ADHESION AND PROLIFERATION OF VASCULAR SMOOTH MUSCLE CELLS ON POLYLACTIDE-POLYETHYLENE OXIDE COPOLYMERS WITH DIFFERENT CONTENT AND LENGTH OF POLY-ETHYLENE OXIDE CHAINS

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Abstract

We evaluated antiadhesive effects of polymer surfaces prepared from PDLLA-PEO copolymers using PEO with a different molecular weight and different PEO content in comparison with the native poly(L-lactide) (PLLA) surface. All PDLLA-PEO copolymers significantly decreased number of initially adhered cells (by 23- 55% in comparison with pure PLLA) as well as spreading area 24 hours after seeding (by 39-79%). Cell proliferation, estimated by cell number on the 6 day after seeding and bromodeoxyuridine (BrdU) labeling index, was significantly lower on PEO-containing copolymers (by 58-96% and 21 - 35%, respectively) compared to pure PLLA surface. Imunofluorescence staining of vinculin showed that the ability of VSMC to form focal adhesion plaques was markedly reduced on surfaces with the highest content of PEO (33 and 44%). Thus, these copolymers are promising for creation of surfaces preventing uncontrolled adsorption of proteins and adhesion of cells. Consecutively, binding of defined ligands for cell adhesion receptors would enable to control cell behaviour on these materials, which could be used for construction of vascular prostheses.

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Introduction

Polylactide based polymers are extensively studied as a potential material for construction of artificial prosthesis due to their biodegradability and biocompatibility (Aframian et al. 2002, Tsuji et al. 2001) as well as physical parameters enabling preparation of polymer scaffolds (Zhang 2000). Adhesion of VSMC on PLLA is similar as on conventional culture plastics (Bačáková et al. 2003). PLLA in a form of nano-fibrous scaffolds increases adsorption of ECM proteins and adhesion of osteoblasts in vitro (Woo et al. 2003) as well as supports bone regeneration in vivo (Meining et al. 1996). PDLLA can serve as a drug carrier (Wildemann et al. 2004, Liang et al. 2004). On the other hand, poly(ethylene oxide) is known for its resistance to adsorption of cell adhesion-mediating proteins due to hydrophilic but uncharged nature of the polymer (Groll et al. 2004). In micelles, it can be used as carries for oral DNA delivery in vivo (Chang et al. 2004), or creating reverse thermo-responsive polymers used as a drug delivery system (Sosnik et al. 2004).

Material and methods

High-molecular-weight homopolymers poly(L-lactide) (PLLA) and poly(DL-lactide) (PDLLA) were synthesized by ring-opening polymerisation of the monomer L-lactide and/or DL-lactide in presence of tin(II) octoate as a catalyst. Alpha-methoxy-w-hydroxy poly(ethylene oxide)s (MeO-PEO) were prepared by anionic polymerization of ethylene oxide. Block copolymers MeO-PEO-b-PDLLA (referred as PDLLA-PEO) were synthesized by controlled ring-opening polymerisation of the monomer DL-lactide with MeO-PEO as a macroinitiator in presence of tin(II) octoate as a catalyst. Preparation of polymers has been described in details by Kubies et al. (2000), Rypáček et al. (2001) and Bačáková et al. (2003). The composition of the copolymers varied as followed: the sample LM235 - M_n (PEO) =11 000, M_n (PDLLA)=20 000; the sample LM 285 - M_n (PEO) = 23 800, Mn (PDLLA) = 20 600; the sample LM 286 - M_n (PEO) = 23 800 and M_n (PDLLA) = 10 000. Therefore, we compared the copolymers with short (M_n=11 000) and long (Mn=23 800) length of PEO chain.

The copolymers 285a, b, c, were deposited on polymeric support, prepared in advance on silanized glass coverslips by spin casting of 0.5% wt PLLA solution in chloroform. The set of the copolymer surfaces with different PEO content diluted with the PDLLA homopolymer was prepared by spin casting of the copolymer solutions (1%wt in acetone) on the polymer PLLA support. The copolymer LM 286 was deposited directly on silanized glass by spin casting from the 0.5%wt micellar solution in dioxane/water = 6/4 vol/vol. The final concentration of the PEO phase on the surface of the polymeric film deposited on glass was as followed: the surface LM 235 with 33% wt of PEO phase, surfaces LM 285a, LM 285b and LM 285c with 44,6 %, 33% and 18%wt of PEO phase, respectively, and surface LM 286 with 70.4% PEO.

