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Spreading-independent Growth of Normal Fibroblasts in Three-Dimensional Cultures*

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Changes of cell shape resulting from cellular flattening on culture substratum have previously been demonstrated to correlate with mitotic activity of normal animal cells in monolayer cultures. Here, we compared the shapes and proliferation of chick embryo fibroblasts cultured either in multicellular, multilayered sheets extended between glass fibres, or in standard monolayers. Fibroblasts in sheets retained the mitotic activity characteristic of that observed in sparse monolayer cultures, i.e. considerably higher that in confluent monolayers. Morphometric analyses revealed, however, that the cells in sheets were considerably less flattened than in monolayer cultures. These observations indicate that the modulation of culture conditions resulting in multidirectional cell stretching leads to the dissociation of flattening and mitotic activity of normal animal cells, so long as an intracellular stress field, generated by contractile cytoskeleton and stabilised by intercellular contacts, is maintained.

Key words: Cell shape, proliferation, cytoskeleton, tension, cell attachment.

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Cell behaviour in tissues depends on the range of intrinsic and extrinsic factors, which regulate basic cellular functions. Mechanical interactions between cells and their environment have been suggested to influence proliferation, differentiation and apoptosis (BEN ZE'EV 1991; CURTIS and SEEHAR 1978; CZYŻ *et al.* 2001; FOLKMAN & MOSCONA 1978; IWIG *et al.* 1995; SINGHVI *et al.* 1994; WATT *et al.* 1988). Cellular spreading on the substratum in monolayer cultures was suggested to alleviate the development of isometric contraction (mechanical tension), which results in cytoskeletal rearrangements such as the formation of microfilament bundles (stress fibers) (BURRIDGE & CHRZANOWSKA-WODNICKA 1996; IRELAND *et al.* 1989). Disorganisation of stress fibers upon cellular detachment from substratum (CZYŻ *et al.* 2001) or treatment with cytochalasin B (IWIG *et al.* 1995) inhibited cell growth indicating that tension-dependent architecture of the cytoskeleton plays a regulatory role in determining mitotic activity. These studies considered the role of organisation and mechanical equilibrium of cytoskeleton in regulation of normal cell proliferation. However, they have left the problem of direct engagement of changes of cell shape in the regulation of proliferation unsolved.

Chick embryo fibroblasts are characterized by a unique ability to spontaneously form multicellular and multilayered sheets suspended between glass fibers. Fibroblasts in sheets form stress fibers and remain mitotically active regardless of their position within a sheet i.e. they synthesize DNA independently of the contact with glass fibers (CZYŻ *et al.* 2001). In this communication, we demonstrate that although fibroblasts in sheets remain less flattened than in monolayer cultures, they retain a

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high mitotic activity. Our observations directly demonstrate that the modulation of culture conditions which promotes multidirectional cell stretching leads to the dissociation of flattening and mitotic activity of normal animal cells, so long as an intracellular stress field, generated by contractile cytoskeleton and stabilised by intercellular contacts, is maintained.

Material and Methods

Cell cultures

All experiments were carried out on fibroblasts isolated from 9-11 days old chick embryos (White Leghorn or Astra D). Primary cultures were maintained as described previously (KOROHODA and CZYŻ 1994). Cells for secondary cultures were harvested by trypsinisation (0.25% trypsin, Gibco) and seeded into 6-well culture flasks (Falcon) at initial density of 10^4 cells/cm^2 and cultured for 2 and 6 days, to obtain sparse (2,5 x 10^4 cells/cm^2) and dense (1,1 x 10^5 cells/cm^2) monolayers, respectively. Sheet cultures of chick embryo fibroblasts were prepared as described previously (CZYŻ *et al.* 2001). 6 day-old cell cultures with well developed sheets were used in all experiments.

DiIC$_{18}$ staining and morphometric analyses

For morphogenic analyses of cell shapes, cells trypsinised from primary cultures were stained with DiIC$_{18}$ (Molecular Probes, 10 μg/ml) for 20 min and mixed with their unstained counterparts at the proportion of 1:10. The resulting cell suspension was used for preparation of secondary cultures as described above. Pictures of 50 randomly chosen regions of 3.7% formaldehyde fixed-cell monolayers were taken with Leitz Orthoplan epifluorescence microscope with 25x NPL Fluotar objective, digitised with a Mustek 800IISP scanner and analysed with custom-designed CYTOP software (author: Ryszard Tokarski).

