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THE FORMATION OF FILOPODIA-LIKE PROTRUSIONS DURING PREPARATION OF CELL SUSPENSIONS FOR SCANNING ELECTRON MICROSCOPY

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Abstract

The standard preparation of cell suspensions (e.g., blood cells, cell suspensions derived from monolayer cultures or from various tissues, etc.) for scanning electron microscopy (SEM) includes fixation of the cells in suspension and subsequent dehydration and critical point drying (CPD) of the cells after their preliminary attachment to the special substrata. In the course of the SEM examination of cell suspensions of various origins, unusual morphological cell surface structures, filopodia-like protrusions (FLP), were consistently detected in 3-20% of the cells in the populations. FLP can be effectively observed only by using a stage tilt angle of no less than 30°. FLP were single or multiple thin threads extending from basal parts of a spherical cell and attaching to the substratum surface used. FLP strikingly resembled substratum-attached filopodia formed by a viable cell at its earliest stages of spreading. The percentages of the cells with FLP were not significantly affected by the character of cell fixation (primary aldehyde fixation alone or primary aldehyde with subsequent OsO_4 post-fixation) or the raising of the temperature and pressure inside the CPD bomb. It seems that the protrusions imitating the natural cell surface structures can probably be formed at later stages of the preparation of cell suspensions for SEM, namely during dehydration and (or) CPD when the cells undergo substantial shrinkage. One of the possible mechanisms by which the cell shrinkage could induce FLP formation follows. A pre-fixed spherical cell which settles down to the substratum sticks to it at some discrete points on the cell surface. As a result of the subsequent cell shrinkage, FLP could be formed and then stretched, connecting the same points on the cell surface with the points of the initial cell-substratum adhesion.

Key Words: Scanning electron microscopy, cell suspensions, cell preparation for scanning electron microscopy, artifacts in biological scanning electron microscopy, cell shrinkage, critical point drying.

Introduction

To study cell surface topography and/or intracellular organization of cells in suspension by scanning electron microscopy (SEM), the cells should be fixed in a suspended state and subsequently attached to some supporting substrata enabling processing and observation (see for reviews: Bell, 1983; Rovensky and Vasiliev, 1984; Malecki and Ris, 1992).

For a number of years, we have performed SEM studies of cell suspensions derived from various cell cultures or tissues, and have consistently discovered cells with unusual surface morphology in populations. Among cells pre-fixed in suspension and thereafter allowed to settle onto and attach to the substratum (e.g., to poly-L-lysine coated coverslips) individual cells with filopodia-like protrusions (FLP) consistently occurred. These protrusions extended from basal parts of a spherical cell to the substratum surface and strikingly resembled substratum-attached filopodia formed by a viable cell at the earliest stages of spreading (Rovensky, 1979). The cells with FLP constituted, as a rule, a small part of the pre-fixed cell population; however, they occurred with high persistence. Their detection was promoted by using a stage tilt angle of no less than 30° in the course of SEM examination and photoregistration.

On careful examination of scanning electron micrographs of cell suspensions published elsewhere, it is often possible to identify cells with FLP although the authors of these publications, as a rule, never mentioned their presence. It seems that the reports by Vergara *et al.* (1977) and Collins and Brunk (1980) prove exceptions. In the first work, the authors note the presence of "elongated protrusions" at the sites of subsequent cell attachment to the substratum in some T-24 cells pre-fixed in suspension. In the second report, the authors indicate the possibility of "artifactual spreading" (formation of substratum-attached protrusions) of Ehrlich ascites tumor cells pre-fixed in suspension and thereafter attached to poly-L-lysine coated surface.

The purpose of the present investigation was to conduct a more detailed study of FLP formation in cells

pre-fixed in suspension and prepared for SEM in order to elucidate the possible role of the following factors in FLP formation: the method of cell fixation, the type of substratum, the raising of the temperature and pressure inside the bomb during critical point drying (CPD).

Materials and Methods

Cell cultures and cell suspension derivation

Normal cells from primary cultures of mouse (MEF) and rat (REF) embryo fibroblasts as well as transformed cells from two lines of mesenchymal origin were used:

REF (LT) - a non-tumorigenic cell line derived from REF as a result of transfection of the cloned gene of the polyoma virus large T-antigen (Komissarova *et al.*, 1988);

PSC-3 - tumorigenic cell line derived from sarcoma developing around the polymeric plate implanted subcutaneously to mouse of CBA-strain (Moizhess, unpublished data).

The cells of 1-2 day monolayer cultures were detached from the bottoms of culture dishes using a warm mixture of 0.25% trypsin solution and 0.02% EDTA solution (1:1). The cells were then resuspended in fresh culture medium and sedimented by centrifugation (no more than 200 *g* for 3 minutes). After washing in Hanks' solution, the sedimented cells were immediately resuspended in a warm (35°-37°C) solution of the fixative.

