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ADHESION AND GROWTH OF VASCULAR SMOOTH MUSCLE CELLS ON PROTEIN ASSEMBLIES FOR BIOMATERIAL COATING

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Abstract

We investigated adhesion and growth of vascular smooth muscle cells (VSMC) on proteins assemblies prepared by coating of thin fibrin net with ultrathin layer of collagen I, fibronectin or laminin. The assemblies were deposited on polystyrene (PS) non-modified with plasma discharge, i.e. a treatment commonly used for creation of surfaces suitable for cell cultivation. All samples with fibrin assemblies improved initial attachment and spreading of VSMC as well as their subsequent growth in comparison with the pristine PS. The adhesion area of VSMC increased on fibrin coated with collagen I and fibronectin, while on laminin it remained similar as that on the pure fibrin. Thin 3D fibrin net markedly changed the morphology of VSMC from polygonal to star-like shape with protrusions. Cells all tested samples formed vinculin-containing focal adhesion plaques and α-actin cytoskeleton, an important marker of VSMC differentiation. The protein assemblies can be used for improvement of cell adhesion and growth on various artificial materials used in tissue engineering, e.g. for construction of bioartificial vascular prostheses.

[Engineering of Biomaterials, 47-53,(2005),9-12]

Introduction

Artificial implants into the human body are often made of bioinert materials not allowing colonization with cells, thus these materials cannot be considered as adequate and long lasting substitutes of the natural tissues. The latter concerns especially currently used vascular prostheses made of polyethylene terephtalate or polytetrafluoroethylene, i.e. highly hydrophobic polymers not supporting vascular tissue regeneration. Therefore, there is an effort to improve the cell adhesion and spreading by various physical and chemical modification of the material surface, e.g. by irradiation with ultraviolet light (Heitz et al. 2003), bombardment with C^+, O^+ and other ions (Bačáková et al. 2001), plasma discharge, covalent binding of ligands for cellular adhesion receptors, e.g. RGD, REDV (Bačáková et al. 2004, Benoit and Anseth 2005) or controlled deposition of layers containing extracellular matrix (ECM) molecules (Brodie et al. 2005).

In the present study, we evaluated the adhesion and growth of VSMC on fibrin assemblies on polystyrene, and the influence of coating these assemblies with collagen I, fibronectin or laminin, important components of the ECM in blood vessels.

Material and methods

Series of biomolecular assemblies on polystyrene surfaces (PS) were prepared at room temperature in wells of tissue culture plates non-treated with plasma discharge (Polystyrene Non-Tissue Treated Plate, 24 wells of diameter 1.5 cm, Falcon Multiwell TM). Five columns, each containing 4 wells, on a plate were coated with different assemblies; the sixth column was left uncoated as a reference sample. Fibrin (Fb) network was attached to PS surface and further coated with fibronectin (FN), laminin (La) or collagen I (CO I), or mixed with CO I. Therefore, we prepared the following samples: 1. PS- Fb, 2. PS- Fb/FN, 3. PS- Fb/La, 4. PS- Fb/CO I (i.e., collagen-coated Fb), 5. PS-(Fb+CO I), i.e. Fb mixed with collagen, 6. PS. The preparation of assemblies on PS was started with adsorption of fibrinogen (Fbg) from TB (TRIS buffer, 50 mM

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0.1 M NaCl, pH 7.4) at 2 µg/ml for 1 hour. Then Fbg molecules in the adsorbed monolayer were converted to Fb with thrombin, 2 NIH U/ml, in TB for 10 minutes. After washing the surface with TB solution of Fbg 200 µg/ml in TB was added. During the following 15 minutes, the Fbg molecules present in solution in the vicinity of the surface were converted to Fb by enzymatic activity of thrombin molecules irreversibly bound to the primary Fb monolayer, and an Fb fiber network grew up from the surface. FN at 50 µg/ml, La at50 µg/ml, and CO I 100 µg/ml were adsorbed from TB onto the Fb networks forming assemblies 2,3, and 4, respectively. Fb network with incorporated CO I (assembly 5) was prepared from TB solution containing 200 µg/ml of Fbg and 100 µg/ml of CO I.

