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Distance-dependent adhesion of vascular cells on biofunctionalized nanostructures

Abstract: Cell-cell and cell-extracellular matrix (ECM) adhesion regulates fundamental cellular functions and is crucial for cell-material contact. Adhesion is influenced by many factors like affinity and specificity of the receptor-ligand interaction or overall ligand concentration and density. To investigate molecular details of cell-ECM and cadherins (cell-cell) interaction in vascular cells functional nanostructured surfaces were used Ligand-functionalized gold nanoparticles (AuNPs) with 6-8 nm diameter, are precisely immobilized on a surface and separated by non-adhesive regions so that individual integrins or cadherins can specifically interact with the ligands on the AuNPs. Using 40 nm and 90 nm distances between the AuNPs and functionalized either with peptide motifs of the extracellular matrix (RGD or REDV) or vascular endothelial-cadherins (VEC), the influence of distance and ligand specificity on spreading and adhesion of endothelial cells (ECs) and smooth muscle cells (SMCs) was investigated. We demonstrate that RGD-dependent adhesion of vascular cells is similar to other cell types and that the distance dependence for integrin binding to ECM-peptides is also valid for the REDV motif. VEC-ligands decrease adhesion significantly on the tested ligand distances. These results may be helpful for future improvements in vascular tissue engineering and for development of implant surfaces.

Keywords: Cell Adhesion, Nanostructure, Vascular Cells, Adhesive Peptides

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1 Introduction

Cell-cell as well as cell-extracellular matrix (ECM) adhesion is vital for fundamental cellular functions including motility, proliferation, differentiation and apoptosis [1]. Responsible for cell-ECM adhesion and mediated signaling are complex multi-molecular assemblies consisting of transmembrane integrin receptors and adaptor proteins linking the receptor to the cytoskeleton [2]. Integrins are known to bind to different amino acid sequences (motifs) present in ECM proteins like RGD. RGD-binding integrins like $\alpha_5\beta_3$ are rather universal among different cell types, e.g. with fibroblasts, endothelial cells (ECs) and smooth muscle cells (SMCs) [3, 4]. Different integrins, some exclusively expressed by specific cell types, are specific to other motifs, such as REDV or VAPG. REDV, also a motif of fibronectin, preferentially binds to ECs via $\alpha_4\beta_1$, VAPG binds to SMCs [5, 6, 7]. In addition to ECM-adhesion, cell-cell contacts via cadherin receptors are important for vascular cells and their function.
When investigating the complex process of these receptor-ligand interactions, for example, to design improved vascular implant surfaces, factors, such as affinity and specificity of the ligands to the particular receptor and overall ligand concentration need to be considered in the experimental setup [1, 3, 8].

To address the precise molecular topology of integrin- and cadherin-ligand interaction, nanostructured surfaces were created [8, 9], on which ligand-functionalized gold nanoparticles with molecular dimensions are separated by non-adhesive regions in a defined manner so that individual integrins or cadherins can interact specifically with the precisely immobilized ligands. The system can, therefore, provide an accurate control of inter-ligand lateral spacing, an essential parameter for cell adhesion *(Fig. 1)*. To interfere with ECs and SMCs three ligand systems were used for functionalization of the AuNPs. RGD, REDV and VE-Cadherins were used to observe specific ligands and/or distance-dependent cell spreading and adhesion. The results,

in accordance with recent studies [8, 9, 10], indicate a ligand-distance dependence in vascular cell adhesion.

2 Materials and methods

Substrate preparation Surface patterning is based on self-assembly of di-block copolymer micelles, as described in [12, 14, 19]. Monomicellar films are prepared by dipping a glass slide into a toluene solution with 5 mg mL^{-1} of PS(x)-b-P2VP(y) di-block copolymers and then retracting the glass slide with a speed of 12 mm min^{-1}. Variation in AuNP distance was achieved by using two different molecular weights of the di-block copolymers (240-b-143 and 1056-b-495). In all cases, a part of the 2VP units were neutralized by the addition of $HAuCl_4$ to the toluene, i.e. PS-b-P[2VP($HAuCl_4$)x]. After evaporating the toluene, the remaining monomicellar polymer films were completely removed with H_2-plasma treatment (Tepla Plasma System). This results in extended and highly regular AuNPs, deposited into a hexagonal pattern on glass (*Fig. 1*).

