DIRECT VISUALIZATION OF THE 10-nm (100-Å)-FILAMENT NETWORK IN WHOLE AND ENUCLEATED CULTURED CELLS

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SUMMARY

Following extraction of actomyosin and tubulin from cultured cells treated with Triton X-100, a cytoskeleton remains which is composed predominantly of the cell nucleus encompassed by a network of 10-nm filaments. After negative staining the dense perinuclear region appears as a densely woven filament net punctuated by patches of high electron density. Enucleation of 3T3 cells with cytochalasin B gives rise to karyoplasts surrounded by 10-nm filaments and cytoplasts in which 10-nm filaments remain situated in the central region of the cytoplasm. While the 10-nm filaments occurred mainly as single filaments in human skin fibroblasts and 3T3 cells, in epithelioid PtK1 and PtK2 cells they were commonly associated in prominent meandering bundles. In addition, in these latter cells after Triton extraction the remaining ribosomes were bound specifically to the 10-nm-filament net.

After exposure of 3T3 cells to cytochalasin B the 10-nm filaments formed branches that radiated from the perinuclear region into the immobile cell extensions. Concavalin A had no marked effect on the distribution of the 10-nm-filament net.

The results suggest that the 10-nm filaments act primarily as structural elements, serving, in particular, to support and constrain the nucleus in its position in the cell.

INTRODUCTION

The finding of 'intermediate' or '10-nm filaments' in a variety of different vertebrate cells (see e.g. Fawcett, 1966; Uehara, Campbell & Burnstock, 1971; Goldman & Knipe, 1973; Ishikawa, 1974; Small & Sobieszek, 1977; Starger & Goldman, 1977) suggests that this filament type may be as ubiquitous as microtubules and actin microfilaments. Morphologically and biochemically the cytoplasmic 10-nm filaments resemble vertebrate neurofilaments. Both filament types show 4 subunits in cross-section (Wuerker, 1970; Small & Squire, 1972), are immunologically cross-reactive (Blose, Shelanski & Chacko, 1977) and are composed of a protein of similar amino acid composition (Small & Sobieszek, 1977) and polypeptide molecular weight (Cooke, 1976; Small & Sobieszek, 1977; Yen, Dahl, Schachner & Shelanski, 1976).

The filament protein from smooth muscle shows a major polypeptide of about 55000 Daltons and a minor component at around 50000 Daltons which appears to derive, at least in part from a degradation of the larger species (Small & Sobieszek, 1977). More recently 10-nm filaments isolated from cultured cells have been shown to exhibit 2 polypeptides of similar molecular weight but in more equal proportions.
Thus far the 10-nm filament protein has been assigned 2 names: skeletin (Small & Sobieszek, 1977) and desmin (Lazarides & Hubbard, 1976). The first has derived from studies on smooth muscle and certain other cell types (Brecher, 1975; Cooke, 1976; Eriksson, Thornell & Stigbrand, 1976; Small & Sobieszek, 1977) which have indicated that the 10-nm filaments probably play a cytoskeletal role, while the second was proposed from observations which suggested a localization of the 55000 mol.wt. polypeptide in the Z-disks of certain skeletal and cardiac muscle myofibrils (Lazarides & Hubbard, 1976).

With the aim of gaining further insight into the cellular functions performed by the 10-nm filaments we have been concerned with developing an effective procedure for revealing, at the level of the electron microscope, the presence and distribution of these filaments in different cultured cell types under normal and imposed conditions. The first stage of this work has been to extend earlier studies of whole Triton-extracted and negatively stained cultured cells (Small & Celis, 1977) to cells from which the microtubules and actomyosin have been extracted. After such extraction procedures, developed initially from studies on smooth muscle (Sobieszek & Small, 1976; Small & Sobieszek, 1977), the 10-nm-filament network was found to be readily revealed.

In this report we show that in the extracted cytoskeleton of all the cultured cells studied the 10-nm filaments form, under normal conditions, a compact network centred around the cell nucleus. From these observations and further experiments on enucleated cells it is concluded that the 10-nm filaments function generally as supportive elements which, in particular, act to constrain the cell nucleus in its position in the cell. The effects of cytochalasin B and concanavalin A on the distribution of the 10-nm-filament network and their significance in relation to other possible roles that these filaments may play are also described and discussed.

