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ARTICLE

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

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Abstract Formation of fibrillar patterns of fibronectin on polymer substrates with gradated physicochemical surface properties was analysed during early stages of endothelial cell adhesion. Fibronectin was pre-adsorbed onto three maleic anhydride copolymer thin films with distinct differences in the protein adsorption strength as verified by heteroexchange experiments. The evolved micrometer scale fibrillar patterns of fibronectin on the compared polymer surfaces were characterized after 50 min of cellular reorganization by an auto-correlation analysis using fluorescence microscopy data. Statistical analysis revealed a decrease of the typical spacings of the fibronectin fibrils from 2.6 to 1.8 μm with decreasing fibronectin adsorption strength to the substrate. Size and density of focal adhesions correlated with this dependence of the fibronectin fibril pattern. From these data a model was developed relating the fibronectin fibril pattern to the fibronectin-substrate adsorption strength through the cytoskeletal force regulation mechanism of the cell.

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 (integrins) to extracellular matrix proteins immobilized on the solid supports (Geiger et al. 2001; Miranti and Brugge 2002; Stupack and Cheresh 2002). 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 (Garcia et al. 1999; Katz 2000; Ingber 2002). 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 therefore 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 (Altankov et al. 1996; Garcia et al. 1999; Katz et al. 2000; Keselowsky et al. 2003; Pompe et al. 2003a; Faucheux et al. 2004).

In this context, the assembly of supramolecular fibrillar structures of FN by cells like fibroblasts and endothelial cells receives attention, since the state of the FN matrix network was found to directly affect cellular behaviour through the formation of specific adhesion sites (Cukierman et al. 2001) which was demonstrated to be an important prerequisite for the differentiation of endothelial cells into microvascular structures (Pompe et al. 2004a). Specific cell binding sites and specific domains of FN could be revealed to be involved in the process of FN fibrillogenesis (Danen et al. 2002; Sottile and Hocking 2002; Wierzbicka-Patynowski and Schwarzbauer 2003). Furthermore, the necessity of exposing cryptic binding sites for FN assembly as well as the influence of intracellular cytoskeletal force were intensively investigated (Hynes 1999; Craig et al. 2001; Vogel et al. 2001; Oberhauser et al. 2002; Ohashi et al. 2002; Li et al. 2003).

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Although a lot of details is already known, a quantitative and mechanistic understanding of FN fibrillogenesis and cell–matrix adhesion remains to be achieved. In earlier works we started to investigate endothelial cell adhesion and FN fibrillogenesis in dependence on the adsorption strength of adsorbed FN to polymer substrates with gradated physiochemical surface characteristics. As the first studies revealed qualitative results on the FN fibrillogenesis in dependence on the polymer substrate characteristic (Pompe et al. 2003a), a subsequent study (Pompe et al. 2005) on nanometer scale features of reorganized FN fibrils demonstrated a clear relation between FN adsorption strength and structural features of FN nanofibrils, which could be further related to the structure of actin stress fibres involved in the FN reorganization. Following this approach the current study is focused on the micrometer scale pattern of FN fibrils to support the previous qualitative findings (Pompe et al. 2003a) with quantitative data. New details of the FN fibrillogenesis are considered for an extended model of the overall force balance of adherent cells to complement the previously derived nanoscale features of this process (Pompe et al. 2005).

