value 263587±12218 cells/cm^2, whereas the values on the samples irradiated for 10, 20 and 30 min were only 182705±3202, 134321±8446, 259976±16765 cells/cm^2. This result, as well as the homogeneous distribution of cells on the material surface, was surprising, because the preferential adhesion of cells on the irradiated microdomains was expected. It is possible that on PET in the acetylene atmosphere, modification of the entire material surface occurred, and the whole surface of the sample gained the physical and chemical characteristics originally supposed only for domains, such as the creation of oxygen-containing groups, changes in the surface wettability etc. On the other hand, the UV light-irradiation in acetylene atmosphere leaded to the formation of hydrogenated amorphous carbon (a-C:H) (Bacakova *et al* 2001, Svorcik *et al.* 2004, Kubova *et al*. 2005). Amorphous carbon has been found to be relatively hydrophobic and not well supporting cell adhesion in comparison with conventionally used cell culture plastics (*Bacakova et al. 2001*, *2004*), so that the cell number was not increased on the irradiated microdomains in comparison with surrounding non-modified polymer.

In all groups of ion-irradiated polyethylene, i.e., at all doses and energies of ions, the cells were growing preferably on the modified domains (FIG.3), although they were found also among them. The selectivity of the cell growth on the domains differed in samples irradiated by ions of different energies and doses.

At the energy of 150 keV, the highest selectivity was observed at a relatively low dose of 10^{13} ions/cm^2. On these samples, 80±0.04% of cells adhered to the domains. The cells on these domains reached the density of 1986325±160223 cells/cm^2, while outside them it was only 4161±1239 cells/cm^2. At the energy 15 keV, the highest selectivity was noticed at the dose of $3\cdot10^{14}$ Ar$^+$/cm^2, where 75.8±0.06% of cells adhered to the domains.

The lowest selectivity at the energy of 150 keV was registered on polymers modified by the highest doses of $3\cdot10^{14}$ ions/cm^2, where only 22.80±0.03% of cells adhered to the domains. At the energy of 15 keV, the lowest selectivity was registered at the lowest doses of $3\cdot10^{12}$ ions/cm^2 (62.4±0.07% of cells on the domains). The lowest doses were probably less sufficient to increase the polymer attractiveness for cell colonization (FIG.4)

Therefore, the cells preferred the domains, on which oxygen-containing functional groups were created by the influence of Ar$^+$ ion irradiation. These groups are known to increase the surface wettability and improve the adhesion and subsequent growth of cells (*Bacakova et al. 1996, 2000, 2001*). Differences between the tested samples in the selectivity of cell adhesion on ion-irradiated domains were caused by the doses of Ar$^+$ ions. At the energy of 150 keV, higher doses of Ar$^+$ might lead to the formation of amorphous carbon, which is known not to increase the material's attractiveness for cells, and thus these domains were less convenient cells colonization (*Bacakova et al. 2001, 2004, Rockova–Hlavackova et al. 2004*).

In case of the ion energy of 15 keV, i.e., a value one order lower, it seems that a lower ion doses were insufficient to create the adhesive microdomains. On the contrary, higher ions doses were better for this purpose and the cells preferred the domains created this way.

Conclusion

The method of irradiation with Ar$^+$ ions appears as convenient for surface modification of polyethylene and other relatively hydrophobic synthetic polymers. Vascular smooth muscle cells preferred the ion irradiation-created microdomains for their growth. However, it is very important to choose appropriate combination of the ion dose and energy, which will be the goal of our subsequent researches.

On the polyethylene terephtalate exposed to UV light in acetylene atmosphere, we obtained a homogeneous colonization of the material surface with vascular smooth muscle cells instead the expected selective adhesion and growth of these cells on the modified microdomains. Nevertheless, on day 3 after seeding, the cell number on irradiated samples increased with the time of exposure to UV light, although on day 7, these differences disappeared. Therefore, these events need further investigation.

