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Fibronectin matrix formation and the biocompatibility of materials

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The formation of an extracellular matrix (ECM) was investigated by the secretion of cellular FN during 72 h incubation of fibroblasts on defined hydrophilic glass and hydrophobic octadecyl glass (ODS). It was found, that the ability of fibroblasts to form their own ECM was inhibited on the hydrophobic ODS in comparison to glass, where significant amounts of FN were deposited in fibrils and clusters. This result was corroborated by the impaired morphology of cells on ODS, visualized by staining of actin micro-filaments and the FN receptor. Moreover, it was found that cell growth was significantly inhibited on the hydrophobic surface. In contrast to these findings, cell morphology and proliferation was not impaired on glass. Precoating of both substrata with FN could restore the cell morphology and enhanced the proliferation on the hydrophobic ODS. ELISA for FN binding using polyclonal and monoclonal antibodies revealed that under these circumstances total FN adsorption, as well as the presence of cell- and heparin-binding domains was much higher on ODS in comparison to glass.

1. Introduction

It is well known that mammalian cells adhere and grow better on wettable surfaces than on non-wettable (hydrophobic) substrata [1, 2]. Although the basis for this difference is still poorly understood, in part, it could be explained by the different conformations of adsorbed fibronectin (FN) [1–5]. FN is a major adhesive protein in the extracellular matrix (ECM) [3–8]. There is abundant evidence that the cellular functions of attachment, motility, growth and synthetic activity are largely regulated by the ECM [9] through the interaction with specific cell surface receptors belonging to the integrin superfamily [10–12]. Some recent data show that formation of FN matrix on the foreign surface might be important for the interaction with cells [13, 14, 16, 17]. Therefore, in a previous study [14] we began to ask the question whether initial remodelling of FN into a matrix [16, 17] may be connected with the biocompatibility of materials. We have demonstrated that fibroblasts incubated on hydrophilic substrata (clean glass) may reorganize preadsorbed FITC-conjugated FN (FFN) in ECM-like fibrilar structures, while this cellular ability was almost abolished when the cells were seeded on a hydrophobic substrate [14]. As hydrophobic substrata bind FN more tightly [5] it seemed reasonable that different binding strengths and conformations of adsorbed FN on hydrophilic and hydrophobic surfaces may affect FN reorganization. It remains to elucidate, however, how cells respond functionally on hydrophobic substrata, and whether there is an alteration of general cellular functions connected with the self-formation of the extracellular matrix.

The aim of the present study is to investigate how fibroblasts organize endogenously synthesized FN in ECM matrix structures [13] when cultured on defined hydrophilic and hydrophobic substrata. To evaluate the effect of FN in the initial stages of cell–material interaction, we studied the organization of the FN receptor and actin filaments. Cell proliferation was estimated as an additional measure for the biocompatibility of the materials. To learn more about the conformation of adsorbed FN we studied the binding of two different monoclonal antibodies, raised to distinct functional groups of the FN molecule, i.e. cell binding and heparin binding domains, and compared to the total FN presence indicated by polyclonal antibody binding.

2. Material and methods

2.1. Cells

Human foreskin fibroblasts were obtained from fresh biopsies and used up to the 9th passage. The cells were

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grown in Dulbecco's MEM containing 10% FBS (Sigma Chemicals Co., St. Louis, MO) in a humidified incubator with 5% CO_2. Fibroblasts from pre-confluent cultures were harvested with 0.05% trypsin/0.6 mM EDTA (Sigma), and trypsin was neutralized with FBS.

2.2. Preparation of fibronectin

Human plasma FN was prepared by affinity chromatography on gelatin-Sepharose 4B [15], and further purified on heparin-Sepharose 4B. Fibronectin was eluted with 0.5 M NaCl in 50 mM Tris pH 7.3 and lyophilized. For the experiments FN was dissolved in distilled water and stored at 4 °C.

2.3. Hydrophilic and hydrophobic surfaces

To obtain a hydrophilic surface, 18 × 18 mm glass coverslips (Menzel, Germany) were cleaned with cold chromium sulfuric acid. These clean glass slides (glass) were stored in double distilled water until use to keep the surface hydrophilic. To render the surface hydrophobic, clean dry glass slides were coated with the octadecyldimethylchlorosilane (ODS). For that purpose, the slides were incubated in 2% (vol/vol) solution of ODS (purchased from Sigma) in chloroform for 24 h, then rinsed with chloroform and finally washed with distilled water. Water contact angles were measured under static conditions by the sessile drop method on three different slides and found to be 25.1 ± 2.7 degrees for hydrophilic glass and 95.1 ± 1.7 degrees for ODS.

