Fibronectin fibril pattern displays the force balance of cell-matrix adhesion

Die Wechselwirkung von Zellen mit künstlichen Materialien hängt sehr stark von der Art der voradsorbierten oder aus der Zelle abgeschiedenen Proteinen sowie deren Konformation und Substratverankerung ab. Dabei spielt die Adhäsion der Zellen mittels ihrer Membranrezeptoren (Integrine) an extrazelluläre Matrixproteine wie Fibronektin eine zentrale Rolle, da durch diesen Prozess das Wachstum und die Differenzierung der Zellen gesteuert werden kann. Vor diesem Hintergrund wurde die Reorganisation von voradsorbiertem Fibronektin auf verschiedenen Maleinsäureanhydridcopolymerfilmen untersucht, welche durch ihre unterschiedlichen physikochemischen Oberflächeneigenschaften eine unterschiedlich starke physisorptive Anbindung von Fibronektin hervorrufen. Mittels Fluoreszenzmikroskopie und Rasterkraftmikroskopie konnte der quantitative Zusammenhang zwischen der Fibronektinkopplungsstärke an das Substrat und den Abständen der Fibronektinfibrillen auf der Mikrometer- und Nanometerskala aufgeklärt werden. Die Ergebnisse erlaubten neue Arbeitshypothesen für das zellregulierte Kraftgleichgewicht bei der Adhäsion von Endothelzellen auf festen Substraten und die damit zusammenhängende Bildung von Fibronektinfibrillen zu erstellen.

Keywords
maleic anhydride copolymer
fibronectin
anchorage strength
fibril formation
focal adhesion
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Introduction

Interactions of cells with materials depend in a general sense on the presence of pre-adsorbed and/or cell-secreted proteins covering the solid surface. Many cell types realize the adhesion to artificial surfaces by binding of cellular membrane receptors (e.g. integrins) to extracellular matrix proteins anchored on the solid supports [1-3]. The function of matrix proteins like fibronectin (FN) is not restricted to the modulation of adhesion and migration of cells but include the presentation of growth factors to the cells and stimulation of various intracellular processes. The conformation of adsorbed matrix proteins as well as their mechanical binding state and lateral distribution were concluded to be relevant for migration and differentiation of myoblasts, endothelial cells, and other cell types [4-6]. Thus, detailed knowledge about the influence of the physicochemical substrate characteristics on the structural and functional status of surface-bound extracellular matrix and the resulting modes of interaction with cells is essential for progress in cellular bioengineering. Numerous studies currently address the impact of physical and chemical characteristics of solid surfaces on the conformation of the immobilized proteins and their availability for specific cellular responses [7, 8].
In the context of these findings we established a thin film polymer platform, which provides a gradated physicochemistry of the substrate surface [9]. Different maleic anhydrides were covalently attached to amine group bearing substrates. With the variation of the co-monomer the density of polar anhydride groups – or carboxylic acid groups in the hydrolyzed state – is changed on the substrate surface leading to a variation in substrate-protein interaction. Early qualitative investigations on the adhesion of endothelial cells onto FN coated substrates revealed significant differences in formation of cell adhesion sites and the reorganization of FN into fibrillar structures [10].

This pattern formation of FN fibrils was studied in more detail, because the assembly of supra-molecular fibrillar structures of FN affects cellular behaviour through the formation of specific adhesion sites [11]. Furthermore we could demonstrate that it is an important prerequisite for the differentiation of endothelial cells into micro-vascular structures [12].
Although a lot of details is already known on FN fibrillogenesis [13, 14], a quantitative and mechanistic understanding of FN fibrillogenesis and cell-matrix adhesion remains to be achieved. Therefore our approach aimed to revealing more details on the force balance of FN-substrate anchorage and the cellular forces applied on the adhesion sites. In detailed studies using protein heteroexchange experiments differences of FN-substrate anchorage strength could be related to the gradated surface physicochemistry of the maleic anhydride thin films [15]. In that sense FN micrometer scale [16] and nanometer scale [17] pattern of FN fibrils were analysed in cell culture experiments. Based on the results we established new working models on the process of FN fibrillogenesis and its dependence on the anchorage of the extracellular matrix.

