Cytochalasin Separates Microtubule Disassembly from Loss of Asymmetric Morphology

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ABSTRACT When neuroblastoma cells bearing neurites are incubated with colchicine or Nocodazole, the cytoplasmic microtubules are depolymerized and concomitantly the neurites retract. We report here that cytochalasin separates the two effects of these drugs: it quantitatively inhibits neurite retraction but does not inhibit microtubule assembly. The neurites that remain contain intermediate filaments and actin but are devoid of microtubules. Depletion of cellular ATP also blocks neurite retraction induced by colchicine or Nocodazole, but some assembled microtubules persist under these conditions. The results suggest that neurite retraction is an active cell process.

The role of microtubules in the organization of the cytoplasm has been studied extensively, in part using manipulations of cultured cells. In classic experiments, cells growing with an essentially round morphology are induced to spread across the substratum and take on a morphology characteristic of the tissue from which they are derived. Exposing these asymmetric cells to drugs that depolymerize their microtubules causes them to revert to a round morphology again. These phenomena have been demonstrated with several cell types (7, 14, 17), but they can be visualized with particular clarity in neuroblastoma cells. In specific culture conditions, neuroblastoma cells extend axonlike neurites (12, 18, 19). The neurites retract rapidly in the presence of the microtubule-depolymerizing drug colchicine. Results of this sort support the notion that the assembly and maintenance of intact microtubules are necessary conditions for the elaboration of asymmetric cell shape (15).

In this report, we further dissect this experimental system. Previous experiments have established that cytochalasin inhibits cell motility; in particular, it blocks neurite extension but does not cause neurite retraction (24). Here, we demonstrate that cytochalasin inhibits the morphological effects of microtubule-depolymerizing drugs. The neurites of neuroblastoma cells incubated with cytochalasin and either colchicine or Nocodazole do not retract, even though the cells are devoid of microtubules. We conclude that depolymerization of microtubules is not sufficient to cause loss of cell shape, and that some other cellular process, inhibited by low concentrations of cytochalasin, is also involved.

MATERIALS AND METHODS

Cell Culture

Mouse neuroblastoma cells were cultured as previously described (21), except at 10^5 cells/35-mm dish. After 8 h, the cells were washed three times with medium containing 0.1% fetal calf serum, and then incubated in that medium for 14–18 h.

Drugs

Cytochalasin B, cytochalasin D, and Nocodazole were added to cells as 100-fold dilutions of stock solutions in dimethyl sulfoxide (DMSO). 2% DMSO had no effect on cell morphology. Colchcine was added to cells as a 100-fold dilution of a stock solution in phosphate-buffered saline with divalent cations. All drugs were purchased from Aldrich Chemical Co., Inc., Milwaukee, Wisc.

Live-cell Microscopy

Cells were observed in live-cell chambers with a Zeiss Universal microscope. The chambers were modified as described previously, to permit exchange of medium (22). Photographs were taken on Pan-X Kodak film, developed in Diafine.

Electron Microscopy

Cells on cover slips were fixed in 2% glutaraldehyde and 0.2% tannic acid (Mallinckrodt Inc., St. Louis, Mo.) in 0.1 M sodium cacodylate and 0.1 M sucrose for 30 min at room temperature (20). Cells were then postfixed with 1% osmium tetroxide in veronal acetate, dehydrated, and flat-embedded in Epon/Araldite (Ted Pella Co., Tustin, Calif.). The blocks were freed of the cover slips by hydrofluoric acid, and silver sections were cut *en face*. The first sections obtained were stained in uranyl acetate and lead acetate for 5 min (16) and examined in a Philips 201 electron microscope.

