Microtubule Disruption Induces the Formation of Actin Stress Fibers and Focal Adhesions in Cultured Cells: Possible Involvement of the Rho Signal Cascade

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Key words: microtubule/actin stress fiber/rho/GTP binding protein/signal transduction

ABSTRACT. To obtain insight into the molecular dynamics and involvement of microtubules and the related signal molecules in the regulation of cell locomotion, we studied the influence of microtubule disruption on actin stress fibers and focal adhesion assembly in addition to cell morphology. We found that all microtubule-disrupting drugs including colcemid and vinblastine rapidly and reversibly induce the formation of actin stress fibers and focal adhesions containing vinculin, accompanied by activated cell motility in serum-starved Balb/c 3T3 cells. In contrast, taxol, a microtubule-stabilizing drug, completely inhibited these effects of the microtubule-disrupting drugs. A microinjection of C3 ADP-ribosyltransferase, a specific inhibitor of rho GTPase, blocked the stress fiber and focal adhesion assembly induced by the microtubule disruption. These results suggested that microtubules contain signal molecules that regulate the formation of stress fibers and focal adhesions by activating the rho signal cascade. We postulate that microtubule-releasing and stress fiber-inducing factors link the intrinsically variable and irregular actin filament dynamics to coordinated and directional locomotion in the process of cell movement.

Cell motility is critically important to many normal and abnormal biological processes, such as embryonic development, wound healing, inflammation and cancer metastasis (9, 40). Many biological active compounds such as growth factors and phorbol esters affect cell motility by disturbing cytoskeletal networks including actin filaments (10, 11, 25). The underlying biological molecular mechanisms have been studied using various types of motile cells including fibroblasts, epithelial cells, leukocytes and neurite growth cones. These studies have shown that the locomotory process of these cells can be divided into essentially three independent, sequential phases: the polar extension of a leading lamella including ruffles and microspikes via the active motility of the anterior, leading edge; the formation of new anterior contacts with the underlying substrate and the retraction of the trailing tail into the advancing cell body (33, 40). Actin and its various associated proteins have been shown to play major roles in all of these processes (6, 7, 15, 22, 41, 42).

In cultured cells, actin filaments exist in three types of structure: the cortical actin network, actin stress fibers and cell surface protrusions such as membrane ruffles and microspikes (38). Actin stress fibers are attached to the endofacial surfaces of the membrane in defined areas of the closest cell-substrate contact, namely, focal contacts or adhesions (2, 13) where integrin, a receptor at focal adhesion, mediates the connection between the cytoplasmic actin filaments and the extracellular matrix (5, 34, 35). Various actin-related proteins including vinculin and talin are specifically localized at the cytoplasmic aspects of focal adhesions, suggesting that they mediate the attachment of actin stress fibers to the plasma membrane at these sites.

Many signal molecules including protein kinases and GTP-binding proteins are co-localized at focal adhesion (24). For example, the activation of protein kinase C (PKC) induces rapid disassembly of actin stress fibers and focal adhesions as well as inhibition of growth factor-induced membrane ruffling, indicating that PKC is involved in the negative regulation of actin stress fibers and focal adhesions (9, 11). A series of recent studies on the biological function of a GTP-binding protein, rho, has revealed that it is an essential component of a signal transduction pathway linking extracellular stimuli to the assembly of focal adhesions and actin stress fibers (3, 30) and forming contractile ring (18). These findings suggested that these signal molecules are involved in the positive and negative regulation of dynamic organization of the contractile actin stress fibers that span the cells between contact foci at the rear and similar contacts at the base of leading edge of locomoting cells. However, little is known of the molecular regulatory
mechanism of directional locomotion during cell movement.

Microtubule disrupting drugs largely abolish cell polarizations and directional locomotion (44). Also, cinematographic and immunocytochemical studies have shown that the free ends of microtubules are often co-localized with focal contact sites along the leading edge of moving fibroblasts (33). These observations suggest the possibility that microtubules are involved in determining net direction of cell locomotion, although the relationship between actin filaments and microtubules in respect to the regulation of coordinated and directional locomotion remains unclear.

