Cell guidance by micropatterned adhesiveness *in vitro*

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Summary

Tracks of adhesiveness are believed to be involved in guiding morphogenetic cell migrations. Here, electronics microfabrication technology was used to manufacture patterns of alternating tracks of adhesive and non-adhesive substratum (untreated fused quartz and adjacent parallel tracks of hydrophobic treatment) of varying period (4, 6, 12, 24 and 50 μm). These experimental substrata were used to model, *in vitro*, possible differentially adhesive guidance cues. The effect of such patterned substrata was assessed using fibroblastic BHK cells and epithelial MDCK cells. Cells were oriented and elongated by these surfaces. Their responsiveness was dependent on cell type, cell-cell interactions, and the geometry of the patterns. Alignment of BHK cells increased with increasing pattern period. Single MDCK cells aligned to all pattern periods, their elongation being period-dependent, whereas colonies were mainly unaffected. These *in vitro* data have important implications, which are discussed with regard to *in vivo* guidance cues. The ability of cells to bridge over non-adhesive regions will influence the effectiveness of linear cues, and will be important for guideposting. The geometry of patterns of differential adhesion is here shown to be an important factor in determining the precision with which local guidance of cells may be controlled.

Key words: cell adhesion, cell guidance, BHK, MDCK, photolithography, contact guidance.

Introduction

Migrations of cells during morphogenesis and regeneration are fundamental to the development and maintenance of the form of multicellular organisms. Such cell migrations are highly stereotyped and are therefore likely to be subject to precise control by guidance cues. The cue or cues involved in control of cell migration are often difficult to determine, even in simple systems, because of the complexity of the environment. One approach to understanding this aspect of the development of multicellularity is to examine the basic behaviour of cells *in vitro* where it is easier to study individual cues. *In vitro* studies of cell behaviour in response to various guidance cues such as cell-cell interactions (Abercrombie, 1982), chemotaxis (see Lackie, 1986), galvanotaxis (Robinson, 1985; McCaig, 1986) and contact guidance (see Dunn, 1982; Curtis and Clark, 1990), have provided much important information as to the fundamental mechanisms by which cell locomotion can be controlled, and provide strong evidence of the appropriateness of a particular cue or combination of cues to many individual *in vivo* systems.

As a tool for examining cell-substratum interactions, the fabrication technology of the microelectronics industry has, in recent years, allowed experimental substrata to be patterned with greater precision to produce micropatterned topography (Brunette, 1986a,b; Dunn and Brown, 1986; Clark et al., 1987, 1990, 1991; Wood, 1988) and micropatterns of differential adhesiveness (O'Neill et al., 1986, 1990; Kleinfeld et al., 1988; Britland et al., 1992). Here we report the use of the techniques for micropatterning cell adhesiveness recently developed in our laboratories (Britland et al., 1992) to assess the effects of repeated patterns of linear tracks of alternating adhesive and non-adhesive substratum on cell behaviour. The fibroblastic BHK cells and epithelial MDCK cells were used to determine the effects of altering the spacing of parallel patterns of adhesiveness of the same dimensions of topographic gratings previously examined as to their effect on these cells' behaviour (Clark et al., 1990). Our findings indicate that the geometry of patterned adhesiveness is an important influence on adhesive guidance, and the implications of this, in relation to both *in vitro* and *in vivo* cell behaviour, are discussed.
Materials and methods

Substratum fabrication

Patterns of hydrophobicity on fused quartz microscope slides were made as previously described (Britland et al., 1992). Briefly, clean quartz slides were spin-coated with photoresist (Shipley, UK), exposed through masks of the desired pattern and developed to give a pattern of photoresist and exposed quartz. These photoresist patterns were then soaked in 2% (v/v) dimethyldichlorosilane in chlorobenzene, rinsed twice in chlorobenzene, and blown dry. The resist pattern was then removed by rinsing in acetone then water, resulting in a final pattern of methyl groups (the hydrophobic surface) covalently coupled to the quartz surface. Repeat patterns of equal-sized hydrophobic lines and untreated quartz spaces were made using masks of repeat spacings 4, 6, 12, 24 and 50 μm (i.e. feature sizes of half these values).