Results

As the basis of the investigations of FN fibrillogenesis, the variation of the anchorage strength of physisorbed FN on three different maleic acid copolymer substrates (hydrolysed anhydride functionalities) was measured by protein heteroexchange experiments with human serum albumin [15]. The copolymer surface of poly(octadecene-*alt*-maleic anhydride) (POMA), poly(propene-*alt*-maleic anhydride) (PPMA), and poly(ethylene-*alt*-maleic anhydride) exhibit a similar surface coverage of FN at the beginning of the experiments of approx. 400 ng·cm^{-2}. The time constants τ_1 and τ_2 of the double-exponential decay functions of FN surface coverage in the exchange experiments could nicely be correlated to the polarity of the surface, i.e. with the density of functional anhydride groups, and related to this with the hydrophilicity of the surfaces. The hydrophobic POMA exhibits the strongest FN-substrate anchorage (large τ_i), while the most hydrophilic PEMA showed the weakest anchorage strength (small τ_i) [16].
At first micrometer scale investigations of FN reorganization by endothelial cells were carried out by fluorescence microscopy. The experiments were restricted to a short time period of 50 min to prevent additional secretion and deposition of matrix proteins by the cells, which could superimpose their dependence on the substrate anchorage strength of the pre-coated FN. The patterns of fluorochrome-conjugated pre-coated FN after 50 min on the different copolymer surfaces is shown in Fig. 1. A qualitative judgment already provides significant differences in density and length of FN fibrils with a higher density as well as long and thin fibrils on the substrates with weaker anchorage strength.

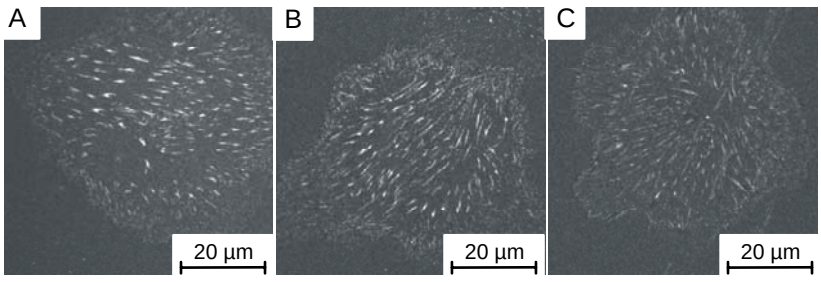

Fig. 1:
Typical images of FN fibrils reorganized by endothelial cell on the three compared copolymer substrates after 50 minutes of cell culture. Fluorescence confocal laser scanning microscopy reveals the pattern formation of the pre-adsorbed rhodamine labelled FN. (A) POMA, (B) PPMA, (C) PEMA.

In order to reveal a more quantitative description of the FN fibril pattern an autocorrelation analysis of the fibril pattern was performed. By analyzing the distance of the first main maximum perpendicular to the fibril orientation in the autocorrelation image the mean periodic spacing of the fibril was determined in regions of a similar fibril orientation – also called polarization of the cells. The outcome of this study is shown in Fig. 2a demonstrating a directly proportional relation of the fibril spacing with FN-substrate anchorage strength, which is expressed by the time constant of the fast exchanged species in the heteroexchange experiments. The periodicity of the FN pattern ranges from 2.6 ± 0.7 µm for the POMA substrate with the high adsorption strength of FN down to 1.8 ± 0.4 µm for the PEMA substrate with lowest FN anchorage.

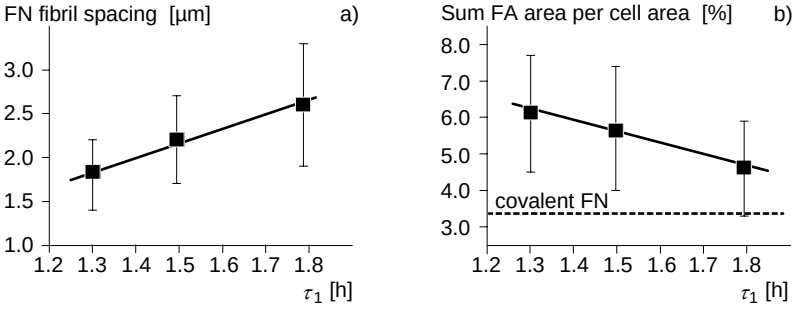

Fig. 2:
(a) Plot showing the FN fibrillar spacing in dependence on the time constant τ_1 of FN exchange.
(b) Plot showing the focal adhesion density in dependence on the time constant τ_1 of FN exchange.

In additional experiments the focal adhesion pattern was analyzed, because the focal adhesions are known as the primary cellular adhesion sites onto the substrates and they act as the initial site of FN fibril formation (Balaban 2001; Pankov 2000). As phospho-tyrosine is predominantly localized in the focal adhesions, its immunofluorescent visualization was used to determine the size and summed area of focal adhesions per cell area. While a similar mean size of around 0.75 µm^2 was found on all three different surfaces, the summed area by cell area showed a dependence on FN anchorage strength as shown in Fig. 2b. Cells grown on the substrate with the highest FN anchorage strength showed the smallest area fraction of focal adhesions. This is in line with earlier findings with cells on covalently attached FN [10] exhibiting an even smaller amount of focal adhesions of 3.4 %.