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HUMAN ENDOTHELIUM ON VASCULAR PROSTHESES MODIFIED BY EXTRACELLULAR MATRIX PROTEINS IN A FLOW EXPERIMENT

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Abstract

Artificial vascular prostheses are used for bypass surgery. Thrombogenicity often cause graft occlusion. Targeted surface alterations including cell seeding may improve the haemocompatibility. Knitted commercial tubular PET (polyethylene terephtalate) vascular prostheses with collagen impregnation were modified by adsorption of laminin (LM) or coating with fibrin network (FB) on the luminal surface. Human endothelial cells were harvested, cultured and seeded at the density of 150x10^3 cells/cm^2 on all grafts. The cell lining was continuously visualized and quantified. The retention was 21%, 37% and only 2% of the seeding density on the unmodified (UM), LM- and FB-coated grafts, respectively. These seeded prostheses were exposed to a laminar shear stress (SS) 15 dynes/cm^2 for 40 minutes (UM, LM, FB) and 120 min (UM, LM) in a chamber simulating blood circulation. The SS was exluded in static (ST) control grafts. After 40 min-SS the cell numbers were 78%, 27% and 72% for the UM, LM and FB prosthesis compared to the ST. The cell densities were 61% and 57% on the UM and LM after 120 min-SS. To conclude, the endothelium formed a confluent layer whereas laminin immobilisation improved endothelial adhesion but not the flow resistance. Reverse effect was observed on fibrin coating.

Key words: *vascular prosthesis, endothelial cells, collagen, laminin, fibrin, adhesion, shear stress*

[Engineering of Biomaterials,58-60,(2006),10-13]

Introduction

Atherosclerosis is the most common disease affecting and damaging human arteries. Surgical intervention may be required to restore the blood supply to endangered organs and tissues. Autogenous or allogenous vessels are ideal natural material for bypass procedure but they are often unavailable or unsuitable for reconstruction. Therefore synthetic vascular prostheses are widely used in vascular surgery. They are usually made of hydrophobic synthetic polymers such as expanded polytetrafluoroethylene (ePTFE) or polyethylene terephtalate (PET) [1]. Despite being considered bioinert, colonisation of undesired cell types, *e.g.* vascular smooth muscle cells (VSMC), platelets or leukocytes leads to neointimal hyperplasia [2] which is a common cause of restenosis. Together with artificial surface thrombogenicity these processes may result in graft occlusion. Inner surface of native vessels is lined with a monolayer of mature endothelial cells (EC) which impart excellent antithrombotic properties. The vascular EC are anchored to extracellular matrix (ECM) proteins of the basal lamina such as collagen, laminin [3] or fibronectin which give to the cells sufficient mechanical support. Several surface modifications are carried out [4, 5] to approximate the synthetic prosthesis to natural vessel, among others adhesion ligand grafting, ECM protein coating and even cell seeding [6,7]. We focused on synthetic commercial PET vascular prostheses of a small diameter, modified them with ECM protein coatings and subsequent EC seeding. We formed the cell monolayer, exposed it to defined flow conditions, *e.g.* laminar shear stress [8,9] and evaluated the cellular retention on the inner grafts surface.

Materials and methods

Knitted crimped tubular PET vascular prostheses with bovine type I collagen impregnation (VUP® Joint-Stock Comp., Brno, Czech Republic) of 6 mm inner diameter were modified on the inner surface by laminin (LM) immobilisation (Engelbreth-Holm-Swarm Murine sarcoma, Sigma®) or by fibrin (FB) network coating prepared by activation of human fibrinogen (Sigma®) with surface attached thrombin (Sigma®) (Institute of Macromolecular Chemistry, Prague, Academy of Sciences of the Czech Republic). Third set of grafts was left unmodified *i.e.* commercial collagen-PET surface (UM). They were all immersed for 12 hours into standard culture medium M199 (Invitrogen®). Human saphenous vein endothelial cells (HSVEC) obtained from coronary bypass surgery were cultured in flasks (Falcon®) and amplified to the passage number p3-5. Culture medium consisted of M199, 20% of foetal calf serum (PAA®), Heparin 50 IU/mL (Choay®), basic fibroblast growth factor b-FGF 10ng/mL (Promocell®) and mixture solution of Penicillin (10x10^3 IU/mL) and Streptomycin (10µg/mL) 1:100 (Sigma®). The cellular suspension was homogenized in fresh medium and injected into the lumen of each prosthesis, both ends were ligated and the grafts were placed into a rotating device (Endostrabilisator, Biegler Co.Ltd., Austria) for 4 hours to achieve homogenous cell seeding. The initial seeding density was 150x10^3 cells/cm^2. Just after seeding a small ring was cut out of the middle of each prosthesis, stained with Live/Dead (L/D) Viability/Cytotoxicity Kit (Molecular Probes®) to roughly see the homogenity of the seeding [Fig. 3C]. The seeding was followed by 48 hours of maturation in culture flasks with fresh complete medium without rotation in a humid incubator with 5% CO_2.

