CONDITIONS FOR FIBROBLAST ADHESION WITHOUT FIBRONECTIN

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SUMMARY
Conditions that permit the adhesion of BHK fibroblasts to a variety of surfaces after inhibition of protein synthesis and competition of any adsorbed fibronectin or vitronectin with the fibronectin cell-binding tetrapeptide, Arg-Gly-Asp-Ser (RGDS), are defined. Exposure of the cells to serum components at any stage in the preparation prevents cell attachment if cycloheximide or fibronectin tetrapeptide is present. If leupeptin is used cell adhesion and spreading occur even when all fibronectin synthesis is suppressed by cycloheximide inhibition, or fibronectin binding by tetrapeptide competition. The adhesions formed under these conditions appear by interference-reflection microscopy and by general properties to be identical to those formed by cells under normal culture conditions. The cell suspensions produced in the presence of leupeptin rather than other trypsin inhibitors show good adhesion at low temperatures, though the cells hardly spread at all. The results suggest that the role of fibronectin in cell adhesion should be reinterpreted in terms of its possible action as an activator rather than as a bonding molecule.

INTRODUCTION
It has been argued by Grinnell (1978) and Grinnell & Feld (1979) that fibronectin is essential for the adhesion and spreading of fibroblasts. They found that cells that were constitutionally unable to synthesize fibronectin were unable to adhere well and spread on polystyrene surfaces unless the medium was supplemented with fibronectin or serum, or unless fibronectin was adsorbed to the substrate. Such experiments would appear to be thorough demonstrations of the essential role played by fibronectin in adhesion and spreading. However, Curtis et al. (1983) showed that BHK cells would adhere to and spread upon modified polystyrene surfaces in the absence of exogenous fibronectin and under conditions of inhibition of protein synthesis in which it was unlikely that fibronectin was being synthesized by the cells. Later Curtis et al. (1986) were able to show that a prime requirement for adhesion and *inter alia* spreading was the presence of a substrate with a fairly, but not very, high density of hydroxyl groups on it.

It could be argued that the demonstration of the lack of synthesis of fibronectin was not wholly adequate in the work by Curtis et al. (1983). It is also possible that the cells might have retained and then secreted small pools of fibronectin during the adhesion stage. Similarly, the results reported in the accompanying paper (Curtis
et al. 1986) on the effects of hydroxylation could be explained as the provision of surfaces suitable for the adsorption of fibronectin from the cells.

This study attempted to investigate these criticisms. In the process of doing so a number of other results generally relevant to the interpretation of fibroblast adhesion have emerged.

The basic approach of this study has been to investigate adhesion and spreading of fibroblasts under conditions in which the action of any fibronectin that might be present has been inhibited by the presence of the low molecular weight fibronectin tetrapeptide, Arg-Gly-Asp-Ser, described by Pierschbacher & Ruoslahti (1984).

MATERIALS AND METHODS

Cells and preparation of cell suspensions

These were exactly the same as those described in the accompanying paper, with the exception that in some experiments trypsin activity was inhibited with foetal calf serum (Biocult, Paisley, UK) at a 5% level, in place of leupeptin.

Substrates

'Bacteriological', 'tissue culture' and chloric acid polystyrene Petri dishes were used exactly as in the accompanying paper.

Measurement of cell adhesion to substrates

The same method as that described in the accompanying paper was used.

Measurement of cell spreading

Cells adherent to culture dishes were fixed in formal saline for 10–20 min. The cultures were then washed in water and stained in 0.1% Coomassie Blue (C.I.42660) in methanol:acetic acid:water (47%:6%:47%, by vol.) for 20 min. The area of each cell was then measured on a Quantimet 720 Image Analysing Computer operating in the feature mode. The cells were visualized with a 10× objective and unspread cells had an area of \( \approx 200 \) pixels (11 pixels = 1 \( \mu m^2 \) under these conditions). About 400 cells were measured for each experimental condition in at least three replicate culture dishes.

