IOPscience iopscience.iop.org

Home Search Collections Journals About Contact us My IOPscience

Quantitative analysis of fibronectin fibrillogenesis by endothelial cells on biomaterials

This article has been downloaded from IOPscience. Please scroll down to see the full text article.

2004 J. Phys.: Condens. Matter 16 S2421

(http://iopscience.iop.org/0953-8984/16/26/015)

View the table of contents for this issue, or go to the journal homepage for more

Download details:
IP Address: 129.252.86.83
The article was downloaded on 27/05/2010 at 15:41

Please note that terms and conditions apply.

INSTITUTE OF PHYSICS PUBLISHING JOURNAL OF PHYSICS: CONDENSED MATTER

J. Phys.: Condens. Matter **16** (2004) S2421–S2426 PII: S0953-8984(04)72148-7

Quantitative analysis of fibronectin fibrillogenesis by endothelial cells on biomaterials

Tilo Pompe[1], **Claudia Mitdank and Carsten Werner**

Institute of Polymer Research Dresden and Max Bergmann Centre of Biomaterials Dresden, Hohe Straße 6, 01069 Dresden, Germany

E-mail: pompe-tilo@ipfdd.de

Received 18 November 2003
Published 18 June 2004
Online at stacks.iop.org/JPhysCM/16/S2421
doi:10.1088/0953-8984/16/26/015

Abstract

The pattern formation during the reorganization of fibronectin into fibrillar structures by endothelial cells was analysed in a quantitative way. Fibronectin was pre-adsorbed onto a maleic anhydride copolymer surface with a defined bond strength of fibronectin to the substrates in order to support its reorganization. Statistical analysis of quantitative image processing including autocorrelation analysis and single object measurement provided distinct parameters for the characterization of the process of fibronectin fibril formation. Periodicity and overall area of reorganized fibrils, and number of long fibrils are suggested as parameters for describing the impact of the variation of the fibronectin–substrate bond strength on the fibrillogenesis by endothelial cells.

1. Introduction

Interaction of cells with biomaterials depends in a general sense on the presence of pre-adsorbed or secreted proteins covering the solid substrates. For many cell types adhesion to artificial surfaces is realized by the binding of cell surface receptors (integrins) to extracellular matrix proteins, which had been immobilized on solid supports [1–3]. Important functions of the extracellular matrix proteins such as collagen and fibronectin (FN) are not restricted to the modulation of adhesion and migration of cells [4]. These proteins also support the presentation of growth factors to the cells and stimulate various intracellular processes. The mechanisms by which the binding of integrins to extracellular matrix proteins influence

[1] Author to whom any correspondence should be addressed.

 © 2004 IOP Publishing Ltd Printed in the UK

cell adhesion, migration, proliferation, and differentiation are intensively investigated in current studies. The conformation of adsorbed proteins as well as their mechanical binding status and lateral distribution are thought to be relevant in influencing cell differentiation of myoblasts, endothelial cells, and several other cell types [5–7]. All these features demonstrate the importance of the extracellular matrix for the growth and differentiation of cells on artificial substrates. Therefore, detailed knowledge of the influence of the physicochemical characteristics of the substrate on the physical and biological status of the extracellular matrix proteins and its effect on the cellular interaction with these proteins is essential for cellular bioengineering. In this context the assembly of supramolecular fibrillar structures of FN by cells such as fibroblasts or endothelial cells is receiving attention, since the process depends on the stretching of the protein by forces exerted from the cell via its cytoskeleton [8]. From that, the interaction strength of adsorbed FN with the underlying substrate can not only strongly influence the assembly of fibrillar supramolecular structures, but also acts as a cellular mechanosensor probing environmental characteristics to adequately switch the proliferation and differentiation of the cells.

In preceding studies a set of thin films of maleic anhydride copolymers with varying surface energy was established to modulate the strength of interaction with adsorbed FN molecules. By variation of the comonomer (octadecene, styrene, propene, ethylene) the advancing static water contact angle could be varied in a range from 100° to 40°. The change in the surface energy can be attributed to the different relative numbers of polar anhydride moieties due to the variation in the size of the comonomer; see [9]. Furthermore, the state of the adsorbed extracellular matrix protein FN was characterized in detail previously [10]. Briefly, similar amounts of approximately 400 ng cm^{-2} of FN could be immobilized onto the different copolymer surfaces. The conformations of the adsorbed FN were proved to be similar on all surfaces by means of immunofluorescence with monoclonal antibodies in specific FN domains. It was shown in displacement experiments on pre-adsorbed FN by proteins of serum containing cell culture media over 48 h that the FN bond strength towards the substrate surface depends on the substrate hydrophobicity, as is already well established [11]. Cell culture experiments on human endothelial cells from the umbilical cord vein grown under standard cell culture conditions demonstrated [10] sensitivity of the cells towards the modulated states of the pre-adsorbed FN. The more pronounced formation of focal adhesions could be correlated with the lower bond strength of the underlying layer of FN to the copolymer substrates. Together with that, a stronger reorganization of the pre-adsorbed monolayer of FN into fibrillar structures was observed. At later stages of culture of the endothelial cells a dependence of the differentiation characteristics into vascular-like structures on the bond strength of FN to the copolymer surfaces was reported [12]. Lower bond strength of FN to the substrate allowed for the development of vascular-like networks of the cells and sometimes small capillaries due to the more intense reorganization of pre-adsorbed and secreted FN into extended fibrillar networks.

