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Nanoscale characterization of cell receptors and binding sites on cell-derived extracellular matrices

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ABSTRACT

Cells are able to adapt their extracellular matrix (ECM) in response to external influences. For instance polymer scaffolds with tunable properties allow for guiding cell adhesion behavior and ECM adaptation in a controlled manner. We propose a new and versatile approach for the investigation of extracellular molecular assemblies at materials interfaces by scanning force microscopy. The distribution of cell adhesion receptors and binding sites of matrix proteins in the investigated ECMs was identified by immunolabeling with 15 nm gold beads. To precisely localize the immunogold in the matrices we utilized electrostatic force microscopy that allows for materials-dependent contrast according to differences in the dielectric properties of the immunolabels. In addition, an image processing routine was developed to localize the immunogold by correlation analysis.

The applicability of our approach for nanoscale characterization of cell-derived ECM was further verified in two independent experiments. We probed the distribution of the cell adhesion receptor $\alpha_5\beta_1$ integrin next to its extracellular ligand fibronectin and the corresponding binding site on the fibronectin molecule.

1. Introduction

The extracellular matrix (ECM) is widely regarded as a key regulator of cell behavior, providing structural and functional support to cells in living tissues, as well as on biomaterials scaffolds. ECM not only provides a physical support but also signals to cells through a range of cell surface receptors which actively couple to various binding sites presented by matrix components [1]. The glycoprotein fibronectin (FN) is one important macromolecular component of the ECM which also carries binding sites for other matrix components. Recent reports have shown that some of these binding sites are cryptic and supposedly exposed through cell-generated tension, which in turn enables the self-assembly of monomeric FN molecules in fibrillar structures [2–4]. The cell adhesion receptor $\alpha_5\beta_1$ integrin binds to the so-called cell binding site consisting of a RGD sequence in the 10th FN repeat domain of type III. Such an interaction allows the cells to actively exert tension, followed by rearrangement of FN to a dense, flexible network.

Biomaterials research relies on biofunctionalization of artificial materials at the interface with living tissue, for instance to achieve biocompatibility of implants. Cells of the connecting tissue respond to the properties of the implant material. Therefore defined physicochemical surface manipulation is used to influence the behavior of individual cells. Such changes most commonly affect a cascade of intracellular physical and signaling events, starting from the cell–substrate interface and finally affecting nuclear gene expression events, else known as mechanotransduction. In turn, this leads to defined changes in the molecular structure of the expressed ECM at the interface with the implants [1,5,6].

One way to characterize these cell adhesion processes on biomaterials is therefore connected to the localization of cell receptors or their corresponding binding sites in the ECM. To trace variations in the arrangement of the ECM on the molecular level it is necessary to attain nanoscale resolution. Recent developments in fluorescence microscopy, such as stochastic optical reconstruction microscopy (STORM) and stimulated emission depletion microscopy (STED) have already been demonstrated to overcome prior limitations in resolution of light microscopy [7–9]. Thus imaging of objects with size of tens of nanometers became possible even under physiological conditions or in living tissue.

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Protein distributions or epitopes are typically found indirectly by closely attaching a label for the respective microscopy technique, like antibodies with fluorochrome labels or genetically modified cells with the expression of fluorescent proteins. Unfortunately such techniques require a very elaborate and expensive setup, as well as specifically designed fluorescent molecules, able to withstand the high-intensity excitation pulses, which may further damage the samples. In electron microscopy (SEM, TEM) nanometer-sized antibody-conjugated gold beads were used for immunohistological staining of epitopes in cells and at the ECM. This approach has already been demonstrated to calculate the spacing and accessibility of domains on fibrillar FN [10,11]. Application of different sized gold beads enables the comparison of distribution and frequency of multiple ECM components simultaneously, e.g. type I collagen and proteoglycans in normal and sclerotic dentin [12]. Nevertheless, almost every application of electron microscopy is restricted to samples under high vacuum conditions and in order to achieve a good contrast, they typically require sample modification steps, such as fixation, heavy metal coating, etc. These conditions restrict the applicability and versatility of these techniques.

In the light of the different restrictions of the mentioned nanoscale microscopy techniques, scanning force microscopy (SFM) provides almost the same flexibility in the choice of imaging conditions and can be used to probe ECM topography and topology at materials interfaces [13]. For instance, receptor sites can be mapped with SFM by prior modification of the probe tip with the corresponding ligand and detection of local attractive forces emerging from specific receptor–ligand interaction [14] as also shown for whole tissue samples [15]. Also immunogold labeling was already demonstrated to be adaptable to SFM. The extracellular domain of $\alpha_{IIb}\beta_3$ integrins reconstituted into planar lipid bilayers was localized in proximity with their corresponding ligands by an immunogold label using SFM [16]. In this imaging mode gold beads were detected by phase shifts in the oscillation of the probe due to varying viscoelastic interactions during the transient tip–sample contact on the hard gold surface in contrast to the pliable lipid bilayer and proteins. The same materials-dependent properties have been used to identify gold-labeled proteins of the ECM network, e.g. different types of collagen [17]. However, conventional phase contrast-based immuno-SFM becomes rather difficult in high resolution applications due to tip size effects.

