Quantitative analysis of fibroblast morphology on microgrooved surfaces with various groove and ridge dimensions

E.T. den Braber*, J.E. de Ruijter*, L.A. Ginsel†, A.F. von Recum‡ and J.A. Jansen*

*University of Nijmegen, Dental School, Laboratory for Biomaterials, POB 9101, 6500 HB Nijmegen, The Netherlands
†University of Nijmegen, Faculty of Medical Sciences, Department of Cell Biology and Histology, POB 9101, 6500 HB Nijmegen, The Netherlands
‡Clemson University, Department of Bioengineering, College of Engineering and Science, 301 Rhodes Research Center, Clemson, SC 29634-0905, USA

Fibroblasts have been shown to respond to substratum surface roughness. The change in cell size, shape and orientation of rat dermal fibroblasts (RDF) was therefore studied using smooth and microtextured silicone rubber substrata. The microtextured substrata possessed parallel surface microgrooves that ranged in width from 1.0 to 10.0 µm, and were separated by ridges of 1.0 to 10.0 µm. The grooves were either 0.45 or 1.00 µm deep. Prior to incubation, the substrata were cleaned and given a radio frequency glow discharge treatment. After surface evaluation with scanning electron microscopy and confocal laser scanning microscopy, RDF were incubated on these substrata for 5 days. During this period of incubation, the RDF were photographed on days 1, 2, 3, 4, and 5, using phase contrast microscopy. Digital image analysis of these images revealed that on surfaces with a ridge width <4.0 µm, cells were highly orientated (<10°) and elongated along the surface grooves. Protrusions contacting the ridges specifically could be seen. If the ridge width was larger than 4.0 µm, cellular orientation was random (<45°) and the shape of the RDF became more circular. Furthermore, results showed that the ridge width is the most important parameter, since varying the groove width and groove depth did not affect the RDF size, shape, nor the angle of cellular orientation.

Keywords: Surface topography, grooves, fibroblast response, quantification, digital image analysis, in vitro

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The field of biomaterials is changing slowly. Although biocompatibility is still defined as the ability of a material to perform with an appropriate host response in a specific application, recent research has shown that various physicochemical and geometrical material surface properties can be used to modulate the accompanying host response. This makes it possible to engineer future biomaterials that provoke a specific biological response, resulting in a unique healing process. Physicochemical properties that can influence the cellular interactions are shape, size and topography of a surface. The latter is not only limited to surface conditions like roughness or curvature, but also includes microtextured surfaces with a standardized surface roughness. For example, in vitro experiments have already demonstrated that surfaces possessing microgrooves induce orientation of fibroblasts. This phenomenon is also known as contact guidance. In two previous studies, we reported that especially surfaces with a 2 µm groove–2.0 µm ridge configuration were able to induce strong orientation and elongation of the fibroblasts cultured on these substrata. Surfaces with 10.0 µm grooves and ridges, however, did orientate the cells. All the grooves of those experiments were 0.45 µm deep. Further, we found that the proliferation rate of the rat dermal fibroblasts cultured on the microtextured surfaces was changed by the wettability of the surface, but not by the different topological micro dimensions on the substratum surface.

Although the influence of microtextured surfaces on the cellular behaviour is evident, very little is known...
about the fundamentals and basic mechanisms of this phenomenon. Several hypotheses have been proposed to explain this specific cellular behaviour. Some investigators suggest that the fibroblasts not only orientate, but also conform to the topography of the biomaterial surface, thus leading to mechanical interlocking. Others argue that cells on microtextured surfaces are able to rearrange their architecture in a three-dimensional orientation and establish an equilibrium of internal and external forces. This could result in a relaxed cytoarchitecture, which favours cellular differentiation.

Considering these theories, it can be questioned whether cells react in a comparable way to surfaces with different geometrical compositions. By varying the groove width, ridge width and groove depth of a standardized parallel groove pattern separately, it will be possible to determine which of these features induces the observed contact guidance. Furthermore, it will be possible to evaluate the impact and importance of the dimensional changes of specific surface features on the cellular behaviour. Therefore, the aim of this study was to quantify the possible changes in fibroblast morphology and orientation after culturing these cells on microgrooved surfaces with various dimensional configurations.

