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Reorganization of substratum-bound fibronectin on hydrophilic and hydrophobic materials is related to biocompatibility

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It is a general trend that mammalian cells interact better with wettable surfaces than with non-wettable surfaces. The basis for this difference is still poorly understood. In this study hydrophilic clean glass and hydrophobic octadecyl glass have been used as model surfaces. We show that fibroblasts on hydrophilic surfaces may reorganize fluorescent fibronectin (FN) in an extracellular matrix-like structure whereas on hydrophobic surfaces no rearrangement of FN occurs. This was accompanied by a high proliferation of fibroblasts on clean glass whereas on octadecyl glass no cell growth occurred. Moreover, it was demonstrated that there are striking differences in the morphology of fibroblasts adhering to hydrophilic and hydrophobic surfaces, judged by the overall cell shape, the organization of FN receptors and actin filaments. Indeed, the preadsorption of FN on these surfaces could almost abolish morphological differences between hydrophilic and hydrophobic surfaces. However, preadsorption of FN could not restore the proliferation of fibroblasts on the hydrophobic surface. Taken together, the results suggest that the method of adsorption and reorganization of FN may be critical for the biocompatibility of materials.

1. Introduction

It is generally agreed that mammalian cells interact better with hydrophilic (wettable) surfaces than with hydrophobic (non-wettable) surfaces [1, 2]. The basis for this difference is still poorly understood [3], but since fibronectin (FN) was identified as a serum component that promotes cell adhesion and spreading [4, 5], this variation in the biological reactivity of materials might be explained by established differences in the FN adsorption on the surfaces [3, 6, 7]. This possibility is supported by the analyses of adsorbed FN with antibody binding assays [3, 6] and ellipsometry [8]. Up to now, however, it is not clear how the cells can recognize such differences in the FN conformation.

On the other hand, cells can modify adsorbed proteins [9–11], which also may be important for the biocompatibility of materials. For example, fibroblasts in serum-containing medium, removed adsorbed FN from glass (hydrophilic surface) and organized it into specific fibrillar structures [9, 10] similar to the FN matrix fibrils [11]. This phenomenon is related to the general property of anchorage-dependent cells, which form an extracellular matrix (ECM) [11]. In this respect the important question arises: how may the above differences in FN adsorption on hydrophilic and hydrophobic surfaces affect FN reorganization by the cells?

Recently we have been studying the ability of fibroblasts to reorganize fluorescent FN (FFN) bound to materials with different surface wettability, assuming this may be related to the biocompatibility. In this study, defined hydrophilic and hydrophobic surfaces have been used as a model, and we have demonstrated that fibroblasts on hydrophilic surfaces may reorganize FFN in ECM-like structures whereas on hydrophobic surfaces almost no rearrangement of FFN occurs. To learn more about the initial stages of cell–material interaction, and particularly the effect of FN, we studied the morphology of cell spreading, and the organization of FN receptors and actin filaments. The subsequent cell proliferation has been measured in order to further characterize the biological reactivity of the surfaces in context with FN organization.

2. Material and methods

2.1. Cells

Human foreskin fibroblasts were prepared from fresh skin biopsy and used up to the seventh passage. The cells were grown in Dulbecco's minimal essential medium (DMEM) containing 10% fetal bovine serum (FBS) (Sigma Chemicals Co., St. Louis, MO) in a humidified incubator with 5% CO_2. For experiments

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the cells were harvested from pre-confluent cultures with 0.05% trypsin/0.6 mM EDTA (Sigma).

2.2. Fibronectin and fluorescent fibronectin preparation

Human plasma FN was prepared by affinity chromatography on gelatin-Sepharose 4B [12], and further purified on heparin-Sepharose 4B. FN was eluted with 0.5 M NaCl, 50 mM Tris pH 7.3 and lyophilized. For experiments FN was dissolved in distilled water and stored at 4 °C.

FN was conjugated with FITC (Sigma) as described [10]. Briefly, 2 mg of FN were incubated with 0.1 mg FITC for 2 h at room temperature in 0.1 M carbonate buffer pH 9.0. Non-reacted dye was removed by gel filtration on Sephadex G-25 (Pharmacia, Sweden). FFN retained complete biological activity based on cell spreading assays (data not shown).