Vascular smooth muscle cells (VSMC) were derived from the thoracic aorta of 8-week- old male Wistar SPF rats by explantation method (Bačáková and Kuneš 1995), and used in passage 5. Cells were seeded at the initial number of 30 000 cells/well (i.e., population density of about 18000 cells/cm^2) into 1.5 ml of Dulbecco-modified Eagle medium (DMEM; Sigma, U.S.A.), supplemented with 10% of fetal bovine serum (Biomedica, Czech Republic) and 40µg/ml of gentamicin (LEK, Ljubljana, Slovenia).

The number of initially adhered and spread VSMC, as well as the size of cell spreading area, were evaluated 24 hours after seeding using either native living cultures or fixed samples stained with Gill`s hematoxylin and eosin (Sigma, St. Louis, MO, U.S.A.). Cells were counted on 4 samples (in total on 31-49 randomly selected fields of 0.14 mm^2, phase-contrast microscope 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 20-26 microscopic fields for each sample (121-176 cells, objective 20x, 0.14mm^2). On day 3 after seeding, 4 samples of each type (in total 54-66 microscopic fields) were used for counting of cell number. On day 7 after seeding, cells on four samples of each type were counted by Coulter Particle Counter (Coulter Electronics Ltd., Florida, U.S.A.). Bromodeoxyuridine (BrdU) labelling index, i.e. a marker of cell proliferation, was measured in cells three days after seeding. After 40 min incubation of cells with BrdU added to the culture media (final concentration $4x10^{-5}$ M), the BrdU incorporated into newly synthesized DNA was visualized using immunoperoxidase method (monoclonal antibody against 5-BrdU, EXBIO Prague, Czech Rep., dilution 1:200). Stained cells were counted in 15-16 randomly selected microscopic fields (0.14 mm^2, objective 20x).

Immunofluorescence staining of vinculin, a protein of focal adhesion plaques, and α-actin, a contractile protein and marker of VSMC differentiation, was performed in 4-day-old cultures. The cells were fixed in methanol (5 min, -20oC), pretreated with 3% foetal bovine serum in PBS containing 0.1% Triton X-100 solution (20 min at room temperature). As primary antibodies, mouse monoclonal anti-human vinculin antibody (dilution 1:100, Sigma, U.S.A.) and mouse monoclonal anti-α-smooth muscle actin, clone 1A4 (dilution 1:200, Sigma, U.S.A) were used for incubation overnight at 4°C. As a secondary antibody, goat anti-mouse IgG fluorescein isothiocyanate (FITC) conjugate (1:200, Sigma, U.S.A.) was applied for 1 hour at room temperature. Digital microphotographs of cells were taken under inverted epifluorescence microscope (Olympus, Japan) by a digital camera (Olympus, DP70, Japan), 20x 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.

Results and discussion

In comparison with the unmodified polystyrene, all protein assemblies significantly improved the VSMC spreading. On day 1 after seeding, the largest cell adhesion area was achieved on fibrin coated with fibronectin, whereas the lowest spreading was found on the pure or laminin-coated fibrin net (FIG.1 and 2A). Interestingly, on the latter sample (i.e., PS-Fb/La), the small cell spreading area was associated with star-like shape of cells, characterized by multiple thin, long and branched protrusions and resembling the morphology of neurons, whereas the well-spread cells on fibronectin-coated fibrin were of polygonal shape typical for cultured VSMC (FIG.1B, C). However, during the degradation of fibrin, which lasted from several hours to about three days, the cells changed gradually their star-like morphology to the standard polygonal shape (FIG.1A, B, D). Laminin or collagen films, deposited on fibrin net, reduced the rate of fibrin degradation, thus the star-like morphology was most persistent on these samples (FIG.1C, E).

The size of cell spreading area is an important marker of cell adhesion and markedly influences the following cell proliferation, differentiation, motility and other cell functions (for review see Bačáková et al 2004). In addition, the fibrin network used in this study represented, at least partly, a 3D system, which has been reported to have an important influence on the morphology and behaviour of cells. For example, 3D collagen matrices supported the differentiation and development of specialized functions in endothelial cells, e.g. vasculogenesis (Davis et al. 2002).

