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

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Abstract

The attractiveness of synthetic polymers for cell colonization can be affected by physical and chemical modification of the polymer surface. In this study, high density polyethylene (HDPE, m.w. 0.952g/cm^3) and low density polyethylene (LDPE, m.w. 0.922g/cm^3) were modified by an Ar plasma discharge using Balzers SCD 050 device (exposure time 10, 50, 150 and 400 seconds, discharge power 1.7W). The material was then seeded with rat aortic smooth muscle cells (RASMC; passages 8 to 9, 17 000 cells/cm^2) and incubated in a DMEM medium with 10% of fetal calf serum. On day 1 after seeding, the number of initially adhered cells was significantly higher on all modified HDPE and LDPE samples. On day 2, this difference persisted in HDPE, whereas in LDPE only the values on the samples modified by 150 and 400 seconds were significantly higher. On the 5th and 7th day, there were no significant differences in cell number among all LDPE samples. However, on the HDPE foils, significant differences were still apparent on the samples modified for 400 seconds. The cell spreading areas measured on day 1 after seeding were significantly larger on all modified LDPE samples, and, on day 2, on the HDPE samples exposed for 150s. The increased cell colonization was probably due to the formation of oxygen-containing chemical functional groups in the polymer. These results suggest that the responsiveness of the cell to the changes in physicochemical surface properties was more pronounced in HDPE than in LDPE. On both types of polyethylene, the most appropriate exposure time for the enhancement of cell adhesion and growth seemed to be 150 and 400 seconds.

***Keywords**: Ar plasma discharge, high density and low density polyethylene, cell adhesion, cell proliferation, vascular smooth muscle cells, biomaterials, tissue engineering.*

[Engineering of Biomaterials, 67-68, (2007), 1-4]

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 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). In this study, we used an Ar plasma discharge for surface modification of high- or low-density polyethylene, i.e. materials that are promising for biomedical use. On the modified polymers, we evaluated the colonization with smooth muscle cells in cultures isolated from the rat aorta.

Materials and methods

Preparation of the polymer samples

High density polyethylene (HDPE, m.w. 0.952g/cm^3) and low density polyethylene (LDPE, m.w. 0.922g/cm^3), which are model materials for the potential development of tissue replacements, were modified by an Ar plasma discharge (gas purity: 99.997%) using a Balzers SCD 050 device for 10, 50, 150 and 400 seconds; the discharge power was 1.7 W.

Cells and culture conditions

The modified materials were cut into square samples 10x10mm in size, placed into 24-well plates (TPP, Switzerland; well diameter 1.5cm) and fixed to the well bottom by polyethylene circles (inner diameter 0.7cm, inner area 0.38465cm^2). Vascular smooth muscle cells were isolated by an explantation method from the aorta of young male rats of the strain Wistar SPF (Bacakova et al. 2000, 2001). In the 8th to 9th passage, the cells were seeded on the samples at a density of 30,000 cells/well (i.e., 17,000 cells/cm^2). The cells were cultivated in 1.5ml of Dulbecco's Modified Eagle Minimum Essential Medium (Sigma, U.S.A.) supplemented with 10% foetal bovine serum (Sebak GmbH,

Aidenbach, Germany) for 1, 2, 5 and 7 days (temperature of 37°C, 5% of CO_2 in a humidified air atmosphere). The cells were then fixed by 70% cold ethanol (-20°C) and stained with hematoxylin and eosin. The number of cells on the sample surface was evaluated on pictures taken under an Olympus IX 50 microscope using an Olympus DP 70 digital camera. The cell spreading areas were determined using Atlas software (Tescan Ltd., Brno, CR). As control materials, non-modified polymers, pristine polystyrene foils and standard tissue culture polystyrene dishes were used.

Statistics

The results were presented as mean ±S.E.M. (Standard Error of Mean). Statistical significance was evaluated by Student's t-test for unpaired data. Values p≤0.05 were considered as significant.

Results and discussion

On the first day after seeding, the numbers of cells on the HDPE samples were significantly higher on all modified foils than on the pure HDPE, and on both types of polystyrene. The highest average cell number, amounting to 13,560±740 cells/cm^2, was found on the sample modified with plasma discharge for 150s. The lowest number of cells was detected on the sample modified with 50s plasma discharge, where we observed only 110726±804cells/cm^2 (FIG.1). Similarly, on the LDPE samples, the numbers of cells on day 1 after seeding were also higher on all modified samples in comparison with the pure LDPE and the tissue culture polystyrene dishes. The highest value of 13,530±1,460cells/cm^2 was reached on the sample modified for 150s, and the lowest cell number of 11140±1350cells/cm^2 was observed on the sample modified for a relatively short time interval of 10s (FIG.2).