Preparation of cell suspensions for SEM

Fixation. Either primary aldehyde fixation alone or aldehyde fixation with subsequent OsO_4 post-fixation was used. In the former case, the cells were fixed in 2% glutaraldehyde in 0.1 M Na-cacodylate buffer with 0.1 M sucrose and pH 7.2 (Collins and Brunk, 1980). Fixation was performed for 1 hour at room temperature and thereafter (for 1-3 weeks) at 4°C. In the latter case, the aldehyde fixed cells were rinsed in Hanks' solution and post-fixed in 1% OsO_4 in Hanks' solution for 1-2 hours at room temperature.

The fixed cells were washed twice in Hanks' solution and resuspended in distilled water.

Attachment to the substratum, dehydration and drying. The cells were prepared for dehydration and drying after the preliminary attachment of pre-fixed cells to substrata. Poly-L-lysine coated coverslips (Sanders *et al.*, 1975), aluminum foil plates (Rovensky, 1978; Samilchuk and Rovensky, 1980), or Millipore filters (Millipore Corp., Bedford, MA) impregnated with an aqueous solution of albumin (Flechon *et al.*, 1975) were used as the substrata to which cells pre-fixed in suspension can effectively attach.

Pre-fixed cell suspensions were placed in tissue culture multiwell plates (Linbro, Flow, Scotland) with the substrata on their bottoms. The cells were allowed to sediment by gravity to the substratum surfaces for 2 hours at room temperature. The cells sedimented onto poly-L-lysine coats or onto aluminum foil proved quite stably attached to the surface of those substrata. Attachment of the pre-fixed cells to the surface of Millipore filters (0.45 μm, Millipore Corp., USA) impregnated with a 50% aqueous solution of albumin was achieved by 2% glutaraldehyde treatment of the filter with sedimented cells, the fixative induced denaturation of albumin attaching the sedimented cells to the filter surface.

The pre-fixed cells attached to the substrata were dehydrated in a graded series of either acetone or ethanol; the substrata were then placed in the CPD apparatus (Balzers Union, Liechtenstein).

The cells were dried using either a routine procedure of CPD from liquid CO_2, or its modification offered by Boyde and Maconnachie (1984) and named by the authors "evaporative drying in a near saturated atmosphere" (EDSA).

Cells on the substrata were coated with gold-palladium by ion sputtering (Balzers Union) and examined in a scanning electron microscope (Philips PSEM-501). Stage tilt angles used for the examination were 0°, 30°, and 45°.

The effectiveness of attachment of the pre-fixed cells to the substratum was evaluated as follows: The number of pre-fixed cell suspensions placed in a well of culture multiwell plate was evaluated by means of a hemocytometer. Proceeding from the number of the cells and the area of the well bottom, it was possible to calculate the expected number of the cells which would be attached to a definite area on the substratum surface. This expected cell density could be evaluated on the assumption that **all** suspended cells which were allowed to sediment will be attached to and distributed evenly on the well bottom. The expected cell density was subsequently compared with the real average number of the cells attached to the same area on the substratum surface observed with the SEM. Percent ratio between the real and expected cell densities, as the effectiveness of attachment of the pre-fixed cells to the substratum, was calculated.

The percentage of the cells with FLP was estimated during SEM examination counting 80-100 cells attached to the substratum surface.

Results

It is evident from Table 1 that the method of prefixation in suspension affects the cells' capacity to attach

Table 1. FLP formation in various conditions of preparation of cell suspensions for SEM.

Cells	Fixation in suspension	Substratum for attachment of prefixed cells	Drying	Percentage of cells attached	Percentage of cells with FLP
MEF	g	p-l	CPD	93 ± 6	8 ± 2
	g+Os	p-l	CPD	16 ± 2	6 ± 1
	g	a.f.	CPD	91 ± 6	11 ± 1
	g+Os	a.f.	CPD	20 ± 3	12 ± 3
	g	a.f.	EDSA	69 ± 4	12 ± 3
	g+Os	a.f.	EDSA	16 ± 3	9 ± 2
	g+Os	p-l	EDSA	36 ± 4	10 ± 3
	g	alb.f.	CPD	-	5 ± 1
REF	g	p-l	CPD	-	8 ± 3
	g	a.f.	CPD	100 ± 7	3 ± 1
REF (LT)	g	p-l	CPD	83 ± 3	15 ± 2
	g+Os	p-l	CPD	17 ± 4	9 ± 2
	g	a.f.	CPD	81 ± 6	13 ± 3
	g+Os	a.f.	CPD	26 ± 3	10 ± 3
	g	p-l	EDSA	75 ± 6	11 ± 4
	g+Os	p-l	EDSA	22 ± 4	12 ± 3
	g	a.f.	EDSA	83 ± 5	12 ± 4
	g+Os	a.f.	EDSA	26 ± 4	10 ± 2
	g	alb.f.	CPD	-	20 ± 3
	g+Os	alb.f.	CPD	-	20 ± 5
PSC-3	g	p-l	CPD	100 ± 4	16 ± 2
	g	a.f.	CPD	100 ± 5	12 ± 3

g - primary glutaraldehyde fixation; g+Os - primary aldehyde fixation and OsO_4 postfixation;
p-l - poly-L-lysine coated coverslip; a.f. - aluminum foil plate;
alb.f. - Millipore filter impregnated with albumin; CPD - critical point drying;
EDSA - "evaporative drying in a near saturated atmosphere"; Mean ± standard error of the mean.