The flow experiment occured in a haemodynamic bench (Inserm U577, Universite Victor Segalen, Bordeaux, France) consisting of a peristaltic pump, water container, heating, flowmeter, tensiometer and silicon tubing circuit. The conditions inside simulate human arterial circulation (temperature 37°C, pulsatile pressure 120/60 mmHg, laminar shear stress (SS) 15 dynes/cm^2, medium viscosity 0,04 Poiseuille (achieved by adding 8% of Dextran, Sigma®). So called dynamic prosthesis was mounted on a special metal conector creating laminar flow and static control prosthesis was placed into a blind branch of the circuit and thus submitted to same conditions of medium, pressure and temperature but excluding shear stress.

There were 4 pieces of each type of seeded grafts (UM, LM, FB). The first one was used to evaluate the seeding, the second and third ones were exposed to dynamic flow conditions for 40 (UM, LM, FB) and 120 (UM, LM) minutes (´) and the fourth one was submitted to static conditions exluding shear stress.

After each step of the experiment (seeding, maturation, flow) a prosthesis fragment was saved for immunocytochemical staining [*e.g.* FIG.2B], another fragment of defined surface area was stained by immunofluorescent L/D Kit to visualize the cells which were then removed from the support by a phosphate buffer saline (PBS) solution with 0,125% Trypsin (Sigma®) and 0,0625% EDTA. M199 medium with 10% FCS was used to stop the Trypsin/EDTA solution. After centrifugation (5 minutes, 300g) and resuspendation the cells were counted in a 1 mm^3 Malassez chamber to obtain the cell number and count the cell density on the prosthesis fragment which was expressed as mean and SEM of 8 independant observations. This was compared to the population density on the corresponding static graft. For statistical evaluation Student t-test for unpaired data was used.

Results

The initial seeding density of HSVEC was 150x10^3 cells/cm^2 on each of the prosthesis. The seeding efficiency was the best on the LM graft where the cell density decreased to

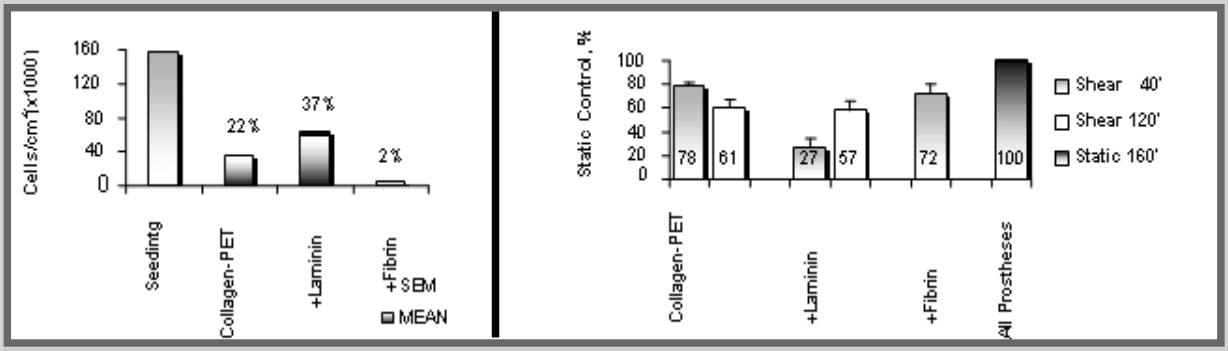

FIG.1A. Human endothelial cells density (HSVEC, p3-5) on modified knitted collagen-PET vascular protheses 48 hours after seeding 150x10³ cells/cm². P<0,001 each of the grafts versus (vs.) seeding density, Student t-test for unpaired data.

FIG.1B. Retention of HSVEC on modified knitted collagen-PET vascular protheses after laminar shear stress (SS=15dynes/cm²), p<0,01 all grafts vs. corresponding static control after both 40 and 120 minutes of SS.