2.4. Cell attachment

Approximately 5×10^5 cells in 3 ml medium containing 10% FBS were incubated for 2 h in 6-well tissue culture plates (Falcon, Becton Dickinson & Company, New Jersey) containing the slides. Depending on the experimental protocol, some slides were precoated with 20 µg/ml FN in PBS for 30 min at 37 °C and then incubated with cells under serum-free conditions. At the end of the incubations the samples were fixed with 3% paraformaldehyde in PBS and viewed under phase contrast with a Zeiss Universal Microscope (Carl Zeiss Jena, Germany).

2.5. Measurement of cell growth

1×10^5 cells in 3 ml medium containing 10% FBS were cultured in a humidified CO_2 incubator for 3 days in 6-well tissue culture plates (Falcon) containing the slides. A MTT Cell Proliferation Kit I (Boehringer Mannheim Biochemica, Germany) was used to measure cell proliferation.

2.6. Distribution of fibronectin, fibronectin receptor and actin

To detect secreted FN (ECM formation) after 3 days of cultivation, fixed fibroblasts on glass and ODS were incubated with a monoclonal mouse anti-FN antibody (Dianova, Germany) diluted in PBS with 1% BSA at 37 °C for 30 min. This was followed by an incubation with FITC-conjugated goat anti-mouse IgG (Dianova, Germany) in the presence of 10% goat serum. To detect the FN receptor and actin, fixed cells were permeabilized with 0.2% Triton X-100 for 5 min. FN receptor was visualized with rabbit anti-β_1 integrin (a gift from Dr Kenneth Yamada, National Institute of Health, Bethesda, USA), diluted in PBS (with Ca^{2+} and Mg^{2+}) containing 1% BSA. Cells were incubated for 30 min at 37 °C, washed with PBS and then for 30 min at 37 °C with rhodamine-conjugated goat anti-rabbit IgG (Dianova, Germany) containing 10% normal goat serum. To detect actin, cells were incubated for 30 min at 37 °C with 5 U/ml FITC-conjugated phalloidin (Sigma). At the end of the incubations, samples were washed and mounted, then observed and photographed with a Jenamed Fluorescence Microscope (Carl Zeiss Jena, Germany).

2.7. Enzyme immuno assay for FN adsorption

The amount of adsorbed FN from a 20 µg/ml solution on the materials was investigated with an ELISA. After incubation at 37 °C for 1 h the surfaces were rinsed three times with PBS and incubated with the following antibody solutions: polyclonal rabbit IgG anti-human FN, or monoclonal mouse IgG anti-human FN directed versus the cell binding domain, or a monoclonal mouse IgG anti-human FN directed versus the heparin-binding domain (all antibodies Biomol GmbH, Germany). The secondary antibodies used were polyclonal goat anti-rabbit IgG conjugated with or goat anti-mouse IgG conjugated with POD (both antibodies from Dianova, Germany). OPD (1 mg/ml) was used as chromogenic substrate. The results are given as optical densities (OD) and allow a semiquantitative measure of the protein adsorption (polyclonal antibody binding) and the presence of binding sites on the surface (monoclonal antibody binding).

3. Results

Fig. 1 shows the morphology of fibroblasts after 2 h attachment on glass (Fig. 1a, c, e, g), and ODS (Fig. 1b, d, f, h). A clear difference in the initial cell spreading on hydrophilic and hydrophobic surfaces has been found. Many of the cells attached on glass possessed normal fibroblast morphology, with typical extended cell shape and prominent actin stress fibres (Fig. 1a). Numerous FN receptor-rich clusters of focal adhesions oriented to the direction of cell polarization were found (Fig. 1c). On hydrophobic ODS, however, cells were less spread. Most of the fibroblasts did not possess actin stress fibres (Fig. 1b) nor extended focal adhesions (Fig. 1d). In contrast, coating the slides with FN tended to restore normal cell morphology. Many of the cells on both hydrophilic and hydrophobic substrata had a similar morphology, with prominent actin stress fibres and focal distribution of the FN receptor activity (see for comparison Fig. 1e, g for