To clarify the molecular dynamics and the involvement of cytoskeletal and signal molecules in the process of regulating directional locomotion, we studied the influence of microtubule disruption on the formation of the focal adhesions and actin stress fibers that are involved in cell locomotion. We found that microtubule disruption rapidly and reversibly induced the formation of focal adhesions containing vinculin and of actin stress fibers, accompanied by activated cell movement in serum-starved Balb/c3T3 cells. Our studies provide more direct evidence for microtubule-mediated regulation of integration of the intrinsically variable and irregular actin filament dynamics to coordinated and directional locomotion during cell movement.

MATERIALS AND METHODS

Materials. Colcemid, colchicine, vinblastine, nocodazole, β-lumicolchicine, cycloheximide, actinomycin D, cytochalasin B and D and fat-free bovine serum albumin (BSA) were obtained from Sigma (USA). Insulin was obtained from Armarsh International. Taxol was from RBI International (USA). C3 exoenzyme and wortmannin were purchased from Biomol Research Lab, Inc (USA). Rhodamine-conjugated phalloidin was from Molecular Probes, Inc. All antibodies were purchased from Sigma except for fluorescein- and rhodamine-conjugated second antibodies obtained from MBL Co. (Japan).

Cell Culture and Microinjection. Balb/c3T3 A31 cells were maintained in Eagle's minimum essential medium (MEM) (Gibco) containing 10% fetal calf serum (FCS) (Bocknek Lab.), as described previously (10). Cells (1 x 10^6) were seeded onto 35 mm Corning plastic dishes for 7 to 10 days, and then starved for 16-20 hr in FCS-free MEM. The inner- and outer-surfaces of glass needles for microinjection were siliconized with Sigma Coat (Sigma) to avoid physical attach-
Fig. 1.
logical changes accompanied by the rearrangement of cell monolayer in confluent, serum-starved Balb/c3T3 cells within 30 min after the treatment (not shown). These observations suggested that they could cause rapid polymerization and redistribution of actins since actin filament dynamics can mainly supply force to cell movement and rearrangement (16, 38, 39). To test this notion, we analyzed the state of the polymerized actin filaments following the addition of colcemid. Serum-starved, confluent Balb/c3T3 cells had very few stress fibers, but filamentous actin was distributed on the plasma membrane in a punctate manner (Fig. 1A, E). On the other hand, many microtubules elongated from the centrosome into the periphery of the cells (Fig. 1B). Vinculins were localized diffusively throughout the cytoplasm in untreated cells (Fig. 1F). Within 5 min of exposure to colcemid (100 ng/ml), there were no apparent changes in actin and microtubule organization. However, actin filaments were markedly redistributed during the following 10-20 min in serum-starved cells, punctate actin was lost, and fine but stress fiber-like actin filaments gradually increased throughout the cytoplasm in parallel with the disappearance of filamentous microtubules. Many new stress fibers were clearly discernible within 30 min, during which most microtubules disappeared and the immuno-staining with anti-tubulin was diffused throughout the cytoplasm (Fig. 1C, D). The diameters of these bundles of stress fibers increased over the following 30 min or so. Actin stress fibers were similarly induced by colchicine, vinblastine and nocodazole (not shown). The colcemid-induced actin stress fibers were maintained for as long as colcemid was present in the culture medium, up to at least 5 days (unpublished data). These findings indicated that microtubule-disruption continuously stimulates and activates signal transduction for stress fiber formation. Similar actin stress fiber formation was induced more rapidly by adding a low concentration of FCS (not shown), confirming the results reported by Ridly and Hall (31). However, the FCS effect disappeared 12 h after adding 0.5% FCS (not shown). Colcemid also caused vinculin (Fig. 1G, H) and talin (not shown) to accumulate into focal adhesions at the ends of the new stress fibers. These were initially detectable within 10 min and abundant by 30 min. The relative amount of vinculin and talin in focal adhesions apparently increased in parallel with the increasing diameter of stress fiber bundles across the cytoplasm (arrows in Fig. 1G, H). The induction of stress fi-

![Fig. 1A](image1.png) ![Fig. 1B](image2.png) ![Fig. 1C](image3.png) ![Fig. 1D](image4.png)