Both the length of PEO chains and percentage of PEO were reported to affect markedly the cell non-adhesive properties of PEO-PDLLA and related copolymers (Kim and Kim 2002).

The covered glass slides were inserted into 24-well-Nunclon Multidishes (Nalge Nunc Int., Denmark, diameter 1.5 cm) for cell experiments. From two to four samples were used for each experimental group and time interval.

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 15 to 19. Cells were seeded at the initial number of 30 000 cells/well (i.e., population density of 16000 cells/cm^2) into 1.5 ml of Dulbecco-modified Eagle medium (DMEM; Sigma, U.S.A.), supplemented with 10% of fetal bovine serum (FBS; Sebak GmbH, Aidenbach, Germany) and 40µg/ml of gentamicin (LEK, Ljubljana, Slovenia).

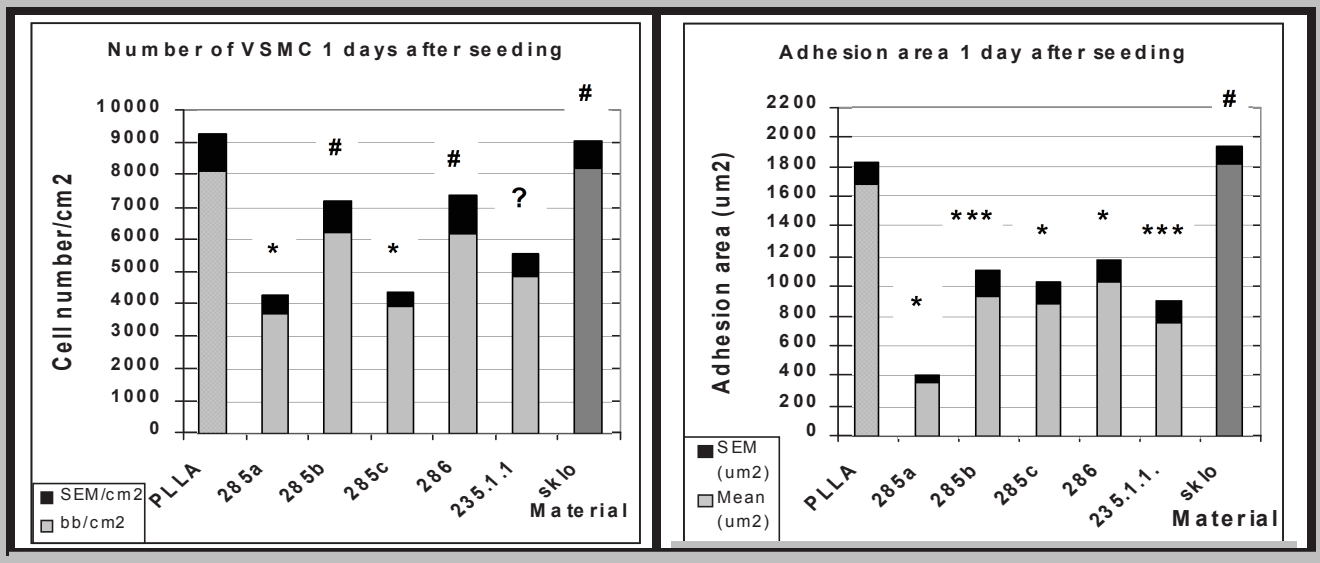

FIG. 1. Population density (A) and adhesion area (B) of VSMC on day 1 after seeding on PDLLA-PEO copolymers with various length of PEO chains and different PEO content. Mean ± SEM, statistical sign.: *** p < 0.001, p < 0.05, ** p < 0.02, * p < 0.01, # p = n.s.

