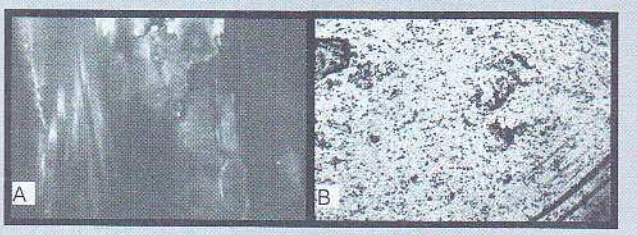

FIG. 2. Adsorption of collagen IV conjugated with Oregon Green 488 on unmodified CFRC (A) or CFRC of group #6 (B; see Material and methods). Confocal laser scanning microscope, obj. 40x.

cence of Oregon Green-conjugated collagen IV (FIG. 2), this finding could be explained by a higher and more homogeneous adsorption of collagen, which can be contained in the serum supplement of the culture media, or secreted by the cells themselves. Immunofluorescence staining of vinculin showed a relatively high formation of focal adhesion plaques in cells on samples #6 (FIG. 3). In these plaques, integrins communicate with a wide spectrum of signaling and cytoskeletal molecules, which control cell viability, growth and differentiation. On unmodified CFRC, a diffuse pattern of vinculin distribution (FIG. 3) indicated a low formation of focal adhesion plaques, which might explain a lower proliferation of MG-63 cells found on these materials in our earlier study [7]. In addition, the cells on modified CFRC, especially on samples #6, showed a tendency to contain more osteocalcin (FIG. 1), a non-collagenous calcium-binding extracellular matrix protein, considered as an important marker of osteoblastic differentiation and bone tissue formation [6, 7]. Concentration of ICAM-1, cell surface adhesion molecule of immunoglobulin type, which bind inflammatory cells [9], was similar in modified and unmodified CFRC; only in cell on samples #2, a slight but non significant tendency to increase was noted. These results suggest that coating the CFRC with pyrolytic graphite, as well as their grinding and polishing with metallographic paper and diamond paste, would not enhance the immunoattractiveness of the cell-material complex.

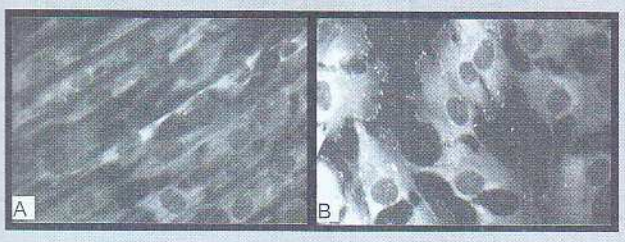

FIG. 3. Immunofluorescence staining of vinculin, a marker of focal adhesion plaques, in MG 63 cells cultured for 3 days on unmodified CFRC (A) or CFRC of group #6 (B; see Material and Methods). Confocal laser scanning microscope, obj. 60x.

Acknowledgements

This study was supported by the Ministry of Education, Youth and Sports of CR (COST, Action 527, grant No. OC/PR 00680), and by a research project No. AVOZ 5011922 of the Inst. Physiol., Acad. Sci CR.

We also thank Mrs. Ivana Zajanová for her excellent technical assistance.

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VASCULAR SMOOTH MUSCLE CELLS IN CULTURES ON LACTIDE BASED POLYMERS FOR POTENTIAL CONSTRUCTION OF ARTIFICIAL VESSEL WALL

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We focused on polymer-cell reaction, specifically on adhesion and spreading of vascular smooth muscle cells (VSMC) on lactide- and polyethylenoxide (PEO)-based polymers, potential materials for construction of vascular prostheses. On poly(DL-lactic acid), PDLLA, the number and spreading of initially attached rat aortic VSMC were similar as on standard cell culture plastics. However, the copolymer of PDLLA and PEO, MeO-PEO-b-PDLLA, almost disabled the cell adhesion and spreading. Grafting of GRGDSG peptide to the copolymer restored the cell adhesion and spreading almost to the values seen on PDLLA. Surprisingly, the concentration of 5% GRGDSG was more effective than a higher concentration of 20%.