We present a new approach for the localization of receptors and binding sites that overcomes some of the above-mentioned limitations and proves an achievable resolution in the lower nanometer range. We developed a procedure that is based on specific labeling of epitopes by immunogold with the combined use of electrostatic force microscopy (EFM) for the recognition of the gold through characteristic interactions with an electric field. To illustrate the applicability of our approach first we investigated the distribution of $\alpha_5\beta_1$ integrins in a reorganized matrix of FN fibrils. In a second experiment we investigated the spacing of cell-binding sites along the FN fibrils, which provides interesting arguments on the stretching state of these proteins during fibril assembly.

2. Materials and methods

2.1. Substrate preparation

For a graded non-covalent anchorage of the adhesion ligand FN borosilicate glass substrates were modified with a thin film coating of maleic acid copolymers, as previously described [18]. Briefly, ultrasonicated glass coverslips (Menzel Gläser, Braunschweig, Germany) with a diameter of 24 mm were oxidized in 5:1:1 mixture of double-distilled water, ammonium hydroxide (28%–30%, Acros Organics, Geel, Belgium) and hydrogen peroxide (35%, Merck, Darmstadt, Germany) for 10 min at 70 °C. Stable immobilization of the polymer chains on the coverslips was achieved by covalent binding to amino groups introduced by modification with (3-aminopropyl)triethoxysilane (Sigma-Aldrich, Steinheim, Germany). Specifically, solutions of 0.3% poly(ethylene-alt-maleic anhydride) (PEMA, M_w=125 000 Sigma-Aldrich, Steinheim, Germany) in tetrahydrofuran (Fluka, Deisenhofen, Germany) or 0.16% poly(octadecene-alt-maleic anhydride) (POMA, 20,000 mol wt, Polysciences Inc., Warrington, PA, US) 1:2 acetone/tetrahydrofuran (Acros Organics, Geel, Belgium) were spin-coated (RC5, Suess Microtec, Garching, Germany) on top of the glass substrates and subsequently thermally annealed for 2 h at 120 °C, followed by a final rinsing with acetone to remove the unbound polymer. The coverslips were autoclaved before use to hydrolyze the anhydride moieties to carboxyl groups in order to ensure noncovalent anchorage of FN to the surface only by unspecific physisorption. On POMA the ligands exhibit much higher anchorage strength as compared to PEMA which was verified by protein exchange experiments [18]. This originates from the large nonpolar side chain of the monomer, which is reflected in the high hydrophobicity of the polymer thin film. FN was purified from human plasma [19] and fluorescently labeled with 5-(and-6)-carboxytetramethyl-rhodamine (Invitrogen, Carlsbad, CA) as described previously [20]. For cell culture experiments FN was solved in phosphate buffered saline (PBS) (Biochrom, Berlin, Germany) until a final concentration of 50 μg/ml, sufficient for a saturated monolayer after 1 h incubation [18].

2.2. Cell culture

Human endothelial cells from the umbilical cord vein (HUVEC) were collected according to the procedure described by Weis et al. [21] and grown in endothelial cell growth medium (Promocell, Heidelberg, Germany) containing 2% fetal calf serum until reaching confluence. Cells from the 2nd–4th passage were used and seeded on the prepared FN-coated polymer surfaces (POMA and PEMA) at a density of 3000 cells/cm^2. The cells formed a subconfluent layer and spread within 1 h of cultivation. After removal of the cell culture medium the cells were gently rinsed with prewarmed to 37 °C PBS solution.

2.3. Immunostaining

Fixation of FN-bound cell receptors was performed according to a previously published procedure by Friedland et al. and Garcia et al. [22,23]. In order to fix the ECM and the attached matrix receptors the cells were incubated for at least 45 min with 1 mM of the membrane-impermeable crosslinker 3,3-dithiobis[sulfosuccinimidylpropionate] DTSSP (Thermo Fisher Scientic, Waltham, MA, US). The crosslinking was stopped by addition of 50 mM Tris(hydroxymethyl)aminomethane (TRIS) (Amersham Biosciences, Piscataway, NJ, US) in PBS (20 min). Afterwards the cells (except for their crosslinked ECM and bound receptors) were solubilized for 15 min with 0.1% sodium dodecyl sulfate (SDS) (Ambion, Austin, TX, US) in PBS. The decellularization was monitored by optical microscopy.

ECM components of interest were labeled by introduction of either fluorescent or immunogold tags. To prevent unspecific binding the sample surface was blocked for 10 min with 1% BSA (Sigma-Aldrich, St. Louis, MO, US) in PBS. To localize the ECM-bound $\alpha_5\beta_1$ integrins, the sample was stained with a monoclonal antibody (CD49e Mouse IgG1, BD Pharmingen, Franklin Lakes, NJ,