The polarization of fibroblasts in early primary cultures is independent of microtubule integrity

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Summary

Fibroblasts in culture apparently require an intact system of microtubules in order to adopt and maintain a polarized morphology. In contrast, the polarization of a number of epithelial cell types has been shown to be microtubule-independent. Reconciliation of these apparently contradictory data is difficult, however, because the epithelial cells were studied in short-term primary cultures while the fibroblasts were studied in secondary or longer-term cultures. To clarify the situation we have examined the effects of the microtubule-disrupting drugs, colcemid and nocodazole, on the polarization of a single cell type, the chick heart fibroblast (HF), maintained in both primary (1°) and secondary (2°) cultures.

Immunofluorescence observations of both types of culture showed that in control medium the cells contained abundant microtubules, which were absent if the cells were cultured in medium containing either colcemid or nocodazole. The effects of microtubule-disrupting drugs on the polarization of the cells were quantified using two measures of cell shape, elongation and dispersion, both of which increase with increasing polarization. The results show that microtubule-disrupting drugs do not have a significant effect on the polarization of HF spreading in 1° culture but significantly reduce the polarization of HF spreading in 2° cultures. The effects of microtubule disruption on HF that had been maintained in 1° culture for 6 h, 24 h or 48 h were also quantified. These measurements demonstrate that the polarization of HF in 1° culture becomes increasingly sensitive to microtubule disruption over this period until, after 48 h, they are as sensitive as HF in 2° cultures to microtubule disruption. The results show that the polarization of HF, cultured immediately after their isolation from the parent tissue is, like that of some epithelial cells, microtubule-independent but that during the first 48 h in culture these fibroblasts change in such a way that their polarization becomes microtubule-dependent.

Key words: microtubules, polarization, colcemid, nocodazole, fibroblasts, primary cultures, secondary cultures.

Introduction

An isolated fibroblast or epithelial cell migrating in culture has a polarized morphology because the protrusive lamellar activity that leads to its translocation is confined to a limited region of the cell periphery (Vasiliev & Gelfand, 1976; Abercrombie, 1980; Brown & Middleton, 1985). If lamellar activity is more uniformly distributed around the cell margin, translocation does not normally occur and the cell lacks an obvious morphological polarity (Vasiliev & Gelfand, 1976; Brown & Middleton, 1985). Understanding the locomotory mechanisms of such cells, therefore, requires a knowledge of the mechanisms responsible for their polarization. The common use of the term 'polarization' in this context is unfortunate because of the alternative use of the word to describe the apical–basolateral polarization of epithelial sheets. Throughout this paper it will be used only to describe the morphology of cultured cells.

In a number of investigations, primarily involving fibroblastic cell types, it has been shown that cells adopt and maintain a polarized morphology only when their cytoplasmic microtubules are intact (e.g. see Vasiliev & Gelfand, 1976). Microtubule-disrupting drugs, such as colcemid and nocodazole, inhibit the polarization of spreading cells (Goldman, 1971; Ivanova et al. 1976) and...
cause previously spread cells to lose their polarization (Vasiliev et al. 1970; Goldman, 1971; Gail & Boone, 1971).

In contrast, a number of other investigations, primarily using epithelial cell types, have shown polarization to be microtubule-independent, since the cells could adopt and maintain a polarized morphology despite the disruption of their microtubules (DiPasquale, 1975; Chernoff & Overton, 1979; Euteneuer & Schliwa, 1984; Middleton et al. 1988). These results have led to the suggestion that the polarization of fibroblasts and epithelial cells may involve different mechanisms (Middleton et al. 1988). This may be an over-simplification, however, since the polarization of cells from two epithelial cell lines is microtubule-dependent and these cells respond like fibroblasts to the disruption of their microtubules (Dommina et al. 1985; Karavanova et al. 1985).

Any interpretation of the existing results is complicated by the wide variety of cell types, culture protocols and experimental conditions used in the various investigations. However, a common feature is that cells exhibiting microtubule-independent polarization have been freshly isolated from the parent tissue and studied in short-term primary cultures (DiPasquale, 1975; Chernoff & Overton, 1979; Euteneuer & Schliwa, 1984; Middleton et al. 1988). On the other hand, those cells exhibiting microtubule-dependent polarization have been maintained in culture for one or more passages (Vasiliev & Gelfand, 1976; Ivanova et al. 1976; Middleton et al. 1988) or have been established cell lines (Gail & Boone, 1971; Goldman, 1971; Dommina et al. 1985; Karavanova et al. 1985). This suggests the possibility that the contrasting results obtained in the various investigations could be due to differences in the length of time for which the cells had been maintained in culture.