Materials and methods

Substrate preparation and analysis

Poly(octadecene- *alt*-maleic anhydride) (POMA) (Polysciences Inc., Warrington, PA), poly(propene- *alt*-maleic anhydride) (PPMA) (special product of Leuna-Werke AG, Germany), and poly(ethylene- *alt*-maleic anhydride) (PEMA) (Aldrich, Munich, Germany) films on top of glass coverslips were produced by spin coating (RC5, Suess Microtec, Garching, Germany). Solutions of 0.08% in tetrahydrofuran (Fluka, Deisenhofen, Germany) for POMA, 0.1% in methylethylketone (Fluka) for PPMA, and 0.12% in a 1 : 2 mixture of tetrahydrofuran and acetone (Fluka) for PEMA were applied. The coverslips (24 mm×24 mm) had been freshly oxidized in a mixture of aqueous solutions of ammonia (Acros Organics, Geel, Belgium) and hydrogen peroxide (Merck, Darmstadt, Germany) and thereafter surface-modified with 3–aminopropyl-dimethylethoxy-silane (ABCR, Karlsruhe, Germany). Details of the preparation and characterization were published elsewhere (Pompe et al. 2003b). Autoclaving induced hydrolysis of the anhydride moieties to provide a surface exclusively bearing carboxylic acid groups which are fully dissociated at the pH of the cell culture medium (Osaki and Werner 2003). The surfaces of the different copolymers exhibited a different surface energy depending on the kind of the comonomer due to the different mass fraction of the maleic acid groups. Accordingly, quasi-static advancing water contact angles (sessile drop method, G40, Kruess, Hamburg, Germany) were determined to 100°, 38°, and 21° (±5°) for POMA, PPMA, and PEMA, respectively.

For the determination of the anhydride density, the copolymer films were immersed in 50 mM solution of methionine amide hydrochloride (Bachem Biochemica, Heidelberg, Germany) in PBS for 1 h. Subsequently, the atomic composition was quantified by X-ray photoelectron spectroscopy using an Amicus spectrometer (Kratos Analytical, UK) equipped with a non-monochromatic Mg Kα X-ray source operated at 12 kV and 240 W. The kinetic energy of photoelectrons was determined using an analyser with a pass energy of 75 eV. The take-off angle, defined as the angle between sample surface normal and the electronoptical axis of the spectrometer, was 0°. Spectra were referenced to the hydrocarbon C 1s peak at a binding energy of 285.00 eV. Quantitative elemental compositions were determined from the peak areas of the high-resolution spectra (C 1s, N 1s, O 1s, S 2p, Si 2p) using experimentally determined sensitivity factors and the spectrometer transmission function. The anhydride surface density was estimated from the elemental composition by linear equation analysis assuming a density of 2.85 g cm^{-3} of the detected SiO_2 from the silicon wafer substrate and a mean attenuation length of the XPS signal in the substrate and the surface layer of about 3 nm (Seah and Dench 1979; Cole et al. 2000). Within this routine the elemental compositions (C, N, S, Si) of the layer components (SiO_2, maleic anhydride copolymer and methionine amide) were fitted to the XPS data in a least square sense.

Prior to cell culture experiments the polymer-modified coverslips were coated with FN (Roche, Basel, Switzerland) or fluorescent labelled FN (FN-RHO) out of a solution of 50 µg/ml in phosphate-buffered saline (PBS) (Biochrom, Berlin, Germany) for 1 h. FN labelling was performed using the FluoReporter Tetramethylrhodamine Protein Labeling Kit (Molecular Probes, Eugene, OR). The average degree of FN labelling was determined according to the kit protocol with a spectrophotometer (Specord S10, Zeiss, Jena, Germany) to be 3.8 (±1.1) mol dye per mol protein.

FN adsorption strength was measured by protein heteroexchange with human serum albumin (HSA) (Sigma, München, Germany), as described in detail elsewhere (Renner et al. 2004). Briefly, after FN-RHO adsorption the surface was rinsed by PBS followed by addition of HSA in PBS at a concentration of 50 µg/ml and the fluorescence intensity of remaining FN-RHO on the surface was measured by fluorescence confocal laser scanning microscopy (TCS SP, Leica, Bensheim, Germany) up to 48 h. Comparison with high pressure liquid chromatography (HPLC)-based protein quantification provided evidence, that the fluorochrome labelling of the proteins did not affect their adsorption and exchange characteristics. HPLC quantification was performed as follows: after acidic vapour phase hydrolysis, amino acids were fluorescence-labelled, separated with a HPLC system and quantified by a fluorescence detector (Series 1100, Agilent Technologies, Böblingen, Germany). The amount of FN was determined by

numerical analysis (in a least square sense) utilizing the known amino acid sequence of FN (see details in Salchert et al. 2003).

