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Contact guidance of fibroblasts on biomaterial surfaces

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The physico-chemical properties of a biomaterial and its surface-texture greatly influence the type of tissue reaction. Grooved substrata provoke cellular orientation which is known as contact guidance. Using gingival fibroblasts it has been demonstrated that microstructured hydrophilic (by glow discharge treatment) silicone also induces cellular alignment. Further analysis of the cell contacts by laser scanning microscopy has revealed that the focal adhesion sites were also oriented along the substratum microstructures. This phenomenon supports earlier hypotheses regarding cellular alignment and may be responsible for the orientation of the whole cell.

1. Introduction

The biocompatibility of implants is characterized by the absence of inflammatory processes on the biomaterial surface. This type of tissue reaction is not only influenced by the physico-chemical properties of the bulk material but also by its surface microstructure. It has been shown that the surface texture considerably alters the type of tissue reaction around subcutaneous implants, and that (probably) interfacial stresses and micromotion result in inflammatory reactions [1]. Only a certain micropore diameter between 1 μm and 3 μm promoted fibroblast attachment and tight adherence to the polymeric filter material without the formation of a fibrous capsule [1, 2].

Several *in vitro* experiments have demonstrated that similar micromorphological features provoke orientation of fibroblasts and other mammalian cells known as "contact guidance" [3–5].

Recently some *in vitro* experiments revealed that fibroblasts not only orientate but also conform to the topography of the material's surface and that this most probably leads to mechanical interlocking [6, 7]. The reason for this alignment is still unknown. Cells attach to the substratum surface in several different contacts known as podosomes, dot contacts, point contacts and focal contacts [8–11]. The latter are aggregates of transmembrane integrin receptors, which bind to extracellular ligands such as fibronectin and also to cytoskeletal proteins like vinculin and talin (for review see [12]). Experimental data suggest that focal contacts may be of crucial importance for cellular orientation [12, 13].

It was the aim of this study to analyse the orientation and attachment of human gingival fibroblasts to microstructured silicone by immunofluorescence and laser scanning microscopy (LSM) using monoclonal anti-vinculin antibodies.

2. Materials and methods

A line pattern with a line distance of 1 and 4 μm was written on the photoresist–covered oxide layer of a silicon wafer using an electron beam with an energy of 20 keV and a mean free half-width (MFHW) of 150 nm. This line pattern was developed in a wet etching process which resulted in a regular surface microstructure with a groove depth of 1 μm and a groove width of 4 μm separated by rectangular ridges of 1 μm. For a more detailed description of the production of the wafer surface see [6].

2.1. Production of microstructured silicone

The original surface pattern of the silicon oxide wafer surface was copied using medical grade elastomere (MDX 4-4210, Dow Corning, USA). One part of curing reagent and ten parts of elastomere were carefully mixed. Microstructured wafer discs of 1 cm^2 together with plain controls were glued to a smooth metal surface and surrounded by a plastic ring with a thickness of 2 mm and a diameter of 5.5 cm. The silicone replicas were produced by casting the liquid material into the mould over the surfaces of the wafer discs. This procedure was performed under dustfree conditions in a clean bench. As soon as the whole surface was covered with silicone the moulds were stored in an exicator and degassed for 20 min to remove all air bubbles. The plates were stored overnight in an incubator at 37 °C.

Subsequently, excess silicone was removed using a scalpel, and under dustfree conditions the polymerized

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silicone sheet was torn off the original wafer surfaces. The sheet was separated into small pieces of about 1 cm^2 size, cleaned and disinfected in 70% ethanol, steam sterilized and hydrophylized in an argon atmosphere at 2000 Pa by glow discharge treatment for 30 s (Harrick PDC 32G).

2.2. Fibroblast culture

Gingival biopsies were obtained from teeth after routine oral surgical procedures (removal of wisdom teeth). The samples were immediately immersed in tissue culture medium and subsequently washed with Dulbecco's phosphate-buffered saline (PBS, Serva, Heidelberg) without Ca^{2+} supplemented with antibiotic/antimycotic solution (500 µl/50 ml culture medium) (Gibco, Eggenstein). The specimens were cut under aspectic conditions into approximately 5 mm^2-sized pieces and incubated in culture medium (see also [6]).