**MATERIALS AND METHODS**

**The substrata**

The experimental substrata were produced as described earlier. Briefly, photolithography was used to produce a total of 10 different textured silicon oxide wafers with different surface configurations.

In order to obtain the final experimental substrata, the smooth and grooved silicon oxide wafers were used as moulds, and covered with polydimethylsiloxane (silicone elastomer MDX 4-4210, Dow Corning) to produce a surface replica. After polymerization, the silicone rubber castings were peeled off the moulds and cut into small round discs of 175 mm². These substrata were then manually washed in a 10% Liquinox solution (Alconox Inc.), rinsed, ultrasonically for 12 h in distilled, deionized water. Subsequently, they were given a Soxhlet rinse for 12 h in distilled, deionized water. Finally, the substrata were air-dried and prepared for cell culture purposes by radio frequency glow discharge (RFGD) treatment (PDC-3XG, Harrick; Argon, 0.15 Torr, 5 min). After RFGD treatment, the quality and dimensions of the micro features on the substrata were confirmed by scanning electron microscopy (SEM; Jeol 6310) and confocal laser scanning microscopy (CLSM; Zeiss LSM 410).

**Cell culture**

Rat dermal fibroblasts (RDF) were isolated from dorsal skin grafts, taken from male Wistar rats, 40 to 43 days of age (100-120 g). After dissociation, these cells were incubated (37°C, 5% CO₂, 95% air) in α-MEM with Earle's Salts and with L-glutamine (Gibco), supplemented with 10% (v/v) heat treated fetal calf serum (Gibco), 2.5 µg ml⁻¹ amphotericin B (Gibco) and 50 µg ml⁻¹ gentamicin (Gibco). After approximately 3 days of culturing the RDF were rinsed with phosphate buffered saline without magnesium and calcium (PBS Dulbecco; pH 7.2), supplemented with 5 µg ml⁻¹ amphotericin B and 100 µg ml⁻¹ gentamicin to remove non-attached cells. Subsequently, the growth medium was added and replaced every two days by fresh growth medium. Upon confluence, the RDF were detached by trypsinization (0.25% (w/v) crude trypsin and 1 mM EDTA (pH 7.2)) and resuspended at a lower cell concentration in fresh growth medium. After identifying the cells as fibroblasts by phase contrast morphology analysis, the fifth generation of these cells was used for all experiments.

Substrata with a smooth or microtextured surface were placed in culture wells of 24-well plates (Greiner). After positioning the substrata, the surface grooves were examined with phase contrast microscopy (Leitz DMIL). Subsequently, approximately 1.0 x 10⁶ viable RDF ml⁻¹, suspended in sterile growth medium, were added to each substratum. RDF cultured in wells containing no substratum served as a control group (CTRL). The cells were incubated on a specific substratum for 5 days (37°C, 5% CO₂, 95% air) under static conditions. Growth medium was changed every two days. Every substratum configuration was tested in quadruplicate.

**Digital image analysis**

The effect of the surface microgeometry on the cellular morphology was quantified by digital image analysis (DIA), as described earlier by den Braber et al. In short, RDF at six evaluation areas were photographed by phase contrast microscopy during incubation on days 1, 2, 3, 4 and 5. The evaluation areas were selected by dividing the substratum surface in 740 possible fields of observation of 584.4 µm x 412.5 µm. Each of these fields was given a number, which was entered in a randomization program. Thus, a total of four randomly selected evaluation areas and the field at the centre of each substratum were photographed. Registration of the coordinates of these areas assured that the same areas were observed and photographed during the entire period of incubation.

The phase contrast photographs were scanned digitally (400 dpi x 400 dpi) and analysed with an Acorn R260 computer (RISC processor), the ArchImage 5 for the HAWK V12 frame grabber software package (Foster Findlay Associates, UK) and additional self programmed software. In-house written routines were used to trace all RDF in each digital phase contrast image and to prepare the resulting data for image analysis with the ArchImage program package. The ArchImage program measured several cell parameters, i.e. the cellular surface area, cellular perimeter, cellular circularity, maximum cell length, cell breadth perpendicular to the maximum length, the angle of cellular orientation relative to the surface grooves and number of pitches spanned by a single cell. A schematic representation of these parameters, except the parameter circularity, is
Digital Image analyses