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to the surface of the substrata used. Following primary fixation with glutaraldehyde the suspended cells attached both to aluminum foil or to poly-L-lysine coated coverslips with equal efficiency (80-100%); after secondary fixation with OsO_4 cell attachment to both substrata was significantly worse. The method of drying (CPD or EDSA) did not affect attachment of the pre-fixed cells to poly-L-lysine coated coverslips or to aluminum foil. The effectiveness of attachment of the pre-fixed (by aldehyde fixation alone or by one with OsO_4 post-fixation) cells to the surfaces of Millipore filters impregnated with albumin was not found to be stable enough and varied considerably in the experiments (the data were not included in Table 1).

In the course of SEM examination of cells pre-fixed in suspension and subsequently attached to the substratum surfaces the cells with filopodia-like protrusions (FLP) were detected in a small percentage of cases but persistently.

FLP looked like single or multiple thin threads extending from basal parts of a spherical cell and attaching with their distal ends to the substratum surface (Fig. 1). FLP length could vary from several to 20 μm, their thickness as a rule was 0.1-0.2 μm. Usually, FLP were straight (Figs. 1a, 1b, and 1c), although sometimes they were curvy (Fig. 1d); bulbous distal ends were frequent (Figs. 1b, 1c, 1d, and 1e). In some cases, starting from the site of contact with the substratum, FLP extended further as an even thinner thread which would spread along the substratum surface (Fig. 1f). With regard to