To investigate this we have examined the effects of microtubule-disrupting drugs on the polarization of a single cell type, embryonic chick heart fibroblasts, maintained both in short-term primary and also in longer-term secondary cultures. Our results, based on a quantitative method of shape analysis, show that the polarization of these fibroblasts is initially microtubule-independent but that after 24-48 h in culture it becomes microtubule-dependent.

Materials and methods

Cell cultures

The medium throughout consisted of Dulbecco's modified Eagle's medium (DMEM) plus Ham's F12 medium (1:1) supplemented with 10% foetal calf serum and containing 100 units ml^{-1} penicillin and 100 μg ml^{-1} streptomycin (all from Gibco Ltd). Cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂ in air.

Suspensions of primary chick heart fibroblasts (1° HF) were obtained from the ventricles of 12-day-old chick embryos. These were minced finely, washed in calcium- and magnesium-free (CMF) Earle's saline and dissociated by incubating in 1-0% (w/v) trypsin in CMF Earle's saline for 20 min at 37°C. The resulting cell suspension was washed in one change of medium before being resuspended in fresh medium and counted.

Suspensions of secondary chick heart fibroblasts (2° HF) were obtained by culturing explants of 12-day-old chick embryo ventricles for 8 or 9 days before harvesting their outgrowths and resuspending the cells in medium as previously described (Middleton et al. 1988).

Cultures for routine histological examination and for cell shape analysis were prepared by plating out suspensions of either 1° or 2° HF into 9 mm diameter glass rings waxed onto glass coverslips. To investigate the effects of microtubule-disrupting drugs on the spreading of the cells, replicate samples (0-2 ml) of suspensions of 1° and 2° HF, containing approximately 3-2×10⁵ cells ml^{-1} and 0-6×10⁵ cells ml^{-1}, respectively, were plated out in this way in both control and drug-containing media (see below). After a 6-h incubation the cultures were fixed in formal saline and stained with Harris' haematoxylin.

To investigate the effects of these drugs on 1° HF after they had been in culture for increasing lengths of time, replicate samples (0-2 ml) of suspensions of the cells, containing approximately 1-6×10⁵ cells ml^{-1}, were plated out, as above, in control medium and incubated for 6 h, 24 h or 48 h. At each time a number of replicate cultures were transferred to drug-containing medium and a number to appropriate control medium (see below). After a further 3-h incubation the cultures were fixed and stained as described above. For comparative purposes a similar experiment was also performed with 2° HF, but only at a single time point (6 h) and in this case the initial cell suspension contained approximately 0-6×10⁵ cells ml^{-1}.

Microtubule-disrupting drugs

Stock solutions of colcemid (1 mg ml^{-1}, demecolcine, Sigma Ltd) in water and of nocodazole (1 mg ml^{-1}, Janssen Pharmaceutical Ltd) in dimethyl sulphoxide (DMSO) were prepared and stored at −20°C. Before use these stock solutions were diluted with medium to a final concentration of 0-5 μg ml^{-1} in the case of colcemid and of 1-0 μg ml^{-1} in the case of nocodazole. Control medium was prepared by adding equivalent quantities of water or DMSO to medium as appropriate.

Fluorescence microscopy

Suspensions of 1° and 2° HF were plated out onto coverslips in control medium and in medium containing microtubule-disrupting drugs. After 6 h incubation the cultures were rinsed in phosphate-buffered saline (PBS) and extracted for 10 s in 0-5% (v/v) Triton X-100 at 37°C, as described by Karsenti et al. (1984). After fixation for 5 min in absolute methanol at −20°C, the cultures were washed in PBS and processed for indirect immunofluorescence microscopy using anti-tubulin antibody as previously described (Middleton et al. 1988).

Cell shape analysis

To quantify the effects of microtubule-disrupting drugs on the morphology of the cells we used two measures of cell shape, elongation and dispersion, which have previously been fully described (Dunn & Brown, 1986; Brown et al. 1989). These measures do not completely describe polarization but they quantify two aspects of cell shape, both of which increase with increasing polarization (Dunn & Brown, 1986; Middleton et al. 1988). Examples of the way in which these measures vary in relation to changes in cell shape are given by Dunn & Brown (1986) and Brown et al. (1989).

Cell shapes were traced onto paper using a drawing tube attached to a Leitz Diaplan microscope and digitized using a Magiscan 2 image analyser (Joyce-Loebl Ltd). The zero-, first- and second-order moments of the cell shapes were then calculated from these binary images using the method of Dunn & Brown (1986). To ensure that the estimated moments were
good approximations of the true moments, the magnifications provided by the microscope and video camera were adjusted to ensure that the smallest cell images contained at least 1000 pixels (see Dunn & Brown, 1986). The average number of pixels per cell image was approximately 4000. Elongation and dispersion were calculated from the moments as described by Dunn & Brown (1986).