2.3. Preparation of hydrophilic and hydrophobic substrata

Glass coverslips of size 18×18 mm^2 (Menzel-Glaser, Germany) were cleaned with cold chromium sulphuric acid for 24 h. The hydrophilic glass was stored in distilled water until use. To render the glass hydrophobic, the slides were incubated in 2% dimethyloctadecyssilane (silane) in chloroform for 24 h. The water contact angles were measured by the sessile drop method in triplicate. According to the experimental protocol above substrata were coated with 20 μg/ml FN or 40 μg/ml FFN in PBS for 30 min at 37 °C.

2.4. Cell attachment and morphology

Approximately 5×10^5 cells were incubated for the times indicated in six-well polystyrene plates (Falcon, Becton Dickinson, USA) containing the slides. At the end of the incubations the cells were fixed with 3% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4 and mounted in Mowiol [13]. To study the FFN reorganization the samples were viewed under both phase contrast and fluorescence microscopy using an inverted microscope Axiovert 100 (Carl Zeiss, Germany).

2.5. Distribution of fibronectin receptor and actin

Fixed samples were permeabilised with 0.5% Triton X-100 for 5 min and saturated with 1% albumin. To detect actin the cells were incubated for 30 min at 37 °C with 5 U/ml FITC-conjugated phalloidin (Sigma) in PBS. To detect the β1 integrin (FN receptor), the cells were incubated for 30 min at 37 °C with rabbit anti β1 (a gift from Dr Kenneth Yamada, National Institutes of Health, Bethesda, USA), diluted in PBS with 1% albumin followed by RITC-conjugated goat anti-rabbit IgG (Dianova, Germany) containing 1% normal goat serum, for 30 min at 37 °C. At the end of the incubations samples were mounted with Mowiol.

2.6. Measurement of cell growth

1×10^5 cells in 3 ml medium containing 10% FBS cultured in a humidified CO_2 incubator for the times indicated in six-well polystyrene plates containing the slides. At the end of the incubations, the slides were rinsed with PBS and the mean value of attached cells per mm^2 were estimated in 15 randomly selected microscopic fields. An ocular micrometric scale (Zeiss) was used to measure the surface area. In some experiments the colorimetric MTT Cell Proliferation Kit I (Boehringer Mannheim Biochemica, Germany) was used to confirm the data.

3. Results

3.1. Cell morphology on hydrophilic and hydrophobic surfaces

Underwater contact angles for hydrophilic glass and silanized glass were found to be 25.1° ± 2.7° and 95.1° ± 1.7°, respectively. FN was adsorbed to both surfaces. FN-coated and uncoated surfaces were incubated with human fibroblasts for 2 h. The overall cell morphology and the organization of FN receptor and actin filaments are shown in Fig. 1. The cells attached more strongly (11.8 ± 3.1 cells/mm^2) and spread more on glass (Fig. 1b) than on silane (Fig. 1a). More than 50% of the cells on glass represent normal fibroblast morphology (Fig. 1b) with a typical extended body and prominent actin filament bundles (Fig. 1d). FN receptor in the spread cells was localized predominantly in focal adhesions, while the rest, less spread cells, represented diffuse FN receptor activity and actin staining (Fig. 1f). Cell attachment was significantly lower on silane (7.0 ± 1.3 cells/mm^2). The cells appeared smaller and "shrunk", because of uncompleted spreading (Fig. 1a, c, e). Most of the fibroblasts possessed neither actin stress fibres (Fig. 1c), nor focal adhesion plaques (compare Fig. 1e to 1f). FN receptor activity and actin were concentrated predominantly on cell edges (Fig. 1e, c). Coating the slides with FN completely restored normal fibroblast morphology on silane (Fig. 2a), but also on glass (Fig. 2b). Apparently, most of the cells (more than 90%) on these preparations looked similar (based on phase contrast pictures (Fig. 2a, b)), with prominent actin fibres (Fig. 2c, d) and focal distribution of FN receptors, although focal adhesions seemed to be shorter and less frequent (Fig. 2e, f) on the hydrophobic surface (silane).