Synthetic polymers can be used for construction of artificial vascular prostheses. Disadvantage of these devices is

excessive growth activation, which can lead to stenosis of prosthesis lumen. We suppose that this excessive VSMC proliferation could be prevented by increase of VSMC adhesion to maximum extent. It is known that the highest cell migration and proliferation are at the intermediate adhesion degree, whereas the maximal adhesion turns down the proliferation of VSMC and starts the differentiation program (Mann and West, 2002). The aim of the present study is to control the adhesion and proliferation of VSMC on bioresorbable lactide- or polyethylenoxide-based polymers, which can be used for construction of vessel wall prostheses. The extent of cell adhesion was regulated by grafting different concentrations of Gly-Arg-Gly-Asp-Ser-Gly (GRGDSG) sequence, which binds to cellular integrin receptors (Kok et al. 2002, Glukhova and Koteliansky 1995), into antiadhesive background formed by PEO. In addition, grafting of short oligopeptides enables to avoid undesirable immune reactions, often associated with adsorption of whole extracellular matrix molecules.

Preparation of polymers was described in detail by Kubies et al. (2000), Rypáček et al. (2001) and Bačáková et al. (2003). Briefly, homopolymer poly(DL-lactic acid), PDLLA (Mw=630 000), was synthesized by ring-opening polymerisation of D,L-lactide. Polyethylene oxide (MeO-PEO) was prepared by anionic polymerization of ethylene oxide. MeO-PEO-b-PDLLA (referred as PDLLA-PEO), a block copolymer of lactide with a poly(ethylene oxide) segment was synthesized by controlled polymerisation of D,L-lactide and MeO-PEO (final concentration of the PEO phase on the copolymer surface was 33%). The peptide-modified surfaces were cast from mixtures of MeO-PEO-b-PDLLA and GRGDSG-(N)-PEO-b-PDLLA. Two mixtures were prepared with different ratio (5%, 20% GRGDSG) of peptide-terminated PEO to neutral Meo-PEO in the film. Films of polymers were deposited by a spin-coating method on silanized glass coverslips (diameter 1 cm; Dispolab, Brno, CR), and inserted into 24-well-Nunclon Multidishes (Nalge Nunc Int., Denmark, diameter 1.5 cm). For each experimental group and time interval, two samples were prepared.

VSMC were derived from the intima- media complex of the thoracic aorta of 8-week-old male Wistar SPF rats by explantation method (Bačáková and Kuneš 1995), and used in passages 2 to 20.

Initial adhesion of VSMC on polymers was evaluated 6 and 24 hours after seeding. The cells were fixed with 10% neutral formol and stained with Gill`s hematoxylin and eosin (Sigma, St. Louis, MO, U.S.A.). The number of initially adhering VSMC was counted in 40 randomly selected fields (0.25 mm^2, objective 20x) homogenously distributed in each sample using a phase-contrast microscope (Opton, Axioplan, Germany) equipped with a calibrated eye-piece grid. The size of cell spreading area was measured on microphotographs taken by a digital camera (Sony, 5.0 MPxl, Japan) using a software Atlas (Tescan, Czech Republic) in 3-7 microscopic fields for each sample (6-17 cells per field, objective 40x, 0.0625mm^2). Quantitative data were given as means ± SEM and statistically evaluated by Student`s t- test for unpaired data, using a 5% error probability criterion.