Fibronectin adsorption to attachment surfaces

Bovine fibronectin was prepared from bovine serum by the collagen affinity column method (Engvall & Ruoslahti, 1977) and stored in 8M-urea. For adsorption to polystyrene surfaces the fibronectin solution was diluted in Hanks' saline to 1 M in urea immediately before addition to the dishes; 100 \( \mu g \) of fibronectin per 18.9 cm\(^2\) dish was added to this solution and the protein was allowed to bind to the dish surface for 30 min. Unbound protein was then washed off with Ham's F10 medium. This treatment gives a surface binding of \( \approx 1200 \) ng cm\(^{-2}\) on chloric-acid-treated polystyrene and about half this level on 'tissue culture' grade polystyrene (Curtis & Forrester, 1984).

Inhibition of attachment and spreading by fibronectin peptide

Fibronectin tetrapeptide (Arg-Gly-Asp-Ser = RGDS) was purchased from Mersey Biochemicals, Liverpool, UK. It was dissolved in Ham's F10 medium at four times desired final concentration and samples of this stock were mixed with the appropriate volume of cell suspension.
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**Interference-reflection microscopy (IRM)**

A Vickers M17 microscope with incident light fluorescence equipment was modified slightly by replacement of a dichroic mirror with a pellicle beam splitter and an additional field iris. Examination of cells adhering and spreading on glass coverslips was carried out with a 100X, NA 1.4, objective.

**Measurement of inhibition of protein synthesis by cycloheximide**

Samples of BHK cell suspensions containing $3 \times 10^5$ cells were placed in 0.3 ml microwells, in 5% foetal calf serum in Ham's F10 medium and cultured for 6 h, either in the presence of cycloheximide ($25 \mu g ml^{-1}$) or in its absence. After this period 1 $\mu$Ci $[^{35}S]$methionine (Amersham International, plc, Amersham, UK; sp. act. 1-2 Ci/umol$^{-1}$) was added per well and the cultures were incubated at 37°C; triplicate samples were assayed every 15 min up to 90 min of incubation. The cells were removed from the culture wells by trypsinization at 25°C, followed by recovery of the cells on filters, and washing in 0.3 M-sodium hydroxide for 30 min at 37°C. The filters were then counted in a Packard Tricarb 300 liquid scintillation counter (Packard Instrument Corp., Downers Grove, Illinois, USA) in Picofluor scintillation fluid (Packard Instrument Corp.).

**RESULTS**

It was shown earlier (Curtis et al. 1983) that BHK cells that had been exposed to $25 \mu g ml^{-1}$ of cycloheximide for 6 h prior to trypsinization would adhere to and spread on chloric-acid-treated polystyrene in serum-free medium. In that experiment leupeptin was used to inhibit the activity of trypsin, thereby ensuring that no exogenous fibronectin (or other protein that promotes adhesion) might be added to the system. However, it is possible that the cells, even after this time of exposure to cycloheximide, might retain sufficient pools of this or other adhesive proteins to permit their adhesion and spreading. Preliminary experiments with fluorescently labelled antibodies to fibronectin or to laminin failed to show any sign of such proteins on surfaces on which cycloheximide-inhibited cells had been cultured, but such negative evidence might have purely technical explanations. Consequently, we investigated whether fibronectin tetrapeptide would compete with the cells for binding to a presumably adsorbed layer of fibronectin, and thus inhibit cell adhesion.

**Effects of fibronectin peptide on adhesion of BHK fibroblasts**

Cell suspensions, prepared by trypsinization followed by leupeptin inhibition of the tryptic activity, were made up in serum-free Ham's F10 medium (containing $25 \mu g ml^{-1}$ of cycloheximide) with and without the fibronectin tetrapeptide at 0.0011 M, and plated out on chloric-acid-treated polystyrene at a cell density of $2 \times 10^6$ ml$^{-1}$, which gave for the volumes used and the geometry of the dishes a maximum possible cell attachment of $3 \times 10^4$ cells cm$^{-2}$. The cultures were allowed to settle at 37°C for 20 min and then non-adherent cells were removed by washing with Ham's F10 saline. The numbers of cells per unit area were counted. Results are shown in Fig. 1.