On day 2 after seeding, the cell numbers on all modified HDPE samples continued to be significantly higher than those on the pristine HDPE (FIG.1). However, on the LDPE foils the cell numbers were significantly higher only on the samples modified at longer exposure times. The lowest cell number of 17,200 ±1,960 cells / cm^2, similar to that found on pure LDPE (18,455±19,08cells/cm^2), was observed on the sample modified for 10s. On the sample modified for 50s, the cell number was 22,030±2,340cells/cm^2, which was still non-significant in comparison with the pristine LDPE. Only on the samples modified for 150 and 400s did the cells reach significantly higher numbers of 26,650±2,300 and 26,750± 1,800cells/cm^2, respectively (FIG.2). Therefore, the cell number showed a tendency to increase with the time of exposure to the plasma discharge.

On the 5th day, the cell populations densities on the HDPE were similar in the case of samples modified for 10, 50s and pure HDPE, pure polystyrene and culture dish polystyrene. Significantly higher values were found only on the samples modified for 150 and 400s 71,950±2,870 and 63,695±4,873 cells/cm^2, respectively). On the LDPE samples the situation was different. The numbers of cells were similar on all samples except for the sample irradiated for 10s, where the cell population density was significantly higher, reaching 151,600±6,020cells/cm^2 (FIG.1,2).

On the 7th day, the numbers of cells on HDPE were significantly higher on all modified samples than on the pure HDPE, and the average cell number increased with the exposure time. Thus, the highest average value of 79,020±6,800cells/cm^2 was found on the sample modified for 400s, whereas the lowest cell population density of 43,090±5,840cells/cm^2 was obtained on the pure HDPE foil. On all LDPE samples, the numbers of cells were similar and without any statistical variances.

