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The significance of integrin ligand nanopatterning on lipid raft clustering in hematopoietic stem cells

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ABSTRACT

Hematopoietic stem cells (HSCs) are the vital, life-long source of all blood cell types. They are found in stem cell niches, specific anatomic locations that offer all the factors and signals necessary for the maintenance of the stem cell potential of HSCs. Much attention has been paid to the biochemical composition of the niches, but only little is known about the influence of physical parameters, such as ligand nanopatterns, on HSCs. To investigate the impact of nanometer-scale spacing between cell ligands on HSC adhesion, integrin distribution and signal transduction, we employed geometrically defined, nanostructured, bio-functionalized surfaces. HSCs proved to be sensitive to the lateral distance between the presented ligands with regard to adhesion and lipid raft clustering, the latter being a prerequisite for the formation of signaling complexes. Furthermore, an extensive redistribution of stem cell markers, integrins and phosphorylated proteins in HSCs was observed. In conclusion, integrin-mediated adhesion and signaling of HSCs proved to depend on the nanostructured presentation of ligands in their environment. In this work, we show that the nanostructure of the matrix is an important parameter influencing HSC behavior that should be integrated into biomaterial-based approaches aiming at HSC multiplication or differentiation.

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1. Introduction

HSCs are the origin of the entire hematopoietic blood system. *In vivo* they reside in stem cell niches, which are specialized microenvironments inside the bone marrow [1]. These niches regulate the maintenance of the HSC number as well as the stem cell potential, which is defined as the ability to self-renew and differentiate into several cell lineages [2]. When HSCs leave their niche and start to proliferate, they also differentiate. The signals which the niche provides to HSCs in order to keep their stem cell character are presented by stromal cells, extracellular matrix (ECM) and soluble factors [3]. The ECM of the niches surrounds the HSCs, providing a major component of their physical microenvironment. Two defining features of the ECM inside the niche are its function as a substrate for cell adhesion and anchorage, and its ability to influence stem cell fate. Details concerning the complex molecular composition and physical properties of the HSC niche ECM are yet to be fully understood [4]. For one prominent protein in the specialized ECM of these stem cell niches, fibronectin, however, effects on HSC proliferation, differentiation and the ability to engraft have been described [5,6]. Interactions between fibronectin and HSCs are mediated through integrins in the plasma membrane of the adhering cell. Integrins are heterodimeric molecules, composed of one α- and one β-chain. Beside their ability to link cells to the ECM, they also function as mechanosensors and bi-directional signaling receptors (outside-in and inside-out signaling) [7]. Integrins recognize bio-active sequences in fibronectin, such as RGD, a thoroughly studied integrin binding-sequence that is (among others) recognized by the integrins αVβ3 and α5β1 [8]. Both of these are expressed by HSCs [9,10].

In order to fulfill their role in signal transduction, integrins seem to depend on lipid raft clustering and functionality [11–13]. Lipid rafts are transient, dynamic, cholesterol- and sphingolipid rich nanoscale membrane domains. They can be labeled with the cholera toxin subunit B (CTB) that binds to a specific lipid raft

Abbrevations: HSC, Hematopoietic stem cell; HSPC, Hematopoietic stem and progenitor cell; ECM, Extracellular matrix; cRGD, Cyclic RGD; CTB, Cholera toxin subunit B.

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Table 1
Charactistics of prepared nanostructured surfaces and the used diblock copolymers. By varying the length of the copolymer and the speed of the dipping process, the distance between the gold particles on the nanostructured surfaces was controlled.

Polymer	Molecular weight [g/mol]	Dipping speed [*V*]	Distance [nm]
PS_{154}–*b*-$P2VP_{33}$	19 500	12	20 ± 6
PS_{240}–*b*-$P2VP_{143}$	40 000	12	32 ± 6
PS_{1056}–*b*-$P2VP_{495}$	162 000	24	58 ± 10
PS_{1056}–*b*-$P2VP_{495}$	162 000	18	90 ± 20

constituent, the ganglioside GM_1 [14]. In response to signaling events, lipid rafts cluster into larger, more stable signal transduction platforms [15]. One important property of lipid rafts is their ability to include or exclude proteins. Thus, they function as platforms which organize the complex interplay between ligands, receptors, kinases and other factors during signal transduction [16]. In HSCs, lipid rafts play an important role in stem cell polarization, migration, differentiation and proliferation [17–22]. Lipid rafts also seem to have an important function in the versatile and complex interactions of HSCs with their niches.

The numerous research projects focused on the identification of the molecular components of the HSC niche microenvironment within the last decades stand in marked contrast to the low number of investigations on the impact of physical parameters, like the ECM structure, on HSCs. The ECM is highly structured down to the level of single molecules (tertiary and quaternary protein structure), in other words, the nanometer range. It is well known that collagen fibers and fibronectin fibrils display nanometer-sized structural features [23,24], and that the geometry of the presentation of cellular ligands in the nanometer range has a strong impact on the behavior of cultured cells [25,26]. For mesenchymal stem cells it has been described that cell fate can depend on the nanostructure of surfaces [27,28]. In addition, HSC adhesion and expansion are enhanced on nanofibers [29]. Therefore, it seems likely that HSCs are able to sense differences in the nanostructure of their environment, to integrate this physical parameter into a biochemical signal, and to react in a specific manner.

In this study we analyzed whether (i) HSCs are sensitive to the nanostructural properties of their microenvironment, and (ii) ligand spacing is necessary and sufficient to control integrin-mediated adhesion of HSCs and lipid raft clustering, which is a prerequisite for signal transduction.