Fig. 2. Reversibility of colcemid-induced formation of stress fibers. Confluent, serum-starved cells were incubated for 5 h with colcemid (100 ng/ml), and then washed three times with fresh, warm MEM medium. The cells were fixed and double-stained with rhodamine-phalloidin (A, C) and anti-tubulin antibody (B, D), 25 min (A, B) and 30 min (C, D) later. Bar: 25 μm.
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bers and focal adhesions containing vinculin was observed at concentrations of more than 20 ng/ml of colcemid in serum-starved Balb/c3T3 cells. Cytochalasin B and D inhibited the colcemid-induced stress fiber formation (not shown). To test whether or not the effect is reversible, colcemid was removed after 5 h from the culture medium. During the first 20 min thereafter, stress fibers did not disappear and no microtubules were formed. However, the stress fibers (Fig. 2A, C) rapidly disappeared accompanied by microtubule re-assembly (Fig. 2B, D) during the following 10 min. The vinculin at focal adhesions also disappeared within 30 min after removing the colcemid (not shown). These results indicated that the colcemid-induced formation of actin stress fibers and focal adhesions is reversible and tightly associated with the disruption of microtubules. Actinomycin D and cycloheximide did not inhibit the colcemid-induced stress fiber and focal adhesion formation, indicating that neither RNA nor protein synthesis is not required for the colcemid effects.

**Taxol prevents colcemid-induced stress fiber and focal adhesion formation.** Taxol stabilizes microtubules in a variety of cells (43) and prevents colcemid-induced biological responses such as the initiation of DNA synthesis and the activation of MAP kinases (35). To determine whether colcemid-induced stress fiber and focal adhesion formation is also prevented by taxol, the cells were incubated for 0.5 h with taxol prior to colcemid stimulation (Fig. 3). Taxol (1.0 μM) itself induced the accumulation of shortened fibrinous microtubules around the center of the cytoplasm but had almost no effects on the status of the actin filaments, although rhodamine staining became slightly dense at the periphery of the taxol-treated cells (Fig. 3A, B). Taxol almost completely inhibited colcemid-induced stress fiber formation in addition to preventing the colcemid-mediated degradation of microtubules (Fig. 3C, D). Similarly, it inhibited the colcemid-induced accumulation of vinculin at focal adhesions (not shown).

**Colcemid itself neither induces membrane ruffling nor prevents growth factor-induced membrane ruffling.** Many exogenous factors such as FCS, Bombesin, PDGF, EGF and insulin induce not only the formation of stress fibers and focal adhesions but also membrane ruffling accompanied by the accumulation of filamentous actins (19, 31). These exogenous factors could induce membrane ruffling in our Balb/c3T3 cells under our cultured conditions (10, 11). To test whether colcemid-induced stress fiber formation by taxol. Confluent, serum starved cells were incubated in the presence of taxol (1.0 μM) (A–D) for 0.5 h, and then treated without (A, B) or with (C, D) colcemid (100 ng/ml) for 1 h. The cells were double-stained with rhodamine-phalloidin (A, C) and anti-tubulin antibody (B, D). Bar: 25 μm.
colcemid itself induces membrane ruffling, and accelerates or prevents growth factor-induced membrane ruffling, we investigated the morphological and immunocytochemical changes caused by colcemid in more detail. To visualize fine and small ruffles, we observed the cells continuously under a phase-contrast microscope at high magnification. However, colcemid itself did not induce detectable membrane ruffles before or after the extensive formation of stress fibers (not shown). Insulin induced membrane ruffling in Balb/c3T3 cells cultured in the presence of fetal calf serum (10). However, very few membrane ruffles were induced by growth factors including FCS, PDGF and insulin in serum-starved Balb/c3T3 cells (10, 11). Consequently, it was very difficult to examine the effect of colcemid on membrane ruffling in serum-starved cells. Therefore, we studied the effect of colcemid on growth factor-induced membrane ruffling in the cells that had been cultured in 2% FCS containing-MEM for 2 days (Fig. 4). Insulin induced membrane ruffling within 5 min (Fig. 4A). Insulin induced membrane ruffling in Balb/c3T3 cells at any time in the presence of colcemid up to the establishment of stress fibers (Fig. 4B).