Analytical scheme based on confocal microscopy was used for planimetric analysis of fibroblasts in sheet cultures. Cell images were registered with Bio-Rad MRC 1024 CLSM unit with 40x water immersion NA 1.15 objective. 514 nm excitation line from an argon ion laser was used and emission was collected with 585LP filter. A series of optical sections including complete cell contour was registered for each selected DiIC$_{18}$ – stained cell (Fig. 1A,C,E) and a contour marking the distinct boundary of cell projection was drawn for each section (Fig. 1B,D,F). Partial contours were merged to obtain a complete cell contour used for planimetric analysis of cell projected surface area (Fig. 1 G).

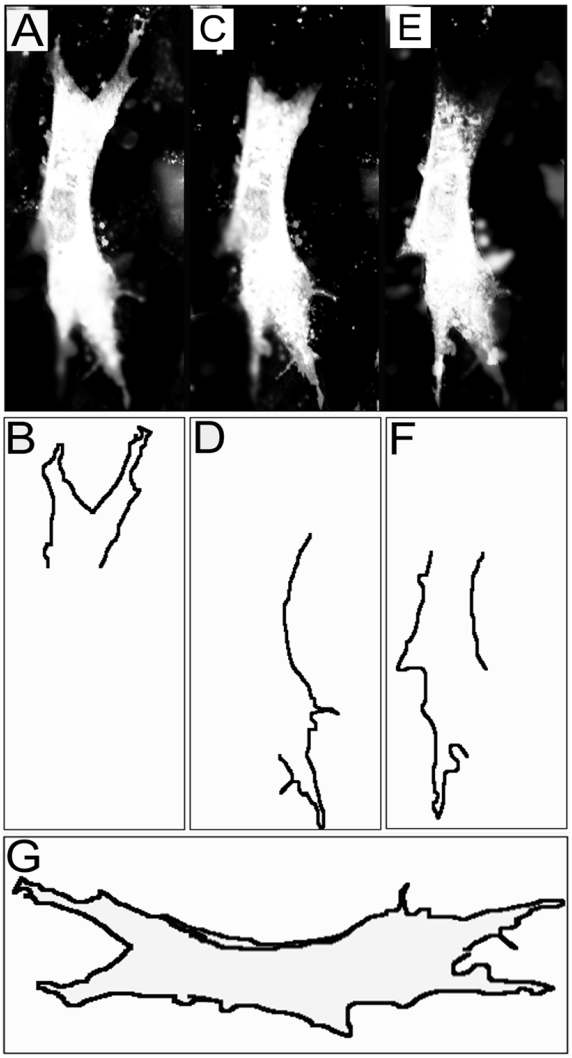

Fig. 1. Confocal microscopy-assisted analysis of cell spreading in multicellular sheets extended between glass fibres. Series of confocal sections of the DiIC$_{18}$ – stained cell (A,C,E) are analysed (B,D,F) to obtain partial contours, which are summed up and the resulting cell contour (G) is then planimetrically analysed.

Cell size was estimated by means of the planimetric method of cell diameter assessment. Suspended cells were stained with DiIC$_{18}$ (Molecular Probes, 10 μg/ml) for 10 min, transferred onto the glass slide, covered by cover glass and observed with an Olympus IMT 2 inverted epifluorescence microscope. The epifluorescence images were recorded with a Hitachi CCD camera, digitised and processed by CYTOP software. Statistical significance was determined by Student's *t*-test or non-parametric Mann-Whitney test with $P<0.05$ considered significant.

DNA content analysis

Flow-cytometric analysis of DNA content was performed with a FACStar cytometr (Becton-Dickinson). The cells were collected by trypsinisa-

tion, fixed with 70% methanol at -20°C, and stained in phosphate-buffered saline (PBS) containing 50 μM propidium iodide (Molecular Probes) and 1 mg/ml ribonuclease A (Sigma) for 1h at 37°C. The excitation wavelength was 488 nm and the fluorescence of DNA-bound propidium iodide was collected using the 575 nm LP filter. Statistical analysis was performed with Mod-Fit 2.0 software (Becton-Dickinson).