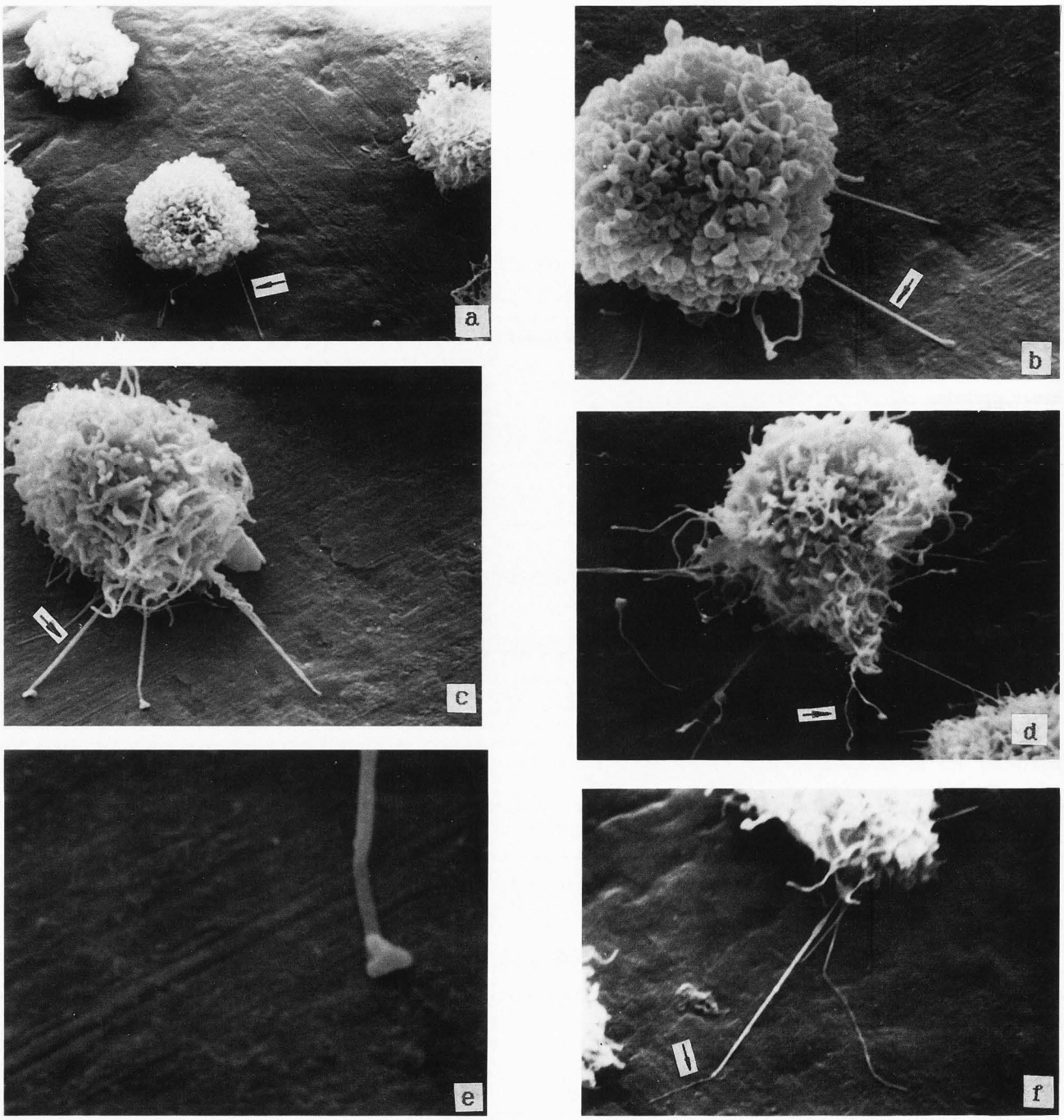

Figure 1. REF (LT) cells pre-fixed in suspension, subsequently attached to aluminum foil plates and critical point dried. (1a, 1b, and 1c) Straight or (**d**) curved and branched FLP (arrows) with bulbous distal ends; (**e**) FLP bulbous distal end; (**f**) FLP from the site of contact with the substratum surface (arrow) extends farther as a thinner thread. Horizontal field width = (**a**) 63 μm, (**b**,**d**,**f**) **33** μm, (**c**) 26 μm, (**e**) 4 μm.

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morphology, FLP strikingly resembled filopodia formed by a viable cell at its earliest stages of spreading on substratum surface (Fig.2).

Table 1 summarizes the percentages of cells with FLP among pre-fixed cells attached to the different substrata. It is evident that these indices in different cell types vary from 3 to 20%. The data presented in Table 1 also show that the percentage of cells with FLP is not significantly affected by such factors as: the mode of cell pre-fixation in suspension (primary glutaraldehyde

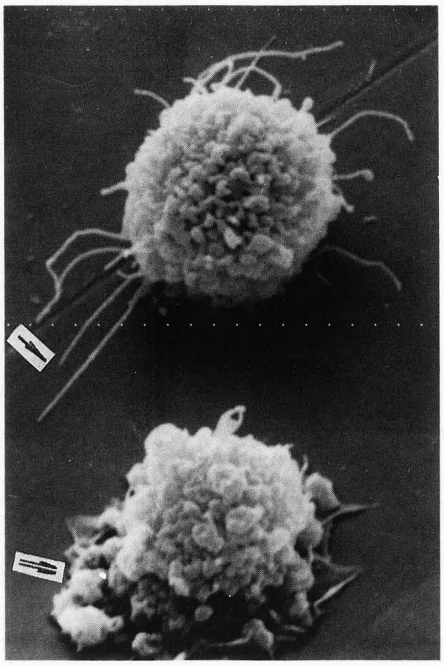

Figure 2. MEF at the early stage (40 minutes after seeding) of spreading on glass coverslip: filopodia (arrow) or lamella (double arrows) formed at the basal parts of the cultured cells. Horizontal field width = 35 μm.

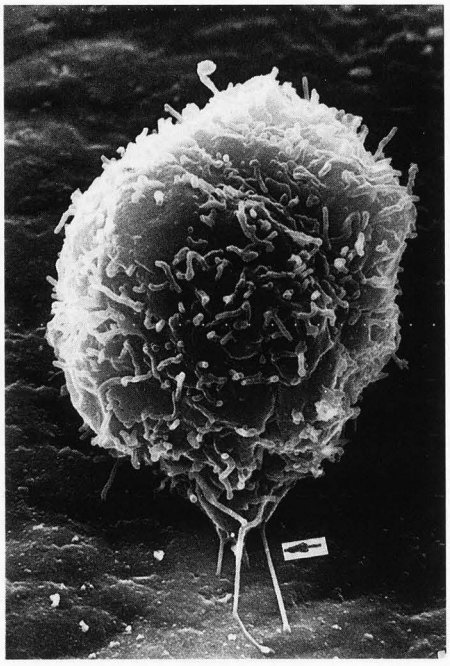

Figure 3. Human lymphocyte pre-fixed in suspension, attached to aluminum foil plate and critical point dried. FLP (arrow). Horizontal field width = 7 μm.

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fixation or one with subsequent OsO_4 post-fixation), the type of substratum used for attachment of pre-fixed cells, or the method of cell drying (CPD or its modification ESDA).

Discussion

Attachment of cells pre-fixed in suspension

The experiments confirmed the data reported earlier that cells pre-fixed in suspension with glutaraldehyde readily attach to the substratum surfaces, namely coverslips coated with poly-L-lysine (Sanders *et al.*, 1975) or aluminum foil plates (Rovensky, 1978). The attachment of pre-fixed cells to polycation coats is accounted for by their electrostatic interaction with this substratum; the mechanism of equally effective attachment to aluminum foil is not quite clear. Presumably, it can also be based on an electrostatic interrelationship, in particular, the interaction between free electrons of metal on the inner surface of the insulating film Al_2O_3 (which covers the surface of the foil) and positively charged regions of the cell surface contacting the film. Attachment of the pre-fixed cells to Millipore filters impregnated with albumin was less effective compared with those attached to the "electrostatic" substrata; the connections between sedimented cells and the filter surfaces caused by the glutaraldehyde induced denaturation of the albumin do not seem to be stable.