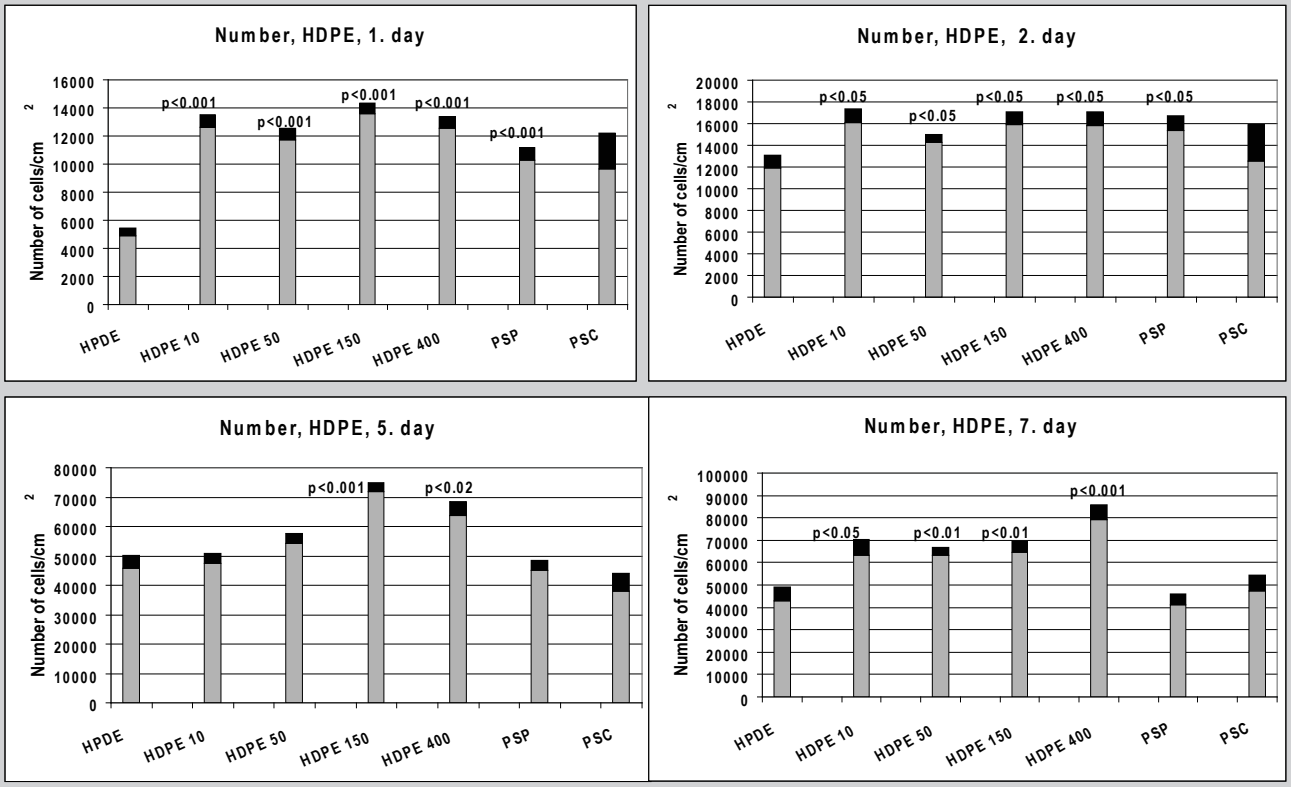

FIG.1. Number of rat aortic smooth muscle cells on day 1, 2, 5 and 7 after seeding on HDPE modified by an Ar plasma discharge. PS = pure polystyrene, PSC = polystyrene culture dish. Mean ± SEM from 20 microscopic fields (0.144 mm^2) obtained from 2 independent samples for each experimental group. Student's t-test for unpaired data. Statistical significance (p≤0.001 etc.) was evaluated in comparison with control unmodified HDPE.

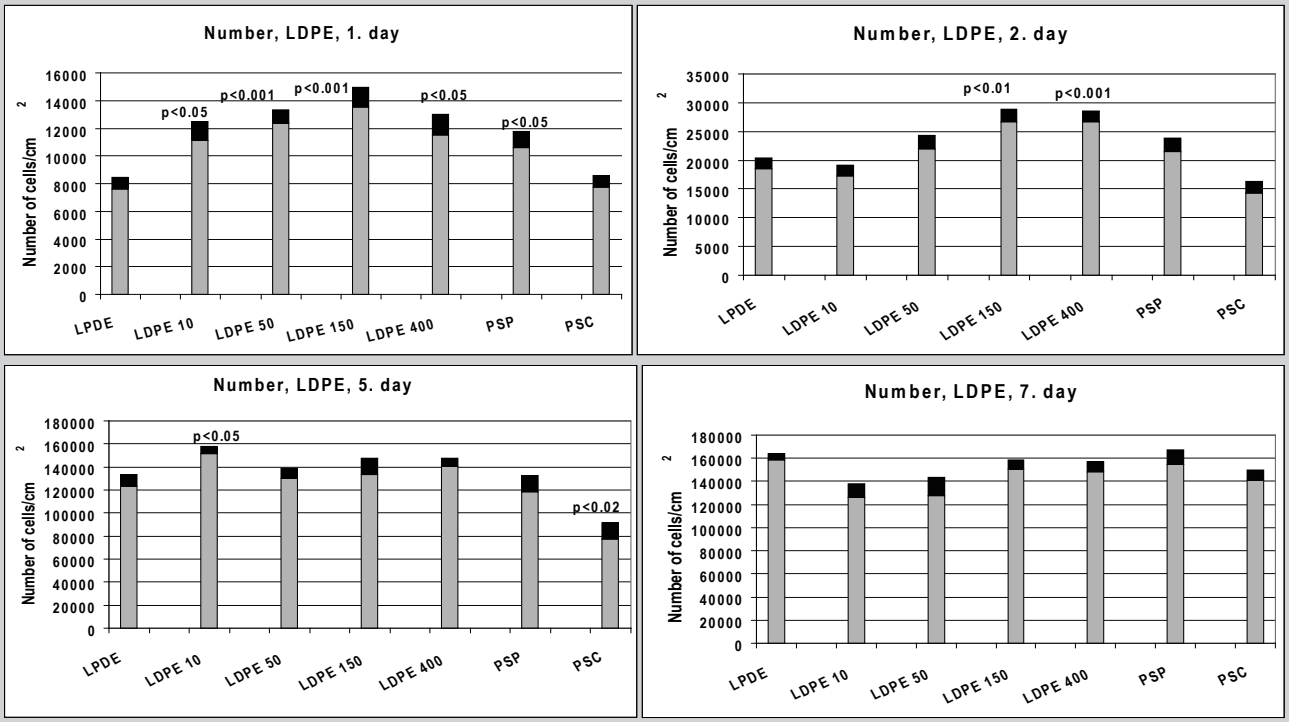

FIG.2. Number of rat aortic smooth muscle cells on day 1, 2, 5 and 7 after seeding on LDPE modified by an Ar plasma discharge. PS - pure polystyrene, PSC - polystyrene culture dish. Mean ± SEM from 20 microscopic fields (0.144 mm²) obtained from 2 independent samples for each experimental group. Student's t-test for unpaired data. Statistical significance (p≤0.001 etc.) was evaluated in comparison with control unmodified HDPE.