Test cells of the fifth passage were used that had been grown in culture flasks (Costar, Fernwald) up to the desired high density. They were trypsinated with 0.05% trypsin solution, and seeded onto the surfaces, which had been placed into the wells of 24-well culture dishes. The culture medium consisted of Dulbecco's modified eagle medium (DMEM, Gibco) together with 25 mM HEPES, 100 i.U ml^{-1} pencillin, 100 µg ml^{-1} streptomycin, 0.2% bicarbonate solution, 4 mM L-glutamin, 1 mM Na-pyruvate and 15% fetal calf serum (all reagents: Gibco). The seeding concentration was 5×10^4 cells ml^{-1} using one-half millilitre for each well in the 24-well dishes (Coster).

2.3 Experimental protocol

Human gingival fibroblasts were incubated on plain and microstructured silicone sheets for 2 days at 37 °C 5% CO_2. Then test and control specimens were stained to identify vinculin-positive attachment sites. All tests were performed at least in triplicate on different days.

2.4 Immunofluorescence staining

The samples were washed with PBS and fixed with 2% paraformalin for 15 min at room temperature. After washing a second time with PBS the samples were permeabilized in 1% Triton X-100 for 5 min at room temperature and washed again in PBS. Then the specimens were incubated with 5% goat serum (Gibco) in a moist chamber for 15 min at room temperature. Vinculin staining was performed with a monoclonal antivinculin antibody (diluted 1:50) from the mouse (Sigma, München) in a moist chamber for 40 min at room temperature. The excess of antibody was washed out with PBS and then the samples were labelled with a second antibody goat-antimouse-TRITC (Dianova), diluted 1:200 for 40 min in the dark at room temperature. Subsequently the samples were washed with PBS and postfixed with 2% paraformalin for 10 min. After a final washing step with PBS the specimens were embedded with Mowiol 4-88 (Höchst, Frankfurt) and stored in the dark overnight.

2.5. Confocal laser scanning microscopy (LSM)

LSM was performed with a Zeiss LSM 410 in the confocal imaging mode. The sample was illuminated with a HeNe laser at a wavelength of 543 nm. For imaging rhodamine fluorescence, the emitted light was detected after passing through a red filter (BP 575–640 nm). For detection of reflection contrast images the reflected light passed through a green filter (BP 515–545 nm) to suppress fluorescence information.

Most images were obtained in dual channel overlay mode. In this mode the sample was illuminated with one laser source (HeNe 543 nm). The returning light was split into two beams using a dichroitic beamsplitter (560 nm) and detected after passing the matching filter by two independent detectors. Each channel was displayed in a separate colour (fluorescence = red, reflection = green) and overlayed in one image.

The topographic reconstruction of the surface structure was made using a function of the laser microscope. The sample is moved through the focus plane of the objective in defined steps (z-direction) while a subsequent series of reflection contrast images (x/y-direction) is evaluated. Out of the vertical distribution of the intensity of the detected light, the maximum is calculated and set in relation to the z-value. The resulting data matrix represents the shape of the surface. The structure is shown in a 3D-profile display.

3. Results

After polymerization the silicone surface was non-wettable and hydrophobic, but glow-discharge treatment rendered the surface completely hydrophilic and wettable with a water contact angle $\sim 0°$ (results not shown).

Optical sectioning of the samples under epi-reflection contrast revealed a regular line pattern of approximately 4-µm-wide ridges separated by 1-µm-wide and deep grooves (Fig. 1). The displayed ratio between

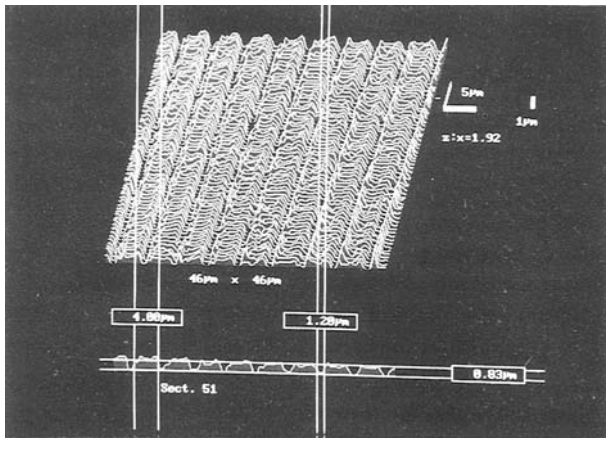

Figure 1 Topographic surface reconstruction of a microstructured silicone sample. Three-dimensional profile display with an area of 46 µm² (total of 256 profile sections). $z : x/y$ ratio = 1.95 : 1. The lower part shows a single profile of one section (No. 51).

z-axis and x/y-axis in Fig. 1 is 2:1, which means that the height of all structures was increased two-fold by the microscope.