For each experimental treatment, and its control, we measured the elongation and dispersion of 35 isolated cells from each of three replicate cultures. All the data points therefore represent means derived from 105 individual measurements. In some cases, to allow comparisons to be made between different culture sets, the experimental values have been normalized by expressing them as a percentage of the control values. Nevertheless, all statistical analyses were performed on the raw data.

Results

Immunofluorescence observations

Immunofluorescence observations using anti-tubulin antibody demonstrated that both primary (1°) and secondary (2°) heart fibroblasts (HF) contained abundant arrays of microtubules when cultured in control medium (Fig. 1A,C). These arrays were absent from the cells when cultured in colcemid-containing medium, although occasional, brightly fluorescent microtubule-organizing centres could be seen (Fig. 1B,D).

The presence of nocodazole in the culture medium also eliminated any microtubules, detectable by immunofluorescence, from both cell types (data not shown).

Effects of microtubule-disrupting drugs on spreading cells

The effects of colcemid on the spreading of these cells were investigated by plating out suspensions of 1° and 2° HF in control and colcemid-containing medium and incubating the cultures for 6h before fixation. The appearance of 1° HF was not affected by the presence of colcemid in the culture medium (Fig. 2A,B) but in contrast the morphology of 2° HF was clearly altered by the drug and the well-polarized cells present in the controls were absent from the colcemid-treated cultures (Fig. 2C,D). These differing responses to colcemid were characterized using two measures of cell shape, elongation and dispersion, which quantify two different

![Fig. 1. Indirect immunofluorescence, using anti-tubulin antibody, of primary (A,B) and secondary (C,D) heart fibroblasts 6 h after plating out the cells in control (A,C) or colcemid-containing (B,D) medium. Only microtubule-organizing centres (arrowheads) are visible in the colcemid-treated cells. Bar, 10 µm.](image)
aspects of morphological polarization and which both increase in value with increasing polarization (see Materials and methods). The presence of colcemid in the culture medium did not significantly affect either the elongation or dispersion of 1° HF but, in contrast, it significantly reduced both these measures for 2° HF (Fig. 3A,B).

Similar results were obtained when this experiment was repeated using nocodazole in place of colcemid (Fig. 4A,B).

Effects of microtubule-disrupting drugs on 1° HF after increasing lengths of time in culture

The above observations suggest that the polarization of 1° HF, in contrast to that of 2° HF, is insensitive to microtubule-disrupting drugs. One possible explanation for this is that polarization of HF does not require an intact system of microtubules until the cells have been maintained in culture for some time. To investigate this HF were maintained in 1° cultures for increasing lengths of time before being exposed to microtubule-disrupting drugs.

Suspensions of the cells were plated out and cultured for 6, 24 or 48 h in control medium before being transferred either to fresh control medium or to colcemid-containing medium for a further 3 h before fixation. For the purpose of comparison, 2° HF were plated out in control medium for 6 h before being treated in the same way.

The morphology of 1° HF transferred to colcemid-containing medium after 6 h in culture was little changed by the drug (Fig. 5A,B). In contrast the morphology of 1° HF transferred to colcemid-containing medium after either 24 or 48 h was clearly altered by the exposure to the drug and the effects were more pronounced in those cultures transferred at the later time point (Fig. 5C,D). The effects of a 3 h exposure to colcemid on the morphology of these 1° cells after 48 h in culture appeared very similar to the effects of the same treatment on the morphology of 2° HF (Fig. 5E,F).

These effects of colcemid were quantified, as before, using the two measures, elongation and dispersion. After 6 h in culture, transferring 1° HF to colcemid-containing medium did not have a significant effect on either measure (Fig. 6A,B). After 24 h in culture the same
treatment caused a significant decrease in the elongation of the cells but not in their dispersion (Fig. 6A,B). However, transferring 1° HF to colcemid-containing medium after 48 h in culture caused highly significant decreases in both their elongation and their dispersion, which were similar to those shown by 2° HF treated in the same way (Fig. 6A,B).

Similar results were obtained when these experiments were repeated using nocodazole in place of colcemid (data not shown).

Discussion

Our results show that the polarization of HF, when initially plated out in 1° cultures, is not significantly affected by microtubule-disrupting drugs, but that these drugs inhibit the polarization of HF when they are plated out in 2° cultures. One possible explanation for this is that the polarization of HF becomes susceptible to microtubule disruption during the first 48 h that they are in culture. By the end of this period, their polarization is as sensitive to microtubule disruption as that of HF in 2° cultures. These results show that the polarization of HF, cultured immediately after their isolation from the parent tissue, is microtubule-independent but that during the first 48 h in culture the cells change in such a way that their polarization, in common with that of a number of other cell types (see Introduction), becomes microtubule-dependent.