To suppress unspecific cell adhesion to the areas between the AuNPs, the glass was passivated by polyethylene glycol (PEG, PEG2000). Glass samples were pre-activated in an O_2-plasma for 2 min and then transferred into a N_2-filled flask and filled with 10 mL dried toluene. A small amount of PEG as well as a drop of trimethylamine was added and the flaks was heated to 80°C. After 24 hours substrates were intensively rinsed with ethyl acetate and methanol and blow-dried with nitrogen

A cyclic peptide with the RGD-motif and thiol-group was used (^{TM}c(RGDfK)-thiols) for functionalization as described [12]. After PEG passivation, substrates were incubated for 2 hours at room temperature with 20 µL of 25 µM RGD-thiol-solution followed by an over-night incubation with Millipore water. For the functionalization with REDV (American Peptide Company Inc. USA), a linker with a thiol group containing carbonic acid was used. The peptide was dissolved in acetonitrile. Glass slides were incubated for 30 min followed by washing with acetonitrile and blow-drying in a steam of nitrogen. Finally, the samples were incubated with 20 µL of a 5 mM REDV-solution for 1 hour and left over night in Millipore water. For the VEC modification, glass samples were incubated at 48°C over night with 20 µL of 100 µM nitrilotriacetic acid (NTA)-ethanol solution. Subsequently they were washed 20 min in Millipore water and 20 min in HBS-buffer. Afterwards the substrates were incubated for 15 min with 10 mM nickel(II)-chloride-solution at RT. After repeated washing with HBS, the protein could be bound to the AuNPs by incubating the slides over night with 30 µL of a 20 µM VEC solution (Recombinant Human VE-Cad/Fc Chimera, R&D Systems, Inc.), in an aqueous 2 mM Ca^{2+} concentration followed by 3 washing repetitions with HBS.

Surface characterization To investigate the ordering parameter of the nanostructures the samples were characterized with scanning electron microscopy (SEM) directly after production of the substrates, as well as randomly after some cell experiments.

Cell culture Human coronary artery endothelial cells (HCAECs) and human coronary artery smooth muscle cells (HCASMCs) from PromoCell (Germany) were cultured at standard conditions with specific low-serum (2.5%) Cell Growth Media (PromoCell). For experiments on nanostructured surfaces medium containing 1% serum was used. Cells had passage numbers less than six.

Cell experiments All functionalized samples were washed several times in PBS. Each surface condition was tested 3 times with two replicates. Cell spreading area and cell number was quantified after one day. Data were acquired by analyzing microscope images of the cells. After fixation with 3.7% paraformaldehyde (Serva) for 10 minutes at room temperature cells were permeabilized in 0.1% Triton X-100 (Sigma) PBS (Gibco) for 5 min. To block unspecific antibody binding, samples were treated with BSA prior to incubation with the first antibody, anti-paxillin ([Y113], Abcam, monochlonal, rabbit, 1:400 in PBS) for 60 minutes. After washing with PBS the secondary antibody (AlexaFluor 568, Invitrogen) in a dilution of 1:200 was added and incubated for 30 minutes.

