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Distribution of Actin and Tubulin in C6 Glioma Cells during Arborization Induced by Cytochalasin D

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ABSTRACT—C6 glioma cells were arborized by cytochalasin D (2 μM), while the processes induced by the drug were destroyed by colcemid (2 μM). Distribution of actin and tubulin during the arborization was investigated by fluorescence microscopy to identify core structures involved in arborization. There were two types of actin filaments in C6 cells: one was oriented radially and the other was concentric. Cytochalasin D destroyed the actin filaments in sequence and transiently left a radial structure in the early stage (the first 3 min) of arborization. Microtubules were also organized similarly. At a later stage (15 min), few radial actin filaments remained, whereas microtubule bundling proceeded and seemed to support a starfish-like cell morphology. In the last stage (2 hr), most actin-staining was found around the plasma membrane undercoat area, whereas the microtubules were bundled in the core part of the processes. Radial actin filaments, which showed higher stability, seemed to be important collectively in the first step of arborization as a transient organizer of the cytoskeleton. Distribution of α-actinin and vinculin on actin filaments was investigated as potential factors stabilizing the radial filaments because of their affinity for the barbed ends of actin filaments for which cytochalasin D also had affinity. Results showed that α-actinin and vinculin were preferentially located on the radial filaments rather than on the concentric filaments.

INTRODUCTION

Astrocytes have multiple elongated processes. While the function of astrocytes is not completely understood, it is likely that their shape is critical to many of them. Astrocytes prepared from neonatal animals and cultured in serum-containing medium can undergo dramatic shape changes resulting in forms characteristic of differentiated cells when treated with membrane-permeable analogues of cAMP. The C6 cell line derived from rat glial tumors (Benda *et al.*, 1968) also differentiate similarly in the stationary phase facilitated with membrane-permeable analogues of cAMP. This mode of morphological differentiation seems to be a type of arborization. The processes form by elongation as well as retraction of cytoplasm around cytoskeletal elements. Little is known about the machinery that results in this dramatic shape change. An intact microtubule system may be required for the change since colchicine was showed to prevent cAMP-induced morphological changes, though the detailed behavior of microtubules during arborization has remained unclear (Goldman and Abramson, 1990). On the other hand, actin filament rearrangement during arborization has been reported by several groups. The most prominent change occurring in response to the cAMP analogues was loss of actin filaments in the cortical layer and their appearance at the tips of the processes (Goldman and Abramson, 1990; Baorto *et al.*, 1992). Furthermore, dihydrocytochalasin B, which disrupted actin filaments in the cell cortex, induced process formation (Baorto *et al.*, 1992). Accordingly, they proposed that process formation can be viewed as "spring-loaded", requiring only disassembly of cortical actin filaments to relieve the constraint on growth. Schliwa (1982) and Weber *et al.* (1976) demonstrated that (a) cytochalasins disrupted actin-based cytoskeletal networks and (b) formed dense actin filament foci in an energy-dependent manner. They proposed that cytochalasins thus accumulated fragments of actin-based cytoskeletal networks in localized cell areas and concomitantly initiated cell retraction and arborization as a result of the "clearing" of large cell areas.

Several questions have been raised in the course of developing their proposal. How were the fragments of actin-based cytoskeletal networks accumulated in localized cell areas? How did the fragments conform appropriately to each cell shape while retraction of cytoplasm around cytoskeletal elements proceeded to form the morphology characteristic of each cell type?

In this paper I describe the distribution of actin and tubulin in C6 cells during cytochalasin D-induced arborization and the possible roles of actin filaments in terms of their constructive aspects other than acting as a mere physical barrier against microtubule organization.

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MATERIALS AND METHODS

Chemical products

Cytochalasin D, colcemid, mouse monoclonal anti-α-actinin antibody and mouse monoclonal anti-vinculin antibody were obtained from Sigma. Mouse monoclonal anti-chick brain β-tubulin antibody was obtained from Amersham and biotinylated rabbit anti-mouse immunoglobulin antibody from DAKO. Rhodamine-phalloidin was obtained from WAKO and glass chamber slides from Nunc.

Cell culture

C6 glioma cells (Benda *et al.*, 1968) were obtained from the Japanese Cancer Research Resources Bank. Cells were cultured at 37°C on glass chamber slides as described elsewhere (Kobayashi, 1994). The cells were routinely used for experiments 12 hr after plating at the cell density of 2×10^4 cells/cm^2 to avoid cell-to-cell contact. Cytoskeletal poisons were added to the cultures as follows: cytochalasin D was dissolved first in dimethyl sulfoxide at 1 mM and diluted with the medium to give the concentration indicated. Dimethyl sulfoxide at 1% (vol/vol) or lower concentrations did not affect the experimental systems used in this study. Colcemid was dissolved first in the medium at 1 mM and diluted with the medium to give the concentration indicated.

