a compound vascular prosthesis similar to that in our study was described by Perego et al. [38]. They synthesized a copolymer of L-lactide and ε -caprolactone (PLLACL), which ensures for biocompatibility and an external Dacron layer was applied for mechanical reinforcement. Surface activation for cell adhesion occurred by hydrolytic etching (NaOH/HCl) and argon/oxygen/air plasma treatment. Human microvascular EC do not adhere to untreated copolymer but form an almost confluent monolayer after activation of PLLACL patches as well as in tubular grafts [38]. Similarly, in our study, the endothelial cell growth was improved after functionalization of PLG and PLGC with cell-adhesive protein

multilayers. In another study, the proliferation rate of bovine aortic EC was compared on commercial textile PET prostheses and on prostheses where industrial Co impregnation was experimentally replaced by an air-spun poly (L-lactic acid) (PLLA) nanofiber mesh [9]. The authors found increased cell proliferation on commercial grafts until day 3; however, from day 5 to day 15 the cell growth was significantly better on the PET-PLLA. They declare that PET sprayed with PLLA provides a nano-featured and more suitable 2-D environment for EC spreading than 3-D commercial PET, which induces cell clustering within the textile matrix [9]. It is known that nanostructured substrates promote the adhesion and growth of cells by supporting physiological conformation of the cell adhesionmediating molecules (e.g., fibronectin, vitronectin), which are spontaneously adsorbed to these substrates from biological fluids including cell culture media [7]. Similarly, modification of PET vascular prostheses with PGA yarns promoted the formation of a layer of endothelial cells in vivo after implantation into dogs [15]. However, our impregnation with PLG and PLGC was rather amorphous and structure less, and thus its effect on the endothelial cell adhesion and growth was less pronounced. Only after deposition of cell-adhesive protein assemblies, the impregnated prostheses became more suitable for endothelialization.

However, not all protein coatings always improved the endothelialization of PET vascular prostheses. Tunstall et al. [39] reported less efficient adherence and growth of Human Umbilical Vein EC (HUVEC) on serum-pretreated PET than on Tissue Culture Polystyrene (TCPS) when normalized for unit surface area. The effective surface area of a knitted PET vascular prosthesis is about 6 times greater than that of smooth surfaces (e.g. PET film or TCPS) due to the interstices present between the fibers (Figure 1A) [39]. In our work, pre-coating PET prosthesis with Co before the attachment of LM, FN, or Fb probably decreased the surface area accessible for cells by reducing the interstices dimensions. However, the pre-coating provided cues for cell adhesion that might have compensated this effect since there were no significant differences in cell growth between pure PET and most of the protein-coated PET (Figure 2A). Additional smoothing of the surface was probably reached by the fibrin gel coating (Figure 1E and 1J). The decrease in the effective surface area probably contributed to moderate decrease in number of growing cells e.g. on PET+Co/Fb on day 4 (Figure 2A). The same effect could contribute to lowering cell numbers on PET impregnated with PLG or PLGC. On the other hand, cells plated on smoother surfaces of PET-PLG and PET-PLGC modified with protein assemblies would most probably have increased their density if the cell culture had extended > 1 week, as they were still sub-confluent on day 7 (Figure 4). Most importantly, subsequent functions of EC on vascular graft such as strength of adhesion and resistance to flow may be enhanced when the cells are seeded on ECM components [40].

Bérard et al. [41] tested the lining of knitted PET prostheses commercially impregnated with type I and type III bovine collagen (i.e. unmodified) with human umbilical cord blood progenitor-derived EC in serum-free conditions. The cells adhere, are maintained, but do not proliferate until day 9. Nevertheless, the application of flow for 6 h restores the endothelial monolayer [41]. In our study, the bovine EC significantly increased in number on both pure PET and proteinmodified PET surfaces during 7 day culture even under static conditions. This discrepancy can be explained by the different cell type, species, seeding density and serum-containing media used in our study. On the other hand, bare knitted PET (without cross-linked sealant), as used in our experiment, would be permeable for blood and

Interestingly, our protein assemblies improved the growth and spreading of endothelial cells, although they were relatively highly hydrophobic (water drop contact angle usually more than 100°). It is generally known that the adhesion and growth of cells is optimum on moderately hydrophilic surfaces. On these surfaces, the cell adhesionmediating molecules are adsorbed in and active, flexible, almost physiological conformation, which enable the binding of specific sited in these molecules (e.g. amino acid sequences such as RGD) to adhesion receptors on the cell membrane. On highly hydrophobic surfaces, the proteins are adsorbed in denatured and rigid conformation, which limits the accessibility of the specific sites by cell adhesion receptors. On the other hand, the material hydrophobicity can be compensated, at least partly, by nanoscale roughness of the material surface [7]. In our study, the good adhesion and growth of endothelial cells on protein-coated hydrophobic surfaces could be explained by controlled attachment of cell adhesion-mediating proteins, which preserved their physiological conformation and accessibility for cell adhesion receptors.

The advantage of combining a synthetic bioresorbable polymer with an ECM protein coating for EC attachment and functioning was demonstrated by several authors [42-45]. Improved endothelial cell lining of PET prosthesis modified with ECM proteins was also reported [46-49]. This supports our concept that bi-component vascular grafts are a prerequisite for sufficient coverage and function of EC seeded onto the lumen.

Tri-component grafts were prepared in our study by adding celladhesive protein assemblies onto a PET graft impregnated with degradable polymers. Gradual resorption of the polymers would theoretically promote trans-mural graft healing [45]. The mismatch in visco-elastic properties between the native artery and the rigid Dacron graft contributes to intimal hyperplasia and graft failure. Integrating synthetic and natural degradable polymers into the vascular graft wall might endow the novel grafts with more appropriate tensile and elastic mechanical properties [23,24,43].