Our data demonstrate that as a result of secondary fixation with OsO_4 the suspended cells lose a considerable amount of their capacity to attach to both poly-L-lysine coats and aluminum foil. Other authors have also reported a less efficient attachment of cells to poly-L-lysine coated coverslips after pre-fixing in an osmium-containing solution (Wen-Lang Lin *et al.*, 1983). Osmium treatment apparently changes the cell surface charge, thereby weakening the electrostatic interaction with the substrata.

Cell drying by the EDSA method, which eliminates the effect of increased temperature and pressure inside the CPD apparatus did not lead to any significant detachment of pre-fixed cells from the substratum surfaces.

FLP formation in cells pre-fixed in suspension

FLP formation was noted in pre-fixed cells in all the populations tested on the substrata used. Although in the given study we used cultured cells of mesenchymal origin, our previous observations (Rovensky, unpublished data) indicate that FLP arise in cells of various epithelial lines pre-fixed in suspension as well as in cells of ascites tumors or in leukocytes (Fig. 3). Consequently, FLP formation can be regarded as a wide-spread phenomenon.

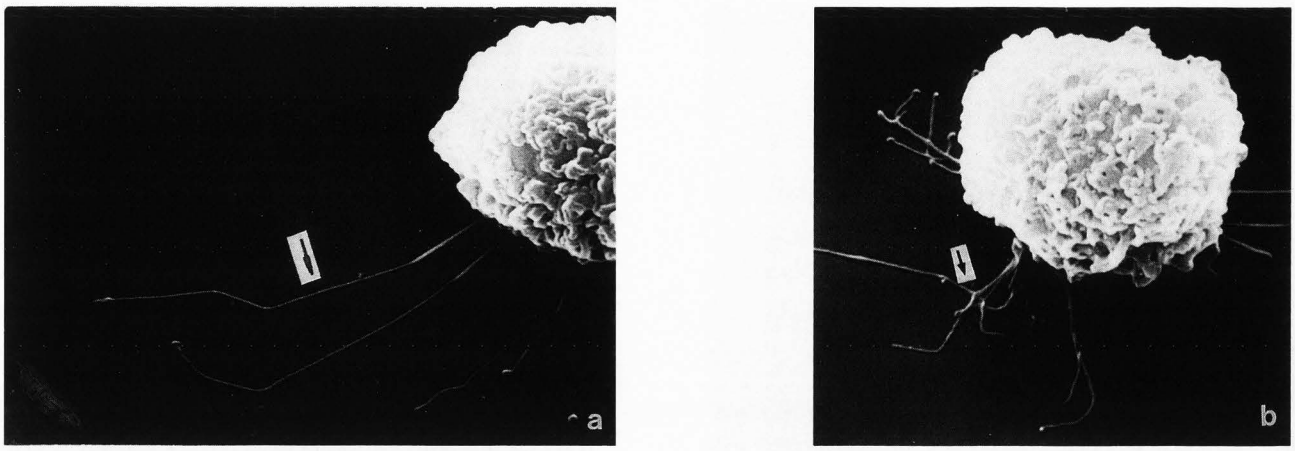

Figure 4. MEF pre-fixed in suspension, attached to poly-L-lysine coated coverslips and air-dried from acetone. (**a**) Straight or (**b**) branched FLP (arrows) with bulbous distal ends. Horizontal field width = (**a**) 13 μm, (**b**) 15 μm.

The percentage of cells with FLP is, as a rule, not large. In our experiments it varied from 3 to 20% in different cell populations. It is impossible to detect any relationship between FLP development and the cell transformation status. In some of our experiments, the percentage of cells with FLP was somewhat higher in transformed cell lines, e.g., PSC-3 and REF(LT), than in primary cultures of normal fibroblasts, e.g., REF or MEF. In other observations, transformed cells, namely 3T3 ras, exhibited a low percentage (3%) of FLP (data not shown).

Can FLP have a natural origin? FLP morphologically resemble the retraction fibrils observed during trypsin or EDTA treatment of cultured cells: during detachment from the substratum, the cell progressively retracts leaving the fibrils attached to substratum surface (Revel *et al.*, 1974; Rovensky, 1979). In our experiments, however, the cells were fixed in suspended state, i.e., after their complete detachment from the substratum surface when the cells had already acquired spherical shapes and lost the retraction fibrils. In some cases, natural microvilli at the basal parts of the cells can be taken for FLP. However, the structures identified as FLP in our experiments were always much longer than microvilli covering the free surface of the same spherical cell. Furthermore, FLP were found not only in cells with abundant microvilli but also in cells of various types in which microvilli were absent and the cell surface relief revealed only blebs or folds.

Can FLP be formed during fixation of cell suspensions? One can conceive that FLP could be formed as a result of contacts of a pre-fixed cell with the substratum. At some points of adhesion of the cell surface to the substratum, filamentous structures could be formed and then stretched. In our experiments (Rovensky, unpublished data), mouse embryo fibroblasts pre-fixed in suspension were allowed to sediment by gravity to the poly-L-lysine coated coverslips in conditions of gentle agitation of the vessel to increase the frequency of cell-substratum interactions and to favor the presumable formation of the filamentous structures. We failed to show any significant increase in the percentages of the cells with FLP or in the linear growth of these structures. Nevertheless, one cannot exclude shrinkage of a pre-fixed attached cell during dehydration or CPD as a cause of FLP formation (see below).