Results of the CLSM surface analysis for B (left) and K (right) substratum. Three-dimensional surface representation of the CLSM surface analysis of B (left) and K (right) substratum. The Y axis represents the groove width (GW) and the groove depth (GD). Reducing the Z section at the bottom represents the groove width (GW) and the groove depth (GD). The measured groove depth is given, which are composed of 22 optical Z sections. Right of the Z surface profiles the size of the scanned area along the X axis. The size of the scanned area along the X axis can be found (1:3.9:6) and (1:4:6). The codes 23, 45, and 67 refer to the X and Y axes, respectively. The coordinates of the scanned area are given, which are composed of 22 optical Z sections. Right of the Z surface profiles the size of the scanned area along the X axis.

After obtaining the numerical data, these parameters were analyzed using univariate, multiple regression, and the non-parametric Kruskal-Wallis models (SAS, release 6.03, SAS Institute Inc., USA).
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Figure 1: Schematic representation of an RDF on a microtextured substrate. The parameters measured during DFA were the RDF surface area (white area within the cellular perimeter), the longest length of the cell (L), the cellular breadth (B), the perimeter (P), and the number of pitches spanned by the cell (n).

By the cell (N) cell:
- cellular orientation (d), the number of pitches spanned by the cell (n) during DFA were the RDF surface area (white area within the cellular perimeter), the longest length of the cell (L), the cellular breadth (B), the perimeter (P), and the number of pitches spanned by the cell (n).

Surface characterization

Results

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<th>Surface</th>
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Circularity = \( \frac{\text{Perimeter}^2}{4\pi \text{Area}} \)

As shown in Figure 1, briefly, this parameter is defined as
protrusions can be seen (Fig. 2). Smaller than the B-ces, the cell edges are clearly outlined, with the surface of the cells appearing to be composed of a single layer of these structures. The surface of the cells is not smooth, but exhibits a rough texture, with the protrusions appearing to be randomly distributed. The surfaces of the cells are irregular, with the protrusions appearing to be randomly distributed.
Finally, careful examination of the phase contrast images also showed that the cells on the textured surfaces seemed to attach to the ridges of the micropattern. This is best demonstrated by the photographs of the RDF on the G and H substrata (Figures 6 and 7). These cells possess several protrusions that end on the (darker coloured) ridges that are situated between grooves (Figure 2).

In order to quantify the DIA parameters (Figure 1), a total of 5217 cells were traced and evaluated. The quantitative analyses proved that the surface area of the RDF on the B, C and F substrata were significantly smaller \((0.001 \leq P \leq 0.0449)\) than the cells on the A, E, or CTRL surfaces (Figure 8). RDF on the D, G, H, J and K substrata did not show a clear difference in surface area, compared with the cells on the surfaces mentioned earlier. For example, the surface area of the RDF on the G substrata was significantly smaller \((0.001 \leq P \leq 0.0127)\) on days 1 to 3 than the area of the cells on the A, E and CTRL surfaces. The area of the cells on the G, and the B and C substrata did not differ significantly. However, on days 4 and 5 the opposite was found, since the area of the RDF on the G, B and C substrata did differ significantly, \((0.001 \leq P \leq 0.0263)\), while the cells on the G, A, E and CTRL substrata did not.

Concerning the measured perimeter and (maximum) length of the RDF (Figure 1) no continuous, strong significant differences were found (data not shown). For the parameter breadth, however (Figure 9), it was found that the cell breadth of the RDF on the B, C, F and G substrata was significantly smaller \((0.001 \leq P \leq 0.0117)\) than the breadth of the cells on the A, E, K and CTRL surfaces. The plots representing the breadth of the cells on the D, H and J substrata (not shown) were positioned in an area between the cell breadth plots of the A, E, K and CTRL surfaces and the B, C, F and G substrata (Figure 9). The breadth of these cells did not differ significantly from the cell breadth plots of the upper (A, E, K, CTRL) or the lower margin (B, C, F, G).

Analysis of the RDF circularity (Figure 10) showed that the cells on the A, E and CTRL surfaces were significantly rounder than the cells on the B, C, F and G substrata \((0.001 \leq P \leq 0.0469)\). The plots of the fibroblasts on the D, H, J and K substrata (not shown) could be found in the area between these plots, with the A, E and CTRL plots marking the upper margin, and the B, C, F and G plots representing the lower margin of this area.