Results

We have shown previously that fibroblasts in multicellular sheets, which are attached to other cells and not to solid substratum, retain a high mitotic activity (CZYŻ *et al.* 2001). Here, we analysed cell cycle characteristics of the cells cultured in sheets, and in sparse and confluent monolayers. Flow-cytometric analyses revealed that the fibroblasts in sheets retained high fractions of mitotically active cells (Fig. 2C). Cell cycle parameters i.e. the relative numbers of cells in G_1, S and G_2 phase of the cell cycle remained similar in sheets and sparse monolayers (Fig. 2A) despite the high local cell densities characteristic for sheet cultures. In contrast, a significant retardation of cell growth was observed in monolayers upon cell crowding (Fig. 2B). Moreover, flow-cytometric and volumetric analyses revealed that culture conditions, while affecting mitotic activity of fibroblasts, exerted no effect on the averaged cell volume. Both light scatter properties (Fig. 2 D,E,F) and averaged diameters (Table 1) estimated for analysed cell populations remained similar.

Further, we investigated the influence of culture conditions on cell spreading. An experimental approach based on differential fluorescent staining with $DiIC_{18}$ was used. It enabled us to delineate cell perimeters in crowded monolayer and sheet cultures where cell borders remained obscure. Cell cultures were established which consisted of subpopulations of $DiIC_{18}$-stained and unstained fibroblasts. $DiIC_{18}$ staining exerted no effect on subsequent cell shape properties of fibroblasts in culture as shown by the comparison of projected areas of unstained and stained cells in sparse monolayers (Fig. 2G,M, Table 1). Planimetric analysis of cell shapes in monolayers revealed that, upon crowding, fibroblasts reduced their degree of spreading. As shown in Fig. 2G,H and summarised in Fig. 2M,N, the majority of fibroblasts in sparse cultures occupied surface areas in the range 1000 μm^2 to 2000 μm^2 whereas, in confluent monolayers only a few cells exceeded 1500 μm^2. The average projected cell area estimated for the cells in confluent monolayers was ca. 30% lower than for the cells from sparse monolayers (Table 1).

Previously, we have shown that multicellular sheets extended between glass fibers consist of several layers, which could impose their multiplanar stretching. To determine how the spatial distribution of cell-substratum attachment points influences fibroblast shapes in sheets, an experimental approach based on the analysis of series of confocal images was adopted. It alleviated the delineation of cell contours and precise quantification of cell shape parameters in sheet cultures. Cell shape analysis revealed that the degree of cellular flattening in sheets was considerably lower than in monolayer cultures. The majority of fibroblasts in sheets was characterised by surface areas below 1000 μm^2 (Fig. 2 I,O, Table 1). Similarly, the nuclear flattening in sheet cultures was significantly lower than in monolayers (not shown). These observations demonstrate that the multilayered structure of sheets (CZYŻ *et al.* 2001), which may impose multidirectional stretching of the cells, reduces the degree of cellular flattening but not the mitotic activity.

Discussion

Upon cell shape changes, numerous phenomena take place including the induction of proliferation,

Table 1

Shape and size parameters of fibroblasts cultured under various conditions

Culture conditions	Sparse monolayer	Confluent monolayer	Sheets extended between glass fibres
Averaged surface area of cell projection (μm^2)	1567 [1] 1603.6 [2) NS)]	1029.8 [1, *)]	721.1 [1,*]
Averaged cell diameter (μm)	14.1	13.9 [NS]	13.4 [NS]

[1] DiI C_{18} -stained cells
[2] DiI C_{18} -unstained cells
[*] Statistically significantly different from unstained sparse cells at $P<0.05$

