Low concentrations of nocodazole interfere with fibroblast locomotion without significantly affecting microtubule level: implications for the role of dynamic microtubules in cell locomotion

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SUMMARY

The role of microtubules (MTs) in cell locomotion is uncertain: while MTs are not essential for motility of certain cells, MTs are necessary for the directed translocation of large cells such as fibroblasts, endothelial cells and neuronal growth cones. Based on previous studies, we hypothesize that cell locomotion may involve MTs in two possible ways: (1) the rate of cell locomotion is proportional to MT level; or (2) cell locomotion is not proportional to MT level but requires a critical level of MTs to proceed. To test these hypotheses, we measured the rate of locomotion of NRK fibroblasts migrating into an in vitro wound, before and after treatment with different concentrations of nocodazole to generate cells with different levels of MTs. Locomotion of cells was monitored directly using timelapse recording and analyzed with an Image-1 image analysis program. Addition of nocodazole (>50 nM) resulted in a rapid reduction in locomotion to a new rate that was maintained for >2 hours. We found that addition of as little as 100 nM nocodazole decreased the rate of locomotion by more than 60%; and that 300 nM nocodazole completely stopped cell locomotion. Although 100 nM nocodazole decreased locomotion over 60%, we detected no qualitative change in MT distribution by immunofluorescence. Quantitative analysis of MT fluorescence in immunofluorescently stained preparations showed that 100 nM nocodazole had no detectable effect on MT levels and that 300 nM nocodazole only decreased MT levels to ~40% of controls. Quantitative analysis of tubulin polymer levels by cell extraction and western blotting yielded results similar to those obtained by quantification of MT fluorescence. A comparison of the locomotion rate measurements with the MT level measurements indicated that over half of the cell locomotion rate could be blocked by nocodazole without significantly affecting MT levels in the cell; the remaining locomotion rate was reduced proportionally to MT levels. These results do not support the notion that a critical level of MTs is required for cell locomotion and suggest that only a portion (<50%) of the speed of the cells is proportional to MT levels. Rather, by analogy with studies of MT antagonists on the mitotic spindle, they suggest a third possibility: that low concentrations of nocodazole interfere with MT dynamics and thus, MT dynamics are critical for the maximal speed of cell locomotion. This notion was further supported by analogous effects of taxol and vinblastine on cell locomotion: at concentrations that reportedly cause little change in the level of MTs, taxol and vinblastine also dramatically decreased the rate of locomotion of NRK cells. In summary, our results establish the relationship between microtubule levels and locomotion rate and suggest that dynamic MTs are rate-limiting for fibroblast locomotion.

Key words: cell motility, wound healing, cytoskeleton, video microscopy, timelapse recording, taxol, vinblastine

INTRODUCTION

Microtubules (MTs) are major components of the cytoskeleton that are present in nearly every eukaryotic cell. Previous studies have found that MTs play a role in the directed cell locomotion of some, but not all cell types. Intact arrays of MTs are clearly necessary for the directed migration of larger cells such as fibroblasts (Vasiliev et al., 1970; Goldman, 1971; Gail and Boone, 1971), endothelial cells (Gotlieb et al., 1983), monocytes (Zakhireh and Malech, 1980), and nerve growth cones (Bamburg et al., 1986). However, in some smaller cells such as fish keratocytes (Euteneuer and Schliwa, 1986) or neutrophils (Zigmond et al., 1981), MTs do not seem to be immediately necessary for locomotion, since complete breakdown of MTs does not affect the short term motility of the cells. From these studies, it seems unlikely that intact MTs are necessary for producing the driving force for motility. Nonetheless, the essential requirement for intact MTs in locomotion of large cells suggests that MTs do play an important role. The correlation between cell size and MT dependence of cell locomotion (Schliwa and Honer, 1993) suggests that MTs may become essential in organizing or contributing to the motile apparatus of the cell as it becomes larger.