3 Results

ECs and SMCs seeded on nano-patterned glass slides (*Fig. 1 A*) were examined after one day by optical phase contrast microscopy. Since only three quarters of the substrates were patterned with adhesive AuNPs and the inter-particle regions were passivated by PEG, a line of cells marking the borderline of the patterned area can be seen (*Fig. 1 B*). Hence the passivation against unspecific cell adhesion was successful and cells could only attach on regions with functionalized nanoparticles. On patterns with different functionalization, we observed that both cell types adhere on the 40 nm patterns, showing a spreading behavior comparable to that seen on a uniform glass surface (*Fig. 2*).
Cell spreading on substrates with 90 nm pattern was reduced. Quiescent cells, presenting a rounded shape that causes scattering of light, and migrating cells with extension could be noticed. After 1 day only a few cells remained attached on 90 nm patterns. Quantitative analysis is given in *Fig. 3*. Both, spreading area and cells/area revealed significant differences between the two AuNPs distances for both cell types and all functionalization. The spreading area is significantly higher

(20% to 50%) on the 40 nm patterns compared to the 90 nm patterns whereas the cell no. is about 50% higher on 40 nm distance patterns than on 90 nm patterns. On all tested linker systems and AuNPs-distances the cells spread to a lesser extent than on uniform glass, except for the SMCs on RGD functionalized AuNPs with 40 nm distances.

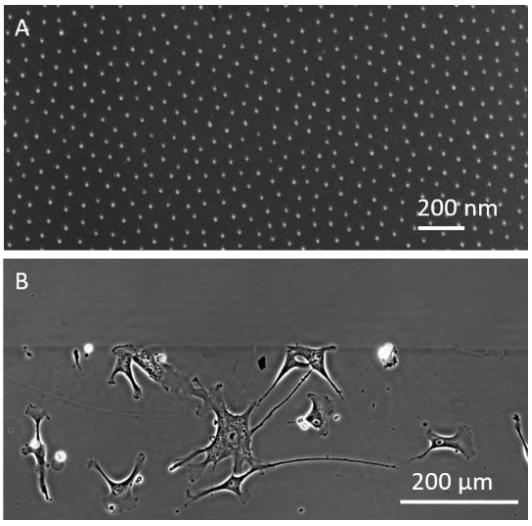

Figure 1: (A) Example SEM micrograph of glass slide decorated with AuNPs, and (B) smooth muscle cells at the dipping line on glass slide decorated with AuNP (here 40 nm distance) and functionalized with RGD. Cell behavior indicates successful passivation in upper part.

The spreading area of both ECs and SMCs is found to be reduced by 20% to 30% on 40 nm distant VEC functionalized surfaces compared to those with RGD and REDV peptides. Though spreading area on surfaces with 90 nm AuNPs is in general smaller compared to the 40 nm patterns, only a significant decrease of cell spreading area for SMCs on VEC compared to the other ligand-systems could be found. The analysis of cells/area indicates differences in ECs and SMCs adhesion. On the VEC functionalized surfaces cell attachment is reduced significantly by more than 40% in comparison to the other two ligands for the same AuNPs distances. On RGD- and REDV-functionalized surfaces with 40 nm distance, both cell types showed similar attachment behaviors as on glass controls. Investigation of focal adhesion formation (data not shown) showed that on 40 nm both cell types built visible focal adhesions, whereas on 90 nm pattern many cells were washed away during the staining procedure. Those left on the surfaces showed poorly recognizable focal adhesions but SMCs, if attached, had clear focal adhesions.

4 Discussion and conclusion

Aiming to gain a better understanding of vascular cell adhesion to specific ligands and density of them, ECs and SMCs were cultured on nano-patterned ECM- or cell-mimicking surfaces.