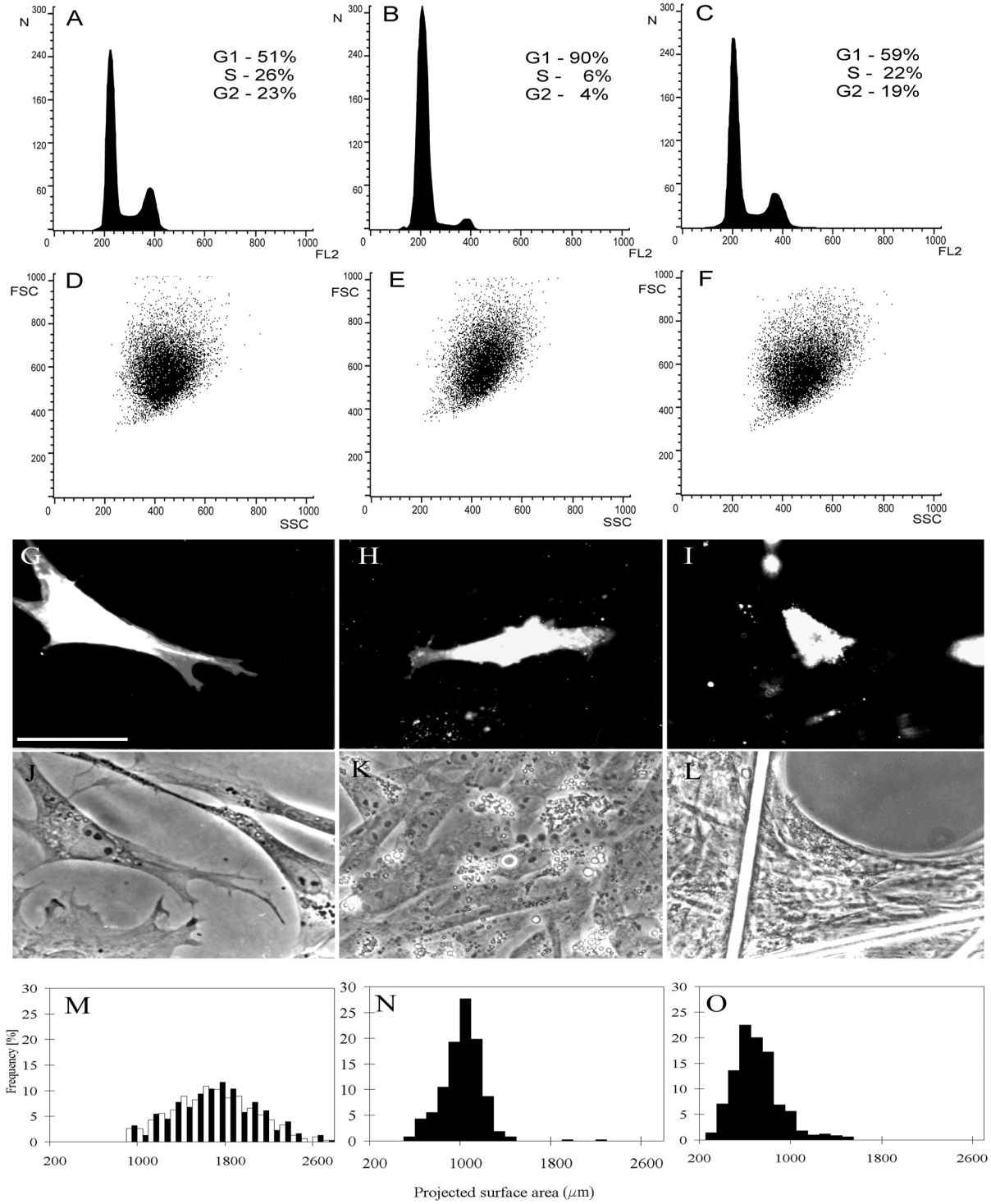

Fig. 2. A-F – Flow-cytometric analysis of mitotic activity (A-C) and light-scatter properties (D-F) of chick embryo fibroblasts cultured in sparse (A,D) and confluent (B,E) monolayer cultures, and in sheets extended between glass fibres (C,F); G-L – Visualisation of the shapes of chick embryo fibroblasts cultured in sparse (G,J) and confluent (H,K) monolayers and in sheet cultures (I,L). Cells were differentially stained with $DiIC_{18}$ and observed in epifluorescence (G-I) or phase contrast mode (J-L, scale bar = 50 μm); M-O – frequency histograms of the spreading of chick embryo fibroblasts cultured in sparse (M) and confluent (N) monolayers and in sheet cultures (O) (open bars – unstained cells; filled bars – $DiIC_{18}$ stained cells).

