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Adhesion and growth of vascular smooth muscle cells in cultures on bioactive RGD peptide-carrying polylactides

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Abstract The surface of poly(L-lactide) (PLLA) films deposited on glass coverslips was modified with poly(DL-lactide) (PDLLA), or 1:4 mixtures of PDLLA and PDLLA-*b*-PEO block copolymers, in which either none, 5% or 20% of the copolymer molecules carried a synthetic extracellular matrix-derived ligand for integrin adhesion receptors, the GRGDSG oligopeptide, attached to the end of the PEO chain. The materials, perspective for vascular tissue engineering, were seeded with rat aortic smooth muscle cells (11,000 cells/cm^2) and the adhesion, spreading, DNA synthesis and proliferation of these cells was followed on inert and bioactive surfaces. In 24-h-old cultures in serum-supplemented media, the number of cells adhering to the PDLLA-*b*-PEO copolymer was almost eight times lower than that on the control PDLLA surface. On the surfaces containing 5% and 20% GRGDSG-PEO-*b*-PDLLA copolymer, the number of cells increased 6- and 3-fold respectively, compared to the PDLLA-*b*-PEO copolymer alone. On PDLLA-*b*-PEO copolymer alone, the cells were typically round and non-spread, whereas on GRGDSG-modified surfaces the cell spreading areas approached those found on PDLLA, reaching values of 991 μm^2 and 611 μm^2 for 5% and 20% GRGDSG respectively, compared to 958 μm^2 for PDLLA. The cells on GRGDSG-grafted copolymers were able to form vinculin-containing focal adhesion plaques, to synthesize DNA and even proliferate in a serum-free medium, which indicates specific binding to the GRGDSG sequences through their adhesion receptors.

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Introduction

The construction of bioactive artificial materials mimicking the natural extracellular matrix (ECM) is a highly advanced approach to tissue engineering. The behavior of cells in contact with biomaterials can be partially controlled indirectly, through the physicochemical surface properties of these materials, such as their surface wettability, electrical charge, roughness or chemical composition [1]. These properties then modulate the spectrum, amount and perhaps the spatial conformation of the adsorbed proteins, including cell-adhesion mediating ECM molecules, and thus modulate the accessibility of specific amino acid

sequences and other ligands for cell adhesion receptors, e.g., integrins [1–3]. However, a direct and more precise control of cell behavior could be better ensured if the bioactive motifs of ECM or other biomolecules, such as hormones or growth and differentiation factors, are attached to a bioinert background resistant to non-specific protein adsorption [4–8]. The cells on these ''templates of natural molecules'' exclusively react to the incorporated signaling groups, and thus their behavior could be effectively controlled by the type, surface density, grouping or lateral distances of the tethered ligands [3, 4, 9–12].

In this study, we focused on the preparation of biomimetic surfaces of biomaterials based on aliphatic polyesters, such as polylactide (PLA), which are the most frequently used biodegradable polymers in tissue engineering. To this end, we used amphiphilic block copolymers composed of polylactide as the hydrophobic block, and poly(ethylene oxide) (PEO) as the hydrophilic block. While the polylactide section ensures good adhesion of the surface-deposited copolymer to the polylactide bulk material, the PEO chain of the copolymer, carrying either an inert methoxy end group or a specific biomimetic group, such as an RGDS peptide sequence, is exposed to the aqueous medium and determines the biomaterial's surface properties. Both inert protein adsorption-resistant surfaces and surfaces with RGDS peptide groups exposed on the inert background were prepared.

Rat vascular smooth muscle cells (VSMC) were used to evaluate the effects of ligand density on the adhesion and subsequent growth of cells, because the newly developed surfaces were intended mainly for vascular tissue engineering. Much less is known about the behavior of VSMC on artificial materials for vascular replacements than about endothelial cells, which are often used for the inner coverage of vascular prostheses. VSMC have usually been excluded from these replacements because of their tendency to excessive proliferation and protein synthesis, resulting in restenosis and occlusion of the graft lumen. However, these cells are the most numerous component of natural blood vessels performing a contractile function, so they should be considered as a necessary component of advanced bioartificial vascular grafts.