**C3 ADP-riboyltransferase inhibits colcemid-induced stress fiber and focal adhesion assembly but not growth factor-mediated ruffling.** FCS- and PDGF-induced stress fibers and focal adhesions are inhibited by the inactivation of rho (30), indicating that endogenous rho proteins mediate the stress fiber and focal adhesion formation induced by exogenous factors (27, 32). The growth factor-induced membrane ruffling is mediated by another small ras-related GTP-binding protein, rac (31). We examined whether or not colcemid-induced stress fiber and focal adhesion formation is also mediated by endogenous rho proteins, using exoenzyme C3 transferase from Clostridium botulinum. This enzyme ADP-riboylates rho proteins on amino acid Asn-41 (36) and renders them biologically inactive (29) but has if little or no effect on rac and other GTP-binding proteins such as cdc 42 and ras (30, 31). When C3 was microinjected into serum-starved Balb/c3T3 cells, it induced morphological rounding of the cells as described (29) but more slowly, suggesting that endogenous rho proteins are active and involved in maintaining cell shape even in serum-starved cells that contain few stress fibers. To examine whether the formation of new actin stress fibers induced by colcemid is dependent on endogenous rho proteins, colcemid was added 10-20 min after C3 microinjection (Fig. 5). C3 at concentrations of more than 50 µg/ml completely inhibited colcemid-induced stress fiber formation (Fig. 5A). Microinjections of 0.1 mg/ml of fat-free bovine serum albumin or 0.25 mg/ml of mouse IgG had no effect (not shown), indicating that microinjection itself did not inhibit stress fiber formation. C3 microinjection had no effect on insulin-induced membrane ruffling (Fig. 5B), thus confirming the findings of Ridley and Hall, that the inactivation of rho by C3 microinjection did not inhibit the actin polymerization leading to membrane ruffles (21). We also examined the effect of wortmannin, a potential inhibitor of phosphoinositide 3-kinase (PI-3k), on colcemid-induced stress fibers and focal adhesions as well as insulin-induced membrane ruffling because it has been shown to inhibit the formation of actin filaments associated with insulin-induced membrane ruffling (19). More than 0.1 µM of wortmannin inhibited insulin-induced membrane ruffling (data not shown), consistent with the findings reported by Kotani et al. (19). However, wortmannin, even at a high concentration (5 µM), did not inhibit colcemid-induced stress fiber formation and microtubule disruption (data not shown), indicating that PI-3K is not involved in colcemid-induced stress fiber formation.

**Fig. 4.** Effect of colcemid on insulin-induced membrane ruffling. Confluent, serum-starved cells were cultured with MEM containing 2% FCS for 2 days. These cells were incubated without (A) or with (B) 200 ng/ml of colcemid for 30 min, and then with 200 ng/ml of insulin for 5 min (A, B). The cells were stained with rhodamine-phalloidin. Arrows indicate insulin-induced ruffles. Bar: 25 µm.
DISCUSSION

The results of this study showed that all microtubule-disrupting drugs rapidly and reversibly induce the formation of focal adhesions containing vinculin and actin stress fibers, accompanied by activated cell motility in serum-starved Balb/c3T3 cells. We further showed that taxol, a microtubule-stabilizing drug, completely inhibits the effects of the microtubule-disrupting drugs. These results suggest that microtubule disruption generates a signal cascade through which the accumulation of vinculin and talin into focal adhesion and the bundling of actin filaments (stress fibers) are simultaneously induced.

As to how microtubules control the intrinsically variable and irregular actomyosin-driven motility of the cells, it is possible that microtubules may contain a signal molecule which can motivate the actin-myosin polymerization and the accumulation of plaque proteins such as vinculin into focal adhesion once it is released from microtubules (Fig. 6). The microtubule-releasing and stress fiber-inducing factor (MRSF) usually tightly associates with microtubules in the resting status (Fig. 6A). Once the microtubules are disassembled totally (Fig. 6B) by drugs such as colcemid, or partially (Fig. 6C) by shrinking through dynamic instability (12, 23), MRSF may then be released to stimulate stress fiber and focal adhesion formation in cooperation with surrounding signal molecules. These released MRSF molecules could again be absorbed into the new microtubules as degraded microtubules start to grow again. Alternatively, MRSF may be released and activated by interaction with signal molecules around microtubules (Fig. 6D). In either situation, microtubules could determine the localization of release of MRSF, and consequently regulate the directional locomotion of motile cells. It is also possible that microtubule disruption itself induces the instability of cell membrane which triggers actin polymerization.