MATERIALS AND METHODS

Cell cultures

The different cultured cells were grown in Eagle's minimal essential medium (MEM) supplemented with 10% foetal calf serum and antibiotics (penicillin 100 I.U./ml; streptomycin 50 μg/ml). The cultured cells included: human skin fibroblasts; mouse 3T3B cells and epithelioid PtK1 and PtK2 cells (American type culture collection). As described previously (Small & Celis, 1977) cells were grown in culture dishes containing coverslips (8 mm x 4 mm) carrying silver electron-microscope grids coated with a plastic-carbon support film. Prior to addition to the dishes the grids and coverslips were sterilized by ultraviolet radiation. Sufficient overgrowth on the grids occurred between 1 and 3 days after plating.

Extraction of actomyosin and tubulin

After appropriate times in culture the coverslips were removed, rinsed in phosphate-buffered saline (PBS) and transferred to 0.1% Triton X-100 in a Ca-free Hanks' solution containing 2 mM MgCl₂, 2 mM EDTA, 5 mM PIPES and with the pH adjusted to 6.5 ("PIPES buffer" see Small & Celis, 1977). Treatment with Triton X-100 was for 45-60 s. After washing twice in the same buffer without Triton the coverslips were dipped for 45-60 s in each of the following ice-cold extraction solutions (see also Small & Sobieszek, 1977): (1) 60 mM KCl, 1 mM EDTA, 2 mM EGTA, 1 mM cysteine, 10 mM ATP, 40 mM imidazole, pH 7.1; (2) 0.6 M
10 nm filament network in cultured cells

KCl, 1 mM EDTA, 2 mM ATP, 1 mM cysteine, 40 mM imidazole, pH 7.1; (3) as for step (1). The coverslips were then rinsed in the PIPES buffer and transferred to 2.5% glutaraldehyde in the same buffer for 10–30 min, prior to processing for electron microscopy.

Electron microscopy

Cells were processed for electron microscopy either after the above extraction procedures or just following the initial treatment with Triton X-100. Following the fixation step in each case the grids were removed individually from the coverslips and negatively stained in the cold as described elsewhere (Small & Celis, 1977). Electron microscopy was carried out in a Siemens Elmiskop 101 or Zeiss 10A operating at 80 kV.

Karyoplasts and cytoplasts from 3T3 cells

Enucleation of 3T3 cells was carried out using 10 μg/ml of cytochalasin B according to the method of Prescott, Meyerson & Wallace, 1972. Karyoplasts were collected from the pellet fraction following the centrifugation of inverted cell cultures grown on circular plastic disks. The cytoplasts were removed from the disks by trypsinization and both the karyoplast and cytoplast fractions were replated on to electron-microscope support grids. After overnight incubation in MEM the grids were processed for electron microscopy as above, either following Triton extraction alone or with the additional extraction procedures.

Treatment with Con A and cytochalasin B

Cells attached to microscope grids and grown in MEM were incubated for various times in the presence of 100 μg/ml of Con A (Sigma) or 10 μg/ml of cytochalasin B (Aldrich Chemicals, Belgium) in the same media. At the end of the incubation the grids were processed for electron microscopy as described above.

RESULTS

Cytoarchitectural elements in cultured skin fibroblasts and 3T3 cells permeated with Triton X-100

The general appearance of skin fibroblasts and 3T3B cells extracted only with Triton and then negatively stained has been described in a previous report (Small & Celis, 1977). Under the conditions employed all 3 filamentous components, the microtubules, actin filaments and 10-nm filaments may be recognized, these being most clearly resolved in the thinner regions of the cytoplasm (see Small & Celis, 1977). In this report we wish to draw attention to the perinuclear region of these cells. After Triton extraction the nucleus is characteristically overlayed and surrounded by a densely stained, patchy region which extends to different extents towards the cell periphery (Figs. 1, 2). In general, this region occupied a larger proportion of the cytoplasm in skin fibroblasts than in 3T3B cells. In favourable instances, the staining in this dense region was sufficient to reveal filamentous elements closer to the cell surface (Fig. 3) and in such cases showed the presence of abundant 10-nm filaments. The 10-nm filaments generally occurred randomly arranged but sometimes were also found in closely parallel arrays, as shown in Fig. 3.

