IMPROVED ADHESION AND GROWTH OF VASCULAR SMOOTH MUSCLE CELLS IN CULTURES ON MODIFIED POLYETHYLENE

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Introduction

Synthetic polymers, such as polyethylene, polystyrene, polyurethane, polytetrafluoroethylene and polyethylene terephtalate, are commonly used in various industrial applications as well as in biology and medicine. They not only serve as growth supports for cell cultures *in vitro*, but can also be used for constructing replacements for various tissues or organs, e.g. non-resorbable or semi-resorbable vascular prostheses, artificial heart valves, bone and joint replacements, and implants for plastic surgery (for a review see Bacakova *et al.* 1996, 2000, 2001).

There are two approaches to the application of these materials. The first approach uses highly hydrophobic or extremely hydrophilic surfaces, which do not allow adhesion and growth of cells. This approach is used for creating bioinert vessel replacements, where permanent blood flow is necessary and thus the adhesion of thrombocytes or immunocompetent cells is not desirable, due to the risk of restenosis of the graft (for a review see Bacakova *et al.* 2000). An alternative approach, widely accepted in recent tissue engineering, is to create surfaces that support colonization with cells and good integration of a replacement with the surrounding tissues of the patient's organism. This concept is used e.g. for constructing bone prostheses that will persist in the patient's organism for many years, and is being developed for the creation of bioartificial replacements of blood vessels, liver, pancreas and even nervous tissue (for a review see Bacakova *et al.* 2000, 2001).

There are various ways of modifying the surfaces of the materials to make them convenient for cell adhesion. For this purpose, the surfaces have been exposed to ultraviolet (UV) irradiation (Svorcik *et al.* 2004), to a beam of various ions (e.g., oxygen, nitrogen, noble gases or halogens for biological applications; Bacakova *et al.* 1996, 2000, 2001) or to a plasma discharge (Turos *et al* 2003). For more pronounced changes in the physicochemical properties of the modified surface, some of these processes can be realised in a gas atmosphere, e.g. in acetylene or ammonia (Svorcik *et al.* 2004). The goal of these irradiation modifications is to create functional chemical groups containing oxygen or nitrogen, like carbonyl, carboxyl or amine groups, on the surface of the material. These groups increase the surface wettability, support the adsorption of cell adhesion-mediating extracellular matrix proteins and stimulate the cell adhesion and growth (Bacakova *et al.* 1996, 2000, 2001, Svorcik *et al.* 2004).

An alternative and more exact approach can be grafting the polymer surfaces directly with various biomolecules, which can influence the cell behavior by a more controllable manner. Therefore, in this study, high- density polyethylene, i.e. material promising for biomedical use, was modified by an Ar plasma discharge and subsequent grafting of glycine (Gly), bovine serum albumin (BSA) or polyethylene glycol (PEG), and/or carbon (C). The aim of these modifications was creation of surfaces attractive for cell colonization. On the modified polymer, we evaluated the adhesion, growth and maturation of vascular smooth muscle cells in cultures isolated from the rat aorta.

Experimental

Preparation of the polymer samples.

High-density polyethylene (HDPE, m.w. 0.952g/cm^3) were modified by an Ar plasma discharge (gas purity: 99.997%) using a Balzers SCD 050 device 50, seconds; the discharge power was 1.7 W. Modified PE was grafted with glycine, bovine serum albumin (BSA) or polyethylene glykol (PEG). Some plasma-irradiated samples and samples grafted with BSA were exposed to carbon

(C and BSA+C, respectively), in a water solution (2 wt.%) for 12 h at room temperature.

Cells and culture conditions.