The number of initially adhered VSMC and spreading area were evaluated 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 VSMC was counted in 21-36 randomly selected fields (0.14 mm^2, Olympus, Japan, objective 20x). The size of cell spreading area was measured on microphotographs taken by a digital camera (Olympus, DP70, Japan) using a software Atlas (Tescan, Czech Rep.) in 10-30 microscopic fields for each sample (1-20 cells per field, objective 20x, 0.14mm^2). On day 6 after seeding, cells were counted on 3-4 samples using hemocytometer.

BrdU labelling index was measured in cells three days after seeding. The VSMC were incubated with BrdU for 40 min and incorporated BrdU was immunolabelled using immunoperoxidase method (monoclonal antibody against 5-BrdU, EXBIO Prague, Czech Rep., dilution 1:200). Positively stained cells were counted in 21-30 randomly selected fields (0.25 mm^2, objective 20x) homogeneously distributed in each sample using a phase-contrast microscope (Opton, Axioplan, Germany) equipped with a calibrated eye-piece grid.

Immunofluorescence staining of vinculin for determination of focal adhesion plaques was performed on 3-day-old cultures. The cells were fixed in methanol (5 min, -20°C), pretreated with 3% fetal bovine serum in PBS containing 0.1% Triton X-100 solution (20 min at room temperature). As a primary antibody, monoclonal mouse anti-human vinculin antibody (dilution 1:50, Sigma, U.S.A.) and as a secondary antibody goat anti-mouse IgG fluorescein isothiocyanate (FITC) conjugate (1:200, Sigma, U.S.A.) were used. Digital photographs of cells were taken under epifluorescence microscope (Opton, Axioplan, Germany) using an oil immersion 100x objective.

Quantitative data were given as means ± SEM and statistically evaluated by Student's t- test for unpaired data, using a 5% error probability criterion.

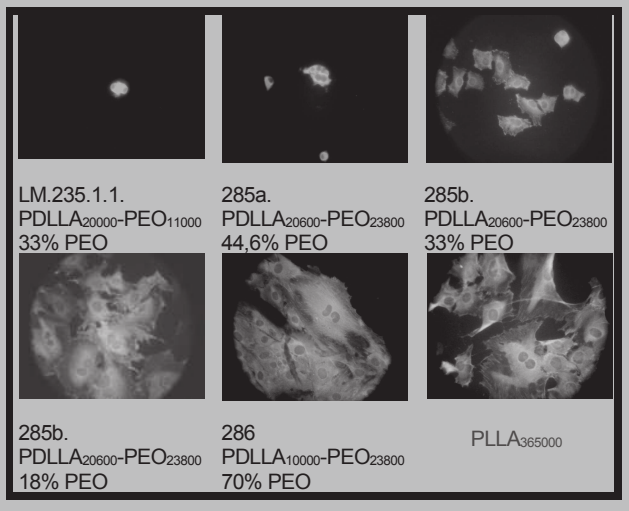

FIG. 2. Immunofluorescence staining of vinculin in rat VSMC on PDLLA-PEO copolymers with various length of PEO chains and different PEO content, 3 days after seeding, microscope Opton Axioplan, objective 100x, immerse oil.

Results and discussion

All types of PDLLA-PEO copolymers showed statistically significant decrease (by 23-55%) in number of initially adhered VSMC 24 hours after seeding (FIG. 1A) in comparison with pure PLLA homopolymer. Spreading area of VSMC was also markedly inhibited by additive of PEO in copolymer (by 39-79%; FIG. 1B). The maximum inhibition of cell adhesion was observed on the sample 285a, i.e. the copolymer with the highest PEO concentration and with the longest PEO chain among samples of the set 285. This could be explained by prevention of adsorption of cell adhesion-mediating proteins from culture medium on PEO in PDLLA-PEO copolymer. Similar results were observed in osteoblast-like cells cultured on poly(L-lactide-co-glycolide) scaffolds modified with PEO (Koegler and Griffith 2004). 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 (Filová et al. 2003).