The type of substratum used for pre-fixed cell attachment may presumably contribute to FLP formation. Collins and Brunk (1980) noted protrusions in basal parts of Ehrlich ascites tumor cells pre-fixed in suspension and attributed this "artifactual spreading" of the cells to the poly-L-lysine coat used as the substratum. However, as shown in our experiments, cells with FLP are detected not only on "electrostatic" substrata (poly-L-lysine coats or aluminum foil) but also on those linked to cells by means of denatured albumin (filters impregnated with albumin).

We suppose the formation of FLP in cells pre-fixed in suspension is most likely to occur during the dehydration and (or) drying procedures. At these steps in the preparation for SEM, cells undergo a serious shrinkage. Cell shrinkage is especially great during air drying due to the compressive forces of surface tension (Bartlett and Burstyn, 1975). We previously demonstrated that following dehydration and air drying, cells pre-fixed in suspension develop long filopodia-like (Fig. 4) and/or lamella-like protrusions which firmly attach to the substratum (Rovensky, 1979; Rovensky and Vasiliev, 1984). Morphologically, these protrusions were strikingly similar to the outgrowths formed by viable cells at

early stages of spreading. We associated the formation of the protrusions with the effect of surface tension compressive forces on the cells during air drying. Although the effect of surface tension is minimized during CPD, cells undergo substantial shrinkage occurring in four stages while within the CPD bomb (Boyde *et al.*, 1977, 1981; Boyde and Maconnachie, 1979; Bastacky *et al.*, 1985). One of these stages is the period in which the temperature inside the CPD bomb is increased above the critical temperature. In our experiments, FLP were formed in cells exposed to the "not quite critical point drying" method (Boyde and Maconnachie, 1984) in which the increase in temperature and pressure inside the bomb is excluded. Others have shown that cell shrinkage is induced by the complex physicochemical processes occurring at different stages of CPD. These processes include a reduction in mutual repulsion of negatively charged sites of protein structures (Boyde and Maconnachie, 1979), changes in the conformation of hydrated proteins, glycoproteins, carbohydrates and mucopolysaccharides; precipitation of dissolved ions; and the formation of voids inside cells (Bastacky *et al.*, 1985). It is still unknown in what way these various processes may induce FLP formation in local sites on a pre-fixed cell surface.

Cell shrinkage during dehydration and/or CPD could give rise to FLP formation in the following way. A pre-fixed spherical cell on settling down to the substratum sticks to it at some discrete points on the cell surface. As a result of the subsequent cell shrinkage, FLP could be formed as the cell body reduces in size while the points of the initial cell-substratum adhesion remain intact.

FLP torn from the substratum could take the shape of unusually long, frequently curved microvilli. Such microvilli-like protrusions (MLP) were markedly distinguished especially against the background of blebbed or folded cell surface relief. In this investigation, MLP were detected in a small percentage of cases in pre-fixed cells attached to substrata (data not shown).

The striking resemblance of FLP and MLP with similar formations of viable cell surfaces should remind one of the autonomous capacity of cellular membrane and cortex to form local outgrowths of filopodia or lamellae. These outgrowths can be generated by a mechanical force applied to the local sites of the cell plasmatic membrane and directed outward (Popov and Margolis, 1988). The outgrowths (in particular, filopodia-like) may arise not only in whole cells but in cytoplasmic fragments (Albrecht-Buehler, 1980). The autonomous pattern of formation of the outgrowths in local sites on the cell surface is probably preserved in a cell pre-fixed in suspension and can be manifested as a result of the influences associated with the drying process.

The data obtained show that the results of SEM studies of cell suspensions of various types should be interpreted carefully. Single long microvilli detected against the background of blebbed or folded microreliefs of the cell surface may not be produced by a viable cell. The category of artifactual formations may include filopodia-like protrusions detected in basal parts of cells pre-fixed in suspension and subsequently attached to the surface of various substrata.