Cell culture

BHK and MDCK cells were suspended from cultures maintained by serial passage as previously described. Briefly, cells were cultured in HEPES buffered Dulbecco's MEM containing 10% foetal calf serum and antibiotics. Cells were suspended by trypsinisation, which was stopped with the above medium, washed by centrifugation, and resuspended in growth medium. Cells were seeded at a density of 0.2×10^6 cells on patterns in 6 cm Petri dishes.

Determination of cell alignment

The degree of alignment of BHK and MDCK cells on grating surfaces was determined as previously described (Clark et al., 1990). Briefly, cells were photographed under phase-contrast optics 24 hours after seeding, and the angle (between 0 and 90°) which the long axis of the cells makes with the grating direction determined within 10° sectors. Between 58 and 65 BHK cells, and between 44 and 71 MDCK cells, were scored for each data point. Alignment was taken to be the proportion of cells whose long axis (maximum caliper length) is less than 10° to the grating direction. In a randomly oriented population of cells this value would be expected to be 0.11. Statistical comparisons were made by determining chi² from 2×2 contingency tables.

Cell lengths of isolated MDCK cells (44 to 71 per sample) were measured from high-power enlargements of phase-contrast micrographs. Controls were cells on unpatterned fused quartz. The significance of differences was determined using Student’s t-test. For all statistical comparisons the significance level was taken to be P≤0.05.

Results

The degree of alignment of BHK cells on patterned substratum adhesiveness was found to be dependent on pattern spacing. Cells cultured on unpatterned quartz and patterns of 4 μm period show little alignment (Fig. 1A,B), whereas those on patterns of larger period are seen to be aligned and elongated in the direction of the lines of adhesiveness (Fig. 1C).

Compared to controls (Fig. 2A), single MDCK cells align in the direction of the lines of adhesiveness regardless of spacing (Fig. 2B-F). The width of cells on 12 and 24 μm wide adhesive lines is restricted to the width of those lines (Fig. 2D,E), whereas on the narrower lines, the width of the aligned cells is greater than the patterned line width (Fig. 2B,C). On 4 and 6 μm patterns, elongated single MDCK cells have formed lamellar protrusions wider than the periods of the patterns (Fig. 2B; arrows in F). At higher densities and
Fig. 2. Phase-contrast light micrographs of MDCK cells cultured on variously treated quartz surfaces. (A) Unpatterned control surface; (B) 4 μm period adhesive pattern; (C) 6 μm period adhesive pattern; (D) 24 μm period adhesive pattern; (E) 50 μm period pattern; (F) 2 μm period pattern. Arrowheads indicate lamellar regions spanning a number of pattern repeats. Bar, 200 μm, A-D; 100 μm, E and F.
The degree of alignment of BHK and MDCK cells cultured on adhesive patterns of various periods.

Fig. 3.

Fig. 4. Cell lengths of single MDCK cells on patterned adhesive substrata of various periods.

after longer periods in culture, long chains of single cells are formed (Fig. 2E), though the restriction of cells to the untreated surface frequently breaks down, such that colonies of cells appear no different from those on unpatterned substrata, except that they may have straight-edged boundaries corresponding to the edge of a line of adhesiveness.

The measured degree of alignment of BHK cells was significantly greater than controls on all patterns and was seen to increase with increasing pattern period such that few cells were not aligned by patterns of period 24 and 50 μm (Fig. 3). Single MDCK cell alignment was maximal on all patterns irrespective of size (Fig. 3). All pattern periods significantly elongated MDCK cells. This elongation increased with period pattern to 24 μm, where cell length is increased to approximately 240% of the control value (Fig. 4). The observed increase in elongation abruptly alters, as cells on 50 μm patterns, though significantly elongated compared to controls (Fig. 4), have average lengths half those of cells on 24 μm patterns (53.14 and 106.95 μm, respectively).