In the more peripheral, thinner regions of the cytoplasm of 3T3B cells the 10-nm filaments were generally not observed after Triton treatment alone. These areas were occupied only by actin filament bundles, single actin filaments and microtubules (see Small & Celis, 1977). In skin fibroblasts the 10-nm filaments extended further towards
Figs. 1, 2. 3T3 cell and skin fibroblast, respectively, after Triton treatment alone. A densely staining patchy region overlays and encompasses the cell nucleus. This region forms 3 lobes in the skin fibroblast of Fig. 2. Fig. 1, × 1050; Fig. 2, × 900.

Fig. 3. Higher magnification of the perinuclear region of a Triton-extracted skin fibroblast. The dense diagonal lines are stress fibres coursing below the 10-nm-filament net. × 53000.
Fig. 4, 5. Human skin fibroblasts after extraction at high and low ionic strength. The dense perinuclear region persists and is sometimes displaced towards one side of the nucleus. Fig. 4, ×1000; Fig. 5, ×900.

Fig. 6. Higher magnification of peripheral part of perinuclear region of cell in Fig. 4, showing the high density of 10-nm filaments. ×53000.
the cell periphery and sometimes they were seen intermingled between actin filaments and microtubules.

The 10-nm-filament network

After further treatment of the cells with buffers of high and low salt concentration (see Materials and methods) the densely stained region around the nucleus, as well as the nucleus itself, was seen to be retained (Figs. 4, 5, 7). With respect to the nucleus this region occurred either symmetrically distributed or shifted to one side (Fig. 5). At the same time the general outline of the cell was still recognizable. Closer examination at higher magnification showed the perinuclear region to be occupied by patches of high stain intensity superimposed on, or interconnected by a tightly packed network of 10-nm filaments (Figs. 6, 8). This network became rapidly more dispersed in regions away from the nucleus and, in general, 10-nm filaments were very sparsely distributed towards the cell perimeter.

In addition to the 10-nm filaments, remnants of the actin filament network were also seen after the present extraction procedure. This included part of the bundle of thin filaments observed at the edges of stationary regions of the cells and smaller actin filament bundles within the cytoplasm, but not the larger stress fibres. Microtubules could not be observed in cells extracted under these conditions.

PtK1 and PtK2 cells

After Triton-extraction PtK2 cells appear in several respects rather different from 3T3 cells and skin fibroblasts. These cells vary from circular to very convoluted in shape and do not, like the latter cells, exhibit ruffling edges or microspikes in the peripheral actin meshwork (see e.g. Small & Celis, 1977). The most prominent feature of these cells is a coarse and extensive interwoven filament net which covers much of the cell area (Fig. 9). After Triton extraction alone the remaining ribosomes in the cells are seen to be mainly confined to the regions occupied by this net and appear to be bound to it (Fig. 10).

The extraction of PtK2 cells with solutions of low and high ionic strength leaves the coarse filament net essentially unchanged in its 2-dimensional appearance (Fig. 11) and allows a clear visualization of the filamentous components. As shown in Fig. 12 this net, like those already described above, is composed of the 10-nm filaments but with these filaments occurring both singly and aggregated into coarse bundles, ranging up to about 0.2 μm in diameter. While these bundles did not, in general, extend to the cell periphery they were often seen to radiate in groups to the apices of the cell.

Less-extensive studies of PtK1 cells showed these to possess the same coarse 10-nm-filament net as described for the cell line PtK2.

3T3B cytoplasts and karyoplasts

Following enucleation of 3T3B cells with cytochalasin B, cytoplasts were obtained which, after spreading, exhibited the general morphology shown in Fig. 13. The nucleus was absent and a densely staining region was observed in the central part of
Fig. 7. 3T3 cell extracted at high and low ionic strength. × 1400.

Fig. 8. Area midway between nucleus and cell periphery of a 3T3 cell extracted as for Fig. 7. Numerous 10-nm filaments course between patches of high electron density. × 44000.
Fig. 9. Epithelioid PtK2 cell extracted only with Triton and showing the predominant and coarse filamentous net characteristic of both this cell line and PtK1 cells. × 40000.