DIA also calculated the angle of cellular orientation relative to the surface grooves (a; Figure 1). The results of these computations (Figure 11) showed that the cells on the B, C, G and H substrata were significantly stronger orientated \((0.001 \leq P \leq 0.0466)\) to the surface grooves than the fibroblasts on the D, E, F, J and K substrata. Orientation of the RDF on the F substrata is more complex. On days 1, 3 and 4 the cellular orientation of these cells was not significantly different compared with the orientation of the RDF on the B, C, G and H substrata \((P \geq 0.1213)\). On the contrary, the orientation of these cells did differ significantly from the orientation of the RDF on the D, E, J and K substrata on days 1, 2, 4 and 5 \((0.001 \leq P \leq 0.0122)\).
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The phase contrast images in Figure 3-7 also show that the RDF were able to span several grooves and ridges on the textured surfaces. DIA counted the number of pitches spanned by a single cell. On day 1, for example, the RDF on the B, C, D, E, F, G, H, J and K substrata spanned 15.51, 12.66, 6.80, 3.94, 10.36, 1.16, 5.15, 2.30 and 1.89 pitches, respectively. Additionally, the average number of pitches that were spanned by a single RDF on day 1 was defined as 100%, thus making comparison between the different textured surfaces possible. The results of these calculations are shown in Figure 12. Although this graph suggests that the number of pitches spanned by the RDF is lower on the B and F surfaces than on the C, D, E, G, H, J and K substrata, statistical evaluation proved that this difference was only significant for the cells on the F substrata up to day 3 (0.0001 ≤ P ≤ 0.0369). Furthermore, Figure 12 shows that pitches spanned by the RDF on the G substrata increases on day 2 and remains on a high level. Since the patterns on the D and F substrata, and the E and G substrata were a direct negative replica of each other (Table 1), direct statistical testing without a conversion to percentile values was possible. These evaluations showed that up to day 4 the pitch span of the RDF on the F substrata was significantly lower (0.0001 ≤ Pday 1-4 ≤ 0.0004) than on the D surfaces. Furthermore, this procedure showed that the pitch span by the cells was significantly lower (0.0001 ≤ Pday 1-5 ≤ 0.0122) on the E substrata than on the G surfaces.

DISCUSSION AND CONCLUSIONS

Results of this study confirm our earlier findings6,7 that a microtextured surface can induce orientation of the RDF cultured on these surfaces. DIA and statistical analysis demonstrate that the degree of cellular orientation relates to the dimensions of the micro features on the surface. This becomes even more evident when the alignment criteria that Clark et al.13 suggested are applied to the data plotted in Figure 11. These investigators defined a population of cells as highly aligned when the long axis of these cells makes an angle of <10° with the direction of the grooves. Review of the data in Figure 11 shows that the cells on the B, C, G and H substrata, and occasionally on the F surfaces, have an orientation which lies between 0° and 10°. Therefore, these cells have to be considered as highly aligned.

Further review of the DIA results concerning RDF size and shape shows that cells cultured on surfaces with small grooves, and especially small ridges like the B, C and F substrata, have a significantly smaller surface area and cell breadth, while no differences were found in cellular perimeter and length. These findings are supported by the measured parameter circularity, which shows that cells cultured on finely grooved surfaces are less circular than RDF cultured on smooth surfaces. The correlation between these results is quite clear. Since more circular cells possess a cell breadth that is equal or almost equal to the maximum cell length, their area will be larger than the area of the elongated cells which possess a smaller cell breadth. This suggests that the elongated cells on microtextured surfaces change their size by reducing their cell breadth. Although these results are rather straightforward, it is important to note that phase contrast microscopy is a method that results in a two-dimensional picture, not giving any information about the volume of the cell. Therefore, it is possible that the elongated RDF are not as flat as the circular cells. This information could be important in determining whether the elongated cells reduce their size, or just change their shape. Size change would mean that the RDF cultured on microtextured surfaces would actually have a smaller cell volume, where shape change suggests altered cell dimensions by a uniform cell volume. Recent reports by other authors suggests that...
optical sectioning with a confocal laser scanning microscope (CLSM) could provide more information on this subject, i.e. cell volume.