Discussion

Any overview of the possible cues controlling the guidance of cells during morphogenesis and regeneration includes differential adhesiveness as an important mechanism by which cells may be guided to distant sites with great precision. There are now many examples where differential adhesiveness is believed to contribute to the observed cell and axon guidance in vivo (Nakatsuji et al., 1982, 1985; Nakatsuji and Johnson, 1984; Silver and Rutishauser, 1984; Rogers et al., 1986; Riggs and Moody, 1987; Zackson and Steinberg, 1988; Mackie et al., 1988; Harris, 1989; Taylor, 1990; ffrench-Constant et al., 1991; Perris et al., 1991; see Dodd and Jessel (1988) and Hynes and Lander (1992) for reviews), though much of the information about basic cell behaviour in response to differential adhesiveness has emerged from in vitro studies (see Dunn, 1982), and more recently, microfabrication technology has been applied to producing adhesive patterns for studying cell behaviour (O'Neill et al., 1986, 1990; Kleinfield et al., 1988; Britland et al., 1992).

Alternating parallel lines of adhesiveness and non-adhesiveness of various spacings of cellular dimensions have been used in this study to examine the effects on cell behaviour of patterned substratum adhesiveness. We have previously quantified the adhesiveness of the different surfaces, when it was shown that BHK cells would not, even when crowded at a border, cross from the more adhesive to the less adhesive surface, in spite of the fact that the less adhesive surface was increasingly capable of promoting adhesion and spreading of cells with time of exposure to the serum-containing culture medium (Britland et al., 1992). Here, we have shown that populations of both BHK and MDCK cells aligned to the direction of patterned adhesiveness, though responsiveness was found to be determined by the spacing of the patterns and cell type. Alignment of BHK cells gradually increased with increasing pattern period; many cells on 4 and 6 μm patterns were seen to span many periods of pattern. Single MDCK cells were aligned on all pattern spacings, but their degree of elongation was dependent on spacing. Colonies of MDCK cells were found to span many periods of the different patterns, the cells comprising them appearing unaligned except at some margins.

An earlier study by Dunn (1982) examined the alignment of chick heart fibroblasts on alternating parallel stripes of non-adhesive cellulose acetate and glass of 10 μm period (though spacing was somewhat variable due to the limitations of the technique of manufacture). It was found that cells were unaligned by this substratum, but were by single isolated stripes of glass approximately 15 μm wide. The present findings confirm this observation. It would appear that fibroblastic cells will produce protrusions which can bridge narrow non-adhesive regions. Differences in the degree of alignment of BHK cells with pattern spacing suggest that the frequency with which this occurs is strongly dependent on the width of the non-adhesive region. BHK cells cross 6 μm areas infrequently and rarely cross non-adhesive areas wider than 12 μm, suggesting that the normal limit of spacing that will reliably guide them is between these two values. Single isolated narrow (3 μm) tracks of preferential adhesiveness have
been shown to align and elongate Swiss mouse 3T3 cells (O'Neill et al., 1990). We predict that such isolated tracks will align BHK cells and are at present undertaking experiments to test this and attempt to find the narrowest width limit which will provide an adhesive substratum for cells. Production of submicrometre features will require technically more sophisticated methods, such as those used previously to produce ultrafine repeat topography (Clark et al., 1991).

The response of single MDCK cells to the adhesive cues in this study, shows that, as with their response to topography (Clark et al., 1990, 1991), these cells are more sensitive to substratum guidance than are BHK cells or cultured primary neurones (unpublished data). On 12 and 25 μm adhesive lines (on 24 and 50 μm period patterns), MDCK cell spreading was limited by the edges of the tracks and was compensated for by elongation along the tracks. The cells spread to the same degree on 12 and 25 μm tracks (i.e. the measured cell length on 12 μm tracks was twice that on 25 μm tracks). On patterns of lesser spacing, single cells span non-adhesive tracks, their elongation decreasing with pattern spacing. Unlike BHK cells, which lose orientation when they are able to bridge non-adhesive areas, single MDCK cells remain oriented, this presumably reflecting a major difference in the motile behaviour of the two cell types. One possibility suggested by preliminary time-lapse studies is that MDCK cells can only produce short protrusions from their lamellae.

