POSSIBLE ROLE OF CENTRIOLES IN STABILIZATION OF CYTOPLASMIC MICROTUBULES

V. I. Gel'fand, O. Yu. Ivanova, L. A. Mittel'man, and O. Yu. Pletyushkina

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Microtubules are one of the principal structural components of the cytoskeleton and they participate in oriented movements of intracellular material and maintenance of the asymmetrical shape of cells. A characteristic feature of microtubules is their polarity, demonstrable on account of the ability of exogenous tubulin to build on to the two ends of pre-existing microtubules [6]. With respect to affinity for tubulin it is customary to distinguish the plus end, at which polymerization takes place more easily (the critical concentration for polymerization is lower), and the minus end, where the affinity for tubulin is is lower (the critical concentration for polymerization is higher).

Microtubules in the cell are rarely in the free state. As a rule, they are attached by one end to special structures, known as organization centers of microtubules. For the cytoplasmic microtubules of animal cells such centers are centrosomes — centrioles with the material surrounding them. Cytoplasmic microtubules are connected with centrosomes by their minus ends [7]. Repair of cytoplasmic microtubules after their destruction takes place from the centrosomes [13]. They remain connected with the centrosomes as a rule even when growth is complete [8].

It can thus be tentatively suggested that centrosomes not only bring about controlled polymerization of tubulin, but also regulate the stability of the microtubules when their total assembly is complete. One possible approach to the verification of this hypothesis is by obtaining fragments of cells not containing a centriolar apparatus and studying the fate of cytoplasmic microtubules in these fragments.

In the present investigation fragments free from centrioles were obtained from mouse embryonic fibroblasts, and it was shown that microtubules in such fragments undergo slow depolymerization. This may be evidence that a constant connection with centrosomes is essential for maintenance of the stability of cytoplasmic microtubules.

EXPERIMENTAL METHOD

Experiments were carried out on secondary cultures of mouse embryonic fibroblasts. The cells were grown on coverslips in medium of the following composition: 0.5% lactalbumin hydrolysate (45%), Eagle's medium (45%), bovine serum (10%). The medium was changed 24 h after transplantation with a density of $5 \cdot 10^4$ cells/ml for medium containing cytochalasin B (from Serva, West Germany) in a concentration of 5 μg/ml. After 18-20 h the culture contained a large number of arborized cells, connected with the substrate only by thin processes. Fragments of these cells were obtained in two ways. To obtain fragments as described by Albrecht-Buehler [3] the culture was shaken three to five times and, as a result, the rounded central part of the cell was mechanically removed, and fragments of the processes, by which the cell was connected to the substrate, remained attached. The second method consisted of obtaining cell fragments by a microsurgical method, for which purpose glass microcapillary tubes with a tip about 0.5 μm in diameter, held in the holder of a micromanipulator, were used. Large processes connected to cells only by an isthmus were separated from the cells.

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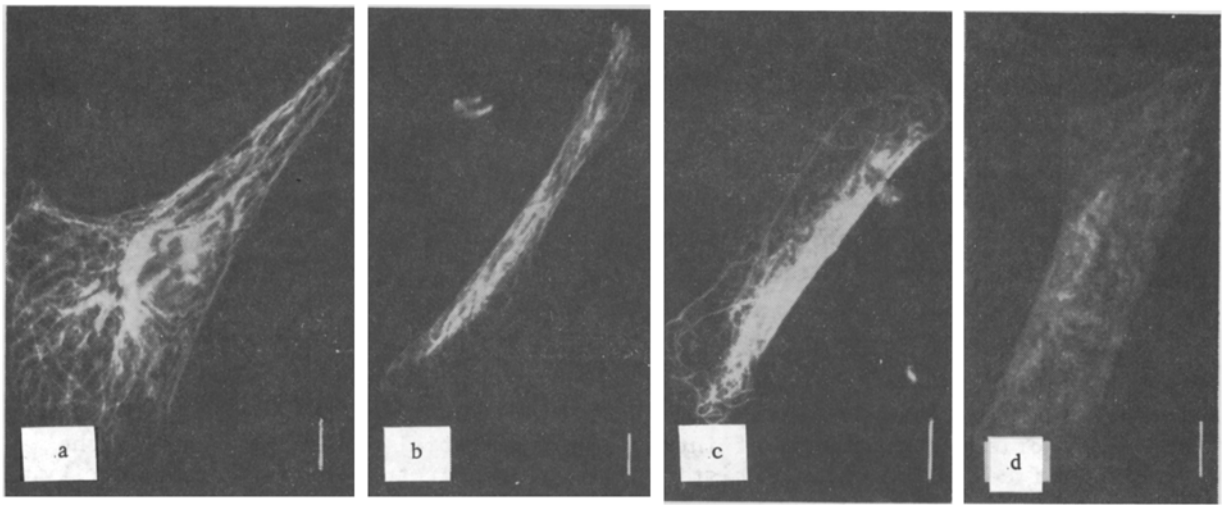