The number of EC on day 7 on PET, PET-PLG or PET-PLGC was highest when seeded on Co/FN matrix (Figure 2). Interestingly, Tjia et al. [42] showed that pretreatment of PLG substrate with Co type I together with cell secreted FN concertedly induces the migration of human keratinocytes to an extent much greater than in the case of FN or Co pretreatment only. They conclude that the roles of ECM proteins are not cross-functional, and that Co-FN interactions may present FN in a suitable conformation, thus promoting cell adhesion and migration [42]. This supports the idea that composite ECM protein assemblies may be better substrata for adhesion, proliferation and retention of cells seeded on vascular grafts [48]. It should be noted that we tested various cell-adhesive protein assemblies in our study and, in addition, EC were seeded in a culture medium containing a serum which can enhance the cell adherence by about 10% however, the differential effect of pre-coated ECM proteins on cell adhesion is still apparent in an experimental setting of this kind [46].

Degradable polymers, including PLG and PLGC, which were used in our study, have recently been evaluated in terms of cytocompatibility towards mesenchymal cells (murine fibroblasts L929 and human osteoblasts MG-63) and immune cells (murine macrophages RAW 264.7). PLG proves to be biocompatible, but PLGC Citation: Chlupac J, Filova E, Riedel T, Brynda E, Pamula E, et al. (2014) Endothelial Cell Lining of PET Vascular Prostheses: Modification with Degradable Polyester-based Copolymers and Adhesive Protein Multi-layers. J Tissue Sci Eng 5: 139. doi:10.4172/2157-7552.1000139

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reduces the adhesion of all cell types and enhances the activation of macrophages. This undesired effect is attributed to the presence of ε -caprolactone units that endow the polymers with low polarity and higher deformability [30,35]. In our present study, PET-PLG and – PLGC were seeded with bovine EC and, consistently with the previous tests, the lowest EC proliferation occurred on PET-PLGC (Figure 2C) even when modified with ECM proteins (Figure 3B and 3E). However, cell growth and morphology on fibrin-containing surfaces PET-PLGC +Co/Fb and +Co/Fb/FN were not that compromised in comparison with other prostheses (Figures 3E, 3F, 4Q and 4R).

Fibrin is a natural biopolymer involved in blood coagulation and wound healing. Moreover, autologous fibrin can be isolated from patients' blood, it is a widely-used substrate in tissue engineering applications and it is known to increase the secretion of ECM proteins by seeded cells [50-52]. Pankajakshan et al. [44] deposited fibrincontaining composite matrix on PCL scaffolds and so promoted the growth and survival of seeded HUVEC. Additionally, production of cell-own matrix (collagen type IV and elastin) is enhanced on this hybrid surface. The authors conclude that the ECM components exhibit maximal activity in combination with each other, thus eliciting integrin-dependent signals and support for the adhering cells [44]. For example, EC can bind fibrin by the aIIbβ3 and avβ3-integrin receptor [53,54]. In our recent study, Filova et al. [55] seeded endothelial CPAE cells on 2-D and 3-D fibrin surfaces combined with ECM proteins. Coating Co, LM or FN on Fb samples preserves excellent EC attachment and growth and enable the formation of more mature focal adhesions containing vinculin and talin on an EC membrane [55]. In accordance with this, the fibrin-containing protein assemblies used in our present experiment appeared to be the most suitable for promoting the growth of EC on PET vascular grafts impregnated with PLG and PLGC.

The approach to tissue engineering of vascular grafts may use (1) cells alone that secrete their own matrix, (2) biomaterials alone that are remodeled by in-growing cells, or (3) a combination of these two approaches. The use of solid non-degradable components in the design of vascular prostheses is associated with several limitations, in particular reduced compliance, reduced vaso activity and the possibility of foreign body reaction [22,56,57]. Nevertheless, firm mechanical reinforcement ensures superior safety by preventing potential graft rupture or aneurysm formation [15]. In addition, degradable polymers may be associated with increased local environment acidity [18]; however, degradation products would be washed out in vascular dynamic conditions, and the advantage of replacing the polymer step-by-step by cell-secreted matrix is self-evident [9].

Thus, certain limitation of our study lies in the fact that this is a purely experimental study investigating only the initial, pre-shear stress cellular behavior under static culture conditions with respect to the newly created composite vascular biomaterial. Further studies will be focused on tubular samples in a dynamic bioreactor, seeded with primary human cells. Some of these studies have been recently published or accepted for publication [49,58]. Also in vivo testing of the modified prostheses in laboratory animal is necessary, particularly after the two-stage endothelialization with autologous endothelium. In advanced tissue engineering, it is also desirable to use short synthetic ECM-derived adhesion oligopeptides instead of entire ECM molecules, which are usually of xenogeneic origin and can evoke immune reaction or can mediate pathogen transmission.

Conclusion

In this study, tri-component vascular grafts were fabricated that comprise commercial semi-finished PET, to provide potential mechanical strength; copolymers PLG or PLGC, for potential semibiodegradability; and cell-adhesive protein assemblies, to provide lining with endothelial cells.

Bovine endothelial cells grew to confluence on PET and on all of the protein-coated PET surfaces. The highest final density was observed on +Co/FN. No marked differences in cell growth were detected among other protein multilayers. Modification of PET grafts by impregnation with degradable copolymers made them less adhesive for EC in the following order PET>PET-PLG>PET-PLGC. However, the additional modification of the impregnated grafts with protein assemblies promoted the establishment of an endothelial lining, especially on fibrin-containing surfaces.

A completely biological tissue-engineered vascular graft is an upcoming substitute for damaged blood vessels, but its clinical use is currently limited due to poor mechanical strength. Polymeric vascular grafts lined with endothelial cells may therefore be a more viable option at the present time.

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