The number of VSMC attached to the PDLLA-PEO copolymer 6 and 24 hours after seeding, was significantly lower (by 49.7 and 86.9%, respectively) than that on control PDLLA surface. On PDLLA-PEO grafted with 5% GRGDSG, the number of adhered cells markedly increased and became similar than that on PDLLA (increase by 44.4% and 496.6%, 6 and 24 hours, respectively, in compare with PDLLA-PEO). Surprisingly, the increase of cell population density on the copolymer grafted with a 20% concentration of GRGDSG was a bit less apparent (by 209%, 24 hours, comparing with PDLLA-PEO), so this density still remained lower than that on PDLLA (by 58.5 and 59.4%, 6 and 24 hours after seeding). Regardless, it was significantly higher compared to that on the PEO-containing copolymer (by 209%, 24 hours, FIG. 1).

Similarly, the adhesion area of VSMC seeded on antiadhesive PEO-containing copolymer was significantly lower (by 81.4 and 56.3%, 6 and 24 hours) in comparison to PDLLA surface. Addition of GRGDSG sequences to this copolymer markedly improved the adhesion ability of VSMC. Six hours after seeding, on the PDLLA-PEO with 5% and 20% of GRGDSG, the cell spreading areas were higher by 1038.2 and 806.4%, respectively, in comparison with non-grafted copolymer, and even higher than on PDLLA (by 68.9% and 112.4%, respectively). On 24 hours after seeding, spreading area of VSMC (polymer with 5 and 20% GRGDSG) nearly reached the value obtained on PDLLA and was significantly higher than that on PDLLA-PEO copolymer (by 132.6 and 60.9%, respectively).

Hematoxylin-eosin staining of VSMC (24 hours after seeding) on different polymeric surfaces showed polygonal VSMC widely spread on PDLLA surface (Fig. 2 A). However, on the PDLLA-PEO copolymer, the cells were usually not spread, round, floating in the culture media and less viable (FIG. 1 B). Attachment of GRGDSG peptide, a ligand for cell integrin receptors, to the copolymer surface markedly improved the cell adhesion capacity (FIG. 2 C, D).

PDLLA represents a polymer with a good cell adhesion, similar to that found on conventional cell culture plastics (Bačáková et al. 2003). PEO, is a nonionic, hydrophilic

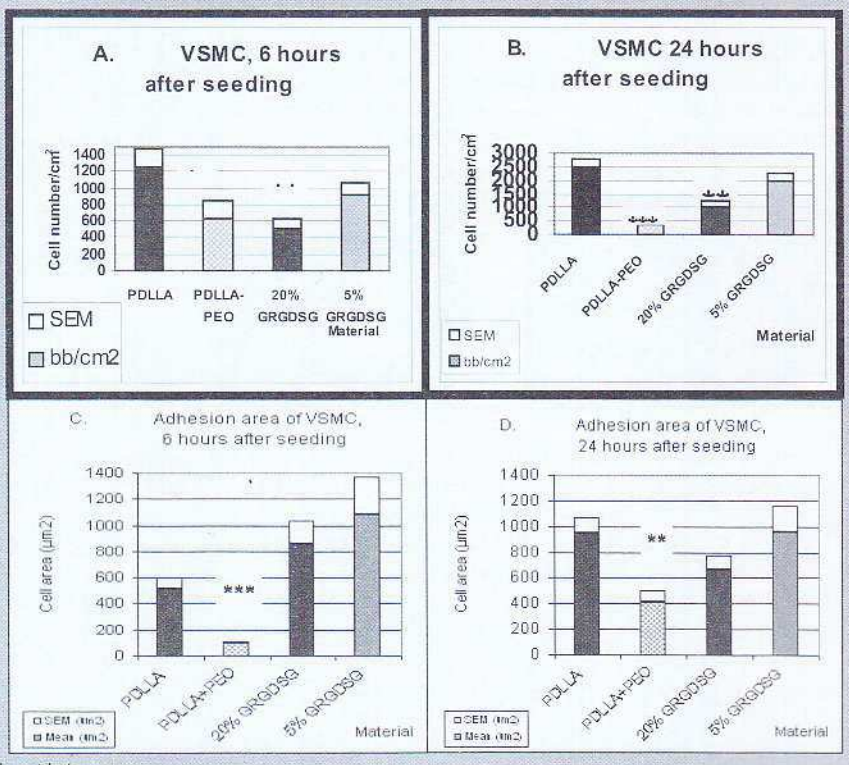

FIG. 1. Population density of VSMC cultivated on different polymeric surfaces on 6 (A) and 24 hours (B) after seeding, adhesion area of VSMC on 6 (C) and 24 hours (D) after seeding. Mean ± SEM from 40 independent fields. Statistical sign.: * p≤ 0.05, ** p≤ 0.01, *** p≤ 0.001