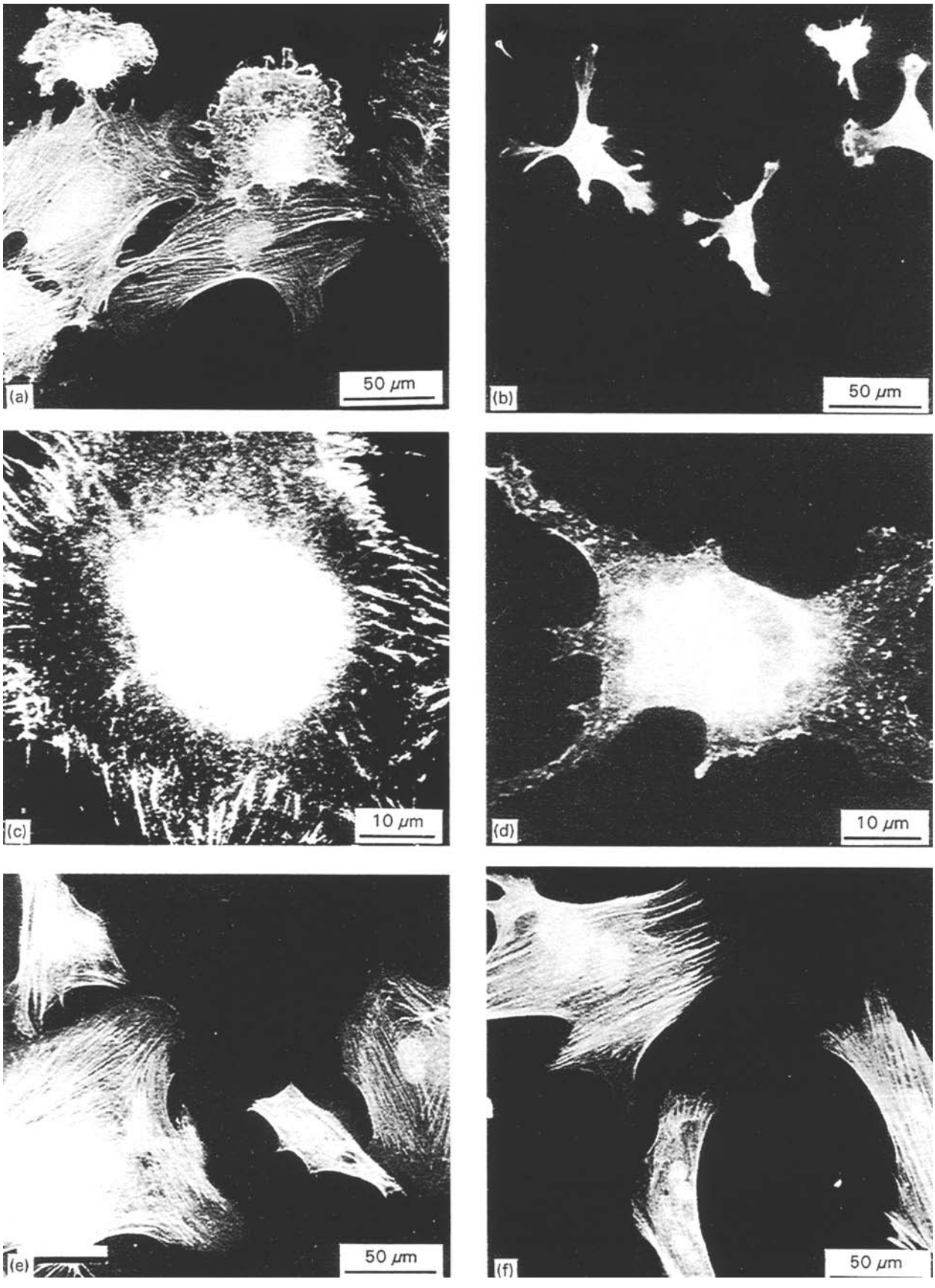
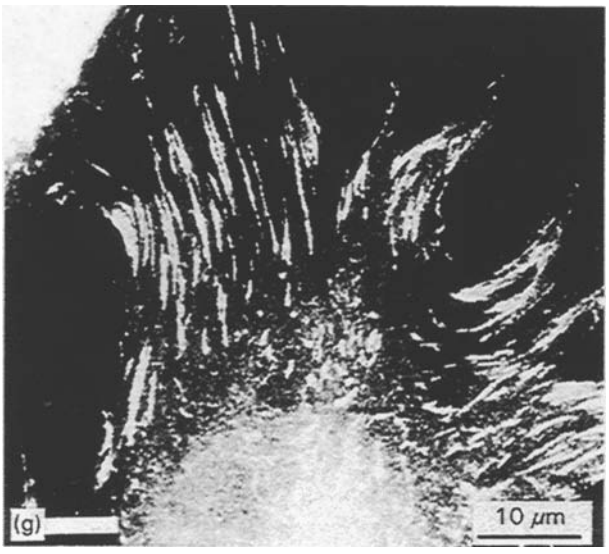