Previous studies on how MTs contribute to cell locomotion
were done almost exclusively by MT ‘knock out’ experiments in which complete MT breakdown was induced by high concentrations of MT antagonists. Breakdown of the bulk of the MTs results in the loss of polarization of the cell as assessed by the presence of ruffling around the entire circumference of the cell rather than in a localized area (Vasiliev et al., 1970; Goldman, 1971). These results are consistent with the idea that without MTs, the cell is incapable of polarizing its actin activity and consequently attempts to go in every direction at once (Vasiliev, 1991). Similar conclusions were drawn from experiments using epithelial cells in which the effect of MT antagonists was to cause the misexpression of actin activity (in this case microvilli rather than ruffles) on basolateral surfaces of the cell where they are not normally assembled (Achler et al., 1989). At this level, one function of MTs in directed cell locomotion seems to be the establishment and maintenance of cell polarity, so that the cell can utilize its actin-based driving force to locomote itself in a unidirectional manner.

The studies described above have explained the role of MTs in cell locomotion by emphasizing the depolarizing effect that depletion of MTs has on actin-based activity. This has been a useful idea for considering the role of MTs in cell locomotion, however, it does not suggest a molecular mechanism by which MTs contribute to cell motility. It also cannot explain the ability of MT antagonists to completely block locomotion of cells at the edge of a wound (see Gotlieb et al., 1983; also this study). In such cells, actin activity is naturally constrained to the portion of the cell not in contact with surrounding cells. If MT antagonists redistribute the actin activity that is polarized at the front of such cells, it should lead to a reduction in the speed of cell locomotion but not a complete inhibition, since some actin activity would still be found at the front of the cells. Indeed, measurements of the effect of MT antagonists on the membrane ruffling of wound-edge fibroblasts have shown that ruffling is decreased 4-6 fold over that in controls, but it is not reduced to zero (Bershadsky et al., 1991).

Other explanations for MT function in cell locomotion suggest that MTs may polarize the delivery of membrane proteins to the leading edge. This hypothesis is supported by numerous studies which have shown that locomoting cells have a propensity for reorienting their microtubule organizing center (MTOC) to a position between the nucleus and the leading edge of the cell (Gotlieb et al., 1981, 1983; Kupfer et al., 1982; Albrecht-Buehler and Bushnell, 1979; Gundersen and Bulinski, 1988). Since the Golgi apparatus is localized at or near the MTOC in most cells, the reorientation of the MTOC also reorients the Golgi apparatus in the cell (Kupfer et al., 1982; Nemere et al., 1985). Protein products from the Golgi are envisioned to travel along MT tracks before insertion into the leading edge. For migrating cells with an oriented MTOC, this would result in the preferential delivery of membrane proteins to the leading edge. Evidence for the polarized insertion of membrane proteins by a MT-dependent mechanism has been obtained in a series of elegant studies using the ts mutant of the G coat protein of VSV virus as a marker for newly delivered membrane glycoproteins (Bergmann et al., 1983; Rogalski and Singer, 1984). Given the preferential orientation of stable, detyrosinated MTs towards the leading edge of wound-edge fibroblasts, it is possible that the delivery of Golgi products could occur on specialized MT tracks (Gundersen and Bulinski, 1988; Nagasaki et al., 1992).

The hypothesis that MTs may be involved in delivering membrane components to the leading edge is supported by substantial structural data. Yet, despite some effort, there are a number of features of this model that have not been demonstrated. One concern is that it is not particularly clear that newly synthesized membrane is necessary for cell locomotion; it is possible that membraneous precursors may come from recycled or stored sources (Erickson and Trinkaus, 1976). Also, studies have failed to detect a population of vesicles near the leading edge that might be candidates for the Golgi-derived insertion vesicles. Other studies designed to measure a polarized ‘flow’ of membrane material rearward from the leading edge, as would be predicted if components were being inserted selectively at the leading edge, have generally failed to produce evidence that such a flow exists (Holfiels et al., 1990; Sheetz et al., 1989; Lee et al., 1990). Finally, additional ideas need to be added to the polarized membrane insertion model to account for the clear effect that MT depolymerization has on polarized actin activity, an activity which is clearly important for locomotion.

Is the role of MTs in cell locomotion explained by the idea that MTs serve as tracks for vesicle delivery? Or is this role instead explained by the ability of MTs to establish intracellular polarity, a function that may require a critical level of MTs? Such questions could be addressed by determining the relationship between MT levels and the rate of cell locomotion. If there is a positive correlation between the MT level and the rate of cell locomotion, it would suggest that MTs play a direct role in determining the migration rate. Such a role would be consistent with the notion that MTs serve in the transport of rate-limiting materials to the leading edge. An alternative hypothesis is that there is a particular level of MTs above which, but not below which, cells can migrate. This ‘critical level’ of MTs may be important for the gross organization and/or maintenance of cellular elements or organelles necessary for the locomotion of a cell. For example, this might include polarizing actin activity (Vasiliev, 1991) or clustering of the Golgi apparatus so that a functional polarity is maintained (Kreis, 1990; Singer and Kupfer, 1986).

