DEFECTIVE ADHESION TO EXTRACELLULAR MATRIX LEADS TO ALTERED SOCIAL BEHAVIOUR IN CULTURED FIBROBLASTS

JOHN D. APLIN* and LYNN J. FODEN
Department of Obstetrics and Gynaecology, University of Manchester, St Mary's Hospital, Manchester M13 0JH, U.K.

SUMMARY

We describe the properties of variant mouse fibroblasts selected for poor adhesion to growth substratum containing subcellular matrix accumulated by adherent cells at confluence. The variant cells adhere to virgin plastic and grow normally to confluence in the presence of serum. After subculture and reseeding onto the same surface the cells initially adhere, but after a further 2 days of growth they retract into aggregates and detach. If the aggregates are dispersed and cells reseeded onto the same surface they remain rounded. However, if the same cells are added back to virgin plastic they adhere and grow normally. The retraction can be abolished by treating the subcellular matrix-coated plastic with papain. This behaviour therefore reflects the ability of the cells to modify the composition of the underlying substratum during growth. The variant cells also exhibit retraction 2 days after seeding on a surface previously containing wild-type cells at confluence, while wild-type cells do not retract on subcellular matrix deposited by variants. This shows that the variant behaviour arises not from a deficiency in the subcellular matrix, but from an alteration in the adhesive capacity of the cells. The results are interpreted in terms of three putative adhesion mechanisms: cell–cell adhesion in confluent monolayers and aggregates; 'early' type cell-substratum adhesion, which occurs during culture on virgin plastic; and 'late' type cell-substratum adhesion occurring on surfaces containing accumulated subcellular matrix. The variant phenotype is characterized by a deficiency in the last of these. It is also associated with an increased ability to grow in suspension culture at high dilution.

INTRODUCTION

Many morphogenetic processes depend on the regulated combination of cell–cell and cell–extracellular matrix adhesive interactions. Considerable evidence now indicates that each of these classes of cell adhesion may occur by means of several distinct mechanisms (Aplin & Hughes, 1982; Aplin, Campbell & Foden, 1984; Edelman, 1983; Franke et al. 1982; Shirayoshi, Okada & Takeichi, 1983; Damsky et al. 1983). One fruitful approach to the dissection of adhesive processes lies in the derivation of variant cell lines deficient in one or another adhesive mechanism (Pouyssegur & Pastan, 1976; Klebe et al. 1977; Pena & Hughes, 1978; Schubert & LaCorbiere, 1980; Harper & Juliano, 1981; Briles & Haskew, 1982; Oppenheimer-Marks, Border & Grinnell, 1984). From a mouse fibroblast cell line (L) we have selected and cloned variants (LL) that adhere to plastic in the presence of serum, grow

*Author to whom correspondence should be addressed.

Key words: fibroblasts, adhesion, extracellular matrix.
to confluence and accumulate an extracellular matrix on the substratum. Later, after subculture and reseeding, the cells modify the matrix to produce a substratum that no longer supports variant cell spreading. Here we show that this can lead to the reversible formation of cell aggregates and may provide a model for progressions from migratory to aggregatory behaviour during development. It may also be relevant to changes occurring in the transition from normal to tumorigenic cells.

MATERIALS AND METHODS

Wild-type cells and selection of variants

The wild-type cells are a subclone of murine L cells originally derived in the laboratory of Dr J. Paul, Glasgow, and obtained from Dr I. Kerr, London. All cells were grown in Dulbecco’s Minimum Essential Medium (Flow) with glutamine (2 mm), non-essential amino acids (1%; Flow), Hepes (20 mm), 10% foetal calf serum (Gibco), benzyl-penicillin (100 units/ml), streptomycin (10 μg/ml) and amphotericin B deoxycholate (2.5 μg/ml). Routine passage was with trypsin (0.005%) containing EDTA (0.002%). Culture plastic was supplied by Gibco.