Detergent-extracted Cytoskeletons

Neuroblastoma cells were extracted with 0.1% Nonidet P-40 in 0.1 M PIPES, 10^{-3} M $MgSO_4$, 2×10^{-3} M EGTA, and 2 M glycerol, pH 6.9. We previously have shown that this buffer preserves most cytoplasmic microtubules and releases the pool of unassembled tubulin (23). For immunofluorescence, these cytoskeleton preparations were fixed in the same buffer without detergent but containing 3.7% formaldehyde (23). Antibodies against vimentin were a gift from R. Hynes (Massachusetts Institute of Technology, Cambridge, Mass.) and antibodies against actin were a gift from K. Burridge (Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.). For determination of assembled tubulin, cytoskeleton

preparations were made from metabolically labeled cells. The microtubules were then depolymerized by a subsequent extraction in the presence of calcium ions, and those extracts were analyzed as radioautographs of SDS polyacrylamide gel electrophoresis slab gels (23).

RESULTS

Experiments by Wessells and co-workers (24) established that 7–10 μg/ml of cytochalasin B blocked elongation of axons in dorsal root ganglia cultures. To determine the minimum concentration of cytochalasin effective on neuroblastoma cells, we incubated cultures with 0.1 μg/ml Nocodazole for 30 min to induce neurite retraction. When the drug was removed, the cells re-extended neurites within 60 min (22). Re-extension was quantitatively inhibited by 1 μg/ml cytochalasin D (CCD) or 2 μg/ml cytochalasin B (CCB). The inhibition was released by washing out the cytochalasin. These concentrations were comparable to those required to inhibit cell motility in other systems (1, 4).

The micrographs in Fig. 1 demonstrate that cytochalasin also inhibited Nocodazole-induced neurite retraction. We began with populations of neuroblastoma in which at least 80% of the cells had extended neurites (Fig. 1 *a*). After 45 min in 0.1 μg/ml Nocodazole and 1 μg/ml CCD, the same percentage of cells still displayed neurites (Fig. 1 *b*). The neurites survived at least 16 h in the presence of both drugs. By comparison, <6% of the cells incubated in Nocodazole alone retain neurites (Fig. 1 *c*). The neurites of cells incubated with both drugs were the original neurites of the cell; that fact was demonstrated by following single cells. The same number of processes, in precisely the same positions, persisted after 45 min in Nocodazole and CCD (compare Fig. 1 *a* and *b*, *insets*). The processes were slightly shorter, and some finer features were no longer visible by phase microscopy. Incubation with cytochalasin alone induced the same effects, both in the dorsal root ganglia system (24) and in neuroblastoma. When the same cell was then incubated with Nocodazole alone, the neurites retracted within 30 min (Fig. 1 *c*, *inset*), which is similar to the behavior of neurites never exposed to cytochalasin. In parallel experiments, the same results were obtained when the Nocodazole was replaced by 5×10^{-5} M colchicine and the CCD by 2 μg/ml CCB.

It is possible that cytochalasin blocked the secondary effect of Nocodazole and colchicine, neurite retraction, by interfering with their primary effect, depolymerization of microtubules. We have examined that possibility by three techniques and find no evidence that assembled microtubules survive the incubation with both drugs. First, microtubules were absent in electron micrographs of thin sections through the neurites of cells treated with both drugs (Fig. 2 *b*), although they were a conspicuous component of the neurite cytoplasm of control cells (Fig. 2 *a*). Similarly, immunofluorescent stain, with anti-tubulin of cells treated with both drugs, showed no tubulin in the neurites (Fig. 3 *c*). Both actin and vimentin were still visualized in these processes by specific antibodies (Fig. 3 *f* and *i*). They remained there even after a 16-h incubation, in contrast to the typical coiling of vimentin about the nucleus after prolonged treatment with microtubule-depolymerizing drugs (8). Finally, a direct biochemical measurement supported the conclusion that no microtubules are present in cells treated with both colchicine and CCD. Detergent-extracted cytoskeletons were prepared under conditions that release unassembled tubulin in the soluble fraction and preserve assembled microtubules in the cytoskeletons (13, 22, 23). No detectable assembled tubulin was present in such preparations from cells treated either with colchicine (Fig. 4, lane *b*), or with colchicine plus CCD (Fig. 4, lane *c*), although there is significant tubulin in the assembled state in untreated cells (Fig. 4, lane *a*).