Fibroblasts showed a typical elongation (Fig. 2). The cellular alignment correlated with an orientation of the vinculin-positive attachment sites (Fig. 3). Numerous focal adhesion sites appeared as strongly fluorescent match-like structures mostly located in the cellular periphery. The surface microstructure was hardly visible and is indicated by a white arrow.

Confocal laser scanning microscopy of one plane in the dual channel mode (reflection contrast plus fluorescence) revealed the underlying microstructure (grey/black) together with the vinculin-positive attachment sites (white) of the cell on the silicone surface (Fig. 4). The area covered by the cell body appeared light grey. The dark lines between the grey represent the grooves in accordance with the design of the structure.

Under higher magnification (Figs 5 and 6) the focal contacts in the cellular periphery showed an orientation to the line pattern similarly to the result in Fig. 3. Moreover, it is obvious that many of the focal adhesions were located on the ridges of the surface or at their edge (Fig. 6). The gap between the ridges was covered by the cell, i.e. the fibroblasts in Figs 4, 5 and 6 bridge one or several grooves. In the slim grooves (1 μm) focal contacts could not be detected.

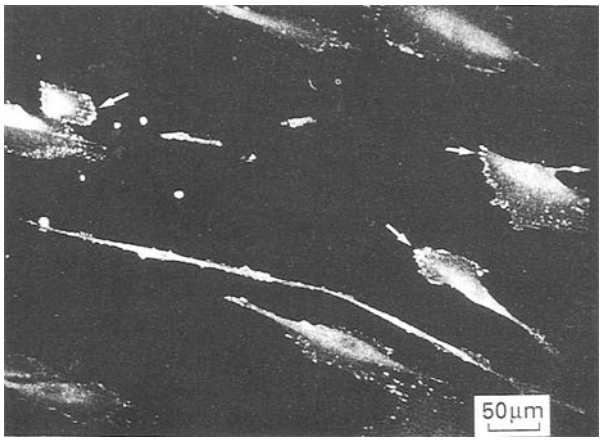

Figure 2 Fluorescence micrograph of human fibroblasts on structured silicone. The white reflection points are focal adhesion sites (arrows).

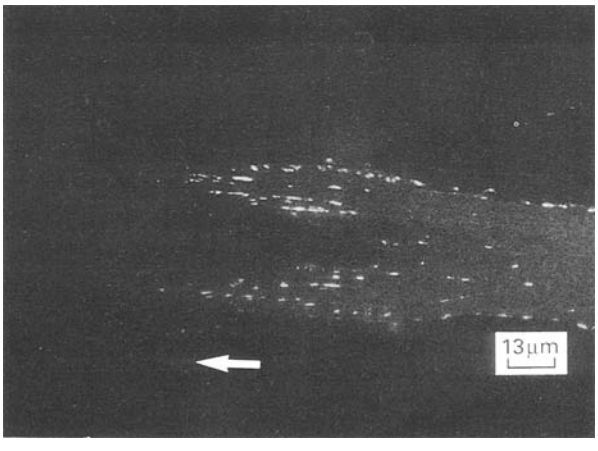

Figure 3 Focal contacts in the cellular periphery of a fibroblast on a microstructured silicone surface. Numerous contacts are visible which are elongated parallel to the long axis (white arrow) of the surface pattern.

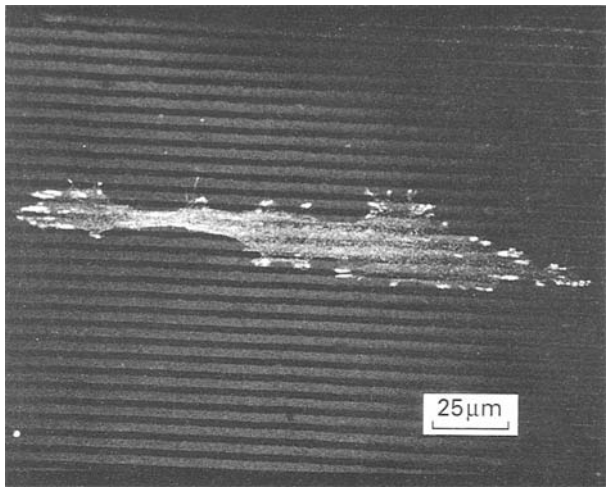

Figure 4 Dual channel overlay image of a fibroblast on a structured silicone surface. The dark grey bars correspond to the ridges of the pattern separated by the black grooves. Within the light grey area the surface is covered by the cell body. Focal adhesions appear as strongly fluorescent white dots or sticks.