Cell culture

Human endothelial cells from the umbilical cord vein were collected and cultured in endothelial cell growth medium ECGM (Promocell, Heidelberg, Germany) with 2% fetal calf serum as described elsewhere (Pompe et al. 2003a). After FN or FN-RHO coating of the substrates, subsequent rinsing with PBS and pre-incubation with cell medium for 10 min (to block unspecific substrate interactions) 3×10^4 cells were seeded and cultured on the samples for 50 min.

Immunofluorescence

After 50 min of culture adherent cells were gently washed with PBS, fixed with 4% paraformaldehyde (Fluka) for 5 min, permeabilized with 0.1% Triton X-100 (Fluka) at 4°C for 10 min, and washed with PBS. Immunostaining was performed with mouse anti-phosphotyrosine (Clone 4G10) (Upstate Biotechnology, Waltham, MA) and secondary antibody staining with Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes). The actin cytoskeleton was stained with FITC-conjugated phalloidin (Sigma) for 20 min. Immunostained samples were analysed by fluorescence confocal laser scanning microscopy (TCS SP, Leica, Bensheim, Germany) equipped with a 40× immersion oil objective.

Image analysis

Using fluorescence confocal laser scanning microscopy the reorganized patterns of FN fibrils were imaged by a 40× immersion oil objective, a pixel size of 61 nm and an image size of 1,024×1,024 pixels. For image processing the freely available program Scion Image (Scion Corporation, Frederick, MD) was utilized. For statistical analysis the patterns of FN fibrils and focal adhesions of 20 cells of three independent experiments were evaluated.

The periodicity of the reorganized FN fibrils was determined by applying a 2d auto-correlation analysis, as previously described elsewhere (Pompe et al. 2004b). Briefly, with the 2D fast Fourier transformation and auto-correlation algorithms of the Scion Image software the 2D auto-correlation image of the analysed fibrillar patterns were calculated. Maxima are expected in the auto-correlation image when the pattern of FN fibrils exhibits characteristic spacings. Areas of a size of 7.8×7.8 μm with similar FN fibril orientation were selected for the auto-correlation analysis. In the auto-correlation image a line section perpendicular to the major fibril orientation through the centre of the image was analysed for the distance between the central main maximum and the first local maximum besides it, which was taken as the typical spacing between the FN fibrils.

For the quantification of focal adhesion complexes thresholding of the phosphotyrosine staining just above the background staining was applied to perform thereafter particle counting and size analysis.

Statistical analysis

Unpaired t-test analysis with a significance level of $p < 0.05$ was performed for the analysis of the size and summed area of the focal adhesions.

Results

The adsorption strength of physisorbed FN on the three different polymer substrates was investigated previously by protein heteroexchange experiments with HSA—for details see (Renner et al. 2004)—since it is known that protein exchange provides a measure of the interaction strength of adsorbed proteins with the underlying substrate (Ball et al. 1998). The initial surface coverage was determined by HPLC analysis with 420 ± 80, 400 ± 25, and 360 ± 35 ng/cm^2 for POMA, PPMA, and PEMA, respectively, showing a dense FN coverage on all substrates at protein amounts exceeding the approximated value for a monolayer of side-on oriented FN molecules (250 ng/cm^2). As reported earlier (Renner et al. 2004) FN exchange from the surfaces in 50 μg/ml HSA solutions in PBS revealed a double exponential exchange characteristics with fast and slow displaced protein species. The most hydrophobic POMA surface showed the slowest and weakest FN exchange while the most hydrophilic PEMA surface showed the fastest and most intense protein exchange. By plotting the time constant of the fast desorbing species τ_1 (as the relevant time scale for current study) over the anhydride density of the copolymer surfaces (determined by XPS) in Fig. 1, the correlation of the FN adsorption strength—as measured by the exchange time—and the number of polar groups being present on the copolymer surface is demonstrated with low exchange rates for lower charge densities measured in terms of anhydride density. As protein interaction is strongly controlled by hydrophobic and electrostatic interaction the gradation of the content of polar surface groups provides a good measure of the protein-substrate interactions.