Staining of actin with rhodamine-phalloidin and indirect immunofluorescence staining of tubulin

Actin was stained fluorescently with rhodamine-phalloidin as described by Barak *et al.* (1981). Tubulin was stained immunofluorescently as described by Byers *et al.* (1980) with minor modification using biotinylated second antibody and FITC-conjugated streptavdin. An Olympus BHS fluorescence microscope was used to observe and photograph the cells.

Double fluorescence staining of actin/α-actinin and actin/vinculin

Fixed cultures were exposed to anti-α-actinin or anti-vinculin for 30 min, washed with PBS for 5 min, exposed to biotinylated anti-mouse IgG for 30 min, washed with PBS for 5 min, exposed to FITC-streptavidin and rhodamine-phalloidin for 30 min, then washed with PBS for 5 min.

RESULTS

Effects of cytochalasin D and colcemid on cell morphology

C6 glioma cells were incubated for 2 hr with 2 μM cytochalasin D and observed by a microscope. They developed a process-bearing spider-shaped morphology (Fig. 1B), similar to that of differentiated C6 cells. Around 4-8 processes were observed on each cell. This altered cell morphology was maintained stably for at least 24 hr in the poison-containing cultures. The effects of cytochalasin D were reversible, so that the arborized cells began to spread soon after the removal of cytochalasin D, resulting in the recovery of their original morphology within 24 hr. Microtubules were necessary for the arborized cell structure because colcemid (2 μM) deformed the cytochalasin D-arborized C6 cells into small disks without a spreading area (Fig. 1C).

Distribution of actin and tubulin in C6 cells

As known well, cytochalasin D induced rapid disorganization of normal microfilament bundles and formation of dense aggregates of condensed microfilaments (Weber *et al.*, 1976; Schliwa, 1982; Goldman and Abramson, 1990; Baorto *et al.*, 1992). Furthermore, microtubules were necessary for cytochalasin D-induced arborization as described above. These results raised the question of whether C6 cells have any intrinsic cytoskeletal structures that constitute the morphology of spider-shaped astrocytes and whether those intrinsic structures, if any, are composed principally of microtubules. Therefore, the cytoskeletal structures were characterized by fluorescence microscopy using rhodamine-phalloidin and anti-tubulin antibody. Figure 2 shows the structure of actin filaments and microtubules in C6 glioma cells. It should be noted that there were at least two types of actin filaments in C6 cells: some were radially distributed in the cytoplasm and others had a concentric distribution (Fig. 2A). Distribution of the radial filaments seemed to correspond to that of processes formed during arborization, whereas microtubules were radially and uniformly distributed, except for those located in the perinuclear regions (Fig. 2B).

Distribution of actin and tubulin in C6 cells during the arborization

The above-described phenomena raised the questions of how actin filaments and microtubules were reorganized

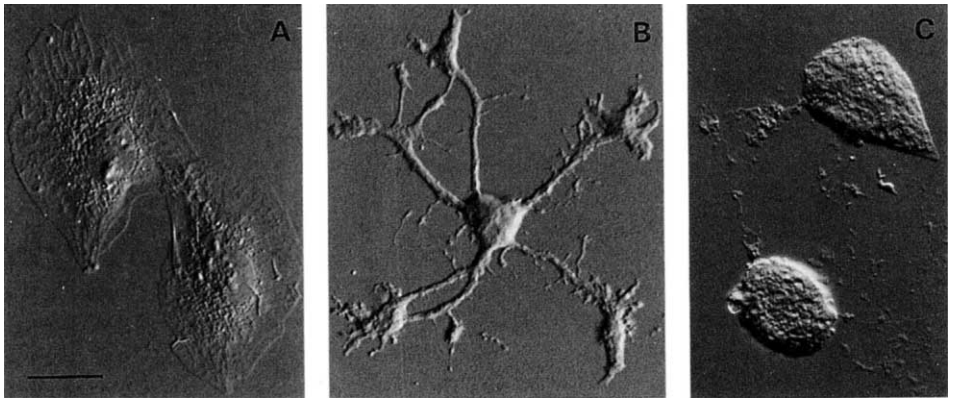

Fig. 1. Effects of cytochalasin D and colcemid on the morphology of C6 cells. Cells were cultured as described in MATERIALS AND METHODS (**A**) for 2 hr without poison, (**B**) for 2 hr with 2 μM cytochalasin D and (**C**) for 2 hr with 2 μM cytochalasin D, then for 2 hr with 2 μM each of cytochalasin D and colcemid. Bar, 25 μm.