Moreover, it follows from our earlier studies on biomaterials with different surface polarity, wettability, roughness, elasticity or spectrum of chemical functional groups, that VSMC react sensitively even to fine changes in the physical and chemical properties of the material surface (for a review, see [1]). Thus they offer a good model for testing the bioactivity of various materials. In addition, these cells are able to produce growth factors, e.g., PDGF-like molecules, in an autocrine manner (for a review, see [1, 13]). This enabled us to stress the role of oligopeptidic ligands with the use of serum-free media not containing cell adhesion-mediating ECM proteins, such as vitronectin or fibronectin, which could interfere with the adhesive oligopeptides incorporated on to the copolymer surface and mask their effects on cell adhesion [4, 11].

Materials and methods

Preparation of the polymer substrates

The polymers, i.e., PLLA, PDLLA, ω-methoxy-poly(ethylene oxide-block-poly(DL-lactide) and GRGDSG-PEO-*b*-PDLLA were synthesized in our laboratory using procedures previously described [14, 15]. The polymer substrates for cell growth were prepared in the form of thin contiguous polymer films cast on a glass support. Circular glass coverslips (12 mm in diameter, Dispolab, Brno, CR) were silanized with dimethyldichlorsilane and a uniform poly-L-lactide (PLLA, M_w = 365,000) film was cast on the silanized surface from a polymer solution in dioxane by spin-coating (PWM32 Precision Spin Coater, Headway Research, USA). The PLLA film in this case represented the surface of a PLLA-based biomaterial scaffold. On top of the PLLA film, a surface-modifying layer with a different polymer composition was cast from acetone solutions. Three types of modifying layer compositions were made: (a) poly(DL-lactide), (PDLLA, M_w = 630,000); (b) an inert, non-adhesive surface formed with a ω-methoxy-poly(ethylene oxide-block-poly(DL-lactide) copolymer (PDLLA-*b*-PEO, with the average molecular weight of the PDLLA and PEO blocks being 18,000 and 11,000, respectively) by depositing a 1:4 (w/w) mixture of PDLLA and PDLLA-*b*-PEO copolymer from acetone solution (the resulting PEO content of the film was 33%). and, by analogy, (c) peptide-modified surfaces, in which a fraction of the PDLLA-*b*-PEO copolymer molecules was substituted with a copolymer containing the GRGDSG peptide attached to the end of the PEO chain, in place of the methoxy end group (GRGDSG-PEO-*b*-PDLLA) [7]. Two series of peptide-modified surfaces were prepared with the same overall PEO content, but with 5% and 20% of the PEO molecules terminated with the GRGDSG peptide.

The polymer-coated glass coverslips were inserted into 24-well-Nunclon Multidishes (Nalge Nunc Int.,

Denmark, diameter 1.5 cm). Two samples were prepared for each experimental group and time interval.

Cell culture on the materials

VSMC were derived from the thoracic aorta of 8-week-old male Wistar SPF rats by explantation [13], and used in passage 20. As revealed by immunofluorescence staining, these cells contained bundles of alpha-actin filaments, which are important markers of VSMC identity and differentiation status (for a review, see [1, 13]). The cells were seeded at a level of 20,000 cells/well (i.e., a population density of about 11,000 cells/cm^2) into 1 ml of Dulbecco-modified Eagle Minimum Essential Medium (DMEM; Sigma, St. Louis, MO, U.S.A.; Cat No. D5648), supplemented with 10% fetal bovine serum (FBS) and 40 μg/ml of gentamicin, or serum-free DMEM with an ITS liquid media supplement (containing insulin, transferrin and selenium; Sigma, Cat. No. I-3146). The cells were cultured for 6–72 h at 37°C in a humidified air atmosphere containing 5% CO_2. The cells were stained with hematoxylin and eosin and then counted in 10–20 randomly selected fields (0.25 mm^2, objective 20×) homogeneously distributed on each sample using a phase-contrast microscope (Opton, Axioplan, Germany) equipped with a calibrated eyepiece grid. The size of the cell spreading area was measured in 24-h-old cultures on microphotographs captured with a DSC-F707 digital camera (Sony Corporation, Tokyo, Japan), using the image processing software Atlas (Tescan s.r.o., Brno, Czech Republic) in 3–7 microscopic fields for each sample (1–8 cells per field, 6–37 cells in total). Cells forming cell–cell contacts were omitted.

The formation of focal adhesion plaques in VSMC on polymers was studied by the immunofluorescence staining of vinculin in 3-day-old cultures as previously described [13]. Briefly, the primary antibody was a mouse monoclonal antibody against human vinculin (Sigma, St. Louis, MO, U.S.A., Cat. No. V-9131; dilution 1:50 in phosphate-buffered saline with 3% FBS, incubation overnight at 4°C). Goat anti-mouse IgG fluorescein isothiocyanate (FITC) conjugate (Sigma, USA, Cat. No. F-8771 was used as the secondary antibody (dilution 1:200, incubation for 1 h at room temperature).