Based on previous qualitative results (Pompe et al. 2003a) FN reorganization in dependence on the adsorption strength of immobilized FN was studied by 2D auto-correlation analysis from fluorescence images (Fig. 2) of FN fibril patterns after 50 min of endothelial cell culture. The fibrillar patterns exhibit differences in the density and length of the visualized structures with fewer and shorter fibrils on the POMA substrate providing a higher FN adsorption strength. Since the cells—due to their polarization—exhibit on any

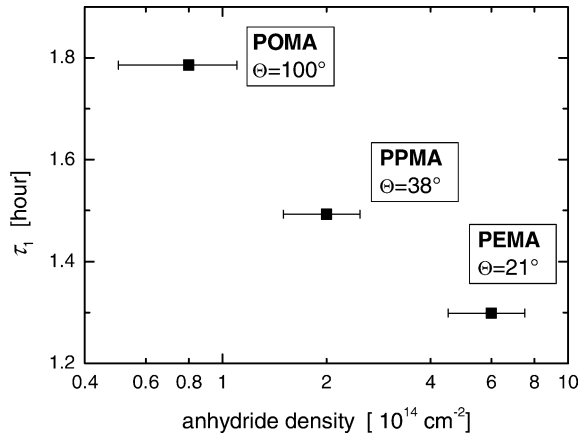

Fig. 1 The time constant τ_1 of the fast displaced FN species of the exponential fit of the FN exchange experiments vs the anhydride surface density of the copolymer surfaces. The *error bar* indicates the mean scatter of three independent measurements

substrate areas of similar fibril orientation, the auto-correlation analysis shows local maxima at distances from the central global maximum, where periodic features are located in the analysed image. Figure 3 gives an example of such an analysis showing the auto-correlation image of the FN fibril pattern on a PPMA surface from the cut-out of Fig. 2b. The cut-out was chosen from an area with similar fibril orientation. By drawing a line section perpendicular to the main orientation of the fibrils in the auto-correlation image—as indicated by the white line in Fig. 3—one remains with the intensity plot showing the central global maximum and the local maxima as shown in Fig. 4. The measured distance of the first major local maximum and the global maximum of 2.2 μm is taken as the periodicity of the FN fibril pattern. For each substrate surface all areas of similar fibril orientation of 20 cells were analysed. The statistical analysis of one typical experiment out of three independent ones is shown in Table 1. As expected from the qualitative evaluation of the data in Fig. 2 the quantitative analysis reveals a clear dependence of the typical FN fibril spacing on the used polymer substrates. 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.

The well-known correlation of FN fibrils and the structure of the actin cytoskeleton (Hynes and Destree 1978; Ohashi et al. 2002) illustrates the direct interplay of intracellular and extracellular components in cell–matrix adhesion and during FN fibrillogenesis. Focal adhesions are the initial sites for cell attachment and generation of FN fibrils (Ohashi et al. 2002; Pankov et al. 2000). The involved integrin receptors link the cell to matrix proteins—such as fibronectin—and act as force sensors to provide a feedback cycle allowing the cells to react on the state of the extracellular matrix (Balaban et al. 2001; Geiger et al. 2001; Miranti and Brugge 2002; Stupack and Cheresh 2002). Previous work (Pompe et al. 2003a) qualitatively indicated significant differences in the formation of focal adhesions due to either covalent or non-covalent FN immobilization. In extension of these findings, focal adhesions in cells grown on FN-coated substrates with gradated physicochemistry were quantitatively analysed based on anti-phosphotyrosine immunofluorescence staining. Phosphotyrosine is known to be predominantly localized in the focal adhesions (Katz et al. 2000). Figure 5 shows typical examples of the focal adhesion pattern on the three different copolymer surfaces. The analysis of the size and summed area per overall cell area of the focal adhesions revealed a gradation in the sum of the focal adhesions in dependence on the substrate by an otherwise similar mean size of around 0.75 μm^2 . Only focal adhesion inside the adherent cell area were analysed leaving the high intensity staining at the outer border out. The reason for this procedure is based on the aim to relate the focal adhesion pattern to the FN fibril pattern, which is predominantly localized inside the cell. The fast evolving and migrating adhesive contacts at the outer border are mostly not colocalized with FN fibrils. Table 1 gives the summed area of the focal adhesions per overall area of the cells. Cells grown on the substrate with the strongest FN adsorption strength show the smallest area fraction of focal adhesions. This is in-line with earlier findings with cells on covalently attached