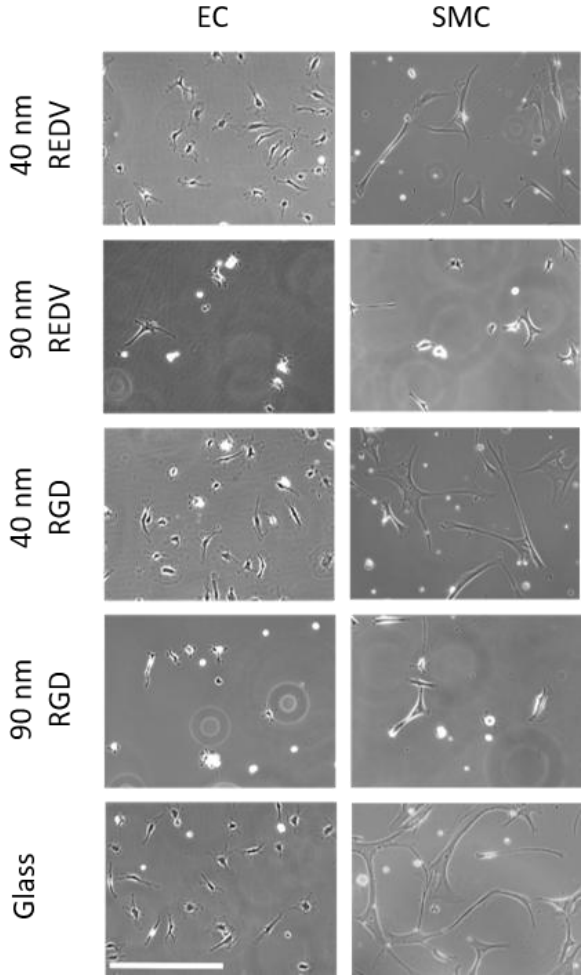

Figure 2: Phase contrast images of ECs and SMCs on given nanostructures and functionalized surfaces as well as on a control glass coverslip surface. Scale bar 400µm.

The array of AuNPs on these surfaces had inter-particle distances of 40 nm or 90 nm and were conjugated either to RGD-, REDV- peptide or VEC. The AuNPs diameter of 8 nm suggests limiting the interaction in a manner that only one receptor-ligand interaction per nano-particle occurs [7, 8, 9]. In previous studies it was shown that functionalization with RGD and specific ligand spacing on the surface is essential for successful assembly of focal adhesions and stress fibers in different cells types. Surfaces with ligand spacing larger than 73 nm failed to induce the formation of such structures [7, 8]. Similar studies using AuNPs arrays functionalized with integrin-specific ligand revealed similar distance-dependencies [10] or demonstrated the ability of

BMP2-functionalized AuNPs arrays to trigger elevated SMAD-signaling pathway activation [11]. In this work we were able to show, that regardless of RGD and REDV-functionalization, NP-distances of 90 nm were less favorable for EC and SMC cell adhesion than distances of 40. This finding is in supported by previously results [8, 9, 10, 11], describing a critical ligand (RGD) density of approximately 67 nm. At larger NP-spacing, integrin adhesion can still take place, yet the adhesions formed fail to develop into stable structures. Our results indicate that previously drawn conclusions about RDG-integrin mediated adhesion might also be found for the REDV-peptide and the associated integrin ($\alpha_4\beta_1$). Additionally, this work suggests that the universal distance-dependence for focal contact formation and cell adhesion, previously shown for RGD and cell types like MC3T3-osteoblasts, REF52-fibroblasts, 3T3-fibroblasts, and B16-melanocytes [7, 8], might be extended to ECs and SMCs. Furthermore, our first results indicate a density-dependence of cell-cell contact stability. Surprisingly, both cell types adhere equally poor on the VE-Cad compared to the integrin-ligands RGD and REDV.