The cell proliferation was evaluated by the shape of growth curves as well as the incorporation of 5-bromo-2-deoxy-uridine (BrdU) into newly synthesized DNA [13]. The percentage of BrdU-labeled cells (i.e., the BrdU-labeling index) was evaluated in ten randomly chosen microscopic fields (0.0625 mm^2, obj. 40× 0–115 cells per field).

Statistical analysis

The quantitative data was presented as mean ± SEM (Standard Error of Mean) from 4 to 20 measurements. Multiple comparison procedures were performed with ANOVA, using the Student–Newman–Keuls method. A value of $p \leq 0.05$ was considered significant.

Results

In serum-supplemented media and 24 h after seeding on PDLLA, the vascular smooth muscle cells (VSMC) attached at a density of 2,510 ± 50 cells/cm^2. On the surfaces made solely of PDLLA-*b*-PEO copolymer, the number of attached cells was strongly reduced, being on average about eight times lower (330 ± 130 cells/cm^2). Modifying this non-adhesive surface to contain 5% or 20% GRGDSG-carrying copolymer increased the number of adhered cells 6- and 3-fold, respectively, in comparison with the pure PDLLA-*b*-PEO copolymer. Thus, on the surface with 5% GRGDSG-PEO-*b*-PDLLA copolymer, the number of cells reached a value of 1,968 ± 8 cells/cm^2, which was close to that found on PDLLA, and on the surfaces with 20% GRGDSG, on average 1,020 ± 420 cells/cm^2 adhered. A similar trend was also observed with the spreading area of VSMC. On PDLLA-*b*-PEO surfaces with 5% and 20% of GRGDSG-PEO-*b*-PDLLA, this area reached 991 ± 238 μm^2 and 611 ± 104 μm^2, respectively, compared to 355 ± 90 μm^2 and 958 ± 133 μm^2 in cells on pure PDLLA-*b*-PEO and PDLLA, respectively. The immunofluorescence staining of vinculin showed numerous streak-like focal adhesion plaques in well-spread VSMC adhering to PDLLA (Fig. 1A), whereas the cells on PDLLA-*b*-PEO were round, often clustered into aggregates and without detectable focal adhesion sites (Fig. 1B). The addition of GRGDSG peptide to the copolymer surface, especially at the lower concentration (5%), markedly restored the ability of the cells to form focal adhesion plaques, although these plaques were usually less numerous, smaller and localized preferentially at the peripheral edges of the cells (Fig. 1C, D). However, the cells on both GRGDSG-containing polymer surfaces were viable, capable of DNA synthesis and proliferation. On day 3 after seeding, their BrdU labeling index amounted to 6.8 ± 2.2% and 21.4 ± 5.7% on the surfaces with 5% and 20% GRGDSG, respectively.

In serum-free media, the number of cells initially adhered to the copolymer surfaces with 5% and 20% GRGDSG-copolymer fraction was only 790 ± 118