The largest cell spreading area on fibronectin-coated fibrin was accompanied by a relatively high number of initially adhered cells on day 1, although the highest cell number was reached on fibrin nets coated with collagen I. Conversely, the lowest cell number was found on the unmodified polystyrene, i.e. the sample with the smallest cell adhesion area (FIG.2B).

Similarly on day 3 after seeding, the cell population densities were also higher on the protein assemblies than on the pure PS surface, namely by 39, 65, 43, 91 and 63% on PS-Fb, PS- Fb/FN, PS- Fb/La, PS- Fb/CO I and PS- (Fb+CO I), respectively. Surprisingly, on the pure PS, the number of BrdU-labeled cells, i.e. cells synthesizing DNA, was similar or even higher in comparison with that on the protein assemblies, which supports the idea that the cells are more active in proliferation at the intermediate rather than at the highest extent of adhesion (for review see Bačáková et al 2004). Relatively high BrdU labelling index was also found in cells on the pure fibrin net, i.e. another sample with relatively low initial cell adhesion and spreading. As a result, the cells on the latter sample reached the highest final population density on day 7 after seeding (FIG.2C). On the other hand, the DNA synthesis was the lowest on the fibrin coated with laminin, also characterized by a low initial cell adhesion, which indicated that the extent of initial cell adhesion is not the only factors controlling the subsequent cell growth (FIG.2D).

Immunofluorescence staining of vinculin performed on day 4 after cell seeding showed that dot-like focal adhesion plaques, i.e. the sites on cell membrane in which the adhesion receptors bind the ECM proteins, were visible in cells on all tested samples, especially on the pure fibrin and fibrin covered or mixed with collagen I. Finer and less detectable focal adhesions were observed in cells on fibrin coated with fibronectin or laminin as well as on the pure PS (FIG.3), i.e. on samples which showed the extreme values of the BrdU labeling index (i.e., the highest on Fn and PS, and the lowest on La). Immunofluorescence staining of α-actin re-

vealed that this contractile protein was present in all VSMC, but the distinct α-actin-containing microfilament bundles were apparent preferentially in cells of a large spreading area.

Conclusion

Protein assemblies composed from fibrin net coated with extracellular matrix proteins collagen I, fibronectin or laminin support and regulate the adhesion and proliferation of VSMC. These layers are promising for surface modifications of artificial materials developed for tissue engineering, such as construction of bioartificial vascular prostheses.
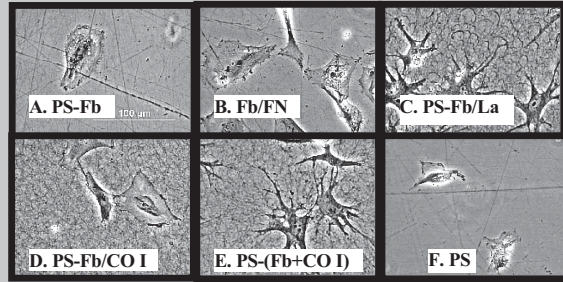
Acknowledgement