Fig. 1

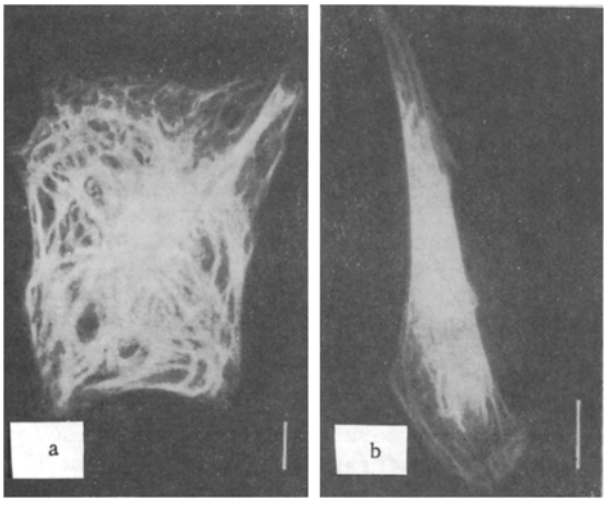

Fig. 2

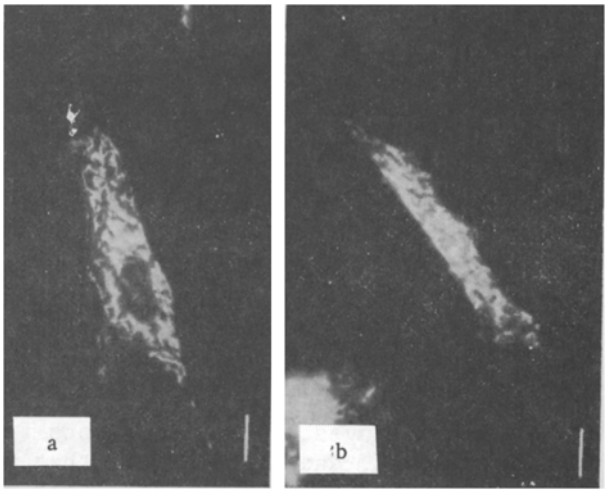

Fig. 3

Fig. 1. Distribution of microtubules: a) embryonic mouse fibroblast; b) fragments of embryonic mouse fibroblast 1.5 h after separation from cell; c) the same, 3 h; d) the same, 6 h after separation. Scale: 20 μm.

Fig. 2. Distribution of intermediate microfilaments: a) embryonic mouse fibroblast; b) fragment of embryonic mouse fibroblast 6 h after separation from cell. Here and in Fig. 3, scale 20 μm.

Fig. 3. Staining of mitochondria with rhodamine 123: a) embryonic mouse fibroblast; b) fragment of embryonic mouse fibroblast 6 h after separation from cell.

The procedure of immunofluorescence demonstration of microtubules and the monospecific antibodies against tubulin used for this purpose were described previously [4]. To reveal intermediate filaments by the indirect immunofluorescence method monoclonal antibodies against intermediate filaments generously supplied by E. B. Mechetner (Laboratory of Immunology of Tumors, All-Union Oncologic Scientific Center, Academy of Medical Sciences of the USSR) were used.

Viability of the cells was assessed by staining with rhodamine 123, as described in [9].

EXPERIMENTAL RESULTS

Cell fragments obtained by shaking the culture treated with cytochalasin, when transferred to medium not containing cytochalasin, began to form a monolayer. The spreading

process was largely complete after 1 h, and subsequently it continued more slowly; monolayer formation was complete after 3 h. Starting from 3 h the morphology of the fragments remained comparatively unchanged for more than 7 h. In some experiments fragments were separated microsurgically from the cell body under control of the microscope. After the operation, areas of cytoplasm of the cell and the processes adjacent to the line of section were pressed, but the region of section itself was evidently covered by a plasma membrane, for under low power of the microscope no sign of outflow of the contents of the cell and process could be observed. On transfer to medium without cytochalasin, these fragments began to spread out in a layer and in their behavior they were indistinguishable from ordinary fragments.