As the preceding work revealed a strong correlation of FN bond strength with the behaviour of endothelial cells, especially as regards FN reorganization, the present work provides a tool for the analysis of the assembly process by which cells reorganize pre-adsorbed and secreted extracellular fibronectin on artificial surfaces. This approach complements the common qualitative comparison of images with a quantitative analysis with measurable statistically significant parameters. To demonstrate the methodology, FN was adsorbed onto poly(propene-*alt*-maleic anhydride) copolymer surfaces, which support the reorganization of FN by endothelial cells as a result of the low bond strength towards FN. By application of different analytical techniques, the pattern formation of FN fibrillar structures was investigated in order to provide a quantitative description of the fibrillogenesis.

2. Experimental set-up

2.1. Substrate preparation and cell culture

Thin films of poly(propene-*alt*-maleic anhydride) were spin coated and covalently fixed onto glass cover-slips as described elsewhere [9]. Fluorescence labelled FN (FN-TRITC) was immobilized from a solution of 50 μg ml^{-1} in phosphate buffered saline over 1 h onto the substrate surface. For details, see [10]. Subsequently, human endothelial cells from the umbilical cord vein were grown under standard cell culture conditions for 50 min on the FN-coated substrates and were fixed with paraformaldehyde prior to microscopy, as described elsewhere [10].

2.2. Image analysis

By means of fluorescence confocal laser scanning microscopy (TCS SP, Leica, Bensheim, Germany) the reorganized patterns of FN fibrils were imaged by a 40× immersion oil objective at a resolution of 1024 × 1024 pixels. For image processing the freely available programs Scion Image (Scion Corporation, Frederick, MD) and UTHSCSA ImageTool (developed at the University of Texas Health Science Centre at San Antonio, TX, and available from the Internet by anonymous FTP from ftp://maxrad6.uthscsa.edu) were utilized. For statistical analysis the fibrillar patterns of 20 cells were evaluated.

The periodicity of the reorganized FN fibrils was determined by applying a 2D autocorrelation analysis. With the 2D fast Fourier transformation and autocorrelation algorithms of the Scion Image software, the 2D autocorrelation images of the fibrillar patterns analysed were calculated. The mathematical relationship between the Fourier transform and the autocorrelation function is the basis of this algorithm:

$$\mathrm{Fourier}[\mathrm{Autocorr}(k)] = |\mathrm{Fourier}[x(i)]|^2. \tag{1}$$

Hence, the autocorrelation function can be calculated as the inverse Fourier transform of the square of the absolute value of the Fourier transform of the function of interest resulting in a real space function with the same periodicity as the original function. The autocorrelation image, as defined from cross-correlation analysis, is the sum of the multiplication of the pixel intensities of the original image with the pixel intensities of the same image plotted over the different translational displacements of the images with respect to each other. Therefore, maxima are expected in the autocorrelation image where the translational displacement leads to an overlap of similar structures.

3. Results

For the better analysis of the FN reorganization behaviour on cell culture substrates with different physicochemical characteristics and for the evaluation of its influence on the proliferation and differentiation of the cells, a method was developed to quantify the degree of FN fibrillogenesis.