Evaluation of the data retrieved during this study also clearly indicates that the width of the ridge is mainly responsible for the contact guidance of the RDF on the microtextured surfaces. This corroborates the findings of Dunn et al. and Green et al. and is supported by the following results of this study. First, the data plotted in Figure 11 show that the average angle of cellular orientation (α) of RDF cultured on the B, C, F, G and H substrata is ≤10°. The micropatterns on these substrata surfaces possess a ridge width of 1.0, 2.0, 1.0, 1.0 and 2.0 μm respectively, but have different groove width and depth. However, if the ridge is ≥4.0 μm, as with the D, E, J and K surfaces (Figure 11), α results in an angle larger than 10°, even when the groove width and/or groove depth are identical to these dimensions on the ‘orientating’ substrata (Table 1). Slight orientation (10°<α<45°) can be found with the RDF on the D substrata which possess a ridge width of 4.0 μm. In contrast to this, the cellular orientation on the surfaces with larger ridges like the E, J and K substrata is random, which can be deduced from the fact that α ≈ 45°. Second, Figure 11 also demonstrates that the surface parameters groove width and groove depth are considerably less important for RDF orientation than the parameter ridge width. The data plotted in this graph show that the RDF on the B, C, F, G and H substrata are closely orientated along the surface grooves, although the groove width measures 1, 2, 4, 8 and 2 μm, respectively. The same principle applies to the groove depth. Although the H substrata possess grooves of only 0.45 μm deep, no significant differences in RDF orientation were observed, when compared with the B, C, F and G substrata with 1.0 μm deep grooves. This is in accordance with reports by Dunn et al., but differs from results published by Clark et al., who concluded that groove depth is the most important dimension of parallel grooved substrata influencing the orientation of cells. However, Curtis and Clark also concluded that these effects vary from one cell type to the other. Third, the phase contrast images show that RDF probably attach specifically to the ridges of the surface pattern. This is particularly clear with the RDF on the G (Figure 6) and H substrata (Figure 7). Careful examination of these photographs reveals that the RDF on these substrata possess cell protrusions that end on, and seem to attach to, the ridges. These possible attachments to the ridges could be associated with surface free energy changes caused by the manufactured, standardized roughness of the substratum surface. If the surface energy is more preferable on the ridges, the deposition pattern of the substratum bound attachment proteins will be influenced. This could result in the formation of cell—substratum bound contacts primarily on the ridges of the surface micropatterns. The significance of this finding is that surface free energy differences are produced on one and the same material by changing the surface topography. The surface free energy differences in the work of others was achieved by differing the surface chemistry. Consequently, the effect of surface free energy and surface chemistry was separated here. This hypothesis is supported by the work of Meyle et al., who reported numerous focal adhesion sites on the cellular periphery of gingival fibroblasts that were cultured on silicone surfaces with parallel surface microgrooves. After producing a reflection contrast/fluorescence image by dual channel CLSM, it could be seen that the vinculin positive attachment sites were located on the ridges of the silicone microtextured substratum. Furthermore, Ohara and Buck suggested that focal adhesion plaques are linear structures of 0.25–0.5 μm wide and 2.0–10.0 μm long. Since the geometrical dimensions of these plaques are so specific, only one major orientation of attachment is possible, which is parallel to the surface grooves and ridges (Figure 13). Accordingly, a cell attaching to a microtextured surface with small ridges will orientate itself parallel to these ridges. Still, it has to be noted that our results show a decrease of RDF orientation on substrata with a ridge size of ≥4.0 μm. This could be explained by the observation of Izzard and Lochnner that there is a possible minimum length of 2.0 μm acquired for focal contacts to provide adhesion. Therefore, if the ridge width increases, the possible orientation of adhesion plaque attachment can increase, thus resulting in cell attachment with a larger angle of cellular orientation (Figure 13). Finally, reviewing the results of this study, it cannot be excluded that the observed cell—substratum interactions are based on the resemblance of these microtextured surfaces with the topography of the fibrillar extracellular matrix, causing the cell to transform and differentiate. If this proves to be true, it is clear that these surfaces could contribute to the process of wound healing around implant surfaces.

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