Cells of MDCK cell colonies have previously been shown to be far less susceptible to substratum topography than single cells (Clark et al., 1990, 1991). It was suggested that contact-induced spreading of epithelial cells (Middleton, 1977) was responsible for this reduction in susceptibility, though the details of the cellular changes associated with cell-cell contact of epithelial cells are unclear. The presence of contacts lateral to the direction of adhesive patterns over-rides the guidance cues for cells of MDCK colonies, though end to end contacts by cells elagated on tracks often resulted in chains of cells being formed. End to end contact on topographic gratings resulted in lateral spreading over-riding the guidance cue (Clark et al., 1990, 1991). The examination of cells spreading on strips of glass exposed by scratching phospholipid-coated glass, suggested that during the initial stages of spreading cell margins are not restricted by adhesion boundries, and micotubule depolymerisation maintained this insensitivity (Ivanova et al., 1976). Similarly, cells can be activated to spread on normally non-adhesive surfaces (Edwards et al., 1988; Curtis et al., 1992), suggesting that integrin-ligand binding is not necessarily the adhesive interaction, but the restriction of spreading of MCDK cells at the some margins of colonies argues against cell-cell contact activation of adhesion on non-adhesive tracks. On these patterned substrata, the non-adhesive area under the colonies would be approximately half the occupied area. Lateral cell-cell contact may simply provide sufficient support, rendering extensive cell-substratum contact redundant.

The ability of cells to bridge non-adhesive areas of multiple parallel adhesive cues has a number of important implications. Cells probably bridge non-adhesive areas by extending protrusions across to make contact with a nearby adhesive area. Differences in the ability of cells to bridge are likely to reflect the length of their protrusions. Significant proportions of neurites from cultured dorsal root ganglion cells were shown to be capable of bridging over regions of low adhesiveness as large as 40 μm and occasionally over 50 μm regions, this ability being related to the length of filopodia produced by the neurite growth cones (Hammerback and Letourneau, 1986). Comparing the effects of spacing of adhesive cues with those of identically spaced topographic cues examined previously (Clark et al., 1990), it can be seen that, as determined by the degree of alignment, the different cues have opposite effects. Increased packing density of cues increases the responsiveness of cells to topographic cues (Clark et al., 1990, 1991; Curtis and Clark, 1990) but decreases their responsiveness to adhesive cues. This clearly suggests that topographical guidance is independent of any adhesive pattern a topographical pattern may contain: they may in fact be antagonistic. If extrapolated to sub-micrometre spacings, this suggests that aligned extracellular matrix, which has been implicated as a guidance cue in development (Lofberg and Ablin, 1978; Lofberg et al., 1980; Nakatsuji and Johnson, 1984; Newgreen, 1989; Wood and Thorsgjod, 1984, 1987; French-Constant et al., 1991), exerts its influence on cell behaviour through topography; the orientation of adhesiveness being irrelevant to guidance, though presumably important in providing a permissive substratum. It has been shown that cells can be extremely sensitive to ultrafine topography (Clark et al., 1991).

It is now clear that the geometry of adhesive guidance cues is important in eliciting a guided response from cells. In order to guide cell migration with any degree of precision, tracks of adhesiveness must be relatively narrow. In the absence of any other directional cues (e.g. chemotactic or haptotactic) a wide track will allow cells to deviate from their intended direction. The present study has also shown that adhesive tracks must be isolated or spaced such that bridging cannot occur, or guidance may be reduced or lost. An adhesive track provides guidance in the two opposite directions of the track. Precise directionality will require a second cue. In many developmental situations, cell-cell interactions could provide directionality. Population pressure, as a result of contact inhibition of locomotion (Abercrombie, 1982), and intrinsic persistence of locomotion of translocating cells (Dunn, 1983) and extending neurites (Katz, 1985), may be sufficient to polarize cell locomotion to a degree which results in precise unidirectional migration. In other words, instead of radially scattering, as they would in an isotropic environment, contact-inhibited cells will be funnelled along an adhesive track. Unidirectional cues such as chemotactic
or haptotactic gradients would not be required for
directionality under these circumstances.

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References


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