Immunocytochemical staining of vinculin, an integrin asso-

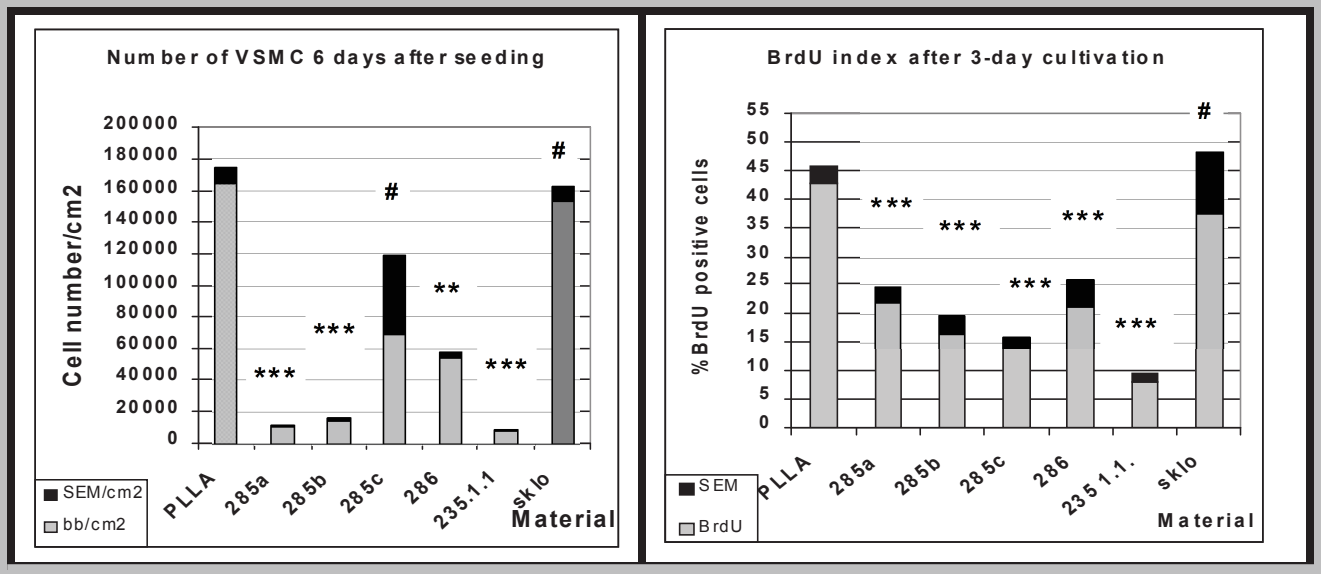

Fig. 3. Population density on day 6 (A) and BrdU labeling index on day 3 (B) after seeding of VSMC on PDLLA-PEO copolymers with various length of PEO chains and different PEO content. Mean ± SEM, statistical sign.: *** p < 0.001, p < 0.05, ** p < 0.02, * p < 0.01, # p = n.s.

ciated protein, revealed that on copolymers 285 the ability of VSMC to form focal adhesion plaques decreases with increasing PEO concentration. On the other hand, the copolymer 286 with very high PEO concentration, i.e. 70%, showed relatively good cell adhesion including formation of focal adhesion plaques. The latter finding, which may be related to different technology of preparation of this material, remain to be elucidated. In addition, on copolymers with comparable PEO content (i.e., LM235.1.1. and 285b, both with 33% PEO), the formation of focal adhesion plaques was better on the copolymer with longer PEO chains (FIG.2), whereas a contrary result was expected (Kim and Kim 2002). On day 6 after seeding, the decrease in cell number on PDLLA-PEO copolymers (by 58-96 % compared to PLLA) after 6-day incubation (Fig. 3A) was even more apparent than that on the first day after seeding (FIG.1) which suggest low or none proliferation activity. Accordingly, BrdU labelling index (FIG.3B), a marker of new synthesized DNA, showed fall (by 21 - 35% compared to PLLA) on samples containing PEO. As cell proliferation is dependent on cell adhesion (reviewed by Bačáková et al. 2004), noticeably, surfaces with very poor adhesion protected cells from proliferation.

Conclusion

On PDLLA-PEO copolymers with 18-44.6% PEO, the reduction of adhesion and proliferation of VSMC is proportional to increasing PEO concentration on the material surface. Thus, the copolymers with higher PEO concentration are promising as inert background for attachment of ligands for cell adhesion receptors in defined concentrations and spatial distribution.

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