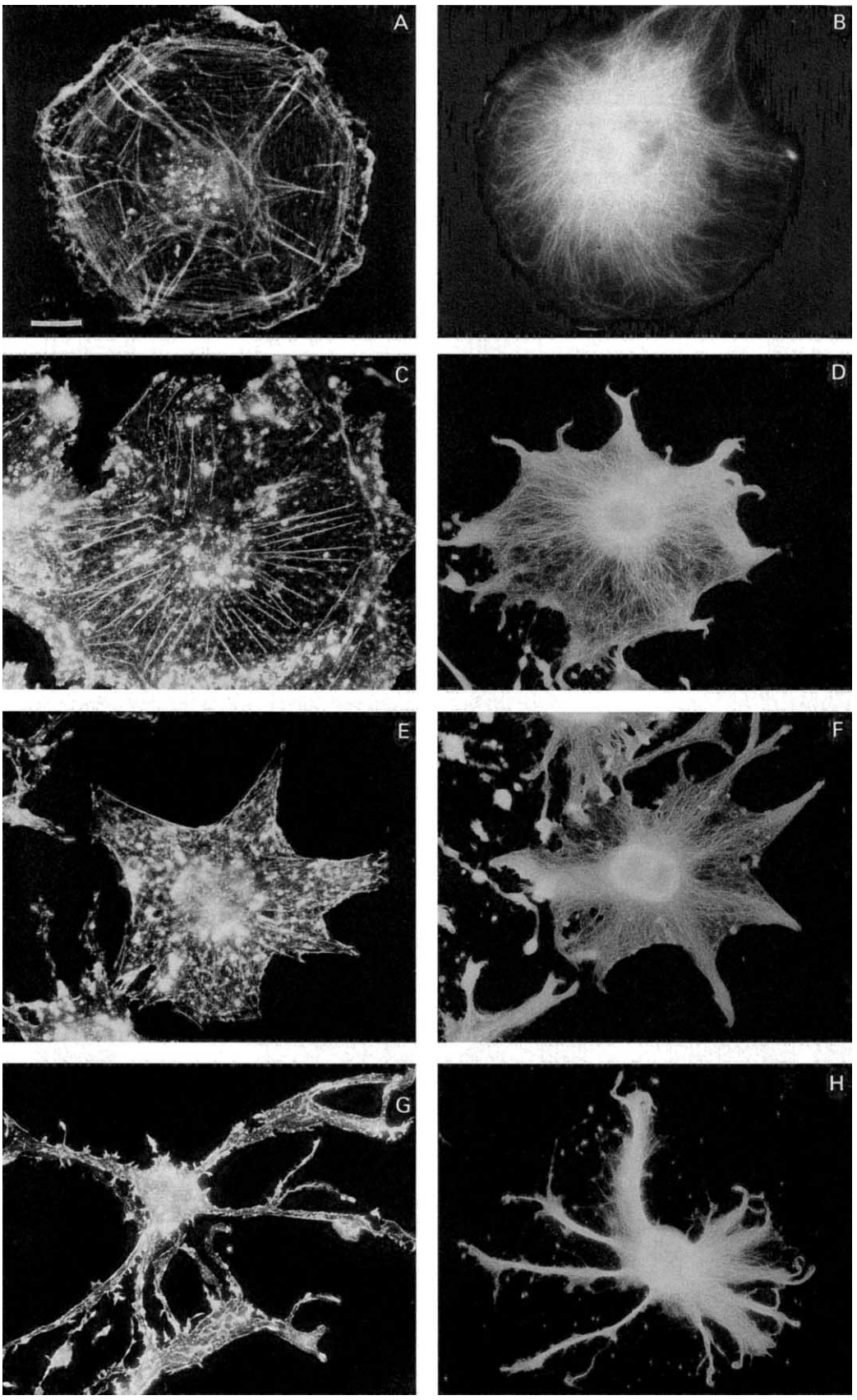

Fig. 2. Distribution of actin and tubulin in C6 glioma cells during arborization induced by cytochalasin D. Cells were treated with 2 μM cytochalasin D for (**A** and **B**) 0 min, (**C** and **D**) 3 min, (**E** and **F**) 15 min and (**G** and **H**) 2 hr. Actin (**A**, **C**, **E** and **G**) and tubulin (**B**, **D**, **F** and **H**) were stained fluorescently as described in MATERIALS AND METHODS. Bar, 10 μm.