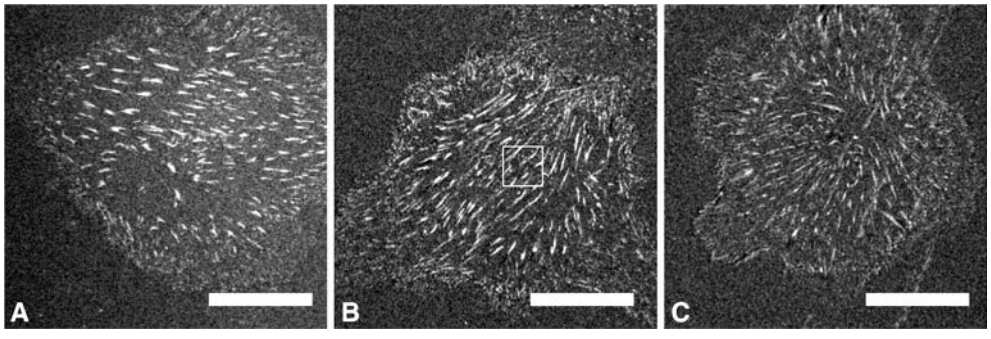

Fig. 2 Typical images of FN fibrils reorganized by endothelial cell on the three compared copolymer substrates after 50 min of cell culture. Fluorescence confocal laser scanning microscopy reveals the pattern formation of the pre-adsorbed FN-RHO. (**a**) POMA, (**b**) PPMA, (**c**) PEMA. *Scale bar*: 20 μm. The *square* in (**b**) indicates the area of similar FN fibril orientation, which was used in the example of the auto-correlation analysis

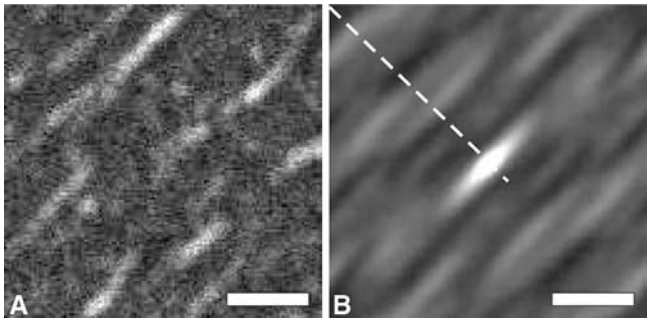

Fig. 3 A cut-out image of Fig. 2b with almost parallel aligned FN fibrils (**a**) and the corresponding auto-correlation image (**b**). The *line* indicates the plotted intensity profile of Fig. 4. *Scale bar*: 2 μm

FN (Pompe et al. 2003a) exhibiting an even smaller amount of focal adhesions. For comparison, these experiments were analysed in an equal manner resulting in a summed area of focal adhesion per cell area for covalent attached FN of 3.4 ± 0.1 %. The data indicate a dependence of the formation of focal adhesions on the FN-substrate adsorption strength, although statistically significant differences ($p < 0.05$) could not always be established for the compared set of the three copolymer substrates.

Discussion

Extracellular matrix structures are linked to cytoskeleton elements by integrin transmembrane receptors (Geiger et al. 2001) and—through this—induce and transduce specific intracellular signals. Since the cellular feedback to the matrix signalling also comprises the reorganization of the matrix components into supramolecular assemblies the formed matrix micropatterns were concluded to reflect the impact of physicochemical properties of the substrate surfaces on the cell–matrix adhesion.