3.2. Cell growth on hydrophilic and hydrophobic surfaces

Proliferation of fibroblasts was much higher on hydrophilic surfaces independently of FN coating: 21.6 ± 6.8 cells/mm^2 for glass and 22.2 ± 5.8 cells/mm^2 for FN-treated glass (Table I). In contrast, no cell proliferation has been found on silane (7.7 ± 2.2 cells/mm^2), but this significantly increased

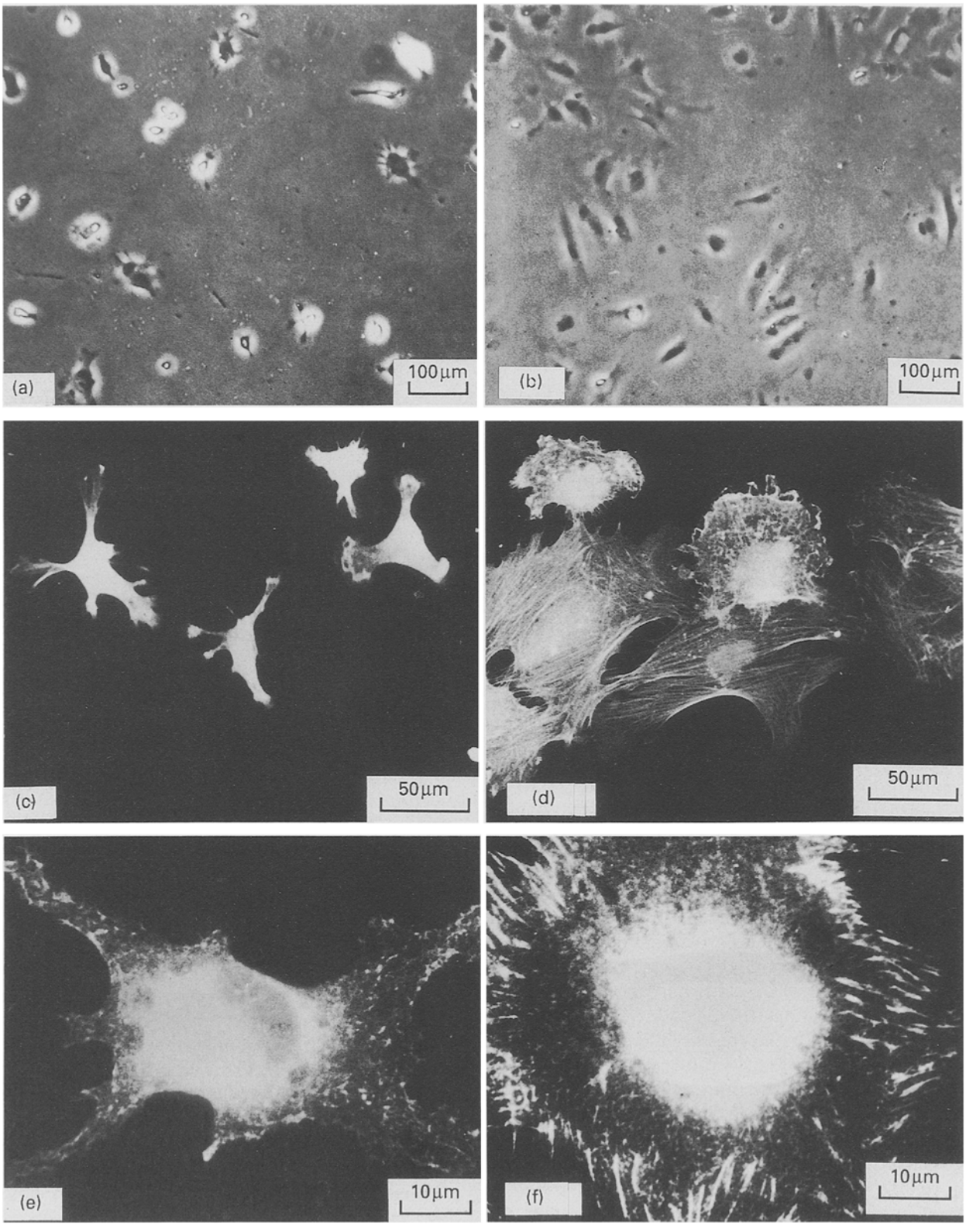

Figure 1 Morphology of human fibroblasts adhered to glass (b, d, f) or silane (a, c, e) in the absence of fibronectin. Fibroblasts were incubated on coverslips for 2 h, fixed and photographed under phase contrast microscopy (a, b) or fluorescence microscopy for actin (c, d) and β1 integrin (e, f).