The results obtained on cell numbers indicate that the sensitivity of the cells to the changes in the physicochemical properties of the polymer surface was more pronounced in HDPE than in LDPE; thus the improvement of the colonization with cells was more apparent on HDPE. However, the cells were better spread on the LDPE foils. On day 1 after seeding, the cell spreading areas were significantly larger on all modified LDPE samples, ranging from 891±65 to1,462±142μm², compared to the values on the non-modified polymer (1,175±77μm²), though on day 2 after seeding these differences disappeared. In contrast, on all HDPE samples the cell spreading areas were similar (from 205±29μm² to 453±46μm²) and, surprisingly, they were relatively small in comparison with those found on the tissue culture polystyrene (546±89μm² respectively). Only on day 2 after seeding was a significantly larger adhesion area found in cells grown on HDPE modified with plasma discharge for 150s (992±52μm²).

Nevertheless, the cells were able to form a confluent layer on all samples except for the pure HDPE. In the non-modified form, the material was not a good substrate for cell adhesion and growth, which may have been due to its relatively high hydrophobicity (water drop contact angle 102.5±2.3° ; Svorcik et al. 2006). Pristine LDPE was more wettable (water drop contact angle 96.6±1.9°) and thus more permissive for cell adhesion. 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

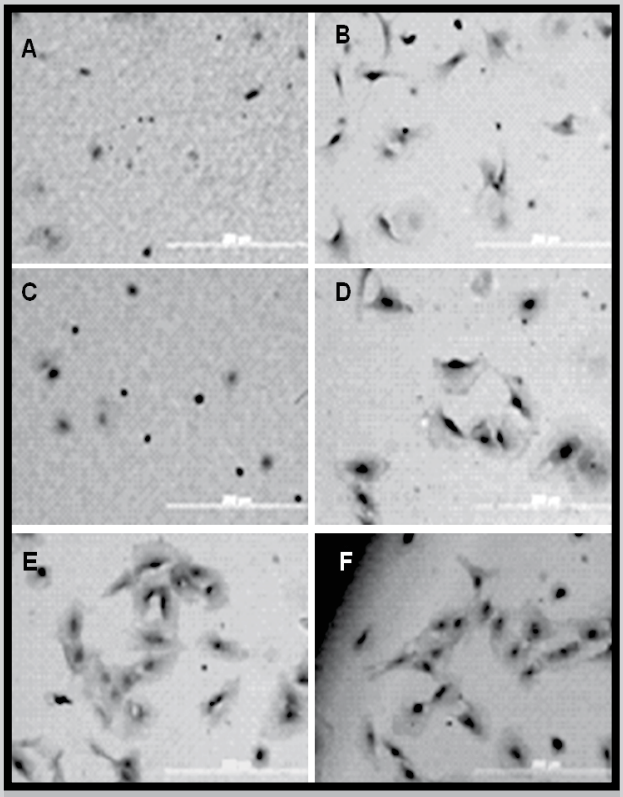

FIG.3. Morphology of rat aortic smooth muscle cells on day 1 after seeding on pure HDPE (A), LDPE (B), HDPE 150s (C), LDPE 150 s (D), pristine polystyrene foil (E) and polystyrene culture dish (F). Stained with hematoxylin and eosin, Olympus IX 50 microscope, bar=200μm.

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 cell adhesion and growth on polymers modified by plasma discharge could be further improved by functionalization of the oxygen-containing and other groups formed on the material surface by amino acids or oligopeptides acting as ligands for cell adhesion receptors and recognized preferentially by vascular smooth muscle cells, such as KQAGDV or VAPG (Mann and West 2002).

Conclusion

Treatment of high- and low-density polyethylene with an Ar plasma discharge increased the population density of vascular smooth muscle cells in cultures on these materials. This effect showed a tendency to be positively correlated with the time of Ar plasma discharge, and was more pronounced in relatively highly hydrophobic HDPE than in more wettable LDPE. However, on the modified LDPE, the cells adhered by a significantly larger spreading area. The improvement of the cell colonization was probably due to the formation of oxidized structures in the polyethylene surface layer and increased material wettability. Thus, plasma discharge proved to be a suitable method for modifying hydrophobic polymer surfaces designed for the construction of tissue replacements, e.g. bioartificial vascular prostheses. This approach deserves further investigation, especially as regards further functionalization of the polymers with bioactive molecules.

Acknowledgements

This study was supported by the Grant Agency of the Acad. Sci. CR (grant No. A 5011301). Mr. Robin Healey (Czech Technical University, Prague) is gratefully acknowledged for the language revision of the manuscript.

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