The results suggest that some cellular process is required for neurite retraction, even in cells containing no intact microtubules. That interpretation is supported by the finding that incubating cells under conditions that blocked ATP production also inhibited the morphological effects of colchicine or No-

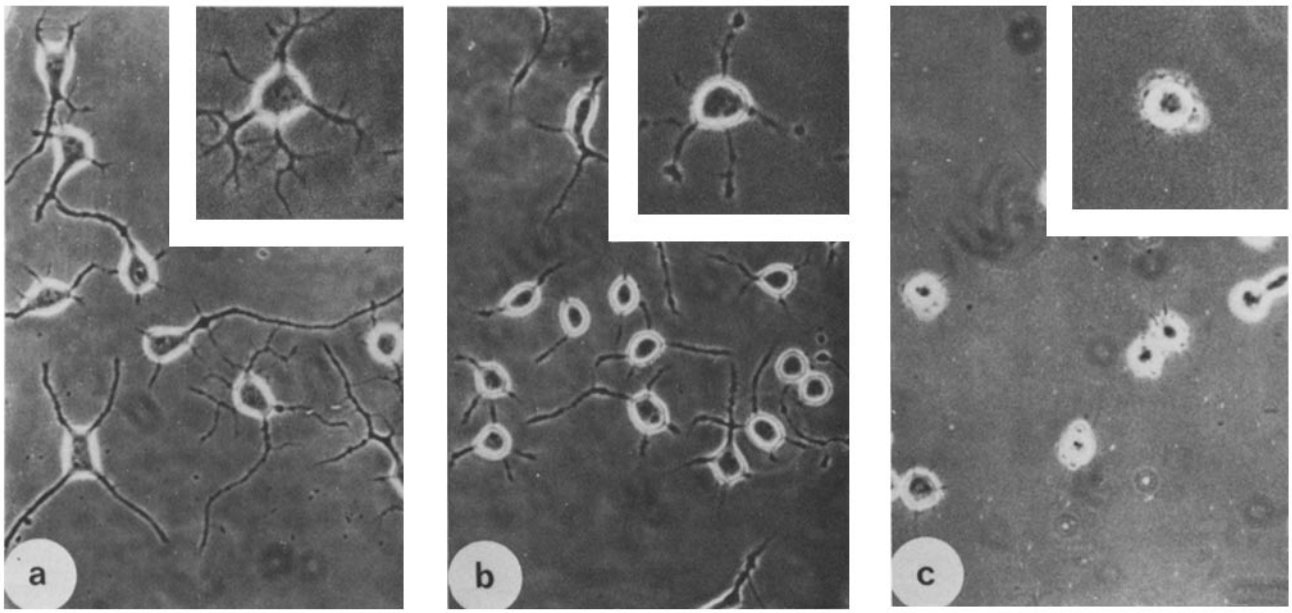

FIGURE 1 Inhibition of neurite retraction by cytochalasin D. Neuroblastoma cells are induced to extend neurites by incubation in low serum (*a*). After 45 min in 0.1 μg Nocodazole and 1 μg/ml cytochalasin D, most cells still have neurites (*b*). Virtually no neurites remain after a parallel incubation in Nocodazole alone (*c*). *Insets*: a single cell is shown before drugs are added (*a*); after 45 min in medium containing both Nocodazole and cytochalasin D (*b*); and 30 min after the cytochalasin, but not the Nocodazole, is removed (*c*).