A parallel set of experiments was carried out in which controls to the above experiments were run out using tissue-culture grade and chloric-acid-treated polystyrene with or without addition of cycloheximide and adsorbed fibronectin. Results are shown in Fig. 2. Comparing controls and experimental sets, it is obvious
Fig. 1. The lack of inhibition of cell adhesion by fibronectin tetrapeptide in the absence of exogenous fibronectin. Histogram of BHK cell attachment (cells cm$^{-2}$) in 20 min at 37°C to chloric-acid-treated polystyrene (Chloric) or to adsorbed fibronectin (FN), in the presence or absence of fibronectin tetrapeptide in solution (+ or −) and in the presence of 25 μg ml$^{-1}$ of cycloheximide (CH). All cell suspensions used were prepared with leupeptin inhibition of trypsin action. Error bar: one standard deviation.

Fig. 2. Controls to Fig. 1. Adhesion of BHK cells in 20 min at 37°C to various surfaces. TC, tissue culture grade polystyrene; FN, adsorbed fibronectin on TC grade polystyrene; Chloric, chloric-acid-treated polystyrene, in serum-free medium (Ham's F10), with in one case cycloheximide at 25 μg ml$^{-1}$. Note that adhesion is appreciable under all conditions. S, serum; LP, leupeptin; FN4P, fibronectin tetrapeptide. Error bar: one standard deviation.
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that while adhesion of the BHK cells is high in controls on both tissue-culture grade and chloric-acid-treated polystyrene surfaces and to adsorbed fibronectin, and is inhibited by fibronectin tetrapeptide when adsorbed fibronectin is present, adhesion is not inhibited by either cycloheximide or fibronectin tetrapeptide or by both together when exogenous fibronectin is absent. Thus adhesion can take place under conditions in which fibronectin is absent or inhibited in its cell-binding activity.

It should be noted that in the experiments described in this paper we have concentrated on adhesion effects rather than on spreading. The short period allowed for attachment is used so that spreading effects will be minimized; nevertheless, under some conditions spreading can be appreciable in 30 min.

Effects of the method of cell-suspension preparation on adhesion and spreading

Since leupeptin rather than serum was used in the work described in the preceding paragraph, to prepare cell suspensions, and since it is well known that serum contains various proteins that inhibit cell adhesion (Curtis & Forrester, 1984), we have compared cell adhesion and spreading in the presence and absence of cycloheximide following leupeptin treatment. Results are shown in Fig. 3. They indicate that if trypsinization was inhibited by serum, cell attachment and spreading are notably reduced compared with the situation after leupeptin inhibition, whether or not cycloheximide is present during the attachment and spreading phase.

Adhesion at low temperatures

Cell suspensions from trypsinizations stopped with leupeptin or with serum were prepared in serum-free Ham's F10 saline at 4°C and then plated out on precooled polystyrene Petri dishes at 4°C. The cell suspensions were allowed to settle onto the culture dish surfaces for 30 min and then cell attachment was assayed. Results are shown in Fig. 4.

Clearly cell adhesion is inhibited at low temperature when the cells are exposed to serum. Inhibition of trypsin activity by serum followed by culture in a serum-free medium leads to partial inhibition of adhesion at 4°C. In the absence of serum components adhesion is extensive but spreading, however, is greatly inhibited under all conditions. The extent of cell attachment is greater on chloric-acid-treated polystyrene surfaces than on tissue-culture grade material.

Interference-reflection microscopy of cell contacts formed on hydroxylated surfaces in the presence of various inhibitors

IRM of cells adhering and spreading on glass coverslips showed that the cells gave the same range of images whether they were in control medium, cycloheximide-containing medium or medium containing the fibronectin tetrapeptide. Results are shown in Figs 5–10. The cells developed fairly close (black or zero order) contacts around their peripheries, and these appear to be of the focal adhesion and close adhesion types (Verschueren, 1985). Polystyrene surfaces cannot be used for this type of examination because the low refractive index of polystyrene results in an image with almost no contrast. The glass used for cell attachment was prepared by
Fig. 3. Adhesion (□) and spreading (■) of BHK cells over 30 min at 37°C after inhibition of trypsin dissociation by either serum or leupeptin in Ham's F10 medium, with or without cycloheximide (25 μg ml⁻¹). Serum inhibition produces significantly less adhesion (P < 0.01%, d.f. 3 and 58) or spreading (P = 0.01, d.f. 3 and 1256) than leupeptin inhibition.