The modified materials were cut into square samples of 10*10 mm in size, sterilized with 70% ethanol for 1 hour, inserted into 24-well plates (TPP, Switzerland; well diameter 1.5 cm) and seeded with smooth muscle cells derived from the rat aorta by an explantation method (passage 3, 17 000 cm²; Bacakova *et al.* 2000, 2001). The cells were cultivated in 1.5 ml Dulbecco´s Modified Eagle Minimum Essential Medium (Sigma, USA) supplemented with 10% foetal bovine serum (Sebak GmbH, Aidenbach, Germany) for 1, 2, 5 or 7 days (temperature of 37°C, humidified atmosphere of 5% of CO_2 in the air). For each experimental group and time interval, four samples were used. The cells on one sample were fixed by 70% cold ethanol (-20°C) and stained with a combination of fluorescent membrane dye Texas Red C2-maleimide (Molecular Probes, Invitrogen, Cat. No. T6008; 20ng/ml) and a nuclear dye Hoechst # 33342 (Sigma, U.S.A.; 5µg/ml). The number and morphology of cells on the sample surface were then evaluated on pictures taken under an Olympus IX 50 microscope using an Olympus DP 70 digital camera. On the remaining three samples, the cells were rinsed by PBS, released with trypsin-EDTA solution (Sigma, Cat. No. T4174) and counted in Cell Viability Analyzer (VI-cell XR, Beckman Coulter). As control materials, non-modified HDPE and standard tissue culture polystyrene dishes (PSC) were used.

On day 7 after seeding, the cells were immunocytochemically stained for several molecular markers of adhesion and maturation, such as talin, vinculin paxillin, alpha-actinin, beta-actin and contractile proteins alpha-actin and SM1 and SM2 myosin (Fig. 1). Fluorescence pictures were taken under a confocal microscope Leica (TCS SP2, Germany). The concentration of these molecules was also measured using enzyme-linked immunosorbent assay (ELISA) in homogenized cells per mg of protein. Absorbance of cell samples taken from modified HDPE foils were given in % of the value obtained in cells on the pure HDPE.

Statistics.

The results were presented as mean ± SEM (Standard Error of Mean). Statistical significance was evaluated by ANOVA, Student-Newman-Keuls method. Values $p \leq 0.05$ were considered as significant.

Results and Discussion

On the 1st day after seeding, the numbers of cells on the HDPE samples were higher on all HDPE foils than on the control PSC. The highest average cell number, amounting to 12,558 ± 2722 cells/cm², was found on the HDPE grafted with PEG.

On day 2 after seeding, the highest numbers of cells were observed on plasma-modified HDPE (16,651 ± 3,440 cells/cm²). The lowest amount of cells was on pure HDPE (7,770 ± 3356 cells/cm²).

On the 5th day, the cell population densities were similar on samples modified by PEG, BSA, C and BSA + C, although the value on HDPE modified by PEG tended to be the highest (268,708 ± 57,936 cells/cm²). The lowest numbers of cells persisted on pure HDPE (97,825 ± 46,223 cells/cm²), pure polystyrene and culture dish polystyrene.

On the 7th day, the numbers of cells on all HDPE samples became similar but lower than the value obtained on polystyrene culture dishes (262,708 ± 114,979cells/cm²). Among HDPE samples, the highest average value was registered on PEG (133,405 ± 72,574 cells/cm²). The lowest cell number was observed on plasma-modified HDPE (71,020 ± 59,609 cells/cm²).

ELISA did not show any significant difference in concentrations of talin, vinculin, paxillin, alpha-actinin, beta-actin, smooth muscle alpha-actin as well as SM1 and SM2 myosin. The concentration of talin tended to be the highest on PSC (229 ± 141 % of the value on pure HDPE). The average concentration of vinculin, SM1/SM2 myosin and especially beta-actin tended to be the highest on BSA + C (265 ± 148 %, 249 ± 143 % and 547 ± 344 %, respectively). The concentration of paxillin in cells on all modified samples was even lower than that on unmodified HDPE, where it as 100 ± 55 %. Both alpha-actinin and alpha-actin attained the highest average concentration on plasma-irradiated HDPE (129 ± 55 % and 229 ± 141 % of the value on pristine HDPE, respectively).