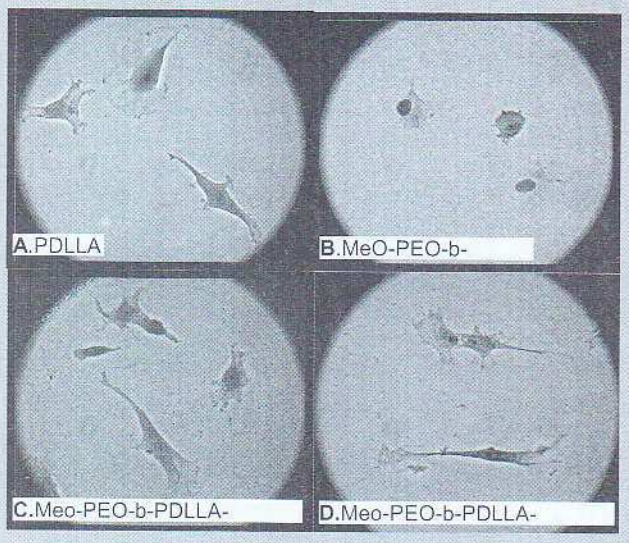

FIG. 2. Morphology of VSMC cultured on different polymeric surfaces. Hematoxylin and eosin staining, 24 hours of cultivation. Phase contrast microscope (Opton, Axioplan, Germany), objective 40x.

antiadhesive polymer which suppresses adsorption of fibronectin (Sofia et al. 1998), fibrinogens (Kim and Kim 2002) and other proteins, mediating cell adhesion to artificial materials through integrin receptors. Because of its excellent inhibiting effects on platelet adhesion (Park and Bae 2002), this polymer is suitable for construction of blood-contacting devices. Copolymer PDLLA-PEO in the present study almost completely prevented cell attachment and spreading on its surface. The adhesion of VSMC on this material was even worse than that on a copolymer of PDLLA with carboxylated PEO in our earlier study (Bačáková et al. 2003). Grafting the PDLLA-PEO with 5 or 20 % of GRGDSG, an oligopeptide present in integrin-binding sites of natural extracellular matrix molecules (e.g., fibronectin, vitronectin, laminin, collagen, osteopontin; Hynes 1999), significantly increased the number and spreading of attached cells almost to the values found on PDLLA or standard cell culture plastics. It was probably caused by specific interaction between GRGDSG and integrin receptors on cells. Surprisingly, the cell adhesion was slightly better on the copolymer with a lower (i.e., 5%) concentration of GRGDSG. This may be explained by more homogeneous distribution or more advantageous spatial conformation and accessibility of this amino acid sequence by cell integrin receptors, and needs further investigation. Nevertheless, this result suggests that the extent of cell adhesion could be regulated by the concentration and distribution of integrin ligands on the artificial polymer surface, which can be applied in prevention of excessive VSMC proliferation on vascular prosthesis.

It can be concluded, that grafting GRGDSG sequence on antiadhesive surface of MeO-PEO-b-PDLLA copolymer can regulate attachment and spreading of VSMC, which gives possibility for further control of proliferation of this cell type on vascular implants.

Acknowledgement

This study was supported by the Grant Agency of the Acad. Sci. CR (grant No. A4050202), Ministry of Education of CR (grant No. LN00A065) and by research project No. AVOZ 5011922 of the Inst. Physiol., Acad. Sci. CR.

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