Variant cells (LL) were selected from mycoplasma-free wild-type cultures. The wild-type cells were grown to confluence in 75 cm² flasks and subcultured using trypsin/EDTA at a 1:5 split ratio. Cells were reseeded into the same flask, grown back to confluence and then selected by gentle tapping to dislodge loose cells. These were grown back to confluence and the procedure repeated until a variant phenotype had developed (three cycles). Cells were then cloned at limiting dilution in microwell trays (Flow). In this way eight variant lines were obtained, all showing similar properties. Variant cells were considered to be derived from the wild type on the basis of: (1) morphology on fresh plastic; (2) similar growth kinetics; (3) karyotype analysis; (4) expression of H2 markers detected by immunofluorescence with monoclonal antibody M1/42 (Stallcup, Springer & Mescher, 1981). Variant cells were passaged as for the wild type, but plated into virgin plastic flasks to avoid aggregation. Alternatively, they could be released using EGTA (2 mm, 10 min, 37°C).

Cloning in soft agar

Stock gels of 5 % agar Noble in phosphate-saline were heated to melt, and 2 ml was added to 18 ml of warmed culture medium with swirling to mix; 1 ml samples were dispensed into 35 mm plastic dishes, and stored at 4°C. Single cell suspensions were prepared by trypsinization, counted, and diluted in medium to the desired density; 4 ml samples of growth medium were warmed to 37°C and 0.5 ml of 3 % agar Noble was added and pipetted to mix. Then 0.5 ml of cell suspension was added and dispersed, and 1 ml was pipetted onto the surface of the previously prepared agar gel. Gelation took place at 4°C for 5 min, then incubation proceeded at 37°C. Dishes were set up to contain 10⁵, 10⁴, 10³ and 10² cells. Colonies containing >20 cells and >50 cells were scored after 9 and 15 days of culture in areas equivalent to half of each plate (Puck & Marcus, 1955).

Enzymic hydrolysis of subcellular matrix

Flasks were cleared of cells by trypsinization after two successive phases of growth to confluence. Papain (Sigma) was added at 2 U/ml in phosphate-saline lacking divalent cations but containing 10 mM-cysteine and 2 mM-EDTA for 24 h at 37°C. Protease-free bacterial collagenase was obtained from Calbiochem and used at 6.25 U/ml phosphate-saline after Millipore filtration. Incubation was for 4 h at 37°C.

Treatment with hyaluronidase followed a slightly different protocol. Testicular hyaluronidase (Sigma) was dissolved in phosphate-saline at 255 U/ml. Variant cell flask cultures at the stage of major retraction (Fig. 3) were first tapped to dislodge loose cells and aggregates, leaving a sparse covering of adherent cells. Hyaluronidase was then added (5 ml per 25 cm² flask) and incubated at 37°C for 30 min. Little further cell detachment occurred during this period. The enzyme solution was decanted and the flasks refed with growth medium.

A separate series of experiments were done to evaluate the possibility of synergism between
Defective fibroblast adhesion

hyaluronidase and trypsin. After the above treatment with hyaluronidase, the enzyme solution was decanted and replaced with trypsin/EDTA causing rapid detachment of the remaining cells. The flasks were then reseeded using 2 × 10^4 to 5 × 10^5 cells per 25 cm² flask, and cell adhesion and growth were observed.

RESULTS

Adhesion and retraction of variant cells on conditioned substrata

Starting from a normally adherent parent fibroblast line, variant cells were selected for poor adhesion to subcellular matrix deposited onto plastic culture surfaces. Eight variant lines were derived and cloned. All of them show similar properties. When seeded onto virgin tissue culture plastic as a trypsinized suspension, the cells settle, attach, spread out (Fig. 1) and grow to confluence (Figs 2, 7). Throughout these events, cell morphologies are similar to those of parent fibroblasts (Figs 8, 9). Confluent cell monolayers may be released by treatment with either trypsin/EDTA or EGTA leaving a 'conditioned' growth surface containing subcellular matrix (SCM) components accumulated by the culture and detectable by radiolabelling techniques (not shown). When trypsinized LL variant cells are reseeded in medium containing serum onto SCM made from confluent, adherent variant cultures, the cells attach and proliferate normally. However, at subconfluence they begin to round and retract into aggregates of various sizes (Figs 3, 7) that eventually detach from the substratum. Retraction occurs whether the substratum consists of trypsin-treated SCM or EGTA-generated SCM. Under these conditions of replating onto SCM, wild-type cells, in contrast, repeatedly grow back to confluence (Figs 8, 9, 10). Variant cells dispersed by trypsinization of confluent cultures (e.g. see Fig. 2) and then reseeded in the presence of serum onto SCM made (with or without trypsin) from retracting cultures (e.g. see Fig. 3) remain largely rounded (Fig. 4). This indicates the presence of a non-adherent substratum since the same cells adhere and flatten on virgin plastic (Fig. 1).