TABLE I Adhesion and proliferation of fibroblasts [cells/mm^2; means ± SD]

	Counted with phase contrast microscopy				MTT assay
Incubation	2 h		72 h		72 h
FN preadsorption	no	yes	no	yes	no
Glass	11.8 ± 3.1	10.7 ± 3.5	21.6 ± 6.8	22.2 ± 5.8	26.6 ± 2.4
Silane	7.0 ± 1.4	11.0 ± 2.5	7.7 ± 2.2	12.0 ± 4.1	11.2 ± 1.0

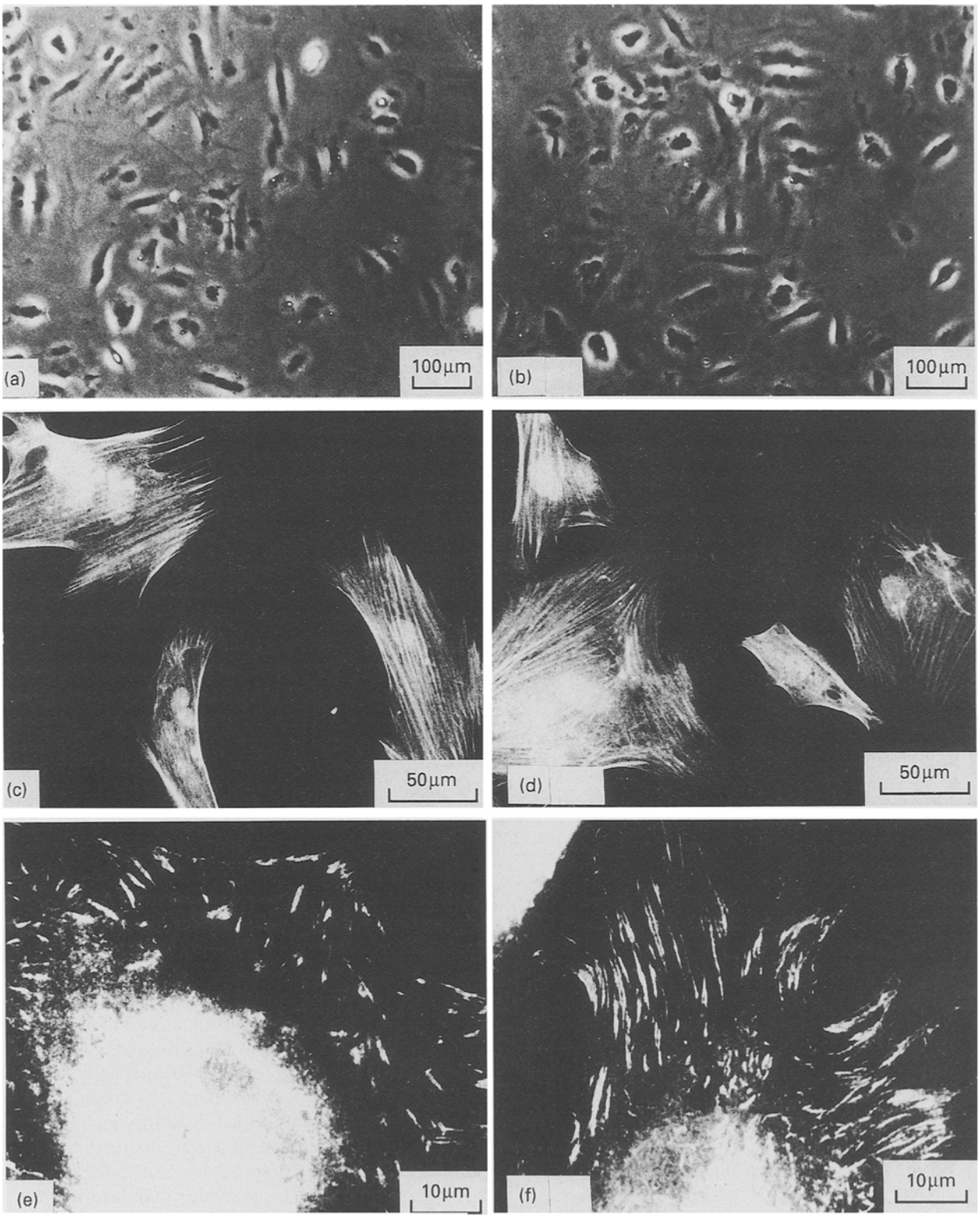

Figure 2 Morphology of human fibroblasts adhered to glass (b, d, f) or silane (a, c, e) in the presence of preadsorbed fibronectin (20 μg/ml). Fibroblasts were incubated on coverslips for 2 h, fixed and photographed under phase contrast microscopy (a, b) or fluorescence microscopy for actin (c, d) and β1 integrin (e, f).

if this surface was treated with FN (12.0 ± 4.1 cells/mm^2). Nevertheless, proliferation was still almost two times less than on glass. The latter results were confirmed by MTT assay (Table I).

3.3. Fluorescent fibronectin reorganization on hydrophilic and hydrophobic surfaces

FFN was adsorbed to the above surfaces, and these substrata were incubated with human fibroblasts for 4 h in the presence of 10% FBS. During that time fibroblasts removed significant amounts of FFN from the hydrophilic glass. This phenomena appears as dark streaks against the bright fluorescent background of adsorbed FFN (Fig. 3a, b). The reorganized FFN appeared in patches and fibrils mostly beneath the cells. By adjusting the plane of the focus, however,