differentiation, apoptosis and modulation of gene expression (BEN ZE'EV 1991; FOLKMAN & MOSCONA 1978; GINIS & FALLER 1997; INGBER 1997; IWIG *et al.* 1995; SINGHVI *et al.* 1994; WATT *et al.* 1988). In this communication, we demonstrate that the modulation of culture conditions resulting in multidirectional cell stretching erases the correlation between cell flattening and mitotic activity in cultures of normal animal cells. Fibroblasts, when cultured in multicellular and multilayered sheets suspended between glass fibers, are characterized by a low degree of flattening but retain a high mitotic activity.

Chick embryo fibroblasts are normal cells which display contact-phenomena such as anchorage-dependence and contact inhibition of growth i.e. they proliferate only when attached to solid substratum and cease to grow upon confluence (KOROHODA & CZYŻ 1994). Analysis of cell shapes in monolayer cultures revealed that mitotically inactive fibroblasts in confluent monolayers were less spread than their proliferating counterparts in sparse monolayers. This observation indicate that, at least in monolayer cultures, mitotic activity of fibroblasts depends on their spreading (CASTOR 1970). In contrast, morphometric analyses of the cells in sheets revealed that fibroblasts cultured under such conditions were considerably less spread that the cells in confluent monolayers but retained a high mitotic activity.

Previous observations indicated that the cessation of growth of fibroblasts in confluent monolayers correlated with disorganisation of actin cytoskeleton and disappearance of stress fibers and focal contacts (KAJSTURA & BEREITER-HAHN 1989). On the other hand, mitotically active fibroblasts in sheets, being less flattened, retained the architecture of actin cytoskeleton similar to sparse monolayer cultures with abundant stress fibers, focal contacts and fibrous deposits of fibronectin (CZYŻ *et al.* 2001). These data indicate that a restriction of cell spreading that results in a disorganisation of cytoskeleton represented by disappearance of stress fibers leads to the inhibition of mitotic activity and, potentially, induction of differentiation and apoptosis (GINIS & FALLER 1997; IRELAND *et al.* 1989; SINGHVI *et al.* 1994; WATT *et al.* 1988). Structural integrity of actin cytoskeleton, which depends on isometric tension (BURRIDGE & CHRZANOWSKA-WODNICKA 1996; CHRZANOWSKA--WODNICKA & BURRIDGE 1996; KOROHODA & KAJSTURA 1982; ZHONG *et al.* 1998) influences the organisation of signalling pathways that regulate mitotic activity of the cells. Therefore, isometric tension generated by contractile activity of actin cytoskeleton may determine cellular competence for proliferation via effect on cytoskeleton architecture.

GALBRAITH and SHEETZ (1998) suggested that cell spreading facilitates the development of internal stress field which determines mechanical equilibrium of cytoplasm and rearrangements of cytoskeleton thus influencing cellular physiology. According to this view, cells in confluent monolayers, being less flattened, are no longer able to develop the stress field of the magnitude sufficient for growth-permissive cytoskeleton rearrangements. On the other hand, fibroblasts in sheets, which consist of several cell layers (CZYŻ *et al.* 2001), are embedded into substratum rather than seeded upon it as in monolayers. Therefore, spatial distribution of cell-extracellulat matrix (ECM) contacts in sheets may impose the multidirectional cell stretching which reduces the degree of cell flattening while retaining conditions for field stress development. Theoretically, in such conditions, the stress field magnitude may reach the values sufficient for "growth permissive" internal rearrangements even in rounded cells, provided the distribution of cell-substratum (or -ECM) contacts remains spatially isotropic.

In conclusion, our observations support the previously published views that the amount of tension or the magnitude of stress field developed by cells is a central factor that determines the quality of signalling pathways implicated in the regulation of cell proliferation (CHICUREL *et al.* 1998; CURTIS & SEEHAR 1978; GALBRAITH & SHEETZ 1998; INGBER 1997). The results presented in this communication directly show that cell shape influences cell behaviour in indirect way and point to the role of cell-generated mechanical forces stabilised by cell-to-cell or cell-substratum contacts in the regulation of cell physiology.

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