Figure 1 Morphology of fibroblasts adhering on plain glass and ODS, or after preadsorption of FN. Human fibroblasts in medium containing 10% FBS were attached for 2 h on plain (a, c) or FN-coated glass (e, g), and on plain (b, d) or FN-coated ODS (f, h). Samples were examined by immunofluorescence microscopy to detect actin (a, b and e, f) or fibronectin receptor (c, d and g, h).

glass and Fig. 1f, h for ODS). Focal adhesions, however, on ODS substrata were observed to be shorter and less frequent (Fig. 1h) in comparison to glass (Fig. 1g).

Plain glass and ODS were incubated with human fibroblasts for 72 h in the presence of 10% FBS for the investigation of FN matrix formation. During that time, on hydrophilic glass, fibroblasts deposited



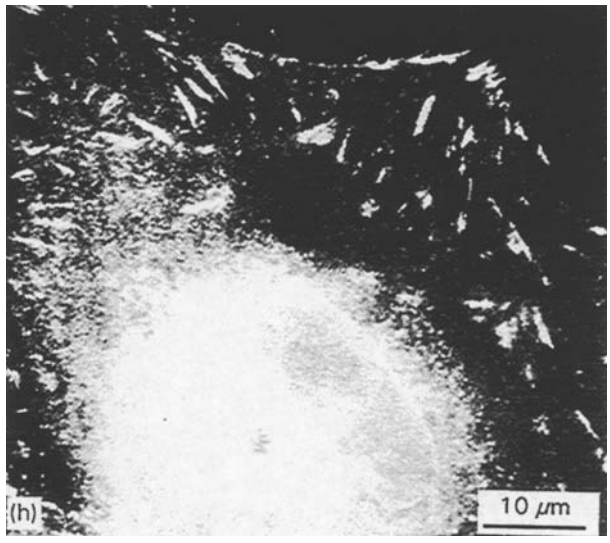


Figure 1 Continued

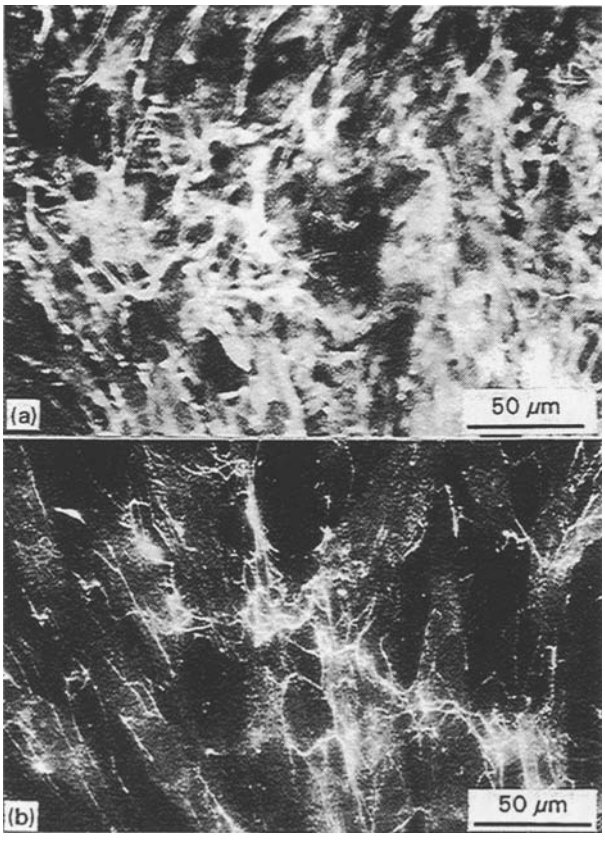


Figure 2 Fibronectin matrix formation on glass (a) and ODS (b). The surfaces were incubated for 72 h with human fibroblasts in medium containing 10% FBS. At the end of incubation, cells were fixed, mounted and processed for immunofluorescence microscopy.