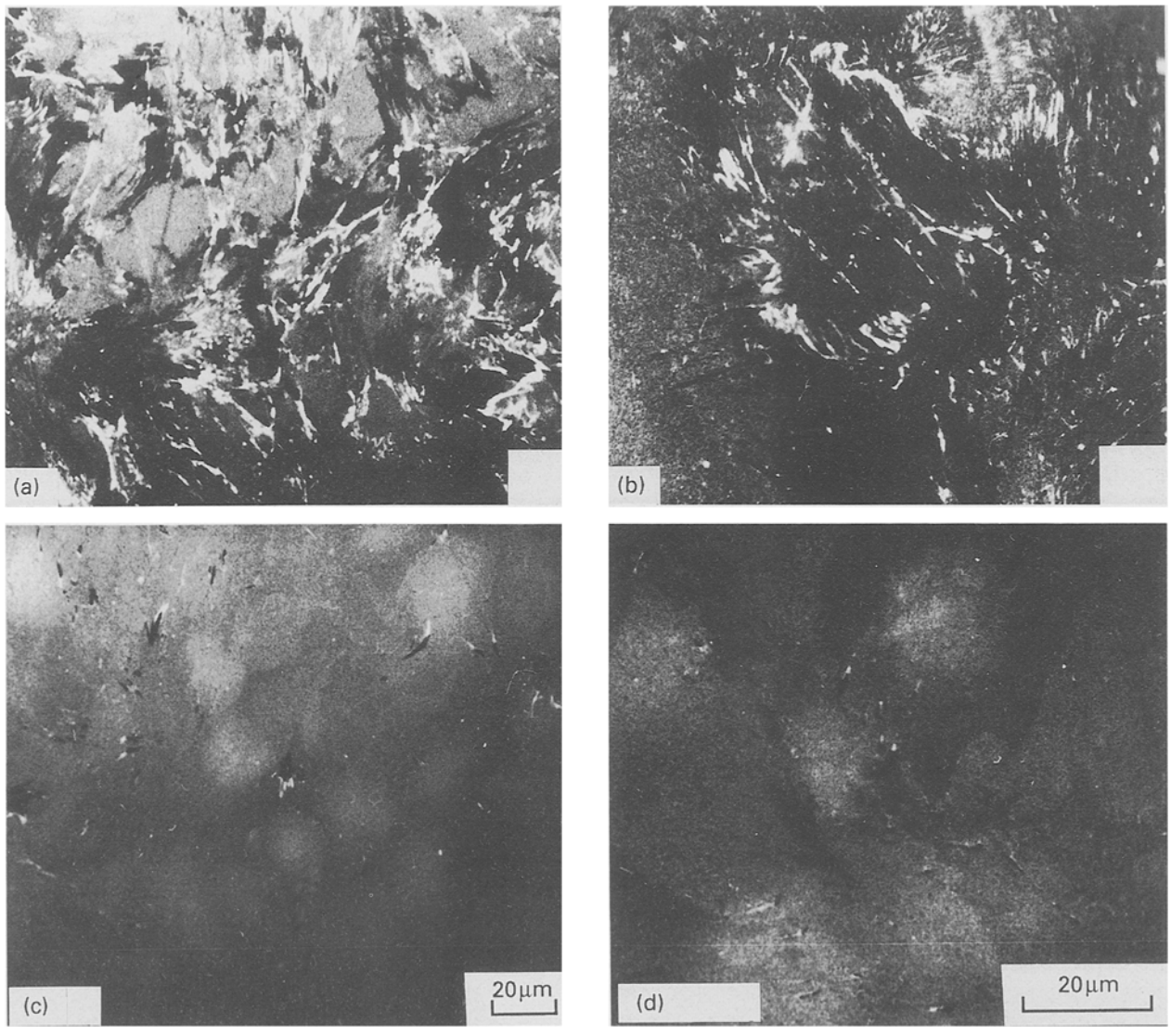

Figure 3 Reorganization of fluorescent fibronectin on glass (a, b) or silane (c, d) in the presence of serum (10% FBS). Fibroblasts were incubated on coverslips preadsorbed with FITC-labelled fibronectin for 4 h. Fibronectin reorganisation was viewed by fluorescence microscopy.

it was evident that many of FFN fibrils were located on the upper cell surface (not shown here). If fibroblasts were cultured on hydrophobic silane, however, essentially no removal of FFN was detected (Fig. 3c, d): only single streaks and fibrils of reorganized FFN could be observed. On the other hand, the cells were spread almost to the same extent as on hydrophilic glass (see Fig. 2a, b). The highest fluorescent background of adsorbed FFN was observed visually on silanized surfaces, but this is not demonstrated in the micrographs in Fig. 3 because of the photobleaching effect and automatic exposure time used.

4. Discussion

In general our results confirm that hydrophilic surfaces possess better biological properties for cell attachment [1, 3, 14], judged by morphological examination of fibroblast spreading and, organization of FN receptor and actin filaments. On hydrophobic surfaces the cells attached significantly less, and show an abnormal cell shape and morphology, most probably because of the uncompleted cell spreading. Coating the slides with FN apparently restored normal cell morphology, making the morphology almost indistinguishable between hydrophilic and hydrophobic surfaces. This suggests that the cells cannot