In this study, we have determined the relationship between cellular MT levels and the rate of fibroblast locomotion. To generate cells with varying levels of MTs, we treated wounded monolayers of NRK fibroblasts with nocodazole, a MT depolymerizing agent, over a range of concentrations. We then determined the rate of cell locomotion and quantified the MT levels in these cells. Our results show that there are two components to the inhibition of cell locomotion by nocodazole: at low concentrations of nocodazole, ~60% of the cell locomotion can be inhibited without any detectable change in the level of MTs; at higher concentrations of nocodazole, the rate of locomotion appears to be correlated with MT level. We found no evidence for a particular critical level of MTs necessary for cell locomotion. Given the likely effects of low doses of nocodazole on MT dynamics (Wilson et al., 1993; Wilson and Jordan, 1994), our results suggest that much of the speed of cell locomotion is related to the dynamics of MTs, not to the MT level. In further experiments, we show that low concentrations of taxol, which stabilize MTs, also inhibited cell locomotion, supporting the idea that dynamic MTs play an important role in fibroblast locomotion. We discuss possible mechanisms by which MTs may contribute to the regulation of cell locomotion rate.
MATERIALS AND METHODS

Reagents
Nocodazole was purchased from Aldrich (Milwaukee, WI); vinblastine, saponin, casein and horse plasma-derived serum (PDS) were purchased from Sigma (St Louis, MO); taxol was a generous gift from Dr Ven L. Narayanan (National Cancer Institute, Bethesda, MD). Nocodazole, vinblastine and taxol were prepared as stock solutions in DMSO. The final concentration of DMSO in cell culture medium never exceeded 0.1% (v/v), and this concentration of DMSO had no effect on NRK cell locomotion or MT levels.

Cell culture
NRK fibroblasts were maintained in DMEM with 10% calf serum as described previously (Nagasaki et al., 1994), and for experiments, cells were seeded on acid-washed glass coverslips (Fisher Scientific, 25 mm diameter for timelapse recording, 18 mm for immunofluorescence). For preparation of MT polymer fractions, cells were plated in 35 mm tissue culture dishes. For all experimental manipulations, cells were incubated for two days to obtain confluent monolayers. ‘Wounds’ were then made by scraping narrow strips of cells from the monolayer with a jeweler’s screwdriver. Medium was immediately removed, and the wounded monolayer of cells was rinsed twice with the appropriate fresh medium before proceeding with timelapse recording, indirect immunofluorescence, or preparation of MT polymer fractions, as described below. For determination of MT levels by immunofluorescence, cells were extracted with 37°C PHEM (60 mM Pipes, 25 mM Hepes, 1 mM EGTA, 2 mM MgCl₂, pH 6.95) containing 0.5 mg/ml saponin and 5 mg/ml casein (dialyzed against PHEM) for 1 minute to remove the monomeric tubulin generated by MT depolymerization drugs. One minute was found to be the minimum time necessary to remove all the monomeric tubulin. We found that inclusion of casein in the extraction buffer reduced the background staining when cells were stained as described below. Immediately after extraction, cells were rinsed once in the buffer and fixed in –20°C methanol for 5 minutes. Methanol-fixed cells were rehydrated in Tris-buffered saline for indirect immunofluorescence (see below).

Timelapse recording of cell locomotion
Timelapse recording was carried out to monitor the effect of MT drugs on the locomotion of NRK cells. A confluent monolayer of NRK cells was wounded with a jeweler’s screwdriver, and rinsed several times with recording medium consisting of DMEM containing 25 mM was wounded with a jeweler’s screw driver, and rinsed several times on the locomotion of NRK cells. A confluent monolayer of NRK cells. Timelapse recording was carried out to monitor the effect of MT drugs (see below).

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For measurement of the rate of cell locomotion, we determined the rate of advance of the wound edge using the ‘track points’ function of an Image-1 image analysis program (Universal Imaging Co., West Chester, PA) as previously described (Nagasaki et al., 1994). The average position of the wound edge was determined from the recordings using images spaced 20 minutes apart. The rate of locomotion before and after switching to medium containing drugs or devoid of drugs (for controls), was determined by a linear regression analysis using the interval between 60 and 0 minutes before the treatment, and the interval between 0 and 120 minutes after the treatment, respectively.