TABLE I Fibroblast proliferation on plain and FN-coated substrata

Material	Proliferation (cells/mm^2) No FN	FN (20 μg/ml)
Glass	110	120
ODS	90	140

TABLE II Binding of FN antibodies (ELISA)

FN antibody specifity	OD at 495 nm Glass	ODS
Polyclonal	0.72	1.24
Cell binding domain	0.73	1.50
Heparin binding domain	0.74	1.54

significant amounts of FN, organized in fibrils and clusters, mostly oriented to the direction of cell polarization (Fig. 2a). If fibroblasts were cultured on hydrophobic ODS, however, essentially less FN fibril formation was observed (Fig. 2b) although cell spreading was almost in the same extent as on hydrophilic glass (not shown).

The results of cell growth measurements are presented in Table I. The estimation of cell proliferation with an MTT assay revealed an inhibition of cell growth on ODS, in comparison to glass. Preadsorption of FN induced a significantly higher proliferation of cells on ODS in comparison not only to the plain substrata, but also to the proliferation on glass.

The measurement of FN adsorption with ELISA demonstrated a significantly higher adsorption of FN on hydrophobic ODS in comparison to glass, as indicated by the higher binding of polyclonal anti FN antibody (Table II). Moreover, a significantly higher binding of monoclonal antibodies to the susceptible cell- and heparin-binding domains of FN was observed on ODS, in comparison to glass.

4. Discussion

In a previous study [14] we have demonstrated that fibroblasts reorganize significantly less amounts of exogenous FN in matrix-like structures [16, 17] when they were incubated for 4 h on hydrophobic ODS in comparison to hydrophilic glass after preadsorption of FN. This fact was explained by the stronger binding of FN to hydrophobic substrata [5], which might

inhibit the necessary exchange and reorganization of FN by other competing serum proteins. It was not clear, however, from these experiments, if the general cellular function, to form its own ECM structure, was altered in response to the hydrophobic environment.

The results from this study show that the wettability of materials may affect directly the ability of fibroblasts to form their own FN matrix. The failure of a hydrophobic substrate such as ODS to support the formation of a natural-like FN matrix may be the reason for the general impaired cell morphology and spreading on plain hydrophobic substrata in comparison to hydrophilic, manifested by the insufficient formation of focal adhesions and actin micro-filaments. Long-term cell adhesion of fibroblasts may be accompanied by endogenous secretion of FN which begins approximately two hours after cell seeding [8]. In this respect, it is also possible that conformational changes of adsorbed, newly synthesized FN may occur on hydrophobic surfaces, which may impair the normal transfer of signals from the substrate to the cell interior. On the other hand, the adsorption of other serum proteins, which occurs before FN secretion starts, may permit the formation of a stable adsorption layer beneath the cells. It is well known that the strength of protein binding on a substrate increases with adsorption time and hydrophobicity [5, 18, 19]. Therefore, the longer the preadsorption and the more hydrophobic the substrate, the less the probability for a secreted FN to displace already adsorbed proteins. Both assumptions are corroborated by the impaired cell morphology and the diminished cell growth observed on plain hydrophobic ODS.

Otherwise, it was demonstrated that the preadsorption of exogenous FN may overcome the deleterious effect of hydrophobicity, most probably because of the much higher initial binding of FN, a fact which is also known from the literature [5, 6], and because of the higher susceptibility of cell- and heparin-binding domains under these conditions demonstrated with corresponding monoclonal antibodies. Thus, preadsorbed FN may provide better starting conditions for the subsequent cell growth [6]. The results of proliferation measurements confirm this assumption, although the restoration of normal fibroblast morphology on FN-coated ODS was not complete. As we showed in our recent study [20] FN preadsorption on hydrophobic surfaces may also enhance the impaired signalling of FN receptor through a tyrosine phosphorylation pathway. Further investigations are necessary to understand the role of matrix proteins and the corresponding signalling via integrins of cell–biomaterial interaction.

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