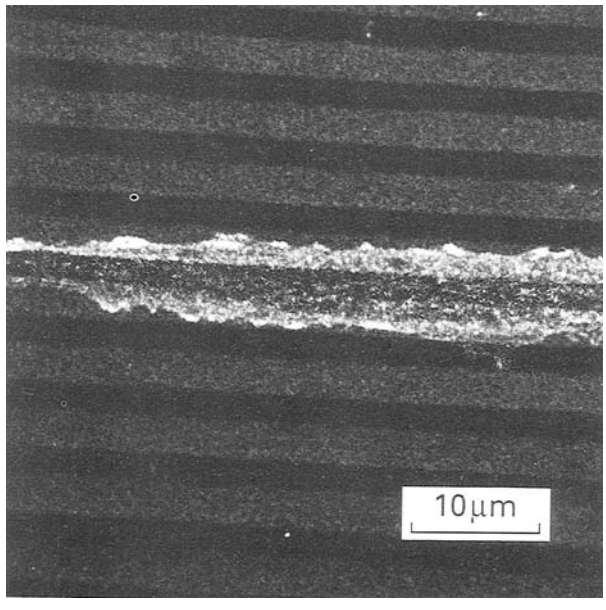

Figure 5 Dual channel overlay image of a cellular process of a fibroblast on the microstructured silicone surface. The dark grey bars correspond to the ridges of the pattern separated by the black grooves. Higher magnification of a slim cellular process bridging one groove.

4. Discussion

Although the three-dimensional display of the microstructured specimen resembled the original surface microstruture, the peaks at the edges of the ridges (Fig. 1) are known as artefacts. These are typical of the optical scanning technique and most probably caused

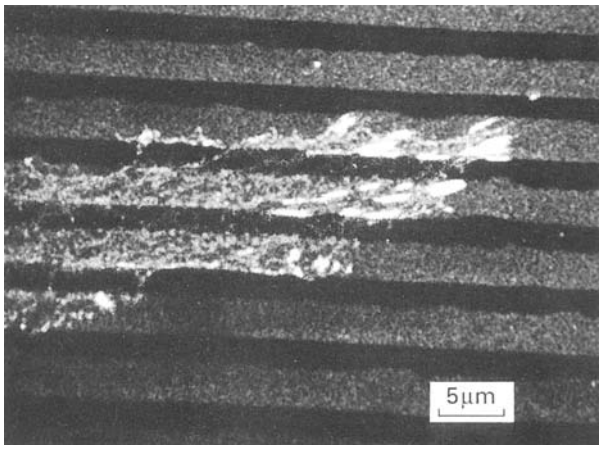

Figure 6 Similar image as in Fig. 5 of a cellular process of a fibroblast on the microstructured silicone surface. Higher magnification of a wide cellular process bridging several grooves with focal contacts on the ridges of the substratum.

by light diffraction. Similarly the nonrectangular shape of the vertical walls of the grooves is also due to the scanning technique of the LSM.

As already known from earlier studies [14–17], fibroblasts form close contacts to the surface, so-called focal adhesion sites, where the distance between the cell membrane and the substratum surface is approximately 10–15 nm. On the cellular side some cytoskeletal proteins like vinculin and talin connect actin fibres with the transmembrane integrin receptors [12].

In our experiments vinculin-positive sites could easily be detected inside the cells with conventional fluorescence microscopy as well as with epifluorescence laser scanning microscopy. Their observed size corresponds well to the dimensions reported by Izzard and Lochner [18, 19].

Depending upon the experimental conditions it can be assumed that plasma proteins like fibronectin and vitronectin covered the hydrophilic silicone surface thus promoting fibroblast attachment and spreading. This led, in some cases, to tremendously extended and elongated cells. In general non-transformed human gingival fibroblasts are much larger than, for example, chick heart fibroblasts [20].

The results of this study confirm that on hydrophilic silicone surfaces fibroblast orientation is mediated by the micromorphology of the surface, most probably by the orientation of the focal adhesion sites on the ridges of the substratum. Also it has been demonstrated that glow discharge treatment results in a hydrophilic silicone surface with similar properties and cellular reactions to those already observed on silicon oxide and araldite [5–7].

Further studies are required to prove whether or not the cellular focal contacts are restricted to the surface of the ridges after a longer period of incubation.

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