Indirect immunofluorescence
Indirect immunofluorescence of fixed cells was performed with a mouse monoclonal antibody (3F3, generously provided by Dr J. Lessard, University of Cincinnati) reactive to all β-tubulin isoforms and a rhodamine-conjugated donkey anti-mouse secondary antibody (Jackson ImmunoResearch, West Grove, PA) as described (Khwaja et al., 1988).

Quantification of MT levels by fluorescence microscopy
Immunofluorescently-stained NRK cells were observed with a Nikon Optiphot epifluorescence microscope using a 63× objective lens (Zeiss, Planapo Chromat, NA 1.40) and a zoom lens (Nikon 0.9-2.25x) at 2.25× to further magnify the image sent to the video camera. Images of cells at the wound edge were captured with a SIT camera (mti-65, Dage-MTI Inc. Precision video, Michigan City, IN) with manual gain and black level controls, adjusted so that background fluorescence was minimal and MTs were the brightest objects in the field. Once the gain and black levels of the SIT camera were optimized, they were not changed for a series of measurements. Images were captured and digitized with Image-1 and stored on a computer optical drive (Panasonic). Fluorescence intensity was quantified using the ‘area brightness measurement’ function of Image-1. A rectangular bar (96 pixels × 1 pixel) was overlaid on cells of interest in such a way that the bar was parallel with the leading edge. With this orientation, virtually all MTs were perpendicular to the long dimension of the bar, thus ensuring that individual MTs cross the bar rather than run along its length. For each measurement, the average pixel intensity (API) was recorded for the bar-covered area in the cell, and the API of the background was measured in a region adjacent to the cell. The background API was subtracted from the API of the corresponding MT measurement. For each nocodazole treatment, the API of the bar-covered area after background subtraction was determined for about 50 cells and the mean was calculated. Standard deviation of these measurements fall between 12 and 20% of the mean fluorescence intensity. For each series of experiments, mean MT fluorescence intensity of nocodazole-treated cells was normalized to that in untreated control cells. As described in the results, we found that making measurements in the leading lamella of cells at 60% of the distance from the nucleus to the leading edge where fluorescence intensity was not saturating was most representative of total MT levels.

Preparation and quantification of MT polymer levels
MT polymer fractions from control and nocodazole-treated cells were prepared and analyzed by SDS-PAGE and western blotting. A 35 mm dish of confluent NRK cells was used for each sample. The confluent monolayers of cells were wounded as described above (15 wounds were made on each dish). After wounding, cells were incubated at 37°C for 1 hour in DMEM (Gibco) supplemented with penicillin, streptomycin, 10 mM Hepes (pH 7.4) and 10% PDS before addition of different doses of nocodazole. The incubation with added nocodazole was for one hour, and then the cells were rinsed once in PEM (100 mM Pipes, 10 mM EGTA and 2 mM MgCl₂, pH 7.0), extracted with PEM containing 0.5 mg/ml saponin for 1 minute, and rinsed twice in PEM buffer (all at 37°C). After thoroughly removing the last rinse, the cells were solubilized in SDS sample buffer containing phenylmethylsulfonyl fluoride. The protein concentration of the MT
polymer fractions was determined with the BCA assay, using BSA as standard (Pierce, Rockford, IL). An equal amount of protein (10 μg) from each sample was run on SDS-PAGE with tubulin standards and then western-blotted as described (Gundersen et al., 1994).

Blots were incubated in monoclonal antibody (3F3) to β-tubulin as described by Gundersen et al. (1994), except that 1/7,500 alkaline phosphatase-conjugated goat anti-mouse IgG was used as the secondary antibody. After development with 5-bromo-4-chloro-3-indolylphosphate and p-nitro-tetrazolium blue, blots were dried in the dark and imaged with a Newvicon video camera (DAGE-mti, 70 series). After digitization and background subtraction with Image-1, the density of the bands was quantified with the ‘area brightness measurement’ function of the Image-1 program. The tubulin standard curve obtained by this method was linear between the range of 25 ng and 200 ng of tubulin, and experimental samples were loaded so that the tubulin levels fell within this range.