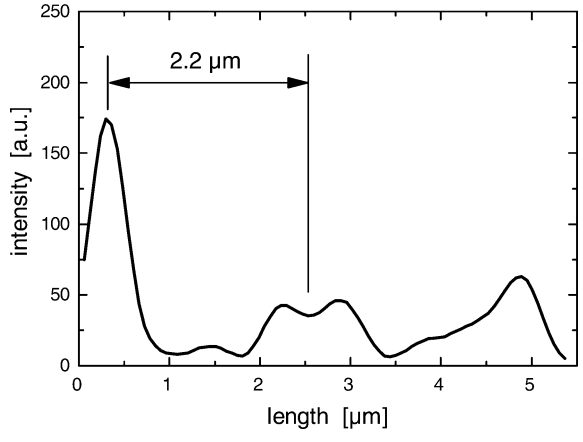

Fig. 4 The intensity profile plot of the auto-correlation image of Fig. 2b perpendicular to the fibril orientation through the centre of the auto-correlation image. The distance between the central global maximum and the first major maximum indicates the typical spacing between the FN fibrils

The current work attempts to quantify the micropattern of extracellular FN fibrils and to link their characteristic to the FN adsorption strength to the substrate, which is controlled by the gradated physicochemistry of the substrate. Based on the measurement of FN adsorption strength by protein heteroexchange studies with HSA (Renner et al. 2004) the earlier qualitative analysis (Pompe et al. 2003a) was extended to provide quantitative data of the mean spacing of reorganized FN fibrils on the different polymer substrates. Figure 6a shows the linear dependence of the FN fibril spacing on the time constant τ_1 of the FN exchange, which characterizes the behaviour of the rapidly displaced species. The exchange of this fraction of surface-bound FN was found to occur in time periods of minutes which is the time scale of the cell culture experiments analysed in this work. Impressively, the cells reorganize the FN fibrils with a typical spacing, which is directly related to the adsorption strength of FN to the substrate. As the process of FN fibrillogenesis is tightly coupled to the force-sensitive focal adhesion/cytoskeleton apparatus of the cell (Hynes 1999; Pankov et al. 2000; Ohashi et al. 2002), it can be concluded, that the cells sense the interaction strength of FN to the substrate and react in an appropriate manner.

A similar sensitivity of the cell cytoskeleton was recently reported as a result of the nanometer scale analysis of FN fibrils by scanning force microscopy on the same polymer surfaces (Pompe et al. 2005). Therein, the dependence of the spacing of paired FN nanofibrils on the FN adsorption strength was related to the inner structure of the intracellular actin stress fibres and their force sensitivity. The actin stress fibres and their force dependent thickness were suggested to act as a template for the FN fibrillogenesis leaving FN nanofibrils with a distinct spacing.

In order to prove the regulation of the FN fibril pattern by FN-substrate adsorption strength via the intracellular force apparatus focal adhesions were analysed as the initial sites for the FN fibril formation (Pankov et al. 2000). The similar mean size of the focal adhesions of 0.75 μm^2 on all polymer substrates distinguishes them as small early focal complexes (Balaban et al. 2001) in line with the short cell culture time of only 50 min. In Fig. 6b the summed area of the focal complexes shows a linear trend over the FN adsorption strength—characterized by the time constant τ_1 of the FN heteroexchange dynamics. As the mean size of the individual focal adhesion sites is invariant on the compared substrates, the results clearly indicate a decreasing number—in line with a larger spacing—of focal adhesions per cell with an increasing FN-substrate adsorption strength. The summed area of focal adhesions in cells on covalently attached FN (data from earlier experiments; Pompe et al. 2003a) is included in the diagram as a limiting value showing an even smaller area fraction and number of adhesion complexes. In addition, a similar trend was seen for the time constant

Table 1 Mean spacing between FN fibrils on the different copolymer substrates

	FN fibril spacing (μm)	FA size (μm^2)	FA sum per cell area (%)
POMA	2.6 ± 0.7	0.7 ± 0.15	4.6 ± 1.3
PPMA	2.2 ± 0.5	0.83 ± 0.15	5.7 ± 1.7
PEMA	1.8 ± 0.4	0.7 ± 0.15	6.1 ± 1.6

A dependence on the FN adsorption strength is observed with the largest spacing for the highest adsorption strength (POMA) and the smallest spacing for the lowest adsorption strength (PEMA). Additionally, mean size and mean density of focal adhesions on the three different copolymer substrates is shown

of the slow exchanged fibronectin species τ_2, which underlines the correlation to the substrate adsorption strength.