Fig. 4. Adhesion at low temperatures. Inhibitory effects of serum. Cell attachment in 30 min at 4°C to chloric-acid-treated polystyrene with either leupeptin (LP) or serum (S) inhibition of trypsin followed by suspension in Ham's F10 medium or a 3% foetal calf serum in Ham's F10 medium (HS) with or without cycloheximide (CH; 25 μg ml⁻¹). Exposure to serum during the inhibition of trypsin stage results in reduced adhesion at 4°C, while exposure to serum during the attachment phase inhibits adhesion. Chl., chloric-acid-treated polystyrene.
Figs 5–10. IRM of BHK cells settled on chloric-acid-treated polystyrene for 30 min at 37°C: with no addition to medium (Fig. 5); in the presence of fibronectin tetrapeptide (Figs 6, 7), cycloheximide (Fig. 8) or fibronectin tetrapeptide + cycloheximide (Figs 9, 10). See Materials and Methods for concentrations used. Bar, 10 µm.
treatment with the chloric acid reagent followed by brief exposure to 2M-sodium hydroxide and then washing with water. This results in a very wettable surface that is presumably silanol-rich.

The extent of inhibition of protein synthesis by cycloheximide

An experimental assumption underlying the design of these experiments is that cycloheximide actually inhibits protein synthesis in these cells under the conditions used. The extent of inhibition of methionine uptake into acid-precipitable material by cycloheximide is at least 95%.

DISCUSSION

The main result reported in this paper shows that extensive cell adhesion of BHK fibroblasts to hydroxyl-group-rich polystyrene can occur when protein synthesis is inhibited and when any residual fibronectin in the system is competing with the fibronectin tetrapeptide RGDS. Secondly, this result cannot be obtained when the cell suspensions used have been prepared with serum.

It might be argued that leupeptin inhibition of trypsin results in an abnormal type of cell adhesion, but secondary experiments show that the cells thus treated have normal morphology and can be maintained through repeated passages with leupeptin inhibition of trypsin and growth in low serum. Furthermore, it is unlikely that the adhesions formed in the presence of cycloheximide and the tetrapeptide inhibitor are abnormal, since IRM examination of the cells shows a normal type of contact.

Since vitronectin-mediated adhesion is claimed to be inhibited by the same peptide sequence (Hayman et al. 1985) it is possible that our results apply to vitronectin-mediated adhesion as well.

Thus there is no essential requirement by these cells for fibronectin in order to form adhesions, provided the cells have not been previously exposed to serum. This finding suggests that claims made by various authors (Grinnell, 1978; Yamada et al. 1982) that fibronectin or vitronectin are essential for cell adhesion are erroneous. Earlier workers such as Pizzey et al. (1983) and Curtis et al. (1983) reported good cell attachment in serum-free conditions in situations in which fibronectin secretion was probably inhibited, but in neither case did they show that trace amounts of fibronectin were not present.

The question then arises, as to the function of fibronectin in stimulating cell adhesion in some circumstances. One solution, following a suggestion by Dr J. G. Edwards, is that fibronectin activates cell adhesion mechanisms. Effects on spreading are less clear but it appears that good if not very extensive cell spreading can occur in 30 min in the absence of fibronectin or when its action is inhibited by competition with the tetrapeptide, provided that some component of serum is not present.

Since exposure of the cells to serum renders them fibronectin-dependent in their adhesion it will be of interest to discover the mechanism of this dependency. It is also of interest to note that in the tissue environment the protein content, which is found in tissue ultrafiltrate (Carter et al. 1974), is substantially different from that found in
serum, particularly in respect of the low concentrations of high molecular weight proteins from serum. Thus it may be that serum represents a really unphysiological component of a cell culture medium, but, of course, in the vascular system it has the properties necessary for maintaining a non-adhesive environment for cells. Curtis & Forrester (1984) have identified five proteins from serum that reduce BHK adhesion, four of which are virtually absent from tissue ultrafiltrate.

Harper et al. (1983) have described two types of fibroblast adhesion: type 1, which is fibronectin-dependent; and type 2, which is independent of fibronectin but requires a high molecular weight glycoprotein on the cell surface and components of extracellular material. It is not yet clear whether the type of independence described in this paper is equivalent to that reported by Harper et al., who detected their type of adhesion in a 10% serum medium.

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REFERENCES


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