When the retracting aggregates detach, they can be reseeded onto virgin plastic in fresh culture medium with serum. Within less than one hour adherent, flattened cells can be seen migrating out from the periphery of the aggregates (Fig. 5) and this process continues subsequently (Fig. 6). If aggregating, retracting cells are dispersed mechanically or with trypsin before being reseeded onto fresh plastic, cell spreading occurs even more rapidly (60 min). This shows that aggregated cells and pre-aggregate cells are equivalent. However, serum is required in the medium for respreading to occur on bare plastic (Fig. 14).

Clonal growth of wild-type and variant cells in soft agar

Data presented in Fig. 7 suggest that in monolayer culture variant cells continue to proliferate even when poorly adherent. This was further investigated by plating single cell suspensions in 0.3% agar in growth medium at four different dilutions; growth of clones was then assessed after 9 or 15 days (Fig. 15). Variants LL11 and LL22 appear to be more clonogenic at higher cell dilutions than the wild type where clones only appeared at the greatest cell concentration. Wild-type clones were
Figs 1-6. Reversible aggregation of variant cells on substrata of different composition. Well-spread LL22 cells are shown 7 h (Fig. 1) and 3 days (Fig. 2) after seeding onto virgin plastic in the presence of 10% serum. Fig. 3 shows, LL22 cells 3 days after seeding in medium with serum onto a trypsin-resistant subcellular matrix (SCM) left by a culture like the one shown in Fig. 2. On the SCM-conditioned surface the cells retract into aggregates before reaching confluence. If a surface carrying retracting cells (as Fig. 3) is cleared of cells by trypsinization, and a new LL22 cell suspension is introduced in 10% serum, most cells remain rounded even after 24 h (Fig. 4). However, if aggregates (as Fig. 3) are allowed to settle in 10% serum on fresh plastic, flattened, migratory cells rapidly appear at the peripheries of the aggregate: Fig. 5, 80 min; Fig. 6, 3 days. Figs 1, 4, 5, ×290; Figs 2, 3, ×140; Fig. 6, ×56.
Defective fibroblast adhesion

Fig. 7. Growth of LL22 variant cells on plastic (●●) and trypsin-treated subcellular matrix made from well-spread confluent cultures of LL22 (✚✚). Proportion of well-spread cells is also shown as a function of time in culture: (○○) on plastic; (摴樗) on SCM.

generally smaller and nearly spherical, while variant cell clones took on a more asymmetric or ragged appearance, with cells apparently capable of migrating outwards from the peripheries (Figs 16, 17, 18). Thus the variant cells are capable of division even when rounded.

Enzyme sensitivity of conditioned substrata

The above experiments suggest that cells in monolayer culture 'condition' the plastic growth substratum by the progressive accumulation of subcellular matrix of which at least a fraction is resistant to trypsin. SCM deposited during the first growth to confluence supports cell–substratum adhesion (Figs 1, 2). However, during the second phase of reseeding and proliferation, the cells appear to modify the SCM further, creating a surface that now fails to retain the cell monolayer. Alternatively, the data could be explained by the idea that, during the second round of growth, the cells simply fill in residual holes in the SCM, creating a more homogeneous non-adhesive surface. The presence of an SCM containing trypsin-resistant protein components was confirmed by the observation that treatment of the surface (either 'primary' adherent or 'modified' non-adherent SCM) with the non-specific protease
204  J. D. Aplin and L. J. Foden