The results clearly demonstrate that the cells can sense the anchorage strength of FN to the substrate by their force-sensitive focal adhesion/cytoskeleton apparatus and react in an appropriate manner to form an adhesion site pattern, which allows for a similar attachment of the spread cells on the different substrates. As shown in Fig. 3 the following working model for the micrometer scale pattern of FN fibril formation can be established. Sketched in a side view in Fig. 3a, the cells adhere onto the surface coated with extracellular matrix proteins, like FN, via their cell membrane integrin receptors. Thus the cells transform into a more flat and spread state, integrins clusters form in the cell membrane resembling at later stages the focal adhesions, and intracellular cytoskeletal elements, like actin stress fibres, bind to those adhesion sites to introduce tensile forces at the adhesion sites. The cells can react to forces applied to the adhesion sites by binding of additional proteins at the focal adhesions and triggering of downstream signalling cascades as well as the inside-out signalling by conformational changes of the integrins. It is assumed that the cells correspond to the variation of FN anchorage strength on the different copolymer substrates by the described adhesion mechanisms.

As visualised in Fig. 3b the results of the experiments indicate that the similar spread cells have to establish a similar force field on the different substrates.
As the extracellular matrix ligand FN is more strongly bound on the hydrophobic substrates like POMA, the cell organise the focal adhesion with a lower density with probably higher tensile force applied at those adhesion sites in comparison to a higher density of focal adhesions with lower applied forces on the more hydrophilic substrates with a weaker FN anchorage strength. As the focal adhesions act as the primary site of FN fibril formation the fibril pattern expresses this behaviour.

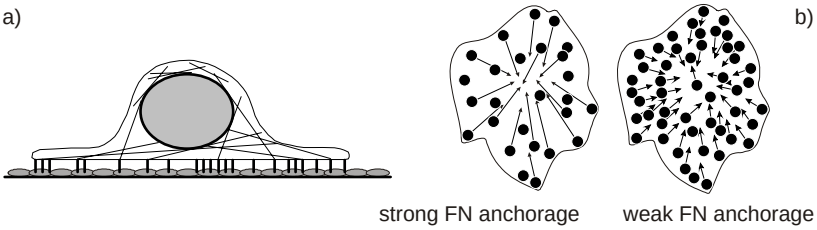

Fig. 3:
Scheme to illustrate (a) the process of cell adhesion and (b) focal adhesion pattern and applied forces therein (arrows) in dependence of FN substrate anchorage strength

Following the micrometer scale investigations, a different approach was used to study the nanometer pattern of FN fibrils. After a similar cell experiment as described above the cells were extracellularily fixed with cross-linker, which does not permeate the cell membrane. Hence, only extracellular protein structures are cross-linked and preserved during a subsequent cell extraction with a strong detergent. The remaining structures were investigated on a combined confocal laser scanning microscope and scanning force microscope (SFM). Due to the fluorochrome-conjugated fibronectin the SFM could be positioned at reorganized fibrillar FN using fluorescence microscopy, and the fibrillar pattern could be visualized by SFM with a nanometer resolution under physiological buffer conditions. This technique allowed to obtain detailed images of FN fibrils. They showed a typical paired structure of FN nano-fibrils, see Fig. 4.

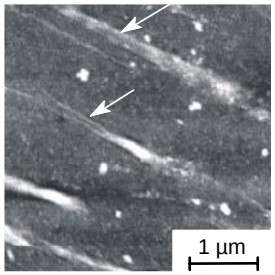

Fig. 4:
SFM height image of FN fibrils on PPMA
Height scale: 70 nm. The arrows indicate paired FN nano-fibrils.

The quantification of the distance between paired nano-fibrils on the different copolymer substrates with a different FN anchorage strength revealed a characteristic distance between 70 nm and 370 nm in dependence on the substrate. The histogram in Fig. 5 summarizes the measurements of the distance between FN nano-fibrils. On substrates with a weak FN anchorage strength the FN nano-fibrils exhibited a lower distance.
Moreover a peak analysis of the histogram proposed a uniform repeating unit for the characteristic distances on the different substrates. This repeating unit was estimated to 71 nm and could be correlated to twice the length of α-actinin, which is an cross-linker of actin stress fibres. Immunofluorescence studies using α-actinin support this idea [17]. The results of the nanometer analysis of FN fibrils illustrate a further influence of the modulation of FN-substrate anchorage strength on the pattern formation of FN fibrils.