recognise well established [3, 6, 7] conformational differences of adsorbed FN. On the other hand, it is difficult to say that coating the slides with FN simply increases the adhesivity of the surface, making it more compatible for cells. The problem is that even in the absence of exogenous FN the cells can modify their local environment by secreting the attachment factors [8, 25], including FN [6, 11, 15, 16]. Therefore, we studied the subsequent cell growth of the adherent cell population as an additional criterion for the materials surface compatibility. These experiments demonstrated complete absence, or insufficient cell growth, of fibroblasts on silane and FN-treated silane, respectively. One possible explanation of this phenomenon could be that loosely adsorbed FN on hydrophilic surfaces [6, 7] provide a better substrate for cell growth [14, 17]. Stronger interaction of FN with hydrophobic substrata [15] might be unfavourable for the dewebbing process during mitosis [18]. Our results also agree with the general trend that cells grow better on glass

and tissue culture polystyrene [14, 17]. However, it is not in agreement with the trend that better growth of 3T3 cells occurs on hydrophobic ethyl metacrylate surfaces [19]. It is noteworthy, that the vitality and morphology of spread fibroblasts on silane after 72 h incubation was similar to hydrophilic glass. This was evident also by corresponding enzymatic activity of fibroblasts mitochondria dehydrogenases (MTT assay) and the number of cells estimated by phase contrast microscopy.