Why the behaviour of the cells should change in this way is unclear. It could either reflect a change that is also shown by the cells in vivo, or it could occur as a result of a process of selection, or, as has previously been suggested (Middleton et al. 1988), it may be associated with the size of the cells.

It seems unlikely merely to reflect a change that occurs simultaneously in vivo because HF isolated from embryos 48 h older than those used here (i.e. from 14-day-old embryos), also show microtubule-independent polarization during the early stages of 1° culture (Middleton, unpublished observations).

A process of selection could, potentially, account for the results if the original cell population contained a small number of cells, undetected by our methods, whose polarization was microtubule-dependent and whose survival and/or growth was favoured by the culture environment. However, this explanation seems unlikely; first, because the change occurs over a relatively short period (48 h) during which there is no detectable cell death in the cultures; and second, because it occurs even if the cells are cultured in the presence of the mitotic inhibitor mitomycin C (Middleton, unpublished).

The size of the cells, however, may well be relevant to our results. As is evident from the scale bars on Fig. 2, HF in 1° cultures are initially very much smaller than the same cells in 2° cultures. The mean spread area (area of

Fig. 3. Values of elongation (A) and dispersion (B) for primary (1°) and secondary (2°) heart fibroblasts cultured for 6 h in control (open bars) or colcemid-containing (shaded bars) medium. Means (n = 105 in all cases) and standard errors are shown. Comparison with control (**), P < 0.001.

Fig. 4. Values of elongation (A) and dispersion (B) for primary (1°) and secondary (2°) heart fibroblasts cultured for 6 h in control (open bars) and nocodazole-containing (shaded bars) medium. Means (n = 105 in all cases) and standard errors are shown. Comparison with control (**), P < 0.001.
substratum occupied) of HF after 6 h in 1° culture is approximately 400 μm² while that of the same cells in 2° culture is approximately 1300 μm² (Middleton et al. 1988). If HF are maintained in 1° culture their mean spread area gradually increases with time until, as can be seen from Fig. 5C, after 48 h they are as large as the...
cells in 2° cultures. As we have shown, it is within this same period that the polarization of the cells becomes microtubule-dependent. These facts could well be related if the polarization of small cells is less dependent on microtubules than that of larger cells, and there is some evidence that this might be the case. For example, the polarization of chick embryo epidermal and corneal epithelial cells, both of which have mean cell spread areas even smaller than that of the 1° HF described here, has been shown to be microtubule-independent (Middleton et al. 1988), and similarly the polarization of small fragments of mouse fibroblasts, unlike that of the intact cells from which they were derived, has been shown to be microtubule-independent (Gelfand et al. 1985). These observations have led to the suggestion that the actin cytoskeleton alone may be responsible for the polarization of such small cells and cell fragments (Vasiliev, 1982, 1987). If this is the case it could provide an explanation for the change from microtubule-independent to microtubule-dependent polarization that we have described in cultures of HF. The actin cytoskeleton alone may be sufficient to maintain the polarization of the small cells initially present in these cultures but as they increase in size their polarization may, in addition, require microtubules.

Our results show that the effect of microtubule disruption on chick HF depends on the length of time for which the cells have been in culture. This finding may explain the apparently contrasting effects of microtubule disruption on fibroblasts and epithelial cells. In those cases where the polarization of epithelial cells has been shown to be microtubule-independent the cells have been in short-term 1° cultures, whilst in those cases where the polarization of fibroblasts has been shown to be microtubule-dependent the cells used have been in 2° or even longer-term cultures (see Introduction). It is not yet clear whether the polarization of epithelial cell types maintained for longer periods in culture becomes, like that of HF, sensitive to microtubule disruption, but it may be relevant in this context that the polarization of cells from two established epithelioid cell lines is sensitive to microtubule disruption (Domnina et al. 1985; Karavanova et al. 1985). Certainly, the suggestion that the polarization of epithelial cells and fibroblasts may involve different mechanisms (Middleton et al. 1988) now seems unnecessary.

Cells in culture become polarized when their protrusive lamellar activity becomes restricted to a limited region of the cell margin and this is closely associated with their ability to translocate (Vasiliev & Gelfand, 1976; Abercrombie, 1980; Brown & Middleton, 1985). We have shown that polarization in HF can, at different stages in culture, be either microtubule-independent or microtubule-dependent. This finding again emphasizes the difficulties involved in interpreting the results of studies in vitro and it is now clearly important that we establish whether or not the polarization of the cells in vitro is microtubule-dependent.

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