FIG. 1. Morphology of VSMC on thin fibrin nets coated with different protein layers (see the Material and Methods) on day 1 after seeding. Living cultures, inversion microscope Olympus, Japan, obj. 20x. PS: polystyrene, Fb: fibrinogen, FN: fibronectin, La: laminin, CO I: collagen I.
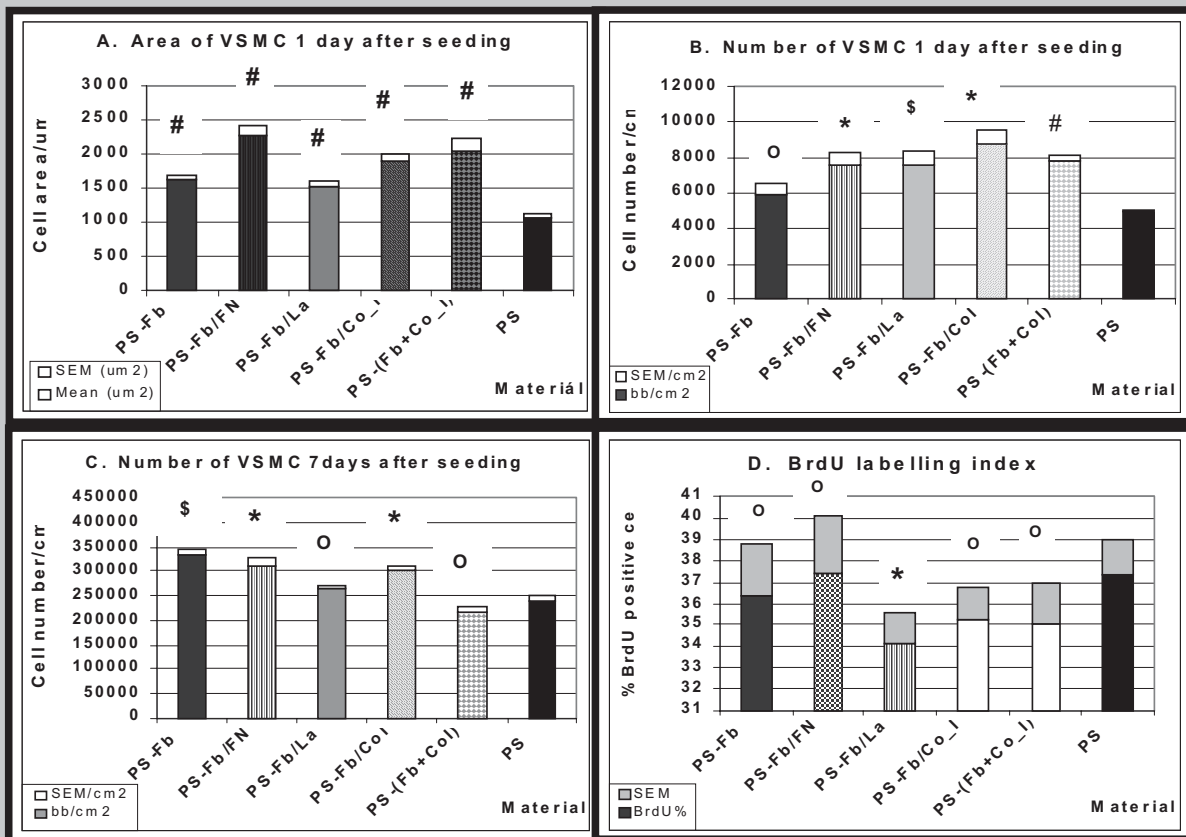

FIG. 2. Spreading area of VSMC 24 hours after seeding (A), cell number 24 hours (B) and 7 days (C) after seeding and BrdU labelling index 3 days after seeding (D) on different protein assemblies (see the Material and Methods). Mean ± SEM, statistical significance: * p<0.01 $ p< 0.02, # p<0.001, O = n.s. in comparison with the values on PS. PS: polystyrene, Fb: fibrinogen, FN: fibronectin, La: laminin, CO I: collagen I.
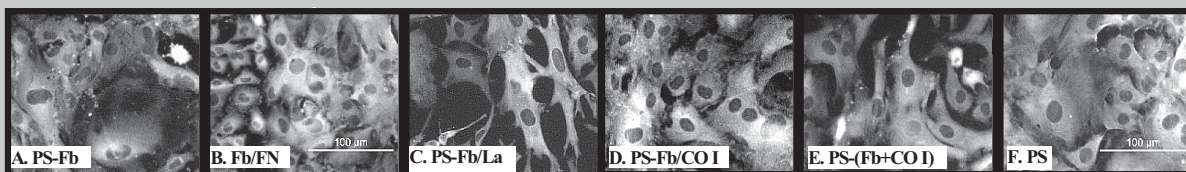

Fig. 3. Immunofluorescence staining of vinculin in VSMC on day 4 after seeding on different protein assemblies (see the Material and Methods). Olympus inversion microscope, obj. 20x. PS: polystyrene, Fb: fibrinogen, FN: fibronectin, La: laminin, CO I: collagen I. Arrows indicate focal adhesion plaques.