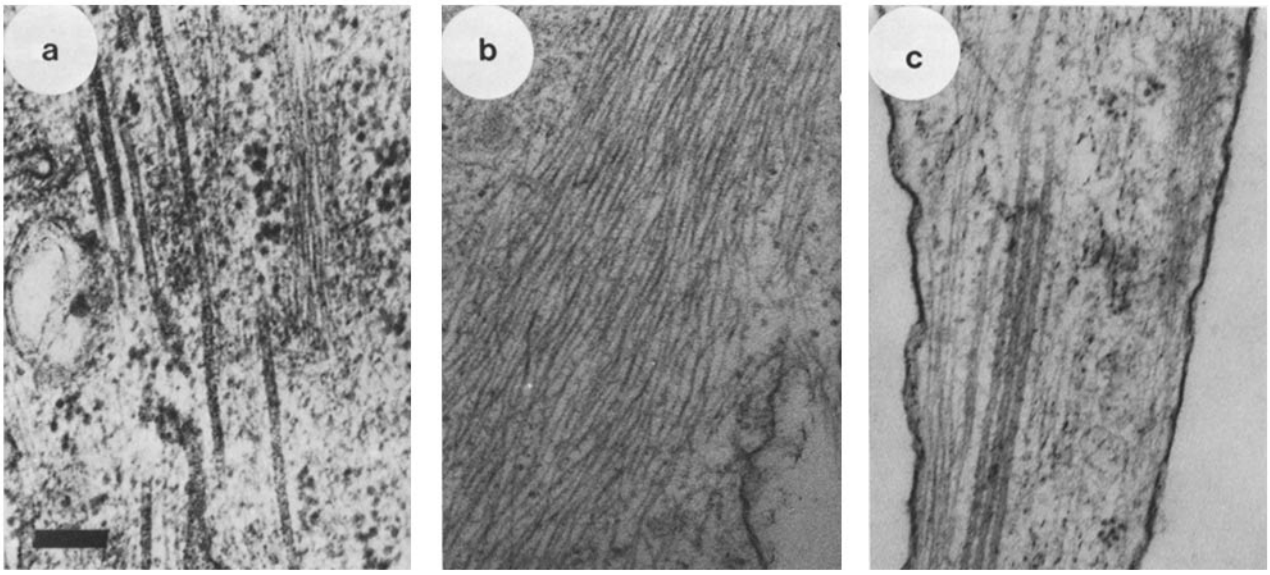

FIGURE 2 Electron microscopy of neuroblastoma neurites. Micrographs show thin sections through neurites. (*a*) Control cells, containing several microtubules and intermediate filaments. (*b*) Cells incubated for 45 min with 0.1 μg/ml Nocodazole plus 1 μg/ml CCD. Bundles of intermediate filaments, as shown, are frequently seen, but no microtubules are visualized. (*c*) Cells incubated for 60 min in 5×10^{-5} M colchicine in glucose-free medium, containing some microtubules. Bar, 2 μm. × 53,000.