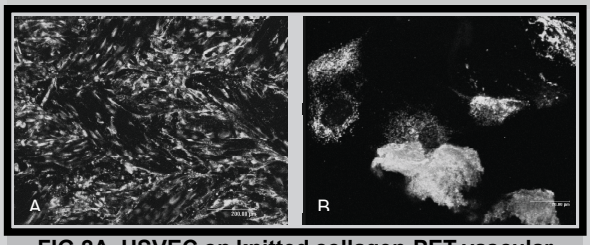

FIG.2A. HSVEC on knitted collagen-PET vascular prosthesis with immobilized laminin. The ultrastructure of the knitted graft is obvious. Anti-von Willebrand Factor Rabbit IgG 1:100 MAb (Sigma®), Propidium Iodide 5µg/mL. Confocal microscope Leica TCS SP2 AOBS 39x10µm, magnif. 10x.
FIG.2B. HSVEC after 40 minutes (´) of shear stress (SS=15 dynes/cm²). Secretion granula with von-Willebrand factor are clearly visible. 25x0,5µm, magnif. 100x.

59±2,6x10³ cells/cm² after seeding and 48 hours of maturation (p<0,001 LM versus (vs.) UM, LM vs. FB). The density on the UM prosthesis was 35±1,0x10³ cells/cm² while only 3,2±0,3x10³ cells/cm² retained on the FB graft (p<0,001 UM vs. FB). Thus the cell retention was 21%, 37% and only 2% of the seeding density on the UM, LM- and FB-coated grafts, respectively (p<0,001 for each of the grafts) [FIG.1A]. After exposing the endothelium to 40 min-laminar shear stress, the cell population density dropped to 78%, 27% and 72% for the UM, LM and FB prosthesis compared to the corresponding static (ST) control (p<0,01 for all grafts). On the UM and LM prostheses the cell numbers were 61% and 57% after 120 min-SS (p<0,01 vs. ST for both grafts) [FIG.1B].

Discussion

Low density of the endothelial cells on the fibrin prosthesis might be caused by technical difficulties and manipulation with dry fibrin network coating rather than by low adhesive interactions between the cells and the support. Higher cell density after 120 minutes than after 40 minutes of shear stress on the laminin graft may have resulted from local inhomogenity in cellular coverage of the tested prosthesis fragment. However, the cell retention was generally low after both 40 and 120 minutes of flow on the laminin grafts compared to the static control.
Gourevitch *et al.* [10] reported EC retention of 73% after 102 min of flow *in vitro* (140 mmHg, 140 mL/s) on gelatin-coated Dacron® (PET) 6 mm ID grafts compared to initial cell density. Our results showed 61% EC retention on collagen-PET prostheses after 120 min-shear stress compared to static control.

Conclusions

Human endothelial cells formed a confluent layer on the luminal surface of both commercial and the laminin modified

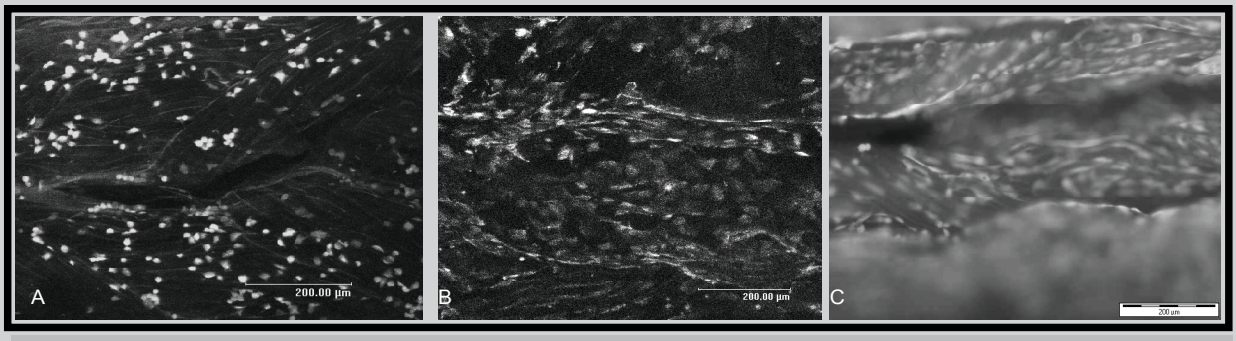

FIG.3A. HSVEC on fibrin network-coated knitted collagen-PET vascular prosthesis 2 hours after seeding (150x10³ cells/cm²). Anti-CD31 human FITC conjugated 1:100 MAb (EXBIO®), Confocal microscope Leica TCS SP2 AOBS, 21x9,5µm, magnif. 10x.
FIG.3B HSVEC on commercial knitted collagen-PET vascular prosthesis after 40´SS (15 dynes/cm²). The cells keep forming a confluent monolayer. Anti-β-actin Mouse IgG MAb 1:200 (Sigma®), Propidium Iodide 5µg/mL, 36x20µm, magnif. 10x.
FIG.3C HSVEC on commercial knitted collagen-PET vascular prosthesis after 120´SS (15 dynes/cm²). The cells still retain confluency. Live/Dead Viability/Cytotoxicity Kit (Molecular Probes®), Light microscope Olympus, magnif. 10x.