Another possible explanation for the differences in cell growth on hydrophilic and hydrophobic surfaces may be that cells need to modify adsorbed FN for their normal function [20]. Therefore they remove and organize FFN from glass into specific fibrillar structures [9, 10], similar to the FN matrix fibrils [11]. As the FN removal is dependent on the exchange of a certain amount of plasma proteins on the substratum [10] it seems reasonable that stronger FN adsorption on hydrophobic surfaces [6] may affect FN removal and reorganization by the cells [16]. Indeed, our results with FFN reorganization by fibroblasts on glass and silane completely confirmed this point of view. No FFN reorganization into a fibril-like structures has been found on hydrophobic surfaces after 4 h of incubation. Only single streaks of FFN were removed from the substratum, which can be considered as "an attempt" by living cells to modify their substrata by a specific contractile activity [10]. It seems that such contractile modification of the ECM is important in embryonic cell migration [21] and connective tissue morphogenesis [20]. Another example of the physiological relevance of such activity of fibroblasts is the reorganization of collagen gels into condensed, dermal-like collagen matrices [22, 23], as well as, the fact that transformed cells are less proficient for the removing of FN from their substrata [24].

5. Conclusions

Summarizing this study, we conclude that the method of adsorption of FN plays a significant role in the biocompatibility of materials. One important event in this process seems to be FN reorganization, which was shown to be dependent on the surface wettability of the material. Further investigations are under way to understand the adsorption behaviour of other matrix proteins, and to elucidate the role of integrin receptors in this process.

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References

1. F. GRINNELL, M. MILAM and P. SPREE, *Arh. Biochem. Biophys.* **153** (1972) 193.
2. *Idem., Biochem. Med.* **7** (1973) 87.
3. F. GRINNELL, *Ann. NY Acad. Sci.* **516** (1987) 280.
4. R. KLEBE, *Nature* **250** (1974) 248.
5. F. GRINNELL and D. HAYS, *Exp. Cell Res.* **115** (1978) 221.
6. F. GRINNELL and M. FELD, *J. Biol. Chem.* **257** (1982) 4888.
7. D. D. MCABEE and F. GRINNELL, *J. Cell. Physiol.* **124** (1985) 240.
8. U. JONSON, B. IVARSON, L. INGEMAR and L. BERGHEM, *J. Colloid Interface Sci.* **90** (1982) 148.
9. J. D. AVNUR and G. GEIGER, *Cell* **25** (1981) 121.
10. F. GRINNELL, *J. Cell. Biol.* **103** (1986) 2697.
11. J. A. MCDONALD, *Annual Rev. Cell. Biol.* **4** (1988) 183.
12. E. ENGVALL and E. RUOSLAHTY, nt. *J. Cancer* **20** (1977) 1.
13. J. RODRIGUEZ and F. DEINHARDT, *Virology* **12** (1960) 316.
14. J. M. SCHAKENRAAD, H. J. BUSSCHER. C. R. H. WILDEVNUR and J. ARENDS, *J. Biomed. Mater. Res.* **20** (1986) 773.
15. P. MCKEOWN and D. F. MOSHER, in "Fibronectin", edited by D. F. Mosher (Academic, New York, 1989) p. 224.
16. F. GRINNELL, personal communication (1993).
17. I. K. KANG, Y. ITO, M. SISIDO and Y. IMAQNISHI, *J. Biomed. Mater. Res.* **23** (1989) 223.
18. R. RAJARAMAN, D. E. ROUNDS, S. P. S. YEN and R. REMBAUM, *Exp. Cell. Res.* **88** (1974) 327.
19. T. A. HORBETT, M. B. SCHWAY and B. D. RATNER, *J. Colloid Interface Sci.* **104** (1985) 28.
20. D. STOPAK and A. K. HARRIS, *EMBO J.* **5** (1986) 665.
21. D. NEWGREEN and J.-P. THIERY, *Cell Tissue Res.* **211** (1980) 269.
22. E. BELL, B. IVARSOON and C. MERRILL, *Proc. Natl. Acad. Sci. USA* **76** (1979) 1277
23. F. GRINNELL and F. R. LEMKE, *J. Cell Sci.* **66** (1984) 51.
24. B. GEIGER, T. VOLK and A. RAZ, *Exp. Biol. Med.* **10** (1985) 39.