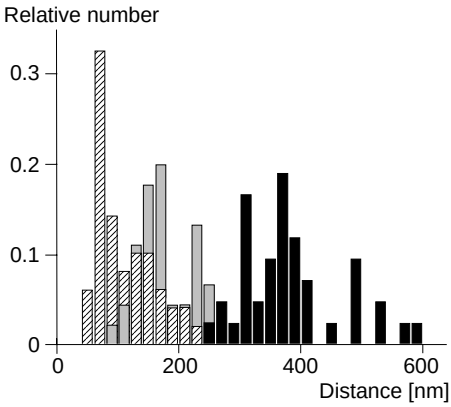

Fig. 5:
Histogram of typical distances of paired FN nano-fibrils on the three different substrates
(POMA, PPMA, PEMA)

Out of the presented data a mechanism (illustrated in Fig. 6) is suggested which relates this feature of FN fibrillogenesis again to the force-sensitive cellular feedback cycle of the focal adhesions: The anchorage strength of FN on the substrate is sensed by the cells via the integrins, other proteins at the focal adhesion and the actin cytoskeleton in a manner as already described above. Accordingly, the cell assembles actin stress fibres triggered by the Rho pathway to exert a force adequate to the anchorage strength of FN, which should manifest itself in the size of the actin stress fibre and probably of the integrin clusters at the focal adhesion. Along the α-actinin cross-linked actin stress fibres structurally related FN fibrils are formed by stretch, transport, and subsequent polymerisation of FN. Hence, the discrete structure of the actin stress fibres – caused by the cross-linking with α-actinin – acts as a template for FN fibril formation. In consequence, a higher anchorage strength of FN to the substrate – i.e. to POMA substrates in comparison to PPMA substrates – results in thicker actin fibres and larger distances between FN nano-fibrils. The occurrence of twice the α-actinin length as the repeating unit originates from the anti-parallel orientation of the actin filaments inside the actin stress fibres and the myosin driven transport and stress evolvement towards the plus ends of the actin filaments.

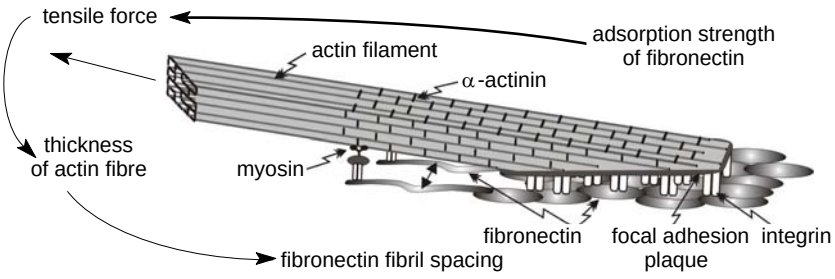

Fig. 6:
Schematic representation of the proposed model for the FN nano-scale fibrillogenesis explaining the typical spacing of FN nano-fibrils and its substrate dependence. Related to the gradated anchorage strength of FN to the different substrates, the tensile force of the cell is adjusted which acts in the actin stress fibres at the focal adhesion. The focal adhesion size and the thickness of actin stress fibres is regulated providing a distinct thickness with a repeating unit due to the α-actinin as the actin cross-linking element. As a result the actin stress fibre acts as a pattern template for the FN fibril formation along the fibres by the myosin driven FN stretch and transport.

Altogether, the results of this work confirm the importance of force balance at the adhesion sites in the process of FN fibrillogenesis. The micrometer and nanometer scale studies proposed a summarized model in the following way:

(i) When the cells adhere to a surface (coated with matrix proteins) they form adhesion sites, where the size and force evolved at those size is regulated by conformational changes of integrins and intracellular signalling cascades, i.e. a high density of focal adhesions and small forces applied therein on substrates with a weak ligand anchorage strength.

(ii) Subsequent FN fibril formation occurs at those adhesion sites resulting in paired nano-fibrils – with actin stress fibre size acting as the template for its distance – and a micrometer scale pattern according to the density of the adhesion sites. We can demonstrate by these data the key role of the physicochemical characteristics of artificial surfaces in the modulation of cell-matrix adhesion via control over FN anchorage. The findings additionally suggest a probable pathway of triggering cellular function, like proliferation and differentiation, by the influence of substrate physicochemistry on the anchorage of extracellular matrix components.

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