Nevertheless, immunofluorescence staining (Fig. 1) revealed that the focal adhesion plaques containing talin, vinculin and paxillin, as well as fibres containing actin and myosin, were in general better developed on modified than on pristine HDPE samples. In the pristine non-modified form, HDPE has been considered as a substrate not very appropriate for cell adhesion and growth, which may be due to its relatively high hydrophobicity (water drop contact angle 102.5 ± 2.3; Svorcik *et al.* 2006). The improved cell adhesion and growth of cells on samples modified by plasma discharge was probably due to the creation of oxygen-containing functional groups. Fourier transform infrared spectroscopy (FTIR) has indicated the presence of peroxide, ester, carbonyl, carboxyl, hydroxyl, amide groups and excessive double bonds in polyethylene modified with a plasma discharge (Svorcik *et al.* 2006). The oxygen-containing groups are known to increase the surface wettability and improve the adsorption of cell adhesion-mediating extracellular matrix molecules (e.g.

vitronectin, fibronectin) from the serum of the culture medium. These molecules are adsorbed in an appropriate amount, flexibility and spatial conformation enabling good accessibility of specific sites on these molecules (e.g., RGD-containing amino acid sequences) by cell adhesion receptors, such as integrins (Bacakova *et al.* 1996, 2000, 2001).

The adhesion, growth and maturation of vacular smooth muscle cells on polymers modified by plasma discharge in our study was improved by functionalization of the polymer surface especially with PEG and BSA + C. It is surprising because both PEG and BSA have been often reported to be non-adhesive for cells [for a review, see Bacakova *et al*. 2007]. However, the antiadhesive action of PEG seems to be dependent on its concentration on the polymer surface and the length of its chain [Bacakova *et al.* 2007]. In the case of BSA, the presence of C might render this molecule to be adhesive for cells, which needs further investigation.

Conclusion

Treatment of high- polyethylene with an Ar plasma discharge had positive effects on the colonization of HDPE with confluent and mature vascular smooth muscle cells. This improvement of the cell colonization was probably due to the formation of oxidized structures in the polyethylene surface layer and increased material wettability. The attractiveness of the material for cell colonization was further intensified by grafting the polymer surface with PEG and BSA + C but this approach needs further investigation.

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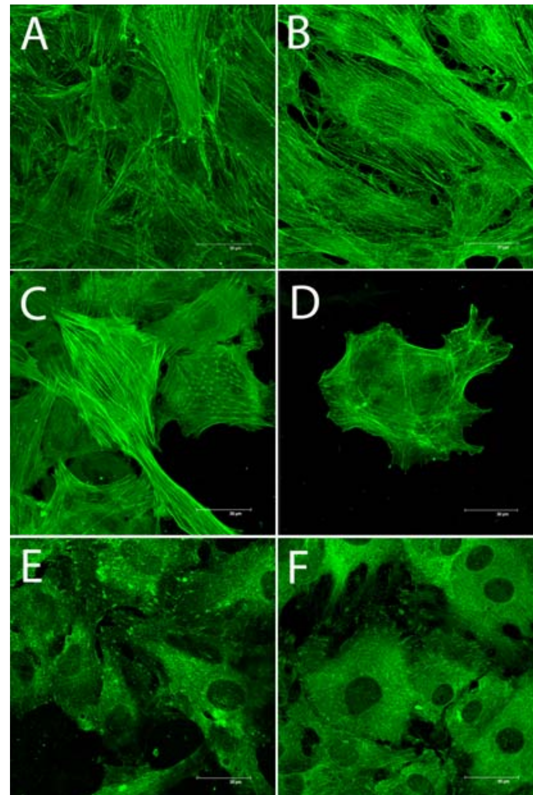

Figure 1: Examples of morphology of vascular smooth muscle cells in cultures on HDPE with various modifications. A) beta-actin on BSA+C, B) beta-actin on C, C) alpha-actin on plasma HDPE, D) alpha–actin on pure HDPE, E) talin on BSAC, D) talin on control glass. Immunofluorescence staining, Confocal microscope Leica (TCS SP2, Germany) obj. 100x, bar = 30 μm