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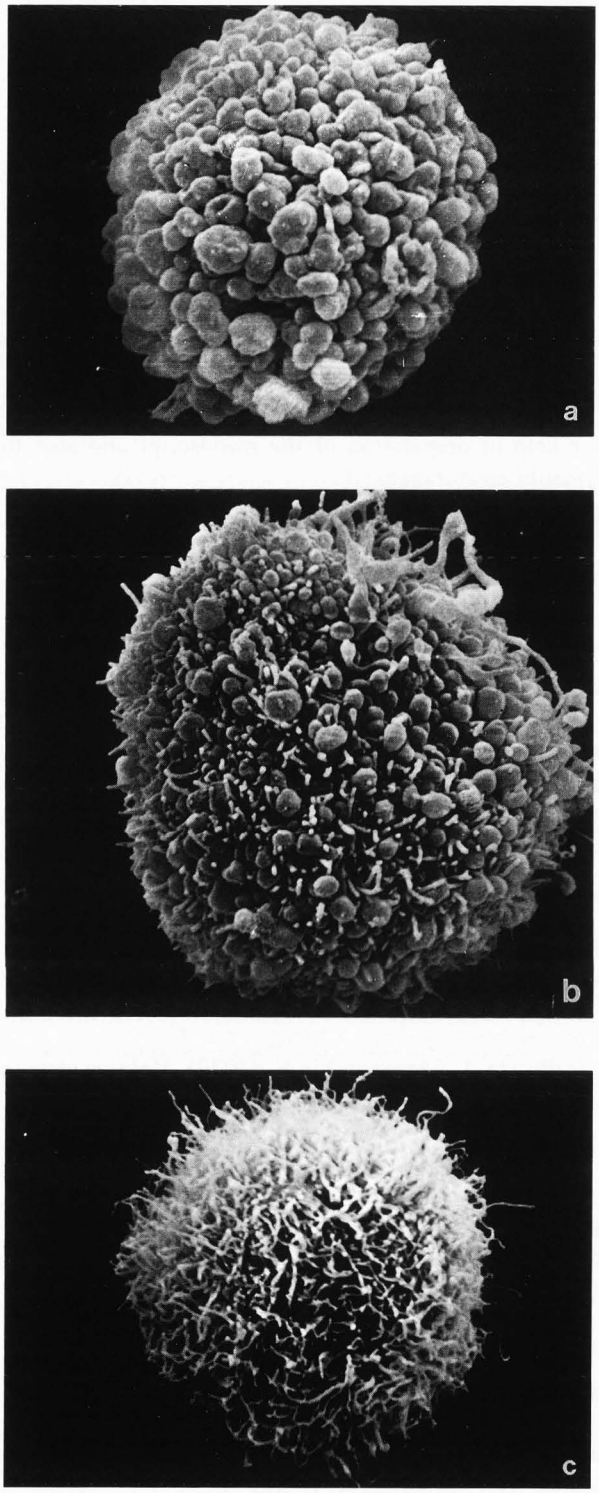

Figure 5. Some surface topography patterns of the cells pre-fixed in suspension, attached to aluminum foil plates (**a**,**b**) or to poly-L-lysine coated coverslip (**c**) and critical point dried. The cells are without FLP. (**a**) MEF, blebbed surface relief; (**b**) MEF, mixed (blebbed and microvillous) surface relief; (**c**) REF (LT), microvillous surface relief. Horizontal field width = 22 μm.

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Discussion with Reviewers

M. Malecki: I suggest that it would be really helpful to include also images of control cells in order to evaluate their surface architecture, but not only those cells having FLPs.

Author: Some surface topography patterns of suspended cells without FLP are presented in Figure 5. The different types of surface topography of suspended cells and the data on the relative concentrations of cells with a definitive type of surface relief in suspensions, derived from various monolayer cultures, were previously described (Rovensky *et al.*, 1992).

M. Malecki: Optical sectioning, with confocal scanning laser microscopy of cells attached after fixation to a substrate, fluorescently labelled with a membrane stain Dil type, and remaining in a buffer, should allow you to

demonstrate cellular filopodia. Application of silanized glass substrates (Malecki and Ris, 1992) should allow you even to pursue optical sectioning with the Nomarski optics. These samples could be further processed to SEM. This way, you could immediately assess your hypothesis. Have you considered undertaking such experiments?

Author: Use of light microscopy for revealing FLP meets with the difficulties caused by the too small (0.1-0.2 μm) thickness of these structures, their extension at an angle to the substratum surface, and "screening" of FLP by the spherical cell body. The additional difficulties of optical identification of FLP arise in case of a microvillous surface topography pattern of the spherical cell. Our efforts to reveal FLP in pre-fixed cells attached to poly-L-lysine coated coverslips, and remaining in Hanks' solution, using video-enhanced differential interference contrast (DIC) microscopy, were unsuccessful. Apparently, confocal laser scanning microscopy (CLSM) could be a more effective method for revealing FLP in cells attached after fixation to a substratum, before their dehydration and drying, but, unfortunately, we have no possibility to use this method.

M. Malecki: Have you considered rapid freezing of cells in suspension (Malecki, 1991) followed by either freeze-fracturing, freeze-drying, or freeze-substitution and CPD processing thought to introduce less shrinkage [e.g., Barlow and Sleigh (1979); Walther and Hentschel (1989)].

Author: We performed the experiments with dehydration and CPD of the cells in suspension state by the method described previously (Jones and Gillett, 1975), and we could not reveal cells with FLP. Now we try to use freezing of the cells in suspension with subsequent freeze-drying (FD) and also FD of pre-fixed cells after their attachment to the substratum.

T.K. Maugel: The "thinner thread" of the FLP terminus in Figure 1f may be due solely to differences in secondary electron yield between processes lying flat on the substrate and those suspended above the substrate. I have often observed a difference in thickness between portions of bacterial flagella that lie on the substrate and adjacent regions of the same flagellum that is suspended above the substrate. Those portions flat to the substrate always appear to be smaller in diameter.