during the arborization and how the cells developed a spider-shaped morphology. C6 cells were incubated with 2 μM cytochalasin D and the distribution of actin and tubulin in the cells was investigated. At an early stage (the first 3 min) of arborization, the cell shape was polygonal (Fig. 2C and 2D). The concentric filaments disappeared, while the radial filaments remained (Fig. 2C). Asterisk-like dense foci of actin (Weber *et al*., 1976; Schliwa, 1982) were observed on the periphery of the cells and in the perinuclear regions. Microtubules were moderately reorganized (Fig. 2D). Most were oriented radially with a tendency to be concentrated according to the arborized configurations. Microtubules surrounding the nucleus formed a ring structure. At a later stage (15 min) of arborization, the cell morphology was starfish-like. The concentric actin filaments disappeared entirely, while a few radial filaments remained (Fig. 2E). Actin distribution was condensed around the plasma membrane undercoat area and there was an increase in the asterisk-like structures of actin in the cyto-

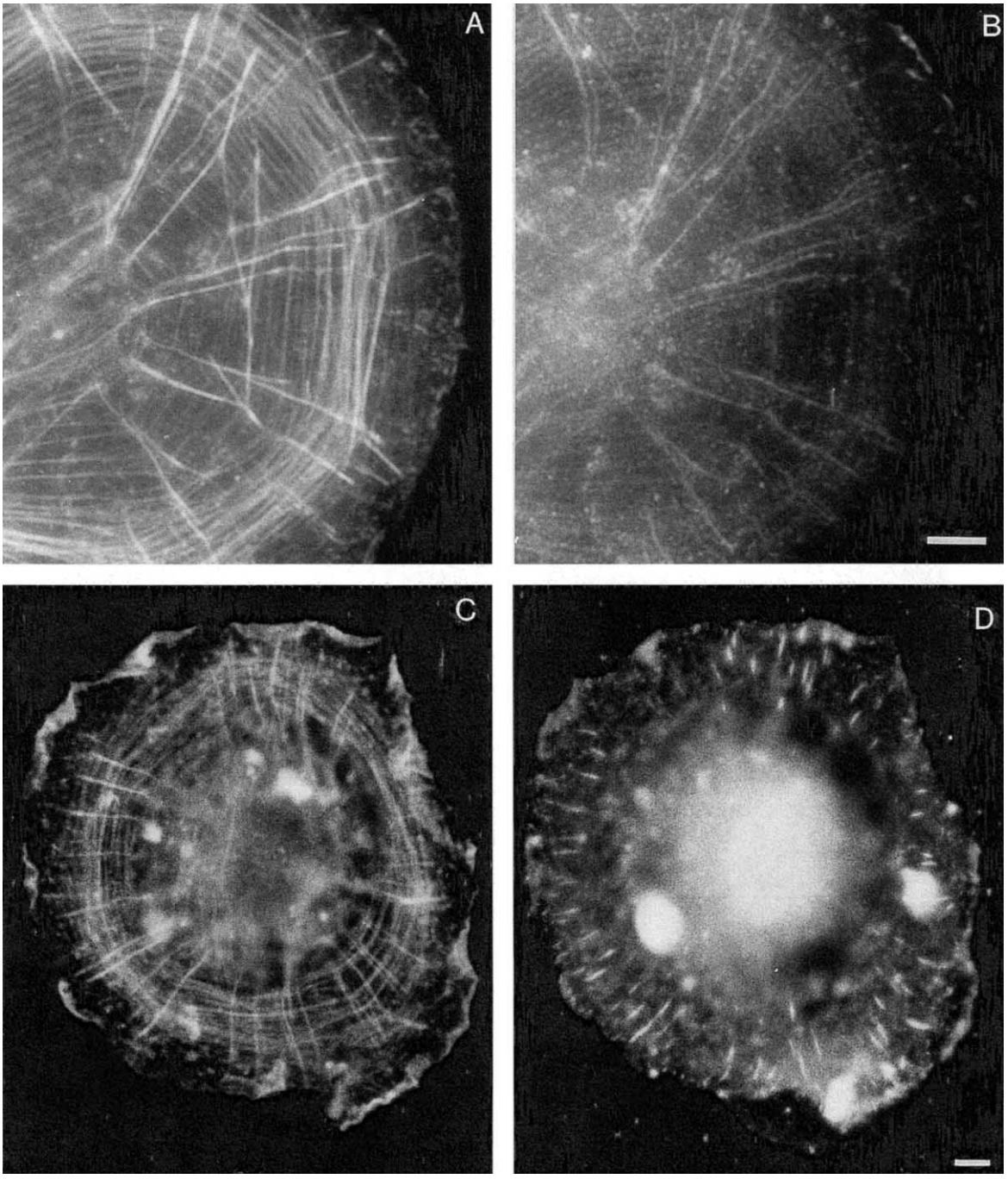

Fig. 3. Distribution of actin, α-actinin and vinculin in C6 glioma cells. Cells were double-stained fluorescently for (**A**) actin and (**B**) α-actinin or (**C**) actin and (**D**) vinculin in the same cells respectively as described in MATERIALS AND METHODS. Bar, 5 μm.

plasm. Microtubule bundling proceeded further to conform to an arborized cell configuration (Fig. 2F). In the last stage (2 hr) of arborization, the cell morphology was spider-like. The central sphere and peripheral processes became distinguishable and both radial and concentric actin filaments disappeared (Fig. 2G). Most actin-staining was found around the plasma membrane undercoat area and at the asterisk structure in the central sphere of the cytoplasm. The microtubule structure became more suitable for conforming to an arborized configuration (Fig. 2H). Microtubules in the peripheral process area were bundled and those in the central sphere area formed a dense basket-like structure surrounding the nuclei.

It should be noted that cytochalasin D destroyed actin filaments in sequence. A radial structure of actin filaments was transiently conformed in the early stage of arborization, while most actin-staining was found around the plasma membrane undercoat area and at the asterisk structure in the central sphere of the cytoplasm in the last stage of arborization.

Distribution of actin, α-actinin and vinculin in C6 glioma cells