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EXTRA-CELLULAR MATRICES FOR TITANIUM IMPLANTS

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Introduction and methods

The success of titanium implants for bone contact is dependent on integration with the surrounding bone tissue, which demands suitable implant surface modification. One approach is the biomimicry of the natural bone extra-cellular matrix (ECM) by coating the implant surface with collagen fibrils, which present binding sites for integrins and are not desorbed when placed in solution. Collagen fibrils can serve as a ground matrix, enabling the inclusion of glycosaminoglycans (GAGs) and proteglycans (PGs) which naturally occur in bone, such as Chondroitin Sulphate (CS), which has been shown to stimulate cell bioactivity in vivo [1], and Decorin. CS and Decorin may be immobilised in collagen fibrils during fibril formation in an appropriate phosphate buffer solution. Synergic effects with growth factors, which can retain their biological activity on collagen, are also possible [2] [3] [4].

In middle ear surgery, adequate mechanical fixation of titanium prostheses to the stapes footplate has not been achieved. Loading an implant whose surface has previously been coated with GAG/PG-containing collagen fibrils with osseoinductive growth factors may result in fixation through osseointegration. The aims of this work are the characterisation of CS- and Decorin-containing fibrils of the collagen types I, II and III for use as coatings, as well as the reaction of primary osteoblasts from human stapes on titanium surfaces coated with these fibrils, both crosslinked and non-crosslinked, in order to select the most suitable coating for animal experiments. The amount of immobilised CS and Decorin in dependence of the ionic strength of the fibrillogenesis buffer was quantified. CS and Decorin were biotinilated and their bioavailability and desorption studied by ELISA. Primary human osteoblasts were obtained from donated human stapes in tissue culture, expanded and plated onto titanium surfaces coated with collagen fibrils. Cellular reactions were investigated with respect to proliferation, alkaline phosphatase (ALP) activity and expression of osteoblastic markers.

Results and discussion

CS/Decorin assays [5], [6] demonstrated the dependence of amount bound per mass unit of fibrils on collagen type and the ionic strength of the fibrillogenesis buffer. Collagen II bound significantly more CS and Decorin than Collagen I and III at both high and low ionic strengths. At high ionic strength a limit for the incorporation of Decorin in Collagen I was reached. Detection of desorbed biotinilated CS and Decorin in phosphate-buffered saline by ELISA showed differences in the desorption profiles of CS and Decorin. Direct ELISA on surfaces coated with fibrils containing biotinilated CS and Decorin showed that both are bioavailable for interactions at the surface.

Osteoblasts from human stapes footplates showed slight differences in proliferation, ALP-activity and expression of osteoblastic markers, depending on the presence of CS or Decorin, the collagen type, and whether crosslinking had been performed. The choice of collagen type appears to have more influence than the presence of Decorin.

Summary

At low buffer ionic strength a higher amount of Decorin and CS is immobilised in fibrils of collagen I, II and III. Collagen type II can bind more CS and Decorin than types I and III. Desorption profiles of CS and Decorin are different. This may affect the release kinetics of growth factors. Primary stapes footplate osteoblast behaviour appears to be influenced by collagen type, crosslinking and presence of CS or Decorin.

Acknowledgements

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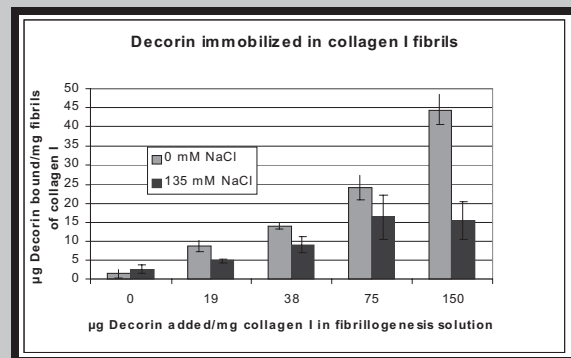[3] Takeuchi Y et al. , *J Biol Chem.* 269(51), (1994), 32634-32638

Diagram: Decorin-binding ability of collagen type I fibrils at low (blue) and high (red) buffer ionic strengths. Increase in ionic strength is achieved by addition of 135 mM NaCl to buffer.