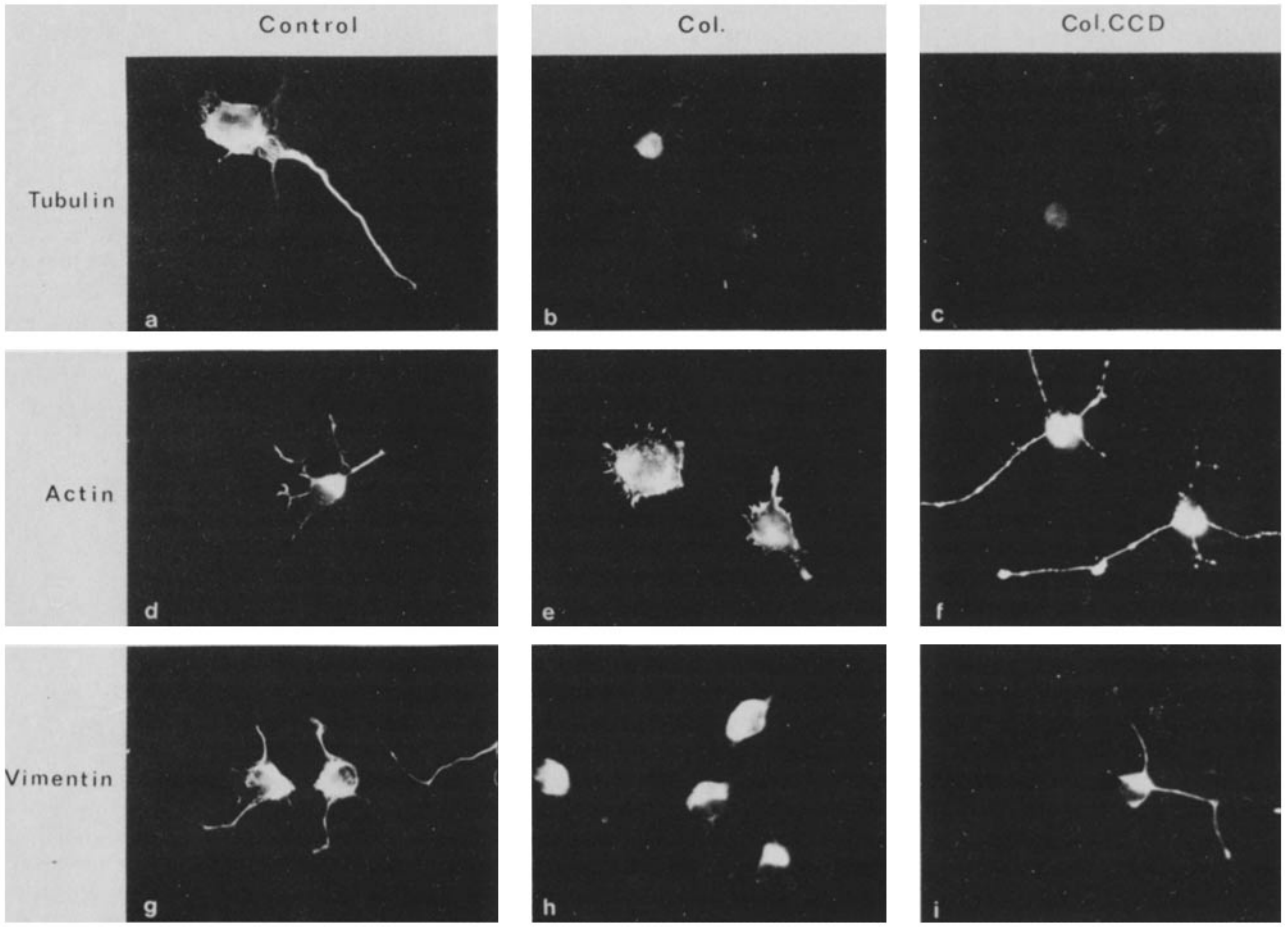

FIGURE 3 Immunofluorescence microscopy. Control cells (*a, d, g*), and cells incubated for 45 min with 5×10^{-5} M colchicine (*col; b, e, h*) or with colchicine plus 1 μg/ml CCD (*c, f, i*) are detergent-extracted in microtubule-stabilizing buffer, fixed, and stained with antitubulin (*a–c*), antiactin (*d–f*), or antivimentin (*g–i*).

codazole. Either 10^{-2} M sodium azide, an inhibitor of electron transport, or 5×10^{-3} M dinitrophenol, an uncoupler of oxidative phosphorylation, in glucose-free medium quantitatively inhibited neurite retraction by microtubule-depolymerizing drugs. The inhbition was not overcome by prolonged incubation nor by increasing the concentration of Nocodazole

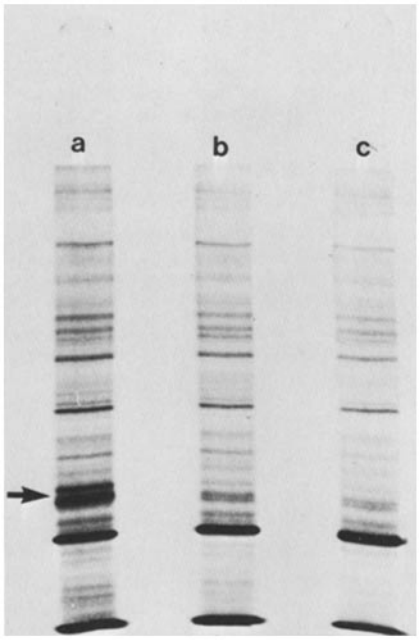

FIGURE 4 Determination of assembled tubulin in cytochalasin-treated cells. Metabolically labeled cells were extracted as described, and the microtubules were depolymerized and analyzed on SDS polyacrylamide gels (7.5% acrylamide). Lane *a*, control cells; lane *b*, cells preincubated with 5×10^{-5} M colchicine and 0.1 μg/ml cytochalasin; lane *c*, cells preincubated with colchicine alone. The arrow indicates the mobility of purified tubulin on the same gel.