Figs 8–14. The parent wild-type cells adhere and grow normally in the presence of serum on their own trypsinized SCM (Fig. 8, 2 days), on fresh plastic (Fig. 9, 3 days) or on trypsinized SCM left by a previous confluent culture of LL22 variant cells (Fig. 10, 3 days). However, variant cells adhere poorly to SCM left by confluent wild-type cells (Fig. 11, 2 days) and eventually retract into aggregates (Fig. 12, 3 days) in a fashion similar to that seen on variant SCM (cf. Fig. 3). Variant cells attach and spread rapidly on fibronectin-coated plastic (Fig. 13, 60 min) in the absence of serum, whilst on bare plastic they attach poorly and do not spread (Fig. 14, 90 min). Figs 8, 11, 12, 13, ×290; Figs 9, 10, 14, ×140.
Defective fibroblast adhesion

Fig. 15. Clonal growth of wild-type cells and two variant lines in soft agar. In each case, initial seeding was at four different cell densities in duplicate plates. Colonies containing >20 cells (open bars) or >50 cells (filled bars) were scored after 9 days (left-hand two bars in each set of four) or 15 days (right-hand two bars; one set only contains one 15-day score). More and larger colonies occur at lower cell densities in the two variant lines compared to the wild type.

papain abolishes the retraction phenomenon, the cells then growing normally back to confluence (Table 1). Application of other matrix-degrading enzymes including bacterial collagenase and testicular hyaluronidase to the SCM fails to alter the behaviour of freshly replated cells.

Behaviour of wild-type cells on variant subcellular matrix

Subsequent experiments were designed to determine whether the variant behaviour arises from deficient adhesive responses in the cells or from the deposition of a defective SCM. Wild-type fibroblasts were seeded onto SCM left by a confluent culture of LL variant cells. Initial adhesion and proliferation proceeded normally, the culture grew to confluence (Fig. 10) and no cell retraction was observed. Thus the cells behaved the same whether they were seeded on their own SCM (Fig. 8), fresh plastic (Fig. 9) or variant cell SCM (Fig. 10). Furthermore, the cell density at confluence was approximately the same in wild-type and variant cultures.

Behaviour of variant cells on wild-type subcellular matrix

When LL variant cells were seeded onto SCM left by confluent wild-type fibroblasts, the cells initially attached and spread, but after 2 days a mixture of
Figs 16–18. Clonal growth of wild-type and variant cells in soft agar. Variant cell colonies (Figs 16, 17) are larger and more 'ragged' than wild-type colonies (Fig. 18). ×155.

Fig. 16. LL22 cell colony. Seeded at 10^3/ml and photographed after 15 days.

Fig. 17. LL11 cell colony. Cells seeded at 10^4/ml and photographed after 15 days.

Fig. 18. L cell colony. Cells seeded at 10^2/ml and photographed after 9 days.

Fig. 19. 'Channels' of bare plastic may be scraped using a rubber policeman onto a surface containing variant SCM. When variant cells are grown on the surface, they align and reach confluence in the absence of SCM while in the unscraped, SCM-containing areas they adhere poorly. ×140.

Fig. 20. Wild-type cells at confluence may be displaced by a gentle stream of medium, whereupon they retain their structure as a sheet, demonstrating the presence of cell–cell attachments. ×140.

rounded and partially flattened cells was observed (Fig. 11). Cells continued to proliferate and eventually retracted into aggregates (Fig. 12). These experiments clearly demonstrate that the variant phenotype differs from the wild type in a cellular property (originating perhaps in cell membrane or cytoskeleton) and, conversely, no difference is observed in the ability of wild-type and variant SCMs to support cell adhesion.