The small GTPase, rho, is involved in the focal adhesion and stress fiber assembly induced by growth factors or FCS in serum-starved Swiss 3T3 cells (30, 31). We also showed that C3 exoenzyme, a specific inhibitor of rho protein, completely inhibited colcemid-induced formation of stress fibers and focal adhesions, indicating that rho plays essential and crucial roles in the induction of stress fibers and focal adhesions by microtubule disruption in serum-starved Balb/c3T3 cells. Two pathways appear to eventually activate rho to induce stress fibers and focal adhesions (28, 30). The first is the lysophosphatidic acid (LPA) induced rho activation cascade which may involve an unknown tyrosine kinase which is sensitive to genistein and tyrphostin (27, 30, 32). We also found that genistein and tyrphostin inhibit the formation of actin stress fibers induced by colcemid (unpublished data). The second is the growth factor-induced and rac-mediated activation of the rho cascade, which may in part, be regulated by PI-3 kinase (19, 31). In addition, rac activation also results in membrane ruffling independent of rho activation (31). However, we found that colcemid-induced microtubule disruption neither induced membrane ruffling nor prevented growth-factor induced membrane ruffling. Furthermore, we showed that C3 exoenzyme inhibited colcemid-induced stress fiber and focal adhesion assembly but not growth factor-mediated ruffling. These findings indicated that microtubule disruption is not involved in rac activation but specifically activate rho cascade. We also observed that wortmannin, a potent PI-3 kinase inhibitor, did not inhibit the colcemid effects, indicating...
Fig. 6. A schematic representation of the regulation of stress fiber and focal adhesion assembly by microtubule releasing and stress fiber-inducing factor (MRSF). MRSF (blue circle) usually binds directly or indirectly to microtubules and moves along them toward the plus or minus end (A). Once the microtubules are disassembled totally (B) by drugs such as colcemid, or partially (C) by shrinking through dynamic instability, MRSF is then released and stimulates stress fiber and focal adhesion formation in cooperation with surrounding signal molecules. These released MRSF molecules could be again absorbed into the new microtubules as degraded microtubules start to grow again. Alternatively, MRSF is released and activated by interaction with signal molecules around the plus ends of microtubules rather than by disassembly through the dynamic instability of microtubules (D).

that PI-3 kinase is not involved in colcemid-induced stress fiber formation. Taken together, it is likely that microtubule disruption may trigger the formation of actin stress fibers and focal adhesions through a mechanism similar to that of rho activation by LPA. However, the signal cascade generated by microtubule degradation may be in part different from that of LPA because taxol has no effect on LPA-generated actin stress fiber and focal adhesion assembly (unpublished data).

Many cellular factors are associated with or involved in microtubules and related phenomena (20). These include microtubule associated proteins (MAPs) and MAP kinases. Focal contacts comprise many signal molecules including c-src and focal adhesion kinase (FAK) in addition to rho and rac (24). Other molecules involved in cytoskeletal dynamics are phospholipids which play very important roles in cytoskeletal organization (4, 14, 17, 39) as well as in the signal transduction of external stimuli (26). For example, Peppelenbosch et al., have shown that leukotriene, a biologically important metabolic product of phospholipids such as PIP$_2$, induces rho-dependent stress fiber assembly (28), indicating that phospholipid metabolism also plays important role in stress fiber and focal adhesion formation. There is the possibility that microtubule disruption may accelerate translocation of rho onto inner surface of membrane since rho seems to be activated through its binding to membrane where phospholipids motivate rho activation (1).

Here, we showed that microtubule disruption rapidly and reversibly induce actin stress fiber and focal adhesion assembly, probably through the activation of rho GTPase by signal molecules such as the above mentioned MRSF. Further experiments need to be done to identify MRSF and elucidate its role in microtubule-mediated regulation of the directional locomotion of motile cells.

Acknowledgement. I thank Dr. Y. Nishizuka for encouragement and critical reading of the manuscript, and Ms. Y. Enomoto for
secretarial assistance. This study was supported by Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture, the Ministry of Science and Technology, Japan.

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(Received for publication, September 12, 1996 and in revised form, September 20, 1996)