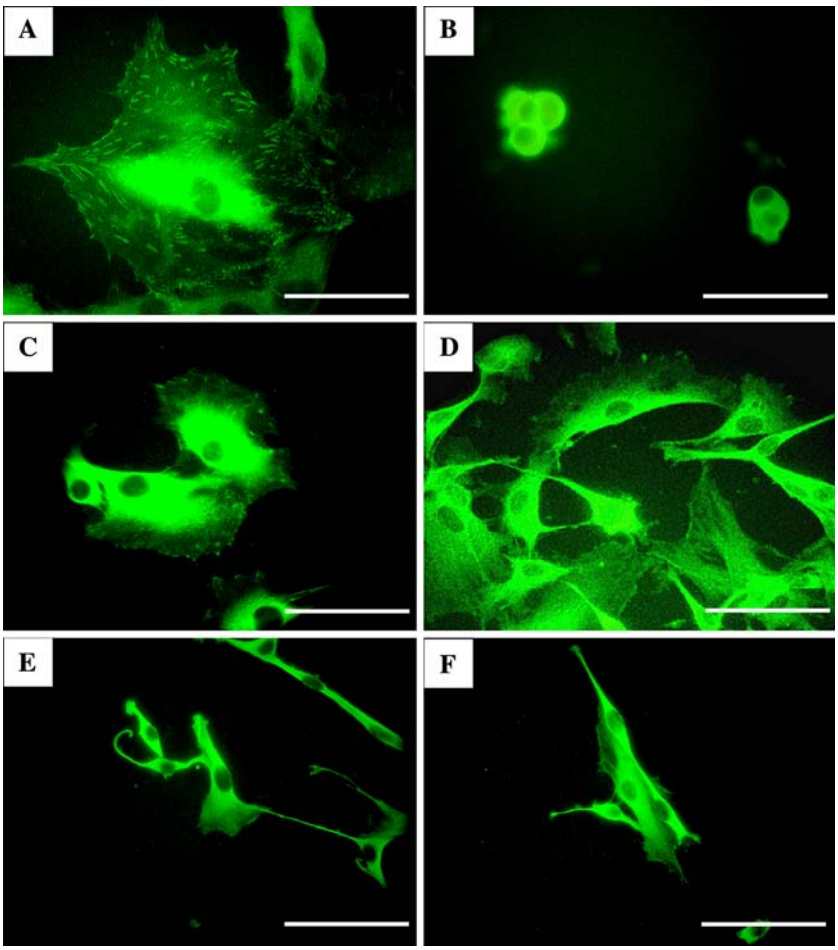

Fig. 1 Immunofluorescence staining of vinculin in vascular smooth muscle cells on day 3 after seeding on polymeric surfaces. (**A**–**D**) Medium supplemented with 10% FBS; (**E**, **F**) serum-free medium. (**A**) Poly(DL-lactic acid), PDLLA; (**B**) Block copolymer of poly(DL-lactic acid) and poly(ethylene oxide), PDLLA-*b*-PEO; (**C**, **E**) PDLLA-*b*-PEO with 5% GRGDSG-PEO-*b*-PDLLA; (**D**, **F**) PDLLA-*b*-PEO with 20% GRGDSG-PEO-*b*-PDLLA. Bar = 100 μm

and 379 ± 99 cells/cm^2, respectively. However, this number was still about four times and twice as high as that on pure PDLLA-*b*-PEO. The vinculin in these cells was distributed both in a homogeneous granular fashion and also organized in tiny focal adhesion plaques, though smaller and less numerous than those in the corresponding cells in serum-supplemented media (Fig. 1E, F). Nevertheless, the cells on the GRGDSG-bearing polymers were relatively well spread. Their spreading areas (348 ± 35 and 707 ± 265 μm^2 for 5% and 20% GRGDS-copolymer fraction, respectively) were significantly larger (about 3.5 and 7 times) than those on PDLLA-*b*-PEO alone (98 ± 18 μm^2). In addition, these cells were spindle-shaped or polygonal, whereas on both PDLLA and PDLLA-*b*-PEO, usually no vinculin-stained cells were found, and if they were, these cells were completely round or fragmented, and vinculin was distributed homogeneously without any signs of assembly into focal adhesion plaques. From 24 to 72 h after seeding, the cells on both GRGDSG-containing surfaces started to incorporate BrdU and divide, whereas on PDLLA and PDLLA-*b*-PEO, they underwent a progressive decline and showed no signs of DNA synthesis (Figs. 2, 3). As a result, on day 3 after seeding into serum-free media, the cells on both GRGDSG-containing surfaces reached a population density 8 to 10 times higher than the values on PDLLA or PDLLA-*b*-PEO alone (Fig. 2).

Discussion

We have demonstrated a versatile method for making the surfaces of synthetic polymer biomaterials biomimetic, in the sense that they provide solid-phase signals to cells through their adhesion receptors and thus stimulate cell adhesion, spreading, and viability in an analogous way to the native extracellular matrix. We focused on synthetic biomaterials based on aliphatic polyesters, a family of polymers including polylactides, polyglycolide (PGA), polycaprolactone (PCL) and related copolymers, e.g. poly(lactide-co-glycolide), PLGA, etc. These polymers are currently probably the most widely used biodegradable synthetic materials