Figure 1 shows a typical image of FN fibrils reorganized by endothelial cells over a time period of 50 min. The surrounding area indicates the typical featureless background of the unorganized pre-adsorbed FN. The reorganized pattern clearly exhibits a pronounced characteristic. In certain regions of the cell, parallel oriented fibrils have been evolved due to the polarization of the cells. These fibrils are formed with a regular spacing. Therefore, the periodicity of the pattern developed was chosen as a parameter for the characterization of the

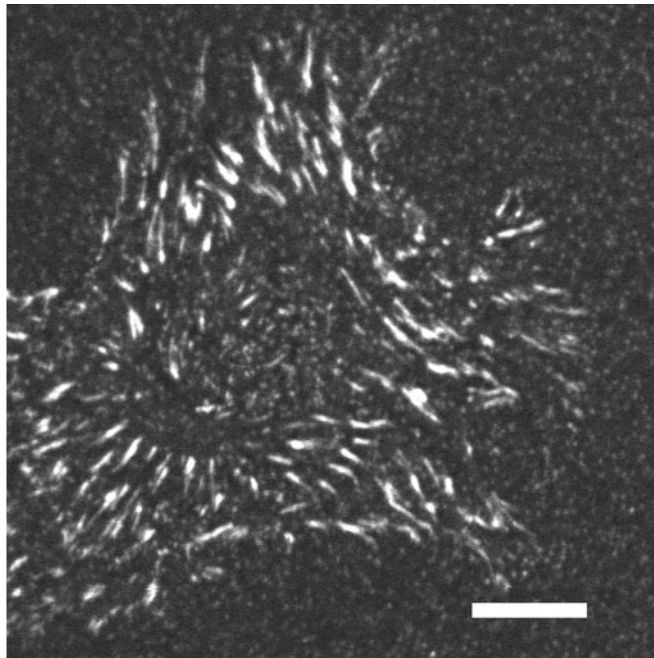

Figure 1. A typical image of FN fibrils reorganized by an endothelial cell on a poly(propene-*alt*-maleic anhydride) copolymer surface after 50 min of cell culture. Fluorescence confocal laser scanning microscopy reveals the pattern formation of the pre-adsorbed fluorochrome (rhodamine) conjugated FN. Scale bar: 10 μm.

FN fibrillogenesis. Furthermore, the size of the fibrils was analysed as defined by area, length, and width of the fibrils.

In a first analysis, the periodicities of the fibrillar patterns were determined by the autocorrelation algorithm of the Scion Image program. To improve the quality of the analysis, parts of the image with a similar fibril orientation were analysed separately from each other. The resulting autocorrelation elucidates a typical distance in the image analysed.

Figure 2 illustrates the image frame analysed with its autocorrelation image. To measure the typical distance of the fibrils a profile along a line perpendicular to the fibril orientation through the centre of the autocorrelation image was plotted as shown in figure 3. By measuring the distance between the central maximum and the first major maximum, the periodicity of the fibrillar pattern could be determined. The typical example reveals a periodicity of the FN fibrils of 2.3 ± 0.2 μm.

In a second analysis step, the area, length, and width of the fibrils were quantified. By usage of the UTHSCSA ImageTool software single fibrils were detected by setting an intensity threshold and further analysed by area measurement and measuring major and minor axes of the object. Unfortunately, the axis measurement is affected by certain problems for thin irregularly shaped objects, i.e. curved fibrils. It measures the largest extent of the object in one direction and then the largest extent perpendicular to that direction. By doing that, it just detects at the coordinates of object points with a maximum difference, but does not observe if the area between these two points is filled by the object. This algorithm leads to an overestimation of the major and minor axes for thin curved objects. Because of the limitations described and the presence of many irregular shaped fibrils, mean values for the area, length, and width gave no

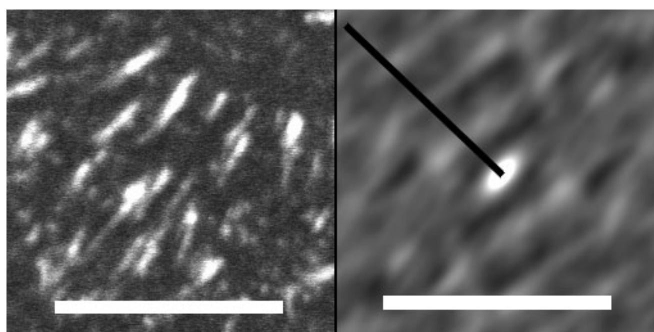

Figure 2. A cut-out image of figure 1 with similarly aligned FN fibrils (left) and the corresponding autocorrelation image (right). The line indicates the plotted profile of figure 3. Scale bar: 10 μm.