Fig. 10. Peripheral region of filament net of a Triton-extracted PtK2 cell. Small, densely staining particles are poly-ribosomes (see inset); these are mainly associated with the 10-nm-filament net. × 7000; inset, × 65000.
Figs. 11, 12. PtK2 cells after extraction at high and low ionic strength. The coarse filament net is seen to be composed of bundles of 10-nm filaments. Single filaments interconnect and lie between the bundles. Fig. 11, x 1800; Fig. 12, x 64 000.
Fig. 13, 14. 3T3 cytoplasts after extraction with Triton alone. Fig. 14 shows a central, densely staining area at higher magnification. Many 10-nm filaments may be recognized together with a few microtubules. Fig. 13, ×1300; Fig. 14, ×63000.
Figs. 15, 16. 3T3 karyoplast after extraction at high and low ionic strength. Higher magnification of one of the branches in Fig. 15 shows the presence of numerous 10-nm filaments. Fig. 15, ×4400; Fig. 16, ×52000.
the cytoplast similar to that seen in the 'whole' cells. Since the cytoplasts were rather planar structures it was normally possible to resolve the filaments within the densely staining region after only Triton extraction. Such an area is shown at higher magnification in Fig. 14. Numerous 10-nm filaments may be recognized intermingled between the cytoplasmic microtubules. The presence of 10-nm filaments in this region confirms earlier observations in the light microscope of a corresponding area of birefringence in cytoplasts of BHK-21 cells (Goldman, Pollack & Hopkins, 1973). Actin filaments, although present in the central areas of the cytoplasts, were not prominent. In the more peripheral parts of the cytoplasts only actin filaments and microtubules were observed. In further experiments it was found that the distribution of 10-nm filaments in cytoplasts extracted with buffers of low and high salt concentration was very similar to that of cytoplasts extracted only with Triton (results not shown). Thus the removal of actomyosin and tubulin did not cause a redistribution of the 10-nm filaments.

Karyoplasts collected during enucleation which were extracted only with Triton and then negatively stained were too electron-dense, even at their edges, to allow an analysis of the presence of any filamentous components. However, following the low and high salt extraction procedure the periphery of the karyoplasts was seen to contain large numbers of 10-nm filaments (Figs. 15, 16). Thus, while numerous 10-nm filaments were retained in the cytoplast, a considerable number also remained bound to the cell nucleus during enucleation.

The effect of concanavalin A and cytochalasin B on the 10-nm-filament network in 3T3 cells

Since intracellular cytoskeletal components have been implicated as likely effectors of surface receptor mobility (see Discussion) it was of interest to test the effect of agents known to cause changes in receptor distribution on the organization of the 10-nm-filament network. While we have not so far made extensive investigations with different agents and conditions, we will report here the results obtained with 3T3 cells with concanavalin A and cytochalasin B.

ConA treatment for 30 min at 100 μg/ml did not produce any marked changes in the distribution of the 10-nm-filament net. In some preparations the patches with interconnecting filaments appeared to be dispersed further towards the cell edges than in controls, but this effect was not observed consistently (see Fig. 17).

With cytochalasin B in doses (10 μg/ml for 15 min) sufficient to produce the cytoplasmic spike-like extensions characteristically seen with this drug a redistribution of the 10-nm filaments was observed. After this treatment the 10-nm filaments formed branches radiating from the perinuclear area into the extensions (Fig. 18).

![Fig. 17. 3T3 cell extracted at low and high ionic strength after treatment for 30 min with 100 μg/ml Con A. In some cases the patches and 10-nm-filament net were more dispersed towards the cell periphery as compared to controls. × 1200.](image1)

![Fig. 18. Effect of treatment of 3T3 cells with cytochalasin B (10 μg/ml for 15 min). 10-nm filaments extend, in parallel arrays into the spike-like extensions formed in reaction to this drug. × 80 000; inset, × 800.](image2)
10 nm filament network in cultured cells
As noted previously (Goldman & Knipe, 1973), these branches also contained microtubules and bundles of actin microfilaments, visualized in preparations treated only with Triton.

**DISCUSSION**

In vascular endothelial cells the 10-nm filaments form a perinuclear ring which can be demonstrated readily by polarization microscopy (Blose & Chacko, 1976), while in various other cells a perinuclear accumulation of 10-nm filaments occurs in response to treatment with microtubule inhibitors (see Starger & Goldman, 1977). And recent studies with naturally occurring antibodies, apparently directed towards the protein of the 10-nm filaments, have indicated a perinuclear concentration of 10-nm filaments in neuroblastoma cells, skin fibroblasts and PtK2 cells (Kurki, Linder, Virtonen & Stenman, 1977; Osborn, Franke & Weber, 1977). In the present studies we have been able to confirm and extend the observations made by polarization and immunofluorescence microscopy. The present method allows the direct demonstration of the localization of 10-nm filaments in cultured cells, thus obviating such problems as antibody specificity, and furthermore permits localization to the level of individual filaments. We have demonstrated that under normal conditions there is a perinuclear concentration of 10-nm filaments in all the cell lines studied. Our unpublished observations on HeLa cells and mouse L cells (Cl1D) show that the same distribution of 10-nm filaments exists also in these cell lines, suggesting that such a distribution may be a general feature, at least of cultured cells.