RESULTS

Low concentrations of nocodazole decrease the rate of locomotion of NRK fibroblasts

We made timelapse recordings to examine the effects of nocodazole on NRK cells migrating into a wound. Before recording, we wounded confluent monolayers of NRK cells (see Materials and Methods) to obtain a uniform population of motile cells. The cells at the wound margin began migration into the wound almost immediately after the wounding and then maintained a constant rate of locomotion. We found that this rate remained constant for several hours (see Nagasaki et al., 1994). Because these wound-edge cells are only capable of migrating in one direction, our study is focused only on the directed translocation activity of cells rather than the random movement of sparse cells, which we found to be intermittent in both duration and direction of movement (unpublished observations).

In a series of experiments, we determined the rate of locomotion before and after different concentrations of nocodazole (25 nM to 400 nM) were perfused into cells in a Sykes-Moore chamber. Fig. 1 shows an example of a timelapse recording of cells migrating into the wound before and after treatment with nocodazole (in this case, 400 nM). As can be seen from the timelapse sequence (Fig. 1), addition of 400 nM nocodazole resulted in a complete inhibition of cell locomotion that was maintained throughout the nocodazole treatment (≥2 hours). If nocodazole was subsequently removed from the medium, cells resumed their migration at about the same rate as before nocodazole addition, indicating that nocodazole did not have a permanent effect on cell locomotion or have any cytotoxic effects on the cells.

A plot of the distance migrated vs time after wounding is shown in Fig. 2 for another experiment (with 300 nM nocodazole). Such a plot shows two significant aspects of the effect of nocodazole on the locomoting cells. One is that the inhibition of locomotion by nocodazole occurred rapidly; within a few minutes of addition of nocodazole, the cells have adopted a new rate of locomotion. The second important result is that the rate of locomotion was constant before, during, and after...
the nocodazole treatments. We found that the rate of locomotion analyzed by linear regression had a correlation coefficient that varied by less than 5% during these intervals. Accordingly, we used a single rate to represent the cell locomotion over the treatment intervals.

In reporting the effect of nocodazole on cell locomotion, we expressed the rate of locomotion as the percentage of the control rate determined in each experiment. This control value was the rate of locomotion in the 60 minutes prior to addition of nocodazole and was determined for each recording. Control values for the rate of locomotion in different experiments were 24.7±3.9 μm/hr (n=21). Nocodazole treatment resulted in a dose-dependent decrease in the rate of locomotion (Fig. 3). We detected a significant inhibition of cell locomotion with concentrations of nocodazole as low as 25 nM (74.4±7.2%, n=2) and the inhibition was more than 60% with 100 nM nocodazole (39.9±4.2%, n=3). Cells stopped forward movement completely after the addition of ≥300 nM nocodazole. Since we perfused cells at a higher rate during nocodazole addition, we checked to see if perfusion of medium without nocodazole (mock treatment) affected cell locomotion. Cells after mock treatment showed only a slight decrease in the locomotion rate (93.6±1.9%, n=3; Fig. 3) and this difference was not statistically significant (P=0.34, Student’s t-test).

To determine if the effect of nocodazole on cell locomotion was attributable to its action on MTs rather than some other action of nocodazole, we tested the effect of low concentrations of vinblastine on NRK cell locomotion in an identical manner. Vinblastine is another MT antagonist that has a completely different structure from nocodazole (reviewed by Dustin, 1984). Previous studies have shown that vinblastine induces depolymerization of MTs and inhibition of cell proliferation at concentrations substantially lower than those of nocodazole (Jordan et al., 1992). We found that vinblastine at a concentration of 5.5 nM reduced the rate of NRK cell locomotion by 78% (data not shown). This supports the idea that nocodazole affects cell locomotion through its action on MTs. Further evidence supporting this conclusion is presented below.

Effects of nocodazole on the level of MTs in NRK cells

Nocodazole has long been established as a MT antagonist which depolymerizes MTs by blocking MT assembly (Hoebeker et al., 1976). More recent in vitro studies have shown that nocodazole decreases the MT dynamic instability parameters of growth rate and shortening rate, and increases the time MTs spend in pause (neither growing nor shrinking) (Wilson et al., 1993; Wilson and Jordan, 1994). To determine the effect of different concentrations of nocodazole on MT levels, we undertook three different approaches: visual inspection of cells immunofluorescently stained with anti-tubulin antibody; determination of MT levels by quantitative measurement of fluorescence in immunofluorescently-stained preparations; and quantification of tubulin polymer levels by western blot analysis.