2. Material and methods

2.1. Cells

Cells of the human acute myelogenous leukemia KG-1a cell line were utilized as a model for immature hematopoietic cells expressing the CD34 antigen. KG-1a cells were purchased from DSMZ (Braunschweig, Germany) and cultured in RPMI 1640 (Invitrogen, Karlsruhe, Germany) supplemented with 20% FBS (Invitrogen) at 37 °C and 5% CO_2. Primary human CD34-positive hematopoietic stem and progenitor cells (HSPCs) were isolated from umbilical cord blood by magnetic activated cell sorting (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer's instructions. Isolated HSPCs were maintained in StemSpan® SFEM Medium (Stem Cell Technologies, Grenoble, France) supplemented with the StemSpan® CC-100 cytokine cocktail (Stem Cell Technologies) at 37 °C and 5% CO_2 for 48 h prior to experiments. Umbilical cord blood was obtained from the DKMS cord blood bank (Dresden, Germany) after informed patient consent.

2.2. Flow cytometry

The purity of isolated HSPCs was tested by flow cytometry analysis of CD34 expression. 2×10^4 cells per sample were washed with PBS/0.1% FBS. Anti-CD34-PC5 (clone 581, Beckman Coulter, Krefeld, Germany) was applied for 30 min on ice. IgG1-PC5 (Beckman Coulter) was used as an isotype control. After washing with PBS/0.1% FBS, cells were subjected directly to flow cytometry (FC 500, Beckman Coulter), or fixed with 1% formaldehyde and stored at 4 °C until analysis.

2.3. Preparation of fibronectin-coated surfaces by adsorption

Fibronectin was isolated from human plasma as described elsewhere [30]. Glass bottom cell culture dishes were covered with 10 μg/ml fibronectin in PBS and left at 4 °C overnight. Substrates were washed once before further application. As a control, glass bottom dishes were coated with 1% (w/v) BSA (Serva, Heidelberg, Germany) in PBS under the same conditions.

2.4. Production and functionalization of nanostructured surfaces by diblock copolymer micellar nanolithography

Nanostructured surfaces were produced as previously described [31,32]. The characteristics of the polystyrene-block-poly(2-vinylpyridine) diblock copolymers (PS-*b*-P2VP) and parameters of the dipping process are included in Table 1. Passivation of glass surfaces was achieved by first activating the surfaces in oxygen plasma for 5–10 min. Then the substrates were immersed in dry toluene (Merck, Darmstadt, Germany) containing ∼ 0.1 mg/ml polyethylene glycol-triethoxysilane (molecular weight 2000 g/mol) and 0.1 μl/ml water under nitrogen atmosphere. Triethylamine (Sigma–Aldrich, Taufkirchen, Germany) was added as a catalyst. The reaction was carried out over night at +80 °C. Finally, the substrates were washed twice with ethyl acetate (Acros Organics, New Jersey, USA), twice with methanol (BASF, Ludwigshafen, Germany) and dried under N_2 flow.

To allow a direct comparison between each nanostructured surface (with ligands spaced apart 20 nm, 32 nm, 58 nm or 90 nm) and the unstructured control (an area on the slide passivated with an anti-adhesive and protein-repellent layer of polyethylene glycol), we produced each sample slide with two distinct parts, consisting of a structured area in the lower part of the slide and an unstructured control area in the upper part (Fig. S1).

The gold particles on the nanostructured surfaces were bio-functionalized with 25 μM of a cyclic RGD peptide (Cys-PEG_6-Nε(Lys-Arg-Gly-Asp-D-Phe)$_{cyclo}$ (cRGD) in water for 2 h at room temperature. Excess cRGD was removed by washing with PBS for 5 min. This washing step was repeated 4 more times.

2.5. Cell adhesion experiments

Prior to adhesion experiments, the nanostructured and bio-functionalized slides were washed once with adhesion medium (RPMI 1640, 1% (v/v) penicillin/streptomycin, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 25 μM $MnCl_2$) and then covered with 3 ml adhesion medium. 2×10^6 KG-1a cells or 1×10^5 primary HSPCs were added and allowed to adhere for 1 h under standard cell culture conditions. Non-adherent cells were removed by washing with PBS. Cell adhesion was assessed with an AxioVert 40 CFL microscope (Zeiss, Jena, Germany) using the AxioVision 4.6 software (Zeiss). The number of adhering cells was determined using the ImageJ 1.42q software (http://rsb.

Table 2
List of the antibodies used for immunofluorescence staining.

Antibody	Clone/Antiserum	Distributor	Dilution applied
Rabbit anti human CD34	Polyclonal	Acris Antibodies (Herford, Germany)	1:100
Rabbit anti human integrin β1	EPR1040Y	Novus Biologicals (Littleton, CO, USA)	1:50
Rabbit anti human CD133	Polyclonal	Sigma–Aldrich (Taufkirchen, Germany)	1:500
Mouse anti human integrin α5	NKI-SAM-1	Immunotools (Friesoythe, Germany)	1:100
Mouse anti human integrin αVβ3	23C6	R&D Systems (Wiesbaden, Germany)	1:50
Mouse anti-phospho-tyrosine	P-Tyr-100	Cell Signaling Technology (Beverly, MA, USA)	1:400
Goat anti rabbit IgG - Alexa Fluor 568	Polyclonal	Invitrogen (Karlsruhe, Germany)	1:400
Rabbit anti mouse IgG - Alexa Fluor 647	Polyclonal	Invitrogen	1:400

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