Since the general outline of the cells, as well as the nucleus, was retained even after extraction of the microtubules and the bulk of the actin component, we conclude that the 10-nm filaments must constitute an important component of the cell cytoskeleton. A cytoskeletal role for these filaments has been suggested previously from studies on smooth muscle (Cooke, 1976; Small & Sobieszek, 1977), purkinje fibres (Eriksson et al. 1976) and PtK1 cells (Brecher, 1975). Furthermore, the nuclear localization of the 10-nm filaments, the retention of the nucleus in the 10-nm-filament cytoskeleton and the presence of 10-nm filaments in 3T3 karyoplasts strongly indicate a tight association of these filaments with the cell nucleus. From these observations we are led to suggest that one role of the 10-nm filaments is to support and constrain the cell nucleus in the cell. In studies on isolated smooth muscle cells in which collagenase was used to degrade the 10-nm-filament network (see Small & Sobieszek, 1977) the cell nucleus was found to be readily lost, suggesting again a dependence of the nucleus on the 10-nm filaments for structural support.

The abundance of 10-nm filaments in epithelioid PtK1 and PtK2 cells is particularly striking. In these cells the common aggregation of the 10-nm filaments into bundles is consistent both with the coarse network of filaments seen by the immunofluorescent method (Osborn et al. 1977) and the observation of tonofilament bundles in ultra-thin sections (Brecher, 1975; Osborn et al. 1977). The extent of the coarse filament net in these epithelioid cells lends support to the suggestion by Brecher (1975) that the 10-nm filaments may be responsible for maintaining them in a
flattened form during mitosis. We found no evidence, however, for local foci of 10-nm filaments that could be designated as possible 'synthesis or organizing centres' (Brecher, 1975). Another marked feature of PtK1 and PtK2 cells was the association of ribosomes with the 10-nm-filament net. In this respect it is interesting to note that Osborn et al. (1977) remarked on a common close association of polyribosomes with the 10-nm filaments in ultrathin sections of fixed and embedded material. Further work will, however, be necessary to show whether or not such an association results from the preparative techniques or is of some cell-physiological relevance.

While one role of the 10-nm filaments would appear to be structural, their intracellular spatial organization is, under certain conditions, rather labile. As already indicated, it has been shown that drugs which disrupt microtubules cause a perinuclear accumulation of 10-nm filaments (Goldman & Knipe, 1973; Holltrop, Raisz & Simmons, 1974; Croop & Holtzer, 1975) which can lead to the formation of 'juxta-nuclear caps' (Bischoff & Holtzer, 1968; Goldman & Knipe, 1973; Blose & Chacko, 1976; Starger & Goldman, 1977). A similar accumulation occurs in resting leukaemic cells under normal conditions (Felix & Sträuli, 1976). This phenomenon of accumulation of the 10-nm filaments in a cap associated with the nucleus is interesting with regard to its similarity to the capping of surface membrane receptors on different cell types which occurs in response to antimitotic agents (see e.g. de Petris, 1975; Nicolson, 1976; Edelman, 1976; Loo, 1976). Although a direct correlation has yet to be demonstrated it is noteworthy that the patches on the 10-nm-filament network are comparable in size to the patches formed by the surface receptors (Smith & Revel, 1972; Rosenblith et al. 1973) and that the general distribution of these patches is not dissimilar to the distribution of Con A receptors on cultured fibroblasts (Vasiliev et al. 1971). We did not in the present investigations note a significant redistribution of the 10-nm filaments in 3T3 cells in response to concanavalin A. However, further correlated light- and electron-microscope studies will be necessary to determine whether or not the 10-nm filament net, where it occurs, may be involved in some way together, for example with actin microfilaments, in the processes underlying membrane fluidity.

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REFERENCES


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