10-fold. The inhibition was rapidly reversed by addition of normal concentrations of glucose to the medium but not of 2-deoxyglucose, and unmetabolized analogue of glucose. The interpretation of these experiments is complicated, however, by the persistence of some microtubules in these cells. Their presence was demonstrated by each of the techniques described above: electron microscopy (Fig. 2 *c*), immunofluorescence, and quantitation of assembled tubulin in extracted cytoskeletons. The electron micrographs showed relatively fewer microtubules in these cells, and only ~30% of the tubulin remained in detergent-extracted cytoskeletons prepared from these cells, as compared with control cells. The retraction of neurites may be prevented by the remaining microtubules. Nevertheless, this result suggests that energy production may be required for microtubule disassembly.

DISCUSSION

The experiments presented above demonstrate that the effects of microtubule-depolymerizing drugs on cell morphology are inhibited by cytochalasin. Cytochalasin does not interfere with the disruption of cytoplasmic microtubules by these drugs. Therefore, the typical loss of asymmetry observed in cells incubated with colchicine or Nocodazole is not solely the passive response to the loss of microtubules. Rather, some other cell process, inhibited by low concentrations of cytochalasin, must also be involved.

The target of cytochalasin in this system remains to be identified. There is an extensive literature on the effects of this drug, but its specificity has not been unambiguously assigned. In many systems, the ability of cytochalasin to block motile events has been interpreted as an effect on microfilament structure and function (1, 5). Recent experiments characterize a direct interaction between cytochalasin and actin filaments in vitro (10). An obvious possibility, then, is that the retraction of neurites requires microfilament function.

That interpretation is supported by our finding that metabolic poisons also inhibit neurite retraction in the presence of colchicine or Nocodazole. A similar result is obtained with fibroblastic cells by DeBrabander and co-workers (3). They find that metabolic poisons prevent the usual effects of microtubule-depolymerizing drugs on these cells: loss of polarity, retraction of the membrane, and appearance of blebs and microvilli. In both neuroblastoma and fibroblastic cells, the effects of metabolic poisons are overridden by the presence of glucose, which enables ATP synthesis by glycolysis. The requirement of ATP for neurite retraction is consistent with involvement of microfilaments. However, these experiments must be interpreted with caution. First, depletion of cellular ATP affects a multitude of cellular processes and structures. Second, we find that some microtubules persist in neuroblastoma cells after incubation with colchicine or Nocodazole in the presence of metabolic inhibitors. The surviving microtubules could be responsible for maintaining the extended neurites. Why there is not complete microtubule depolymerization under these conditions is an interesting question. A change in cell permeability is an unlikely explanation, because increasing the concentration of depolymerizing drugs 10-fold and the time of incubation 20-fold does not disrupt all the microtubules. Reasoning by analogy to recent analyses of the polymer formation and breakdown reactions in vitro (2, 6, 9, 11), we believe one possibility is that nucleotide triphosphates are involved in microtubule disassembly in vivo. This question is presently under investigation.

Our results demonstrate that cell polarity does not collapse simply because microtubules are disassembled; some other cellular functions are required. Similarly, the disruption of intermediate filament distribution that frequently accompanies microtubule disassembly is not passive, because these structures remain in the neurites without microtubules. In one sense, these experiments demonstrate a parallel between neurite extension and neurite retraction: both are inhibited by cytochalasin, and neither is solely a function of the state of microtubules.

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