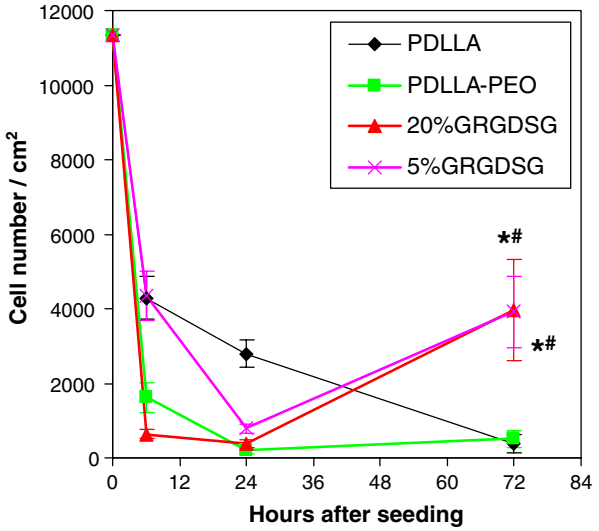

Fig. 2 Growth curves of VSMC cultured in a serum-free medium on poly(DL-lactic acid) (PDLLA), a block copolymer of poly(DL-lactic acid) and poly(ethylene oxide) (PDLLA-*b*-PEO), and the PDLLA-*b*-PEO copolymer in mixture containing 5% or 20% GRGDSG-PEO-*b*-PDLLA. Mean ± SEM from 20 to 40 microscopic fields homogeneously distributed on two samples. Statistical significance: $p \leq 0.05$ in comparison with PDLLA (*) and PDLLA-*b*-PEO (#), respectively; evaluated by ANOVA

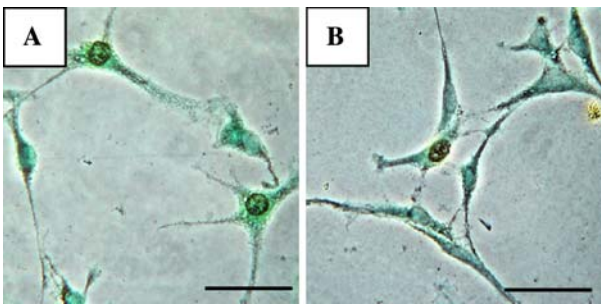

Fig. 3 Immunoperoxidase staining of bromodeoxyuridine (BrdU) incorporated into DNA newly synthesized in VSMC cultured for 3 days in serum-free medium on PDLLA-*b*-PEO with 5% (**A**) or 20% (**B**) GRGDSG-PEO-*b*-PDLLA. Samples counterstained with light green. Bar = 100 μm. BrdU labeling index: 5.4 ± 3.8% (**C**) and 7.1 ± 5.2% (**D**)

for various biomedical applications, and are highly relevant as materials for scaffolds in tissue engineering [16]. Polylactides, i.e. PLLA and PDLLA, were chosen as representatives of this group because of their good biocompatibility and safety for living cells [10]. In comparison with polyglycolides, these materials are more resistant to mechanical stress [11] as well as hydrolytic degradation [12, 17].

Both PLLA and PDLLA exhibit good cell adhesion by supporting the adsorption of ECM proteins contained in the serum supplement of the culture medium. The cells bind to specific amino acid sequences, e.g. Arg-Gly-Asp (RGD) in the structure of ECM proteins such as vitronectin and fibronectin, through their adhesion receptors, mainly integrins [1–3, 12, 18]. However, the adsorption of proteins to biomaterial surfaces is random, and their effect on cell adhesion is rather unspecific. To get more specific control over the nature of signal communication at the biomaterial–cell interface, better defined biomimetic surfaces that contain specific peptide segments are sought. The role of surface-exposed adhesion peptide sequences on specific integrin-mediated cell adhesion and cell phenotype development has been proved in various model systems, where the peptides have been attached to silica, glass or gold surfaces, the substrates also enabling control over their surface distribution by using advanced surface patterning techniques [19–21].

In the work presented here, we prepared biomimetic polylactide surfaces by the surface deposition of amphiphilic block copolymers containing a polylactide block. While the PDLLA chains of the copolymers adhere well and/or entangle with the PLLA or PDLLA bulk material underneath, the hydrophilic PEO blocks of the copolymer molecules, when placed in an aqueous environment, form a tethered PEO layer, which can either be inert or can carry biomimetic peptides on the end of some of the PEO chain. By combining different copolymers in selected proportions, the desired surface mosaic of biomimetic groups can be achieved. To reveal the function of exposed peptide ligands, the unspecific adsorption of proteins that would otherwise screen the effect of the attached peptides has to be suppressed. This is achieved by using a PEO-*b*-PDLLA copolymer with suitable molecular parameters as an inert non-adhesive background.