Cell adhesion on fibronectin

Fibronectin is a component of fibroblast and other extracellular matrices that has been associated with the promotion of cell spreading and migration (Aplin & Hughes, 1982; Hynes, 1981). We therefore tested the ability of fibronectin 'carpets' to promote
Table 1. Growth and morphology of LL22 cells in the presence of serum on enzyme-treated subcellular matrices

<table>
<thead>
<tr>
<th>Substratum</th>
<th>1 day</th>
<th>2 days</th>
<th>3 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>New plastic</td>
<td>Sparse, spread</td>
<td>Semiconfluent, spread</td>
<td>Subconfluent, spread</td>
</tr>
<tr>
<td>tSCM</td>
<td>Sparse, spread</td>
<td>Semiconfluent, spread</td>
<td>Retracting</td>
</tr>
<tr>
<td>tSCM/collagenase</td>
<td>Sparse, spread</td>
<td>Semiconfluent, spread</td>
<td>Retracting</td>
</tr>
<tr>
<td>tSCM/papain</td>
<td>Sparse, spread</td>
<td>Semiconfluent, spread</td>
<td>Subconfluent, spread</td>
</tr>
<tr>
<td>ttSCM</td>
<td>Sparse, 50% rounded</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>tt/SCM/hyaluronidase</td>
<td>Sparse, 60% rounded</td>
<td>Retracting</td>
<td>—</td>
</tr>
<tr>
<td>ttSCM/hyaluronidase/trypsin</td>
<td>Sparse, rounded</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>ttSCM/papain</td>
<td>Sparse, spread</td>
<td>Semiconfluent, spread</td>
<td>Subconfluent, spread</td>
</tr>
</tbody>
</table>

tSCM, matrix remaining after trypsinization of a confluent well-spread culture;
ttSCM, matrix remaining after trypsinization of a retracting culture.
the attachment and spreading of variant and wild-type cells under serum-free conditions. Both cell types attach and spread rapidly (<60 min) on fibronectin (Fig. 13). In the absence of fibronectin the cells remain rounded (Fig. 14). As fibronectin is present in serum, this response may be important in the spreading of cells on virgin plastic (Fig. 1) or the outgrowth of cells from aggregates onto fresh plastic (Figs 5, 6). However, fibronectin does not appear to be involved in readhesion of cells to SCM made by trypsinizing adherent confluent cultures; we have failed (using immunological methods or radioiodination) to detect either endogenous or serum fibronectin in 'primary' or 'modified' SCM before or after adding a serum-containing supernatant.

**Pattern formation directed by subcellular matrix**

Since deposits of extracellular matrix may guide migrating cells in the developing embryo (Boucaut, Darribere, Boulekbache & Thiery, 1984; Hynes, 1981), it was of interest to determine whether the variant behaviour could give rise to subcellular matrix-determined pattern formation. Surfaces containing SCM derived from confluent cells were scraped with a rubber policeman to form a cross-hatched pattern. Preliminary experiments had shown that this procedure removes the SCM, restoring the adhesive properties of the surface to those of new plastic. Variant cells were seeded at low density and allowed to grow on the cross-hatched surface. Where the surface had been scraped free of SCM, cells adhered and aligned along the axis of the 'channel' and became confluent and well spread. Elsewhere, cells were sparser and either aggregated, rounded or randomly aligned (Fig. 19).

**Cell-cell adhesion in monolayer cultures**

One interpretation of these results is that reduction of the strength of adhesive interactions between cells and their substratum could lead to traction forces, generated by cytoskeleton and transmitted through cell–cell adhesive contacts, drawing the cells away from the substratum into aggregates. A formally analogous situation in which traction exerted by fibroblasts on a three-dimensional extracellular matrix gives rise to cell condensations in various patterns has recently been described by Oster, Murray & Harris (1983). The apparent normality of cell–cell interactions in the variant cultures suggests that cell–cell and cell–substratum adhesion are probably biochemically distinct. To demonstrate the presence of cell–cell adhesions in the parent cells, we gently disrupted a confluent monolayer with a stream of buffer. When adhesions to substratum were thus abolished, the culture retracted as a sheet in which cell–cell adhesions were evidently intact (Fig. 20).