It is interesting to discuss these findings in the context of other studies about size and force dependence of focal adhesion. As a direct linear size–force relationship of mature focal adhesion is reported (Katz et al. 2000; Balaban et al. 2001; Tan et al. 2003) with about 5 nN μm^{-2}, there are different findings for small focal contacts and nascent focal adhesions at leading edge or tail of migrating fibroblasts (Beningo et al. 2001; Tan et al. 2003). Furthermore, it is important to note that FN was strongly adsorbed in the cited study—almost like non-displaceable FN—in contrast to our work using weakly to moderately adsorbed FN molecules only. This feature might create a different aspect in the cytoskeletal response behaviour of the cells. Thus, the herein analysed focal adhesions represent according to size and assembly time (cell culture 50 min) small focal complexes, however, they are already characterized by phosphotyrosine expression and are localized at the inner cell contact area. As they do not exhibit a size dependence, we suggest that at this early stage the density of the focal complexes is a regulatory mechanism for the response of the cell to the different substrate adsorption strength of the adhesive ligand FN.

This idea is based on existing models for cell attachment, where cell spreading and adhesion by integrin-adhesion ligand linkages are treated in a physical sense as a wetting phenomenon—the process resembles the spreading of a droplet on a partially wettable substrate due differences in surface energies (Sackmann and Bruinsma 2002). In this process clustered integrins and focal complexes evolve, which represent the primary attachment points to the substrate. Using these structures, the cell stabilizes the spread state through the application of forces to the matrix environment. Growing on substrates with a different adsorption strength of ligand proteins, the cells can only remain attached if they apply a force through the individual integrins not exceeding the adsorption strength of the ligand to the substrate. Since the cell can intracellularly sense the applicable force for individual integrins it is capable to arrange the frequency of its attachment points within the intracellular tensile actin cytoskeleton in a way to achieve an equilibrium force balance. In line with that view it is obvious that more attachment points are necessary to maintain the spread state of an adherent cell on a substrate with lower ligand–substrate adsorption strength. This was observed in the reported case for endothelial cells growing on maleic acid copolymers with gradated physicochemical surface properties. Subsequently, the focal adhesions as the primary attachment points for the cells act as the sites of FN fibrillogenesis. By that mechanism the periodicity found in the FN fibril pattern represents the equilibrium attachment pattern of the focal adhesions.

Using this model assumption and the measured data we obtain rough estimates for the forces involved in cell–matrix adhesion and, in turn, can conclude on the adsorption strength of the FN molecules to the polymer substrates. For covalently attached FN it is assumed that the forces applied by the cells to the focal adhesions are in the range of the values reported in literature for small focal complexes of roughly 5 nN μm^{-2} (Balaban et al. 2001; Tan et al. 2003). From the ratios of the summed areas of the focal adhesion (Table 1) it is possible to calculate the applied forces using an integrin receptor density of around 1,000 μm^{-2} (Wiseman et al. 2004) under the assumption of a constant overall tension applied by the cells via its focal adhesions of variable density on the different substrates. This results in a force per receptor–ligand pair for the three different polymer substrates of 3.6 pN down to 2.85 pN. As these forces are assumed to be the result of the cell regulation mechanism for sensing the FN adsorption strength, they should represent somehow a quantitative measure for the adsorption strength of FN to the polymer surfaces.

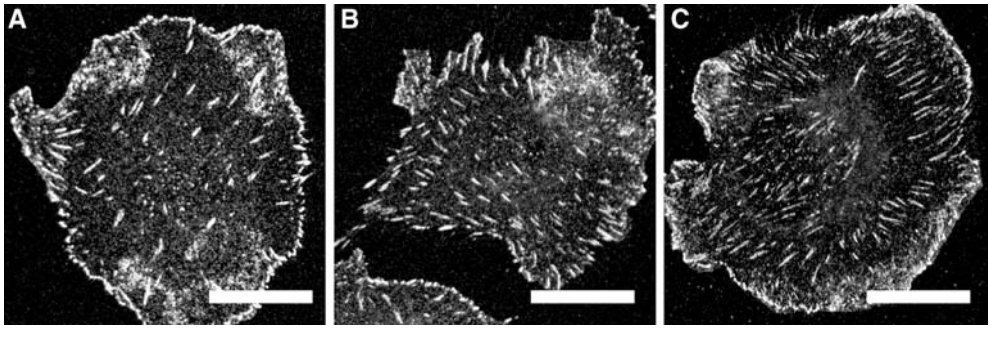

Fig. 5 Typical images of focal adhesions of endothelial cell on the three compared copolymer substrates after 50 min of cell culture: (**a**) POMA, (**b**) PPMA, (**c**) PEMA. *Scale bar*: 20 μm