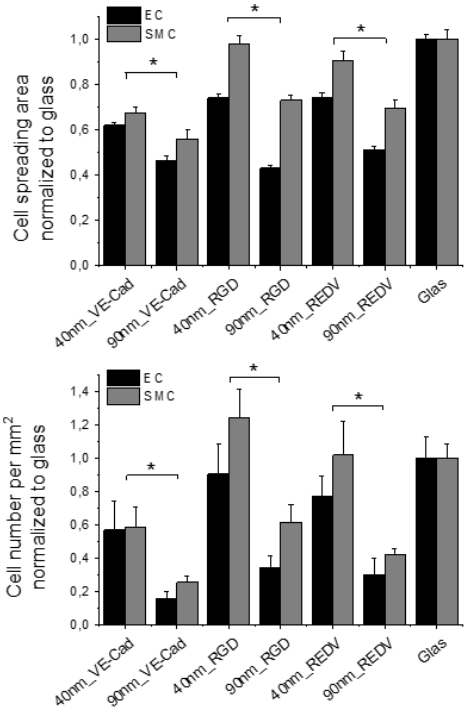

Figure 3: Cell spreading area (top) and cell number (bottom) of endothelial (ECs) and smooth muscle cells (SMCs) on gold structures with several linker systems: VE-Cadherin, RGD, REDV and glass as control; two different AuNP distances per linker system, 40 nm and 90 nm. * indicates significant differences (p=0.05).

Further investigation need to reveal if SMC adhesion on VEC-AuNPs is due to co-adhesion of other ECM molecules to the particles during the experiment. In summary, nanolithography in combination with specific ligand functionalization is an impressive tool for studying cell adhesion phenomena. Due to its simplicity it also has the potential to contribute to the functionalization of implant surfaces.

Author's Statement

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References

[1] Geiger B, Spatz JP, Bershadsky AD. Environmental sensing through focal adhesions. Nat Rev Mol Cell Biol 2009:10:21–33.
[2] Critchley DR. Focal adhesions - the cytoskeletal connection. Curr Opin Cell Biol 2000:12(1):133–139.
[3] Hersel U, Dahmen C, Kessler H. Rgd modified polymers: biomaterials for stimulated cell adhesion and beyond. Biomaterials 2003:24(24):4385–4415.
[4] Massia SP, Hubbell JA. Vascular endothelial cell adhesion and spreading promoted by the peptide redv of the iiics region of plasma fibronectin is mediated by integrin α4β1. J Biol Chem 1992:267(20):14019–14026.
[5] Plouffe BD, Njoka DN, Harris J, Liao J, Horick NK, Radisic M, Murthy SK. Peptide-mediated selective adhesion of smooth muscle and endothelial cells in microfluidic shear flow. Langmuir 2007:23(9):5050–5055.
[6] Gobin AS, West JL. Val-ala-pro-gly, an elastin-derived non-integrin ligand: smooth muscle cell adhesion and specificity. J Biomed Mater Res A 2003:67(1):255–259.
[7] Cavalcanti-Adam EA, Volberg T, Micoulet A, Kessler H., Geiger B, Spatz JP. Cell spreading and focal adhesion dynamics are regulated by spacing of integrin ligands. Biophys J 2007:92(8):2964–2974.
[8] Arnold M., Cavalcanti-Adam EA, Glass R, Blümmel J, Eckn W, Kantlehner M, Kessler H, Spatz JP. Activation of integrin function by nanopatterned adhesive interfaces. ChemPhysChem 2004:5(3):383–388.
[9] Arnold M, Hirschfeld-Warneken VC, Lohmüller T, Heil P, Blümmel J, Cavalcanti-Adam EA, López-García M, Walther P, Kessler H, Geiger B, Spatz JP. Induction of cell polarization and migration by a gradient of nanoscale variations in adhesive ligand spacing. Nano Lett 2008:8(7):2063–2069.
[10] Schaufler V, Czichos-Medda H, Hirschfled-Warnecken V, Neubauer S, Rechenmacher F, Medda R, Kessler H, Geiger B, Spatz JP, Cavalcanti-Adam EA. Selective binding and lateral clustering of α5β1 and αvβ3 integrins: Unraveling the special requirements for cell spreading and focal adhesion assembly. Cell Adh Migr. 2016:10(5):505-515.
[11] Schwab EH, Pohl TL, Haraszti T, Schwaerzer GK, Hiepen C, Spatz JP, Knaus P, Cavalcanti-Adam EA. Nanoscale control of surface immobilized BMP-2: toward a quantitative Assessment of BMP-mediated signaling events. Nano Lett. 2015:15(3):1526-34.