**DISCUSSION**

These results confirm that multiple mechanisms of adhesion to substratum are available to cultured fibroblasts (Harper & Juliano, 1981) and suggest that adhesion to plastic and to the cells' own subcellular matrix are mechanically distinct. Attachment of wild-type or variant cells to plastic in the absence of serum is not followed by spreading out, suggesting that adsorbed serum components (probably including
Defective fibroblast adhesion

fibronectin; Grinnell & Feld, 1982) may be required to promote spreading. Following this initial phase cells enter the growth cycle and reach confluence. After subculture and reseeding on the same culture substratum the variant cells attach and spread, but later, after further growth and division, lose their adhesive contacts with the substratum, round and retract into aggregates. Wild-type cells meanwhile remain adherent. These observations are consistent with a model in which, after the initial phase of adhesion to a virgin plastic surface in the presence of serum, the cells (wild type or variant) progressively modify the substrate – probably by secretion or recruitment from serum in the supernatant – to create a new type of adhesive interaction between cell surface and substratum. This idea is consistent with the observation that fibroblasts in culture continually break and reform adhesive contacts as migration and cell division occur (Abercrombie, 1980). It also agrees with data showing that newly formed and older cell contact areas differ in composition (Lark & Culp, 1984). Only after one round of growth to confluence and readhesion does the transition from ‘early’ to ‘late’-type adhesion become sufficiently complete to lead to retraction or detachment of variant cells. Observations made using variant cells on wild-type subcellular matrix, and vice versa, show clearly that the deficiency is at the cellular level, perhaps cell surface or cytoskeleton, rather than in the deposited material present on the substratum.

Lack of ability to form stable ‘late-type’ adhesions in these cell lines is associated with an enhancement of clonal growth in suspension (Figs 15–18). Such behaviour is characteristic of malignant cells. Outgrowth or breaking off of cells from islands growing in suspension culture in soft agar (Figs 16, 17) also suggests parallels with invasive or malignant behaviour. Since recent results (Land, Parada & Weinberg, 1983) suggest that carcinogenesis is a multi-step process, and since other observations have frequently suggested altered adhesive properties (Grinnell, 1978; Knox & Griffiths, 1982) and loss of attachment dependence of growth (Stoker, O’Neill, Berryman & Waxman, 1968) to be features of tumour cells, loss or modulation of one of several adhesive mechanisms available to cells might have far-reaching consequences in terms of invasion of neighbouring tissue.

This system also provides a model of developmental processes that involve adhesion, migration and aggregation. One example of this is in neural crest cell migration, where extracellular matrix (probably fibronectin) plays a permissive role (Hynes, 1981; Bonner-Fraser & Cohen, 1980; Newgreen & Thiery, 1980). Later an aggregation phase ensues in the formation of early sensory ganglia. It is not yet clear how these processes are controlled. However, data obtained in vitro suggest that cell migration depends on either cell spreading and polarization (Abercrombie, 1980) or at least on the extension and retraction of cytoplasmic processes along the substratum (Campbell, Allen & Aplin, 1984). One of several ways in which migration may be directed is along tracks of extracellular matrix (Newgreen & Thiery, 1980). Here we have shown that reversible cell aggregation can occur, depending on the adhesive properties of the substratum; that adherent cells can themselves modify these properties; and that a preformed pattern of subcellular matrix can give rise to sorting out of adherent cells into corresponding patterns. This may be analogous to previous
experiments showing that cells can migrate up a gradient of increasing substrate adhesiveness (Harris, 1973).

Thus, although our observations have been made using stable variant cell lines, they raise the possibility that a programmed change in the intrinsic ability of cells to adhere to local extracellular matrix may allow a switch from monolayer or migratory to aggregatory behaviour. Such suggestions are in agreement with other direct evidence of changes in cell surface phenotypes that correlate with the onset of aggregation in developing tissues (Edelman, 1983).

This work was supported by a grant from the MRC to J.D.A. We thank Mr Malcolm Plant for karyotyping, Dr Malcolm Taylor for the gift of anti-H2 antibodies, Dr Anne Wilson for help with cloning in soft agar and Dr Steve Campbell for useful discussion.

REFERENCES
Defective fibroblast adhesion


(Received 7 December 1984 – Accepted 28 January 1985)