Author: Really, as a rule, the proximal, non-attached to the substratum, part of FLP looks more bright than the distant part lying on the substratum in SEM examination. However, I think that the distinct differences in thickness between the proximal and distant parts of FLP, observed with SEM, could not be due solely to differences in secondary electron yield. On the contrary, at high magnifications (but within the limits of the SEM resolution) we could see a blurred image of FLP due to a halo of scattered light on the SEM screen. But as seen in Figure 1e (taken at an original magnification of 20000x), the FLP image has clear contours, and it is possible to discriminate some details of its surface structure. Therefore, we suppose that the observed decrease in the thickness of FLP part lying on the substratum is a true one.

P.B. Bell, Jr.: Please explain what a polymeric plate is and how it was used to produce the PSC-3 cell line.

Author: In studies of foreign-body carcinogenesis (Moizhess and Vasiliev, 1989), polyvinyl chloride plates were used to induce the development of tumors in mice of CBA-strain. Eight to fifteen months after subcutaneous implantation of the plates, sarcomas developed, and the cells derived from the sarcoma were cultivated. This culture was established as the PSC-3 cell line.

P.B. Bell, Jr.: "Can FLP have a natural origin?" The use of "natural" is unclear. The question should be, "Can FLP form from pre-existing structures, such as microvilli, or do they form *de novo* from the cell surface." The author concludes that the latter is exclusively the case. However, the photographic evidence is equivocal. I was struck by the presence of long microvilli-like structures on many of the cells that are shown that were not in contact with the substratum. No data are presented to rule out some of the FLP having been formed by the attachment of some of these microvilli to the substratum, with secondary stretching caused when the cells shrink during dehydration and drying. In fact, it seems to me that this is a likely occurrence, given the greater ability of punctate structures to overcome electrostatic repulsion and adhere with the substratum. Of course, this may not be an either-or situation as the author concludes. It could be that in some cases the FLP are from pre-existing structures and, in other cases, *de novo*. The author, in contrast, proposes that the long microvilli are formed when the FLP are torn loose from the substratum. The data presented do not allow one to choose among the possibilities. Please comment.

Author: Discussing the possible "natural origin" of FLP, I meant the natural cell surface structures, e.g., microvilli, which could be taken for FLP. However, FLP differ clearly from microvilli of the same cell. Though FLP are not microvilli, I do not exclude, in any way, the idea that FLP could be formed from microvilli attached to the substratum and subsequently stretched as a result of cell shrinkage during dehydration and drying. At the same time, according to my observations, FLP can be found in cells in which microvilli are totally absent, namely, in cells with blebbed (Fig. 6) or folded

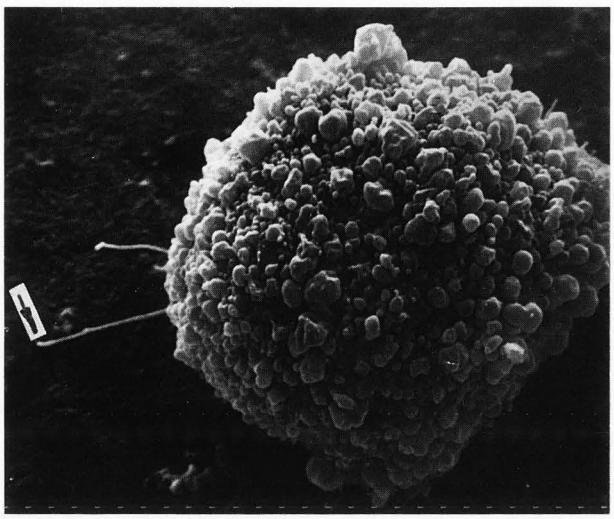

Figure 6. MEF pre-fixed in suspension, attached to aluminum foil plate and critical point dried. Blebbed cell surface relief. FLP (arrow). Horizontal field width = 33 μm.

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surface reliefs. In these cases, FLP could apparently have formed *de novo* at some discrete points of the cell surface attached to the substratum.

With respect to "long microvilli": of course, in a microvillous cell surface relief, both short and long microvilli can be present. However, to my mind, one or several long microvilli can be clearly distinguished against the background of blebbed or folded cell surface relief, and points rather to the artefactual origin of these structures (MLP, microvilli-like protrusions) formed presumably as a result of FLP tearing from the substratum.

At the same time, the formation of FLP (and also MLP), besides the mechanism of "attaching-stretching," can be a result of the autonomous capacity of the domains in the cell plasmatic membrane to form filopodia-like and/or lamellae-like outgrowths. Though the assumption that this capacity is preserved in a pre-fixed cell seems to be hardly probable, it has been previously shown (Rovensky and Vasiliev, 1984) that air-drying could induce formation of not only FLP, but also circular lamellae at the bases of some cells pre-fixed in suspension. The nature of the influences, associated with the drying process, which could induce the manifestation of the autonomous capacity of the cell membrane to form the protrusions, remains unknown.

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