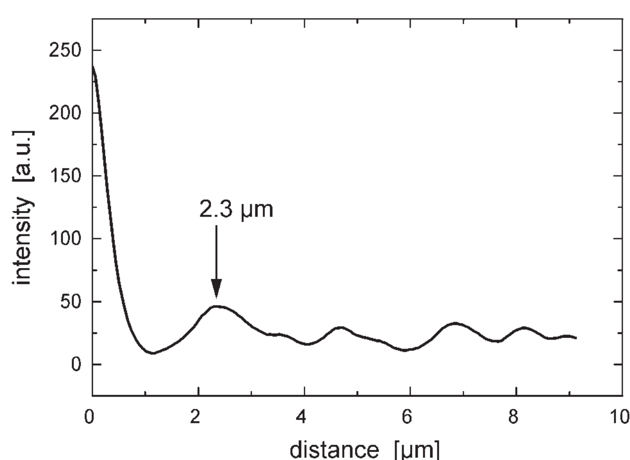

Figure 3. The profile plot of the autocorrelation image of figure 2 perpendicular to the fibril orientation through the centre of the autocorrelation image. The distance between the central maximum and the first major maximum indicates the typical distance between the fibrils.

distinct description of the FN fibrillogenesis. However, by analysing the sum of fibril areas per cell the degree of fibrillogenesis could be quantified. Furthermore, the number of long thin objects per cell exhibits a characteristic behaviour for FN reorganization, which was quantified by size exclusion for fibrils longer than 3 μm and thinner than 1.5 μm. Typical numbers for a statistical analysis of FN patterns after 50 min of reorganization by endothelial cells on a hydrolysed poly(propene-*alt*-maleic anhydride) copolymer surface are 250 $\pm$ 90 μm^2 for the summed area of FN fibrils per cell and 24 $\pm$ 10 large thin fibrils per cell.

The ongoing analysis of FN patterns on other maleic anhydride copolymer surfaces with a different FN bond strengths already indicates that the parameters reported can be used to quantify the FN reorganization behaviour as regards its dependence on the substrate surface properties. The FN patterns reorganized by the endothelial cells indicate distinct differences in periodicity, overall area of FN fibrils, and number of long thin fibrils as regards the dependence on the bond strength of FN to the substrates of different hydrophobicity. Detailed analysis of these observations will be reported in a forthcoming paper.

4. Conclusions

A method was described which allows a quantitative data evaluation for FN fibrillogenesis by endothelial cells on biomaterials. Qualitative comparison of different reorganization patterns by means of image evaluation can now be supported by a quantitative and statistical analysis of distinct features of pattern formation.

The quantitative characterization of FN fibrillogenesis should allow one to link the physicochemical properties of the substrate surface to cellular processes in a better way by addressing the influence of the extracellular matrix. The focus is on the structural contribution of the extracellular matrix, via the overall cell shape and the detailed structure of the cytoskeleton, to intracellular processes affecting proliferation and differentiation. It is well known that the extracellular matrix components are linked with cytoskeleton elements such as the actin filaments via the integrins and other intracellular proteins such as actinin, vinculin, and talin [1] and can—through this—induce and transduce specific intracellular signals. Importantly, the local structure and overall shape of the cytoskeleton controls cellular fate decisions such as proliferation, differentiation, or apoptosis [13]. Therefore, quantitative analysis of the pattern formation of extracellular matrix components can provide most valuable information about the intracellular response to different states of extracellular matrix attached to biomaterial substrates and may clarify the effect of physicochemical properties of the substrate surfaces on the cellular behaviour.

References

[1] Geiger B, Bershadsky A, Pankov R and Yamada K M 2001 *Nat. Rev. Mol. Cell Biol.* **2** 793
[2] Stupack D G and Cheresh D A 2002 *J. Cell Sci.* **115** 3729
[3] Miranti C K and Brugge J S 2002 *Nat. Cell. Biol.* **4** 83
[4] Maheshwari G, Brown G, Lauffenburger D A, Wells A and Griffith L G 2000 *J. Cell Sci.* **113** 1677
[5] Garcia A J, Vega M D and Boettiger D 1999 *Mol. Biol. Cell* **10** 785
[6] Katz B Z, Zamir E, Bershadsky A, Kam Z, Yamada K M and Geiger B 2000 *Mol. Biol. Cell.* **11** 1047
[7] Ingber D E 2002 *Circ. Res.* **15** 877
[8] Vogel V, Thomas W E, Craig D W, Krammer A and Baneyx G 2001 *Trends Biotechnol.* **19** 416
[9] Pompe T, Zschoche S, Salchert K, Herold N, Gouzy M F, Sperling C and Werner C 2003 *Biomacromolecules* **4** 1072
[10] Pompe T, Kobe F, Salchert K, Jørgensen B, Oswald J and Werner C 2003 *J. Biomed. Mater. Res.* A **67** 647
[11] Haynes C A and Norde W 1994 *Colloids Surf.* B **2** 517
[12] Pompe T, Markowski M and Werner C 2004 *Tissue Eng.* **10** at press
[13] Chen C S, Mrksich M, Huang S, Whitesides G M and Ingber D E 1997 *Science* **276** 1425