collagen-PET vascular prostheses after amplification and seeding in high density [FIG.2A, 3B]. After exposure to short-term laminar shear stress similar to that in human vessels the cells showed good retention. Even if the cell detachement degree was statistically significant, the endothelium retained its confluent form on many of the investigated grafts [FIG.3B]. Immobilisation of the extracellular matrix protein laminin on the inner surface improved the cellular adhesion [FIG.2A] but decreased their shear stress resistance compared to the unmodified and fibrin-coated prostheses. Vice versa fibrin network coating [FIG.3A] resulted in worse adherence but better flow resistance compared to the graft with immobilized laminin.

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3D EVALUATION OF THE SURFACE ROUGHNESS USING STEREO IMAGES MADE IN SEM – INFLUENCE ON OSTEOBLAST CELL GROWTH

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The surface roughness creates one of the main conditions for contact of the living tissue with material. The cells adhesion, proliferation and differentiation are usually influenced by the surface morphology very strongly. Now, for biomaterial applications, it is necessary to better understand this influence and to define the roughness parameters, which control these interactions at the cell-material interface.
The surface roughness can be measured by number of methods; often, the contact profilometer can measure a number of parameters characterising the roughness. The disadvantage of this measurement is the line recording of the surface, which is principally two dimensional, and gives us information just from the path in one direction. The other possibility is to create the 3D reconstruction of a surface by suitable code from stereo-pair of images. For this method, using of Scanning Electron Microscope is very suitable and effective for its large depth of focus and simple change of imaging conditions.
We observed the osteoblast-like cells growing on carbon-carbon composites with various surface layers and also plain ones. Particular layers, which would by evaluated are pyrolytic graphite (PyG), titanium-carbon layer (Ti:C-H), diamond like carbon (DLC) and zircon nitride (ZrN). These materials were studied in the form of films prepared either by PACVD method or by pyrolysis on 2D C-C composites. The osteoblast-like cells were grown on the surface for defined time and generally adhesion, doubling time and spreading of cells were evaluated
The SEM images were obtained using JSM5410 scanning electron microscope (JEOL, Japan) and the *Scandium* software (Olympus Soft Imaging Solutions, Germany), which was used for 3-D surface reconstruction of images. Firstly, we compared the roughness parameters measured by line profilometer (Hommel Tester T 1000, Hommelwerke

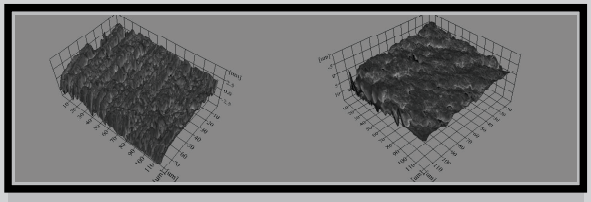

FIG.1. 3D reconstructed surface of ZrN layer deposited on C-C composite by the magnetron sputtering.

GmbH, VS-Schwenningen, Germany) and calculated by Scandium code. We obtained reasonable agreement, even though the length of measurement is substantially different. Than after we studied the dependence of cell spreading on single roughness parameters. For pyrolytic carbon, the optimal value of Ra was obtained, and also the particular correlation between spreading and roughness parameter Rsk was found.

The particular results achieved from the pyrolytic carbon layer shows TABLE 1.

The skewness (Rsk), describing the asymmetry of the

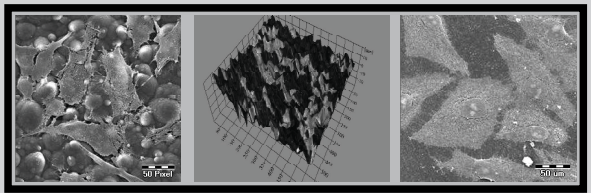

FIG.2. Osteoblast-like cells growth on the PyG layer, SEM; SEM – Scandium.