As described above, arborization involved drastic rearrangement of the actin filaments. The concentric filaments disappeared quickly in the early stage of arborization, while the radial filaments were more stable and remained intact until a later stage. How are the radial filaments different from the concentric filaments? Cytochalasins exert their effects through capping actin filaments on barbed ends (Maclean-Fletcher and Pollard, 1980) or by inhibiting gelation by fragmentation of the filaments (Hartwig and Stossel, 1979; Schliwa, 1982). Therefore, protein assembly around the barbed ends may protect actin filaments from cytocalasins. As known well, some of the barbed ends interact with the cell membrane to organize special structures such as focal adhesions and focal adhesions contain several actin-binding proteins including α-actinin and vinculin, though the distribution of α-actinin is not restricted to focal adhesions (Lazarides and Burridge, 1975; Geiger, 1979; Luna and Hitt, 1992). Accordingly, the distributions of the two actin-binding proteins were studied. Figure 3 shows the distribution of actin (A) and α-actinin (B) in a cell and actin (C) and vinculin (D) in another cell. α-Actinin was distributed densely along the radial actin filaments, whereas its distribution along the concentric filaments was diminished. Vinculin was located at the peripheral ends of the radial filaments, but not on the concentric filaments. These data clearly demonstrated that both α-actinin and vinculin were concentrated on the radial actin filaments.

DISCUSSION

Based upon recent research (Schliwa, 1982; Goldman and Abramson, 1990; Baorto *et al.*, 1992), it has been proposed that cytochalasins fragment actin-based cytoskeletal networks and induce cell arborization as a result of the "clearing" of large cell areas. However, this has raised several questions about the mechanism of redistribution of actin-based cytoskeletal fragments in localized cell areas and the mechanism by which the fragmentation of actin filaments rearranged cytoskeletons suitable to each cell shape while cytochalasins converted the cell morphology characteristic to each cell type (Carter, 1967; Spooner *et al.*, 1971; Wessells *et al.*, 1971; Sanger, 1974).

In this paper, I demonstrated that cytochalasin D fragmented actin filaments in sequence and transiently left a radial structure, suggesting a possible role of these filaments collectively as a transient organizer of cytoskeletons through reactions such as an actin-filament-microtubule interaction (Griffith and Pollard, 1978) to conform to the arborized cells. These phenomena, including the transient radial structure of actin filaments, were not addressed by the early researchers, presumably because: (a) the early stage phenomena in the arborization were disregarded and (b) cytoskeletal filaments for cell contact disrupted the recognition of filaments for cell arborization in a cell system driven by cAMP which usually arborizes stationary phase cells bearing cell-to-cell contact machinery. It should be noted that cytochalasin D provided a cell arborization system consisting of exponentially growing cells without cell-to-cell contact machinery and favored the transient radial structure of actin filaments during the arborization.

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