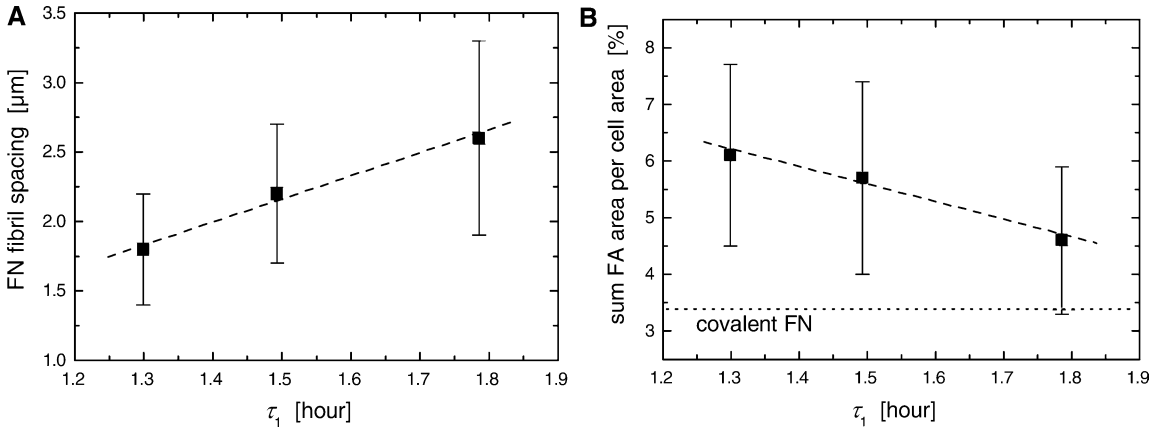

Fig. 6 (**a**) Plot showing the FN fibrillar spacing in dependence on the time constant τ_1 of FN exchange. (**b**) Plot showing the focal adhesion density in dependence on the time constant τ_1 of FN exchange

However, at this end it is hard to compare those numbers with real adsorption energies of the adsorbed extracellular matrix proteins, because of the complex nature of substrate–protein interaction involving a mixture of polar forces, van der Waals interactions, hydrogen bonding, and entropical forces (Haynes and Norde 1994), multiple interaction sites, and the conformational changes the proteins undergo during adsorption leading to severe problems in the experimental determination of those adsorption energies.

Discussing the estimates for the force applied to FN molecules in the light of known values for the $\alpha_5\beta_1$-integrin bond to the FN fragments of the cell binding region (Li et al. 2003), or measurements and simulation results of forces involved in the stretching of FN type III domains (Craig et al. 2001; Oberhauser et al. 2002)—which are thought to be essential for exposing cryptic sites for the assembly of FN molecules during FN fibrillogenesis—it is obvious that the integrin–FN bond remains very stable under these conditions and the process of FN fibrillogenesis has solely to be determined by cellular forces and mechanisms. Thus, the FN–substrate interaction affects the FN fibril pattern only indirectly via the earlier described model of the focal adhesion density regulation.

In summary, this study could reveal a quantitative correlation of the FN–substrate anchorage with cellular FN fibrillogenesis. The force sensitivity of the cell in acquiring focal adhesions for the stabilization of the spread state was found to play the dominant role for the FN micrometer scale pattern. It was demonstrated that the protein–substrate adsorption strength—measured by FN heteroexchange experiments—is proportional to the focal adhesion density and the typical spacing of the FN fibril patterns. This observation implies a cellular feedback cycle controlling the applied force onto the extracellular matrix ligand proteins via the density of the focal complexes in dependence on the adsorption strength of the proteins on the substrate.

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