Immunofluorescence staining with antibodies against tubulin revealed a dense network of cytoplasmic microtubules in the cells, filling the whole cytoplasm (Fig. 1a). In the early stages of separation of fragments from the cells, detection of microtubules in them by the immunofluorescence method did not seem possible, because the fragments were very poorly spread out on the substrate. In areas not yet spread out the detection of fine structures such as microtubules was difficult because of the presence of a diffusion background in the cytoplasm. Meanwhile, 1.5 h after the operation, in medium without cytochalasin the cells spread out reasonably completely, and it was easy to see many microtubules in the fragments (Fig. 1b). By this time there were practically no fragments not containing microtubules in the culture. The fragments still remained spread out 3 h after separation, but the number of microtubules in them was sharply reduced (Fig. 1c). Fragments containing only single microtubules began to appear at this time, but a few fragments contained no microtubules whatever. Immunofluorescence staining by means of antibodies against tubulin 6 h after the operation did not reveal any microtubules in the cell fragments (Fig. 1d). Only occasionally could fragments be seen in which single microtubules were still present. Meanwhile, microtubules were preserved in the cells throughout the period of observation.

A similar decrease in the number of microtubules also was observed in fragments obtained microsurgically. Incidentally, in the cells from which the test fragment was obtained microsurgically, the normal distribution and density of the microtubules were still preserved 6 h after the operation.

To study the specificity of the destruction of microtubules in the fragments, and whether it affects other components of the cytoskeleton, the distribution of intermediate filaments — a component of the cytoskeleton closely connected with microtubules — in the fragments was studied. Intermediate filaments in the fragments were found to be preserved throughout the period of the experiment (Fig. 2b). The only difference in the distribution of intermediate filaments in the fragments and whole cells was that intermediate fragments in the cell were found in virtually all areas of the cytoplasm (Fig. 2a), whereas in the fragments, especially in the late stages after separation from the cells, they were concentrated chiefly in the central part.

Since destruction of the microtubules in the fragments was complete only after 6 h, it might be supposed that they are simply one sign of commencing death of the fragments. To test this hypothesis it was decided to study whether the membrane potential of the mitochondria is preserved in the fragments. The absence of a membrane potential indicates a disturbance of the energy metabolism of the cells and is one of the early signs of disturbance of their viability [5]. The presence of a mitochondrial membrane potential was studied on the basis of their staining by penetrating fluorescent cation rhodamine 123. In intact cells rhodamine 123 stains numerous mitochondria, which are threadlike in shape and of varied length (Fig. 2c). In all the fragments tested, throughout the period of observation, rhodamine 123 also stained many mitochondria (Fig. 2d). The fragments studied thus remained completely viable throughout the period of measurement (Fig. 3).

The results of this investigation thus indicate that microtubules in cell fragments are unstable and are destroyed during incubation for 6 h. Such destruction is specific, for destruction of microtubules was not accompanied by the destruction of another component of the cytoskeleton, namely intermediate filaments, and was not associated with any decrease in viability of the fragments.

Disassembly of microtubules in cell fragments is connected with the absence of centrosomes in them. It is well known that centrioles in interphase cells are located near the cell nucleus and are connected with it [11, 12]. Since the cell fragments studied arise

from the peripheral part of the cytoplasm, they evidently do not contain centrioles. Microtubules in fragments are therefore not connected with centrioles and, in our opinion, this leads to their destruction. The low stability of free (unconnected with centrioles) microtubules can be explained in several different ways. In particular, as was pointed out in [10], if the concentration of free tubulin in the cell falls below the critical concentration for polymerization for the minus end, but is higher than that for the plus end, the free microtubules in the cell will not be stable and will depolymerize, whereas microtubules which are connected with centrioles by their minus ends remain assembled. This is explained on the grounds that disassembly at the minus end will not take place because it is blocked by the connection with the centriole, and the plus end will be stable, for the tubulin concentration in the cytoplasm exceeds the critical concentration for this end. This was evidently the situation in the present case, and separation of the microtubules from the centriole therefore led to their depolymerization.

Such an explanation is not of course the only one possible. For example, it may be that microtubules in fibroblasts are only polymerized on centrosomes, and after the end of assembly they separate from them [2]. In that case, our results could mean that a time of about 6 h is the upper limit for existence of cytoplasmic microtubules after completion of assembly.

In principle, the possibility cannot be ruled out that microtubules in cell fragments are destroyed for reasons totally unconnected with centrioles. However, such a suggestion seems improbable because destruction of microtubules in cell fragments is specific in character, and other components of the cytoskeleton, namely intermediate filaments and bundles of microfilaments [1], are preserved.

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