The mechanism of the protein resistance of PEO-tethered coatings stems from the low affinity between PEO and proteins, which is attributed to the high hydration and mobility of the PEO chains [22]. A tethered PEO layer generates an activation barrier against the protein's approach to the surface due to steric hindrance, and thus markedly slows down the adsorption. A continuous barrier is formed when the layer is in a brush state [23]. The efficacy of PDLLA/PEO block copolymers in changing the surface hydrophilicity and protein resistance depends on the molecular parameters of the copolymers, the details of which are reported elsewhere [14, 24]. In accordance with the above assumptions, the modification of PLA surfaces with pendant PEO chains rendered the PLA surface non-adhesive for VSMC in this study. This result can be explained by an increased surface hydrophilicity and the steric effects of tethered PEO chains, which do

not allow the stable adsorption of cell adhesion-mediating ECM molecules [6, 25]. Only sparse residual cell adhesion has been found on the PDLLA-*b*-PEO copolymer, which might be due to a certain discontinuity of PEO coverage and partial exposure of PDLLA on the surface [9].

When a fraction of the MeO-PEO chains were replaced with PEO chains modified with ligands for integrin receptors, i.e. the GRGDSG oligopeptide, the cell adhesion ability of the surface was almost fully restored. The effect of the GRGDSG peptide exposed on an otherwise cell non-adhesive background became even more evident in a serum-free medium, where only on GRGDSG-modified surfaces the cells were able to assemble vinculin-containing focal adhesion plaques, synthesize DNA and proliferate, which are signs of specific functional binding and signal transduction through adhesion receptors [1–3, 12, 18]. Nevertheless, the affinity of cells to our GRGDSG-modified copolymer was lower than to the polylactide in a serum-supplemented medium, which adsorbed entire natural ECM molecules. The lower bioactivity could be either due to the lack of a synergistic sequence, such as Pro-His-Ser-Arg-Asn (PHSRN) on the fibronectin molecule, or other RGD-cooperating sequences, which help maintain the appropriate spatial conformation of both integrin receptors and their ligands [3, 18, 26], or due to improper surface distribution or spacing of the GRGDSG peptides, or their insufficient accessibility at the copolymer surface. The need for the optimum ligand spacing is also indicated by the difference between surfaces with 5% and 20% GRGDSG-copolymer fraction.

The attachment and spreading of VSMC on GRGDSG-containing polymers was better in serum-containing than in serum-free media. It is assumed that in the serum-supplemented medium, a residual adsorption of cell adhesion-mediating ECM molecules to the surfaces can contribute to cell adhesion [4, 11]. We believe that the versatility of systems based on combining block copolymers with different functionalities would enable a more detailed study of these factors.

In addition, the use of different functionalities, preferred by certain cell types, could help us in obtaining regionally-selective adhesion of endothelial and vascular smooth muscle cells in three-dimensional bioartificial vascular grafts. Preferential adhesion of endothelial cells on the luminal surface of the graft could be achieved by attaching oligopeptides containing the amino acid sequence REDV, whereas the tunica media could be reconstructed by binding oligopeptides containing VAPG or KQAGDV, preferred by VSMC, into polymeric scaffolds [3, 9, 12].

After vascular damage in vivo or explantation in cell culture conditions, VSMC usually undergo so-called phenotypic modulation, i.e., a transition from the contractile to the synthetic phenotype, and activate their proliferation. However, we suppose that the growth of VSMC could be controlled by appropriate concentration, spacing and distribution of adhesion ligands, enabling good spreading and high adhesion strength of these cells. It is known that the migration and proliferation of cells including VSMC is highest at intermediate adhesion strength. If the cells adhere at high initial numbers, with a large spreading area and with numerous focal cell-substrate contacts, they could skip the migratory and proliferative phase and enter the differentiation program [1, 12, 13]. The growth control of VSMC could be further enhanced by long-term release of antimigratory and antiproliferative agents incorporated into the prosthesis material (for a review, see [1]).

Conclusions

A synthetic polymer biomaterial mimicking the function of the ECM was designed, consisting of PLLA bulk material coated with surface-deposited PDLLA-*b*-PEO copolymers functionalized with GRGDSG peptides. When the ECM biomimetic motifs were present, they were recognized and bound by the adhesion receptors of vascular smooth muscle cells, rendering the cells able to adhere, spread and proliferate even in a serum-free medium. Though the effect of biomimetic peptides alone was still lower than that of natural ECM molecules adsorbed on polylactide from the serum-supplemented culture media, the versatility of the system based on the surface deposition of functional block copolymers represents a promising approach to a new generation of biomaterials for tissue engineering, e.g. for the reconstruction of the blood vessel wall.

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