Incubation of cells with 100 nM nocodazole, which reduced cell locomotion more than 60%, did not result in any obvious qualitative change in the level or distribution of MTs in cells at the wound edge. As assessed by immunofluorescence in controls, as well as in cells treated with 100 nM nocodazole (for 1 hour), MTs appear to fill the cytoplasm and extend virtually the entire distance to the leading edge of the cell (Fig. 4a and b). The close approach of the MTs to the leading edge in both controls and 100 nM nocodazole-treated cells is shown for representative cells in Fig. 5. By comparing the MT distributions in the immunofluorescence images (Fig. 5a and c) with the phase images of the leading edges (Fig. 5b and d, respectively), it is clear that a number of MTs in both types of cells closely approach the limiting membrane of the cell edge. In cells treated with 200 nM or 300 nM nocodazole (Fig. 4c and
d), MTs were still detected but they appeared fewer in number and were shorter than in controls. Cells treated with $\geq 200$ nM nocodazole were also less extended than control cells, which may be related to the presence of shorter MTs. We did not observe obvious variations in MT levels during the course of the nocodazole treatment (0.5 hours to 2 hours) (data not shown), so throughout our study, we measured MT levels in cells after 1 hour nocodazole treatment.

At the level of resolution afforded by light microscopy, these observations suggest that low concentrations of nocodazole ($\leq 100$ nM), which were effective in inhibiting cell locomotion (Fig. 3), did not have a significant effect on the distribution of MTs in NRK cells. However, quantitative information concerning the level of MTs in cells at the wound edge after such treatments cannot be obtained from visual inspection of stained MTs. To acquire quantitative data, we have developed a novel microscopic assay that is based on measuring the fluorescence intensity of MTs in cells that have been immunofluorescently stained with tubulin antibodies (see Materials and Methods). Briefly, images of immunofluorescently stained cells obtained with a SIT camera were digitized, and the MT level was quantified using the ‘area brightness measurement’ function of the Image-1 program. As described below, by paying attention to the cellular location at which the measurement is made, we have been able to derive quantitative values that reflect the levels of MTs in cells.

Because of the radial distribution of MTs in cultured cells, an important consideration in attempting to sample the MT fluorescence in stained cells is that MT density is dependent on the distance from the centrosome. In the extracted cells that we used for measurements, the MT array is constrained to essentially two-dimensions so the MT density would fall off as the

**Fig. 4.** Distribution of MTs in motile NRK cells after incubation with different concentrations of nocodazole. Monolayers of NRK cells were wounded and after a recovery period of 1 hour, were further incubated either in the absence or presence of nocodazole for 1 hour. At the end of the incubation, cells were extracted, fixed with methanol and processed for tubulin immunofluorescence staining. (a) Control cells without nocodazole treatment; (b,c,d) cells treated with 100, 200, and 300 nM nocodazole, respectively. Bar, 20 $\mu$m.
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square of the distance from the centrosome. Accordingly, we made all our measurements of fluorescence intensity with reference to the distance between the centrosome and the leading edge. To determine the optimal distance from the centrosome at which to make our measurements, we considered two factors: (1) as one moves towards the centrosome, the density of MTs increases and at some point, MTs overlap spatially and cannot be resolved by the optical system and/or saturate the detector; and (2) the measurement should be made as close as possible to the centrosome to include the greatest number of MTs, which increases the sensitivity and precision of the measurement.

With these criteria in mind, we first established the relationship between MT fluorescence intensity and the distance from nucleus. The fluorescence intensity within a 1×96 pixel bar (this bar was 1/3-3/4 the width of the cells at the magnification used; see Materials and Methods) was recorded at every pixel along the shortest line from the nucleus to the leading edge (phase was used to detect the leading edge). The fluorescence intensity was plotted against both the absolute and relative distance from the nucleus (Fig. 6). As expected for a radial-based array of MTs, the fluorescence intensity decreased with increased distances from the nucleus. Yet because of difference in cell length, the fluorescence intensity decreased at different absolute distances from the nucleus (Fig. 6a). However, when the data were plotted as fluorescence intensity vs relative distance from the nucleus, despite differences in the shape, size and length of cells, the traces fell within a relatively narrow range of values and showed a similar overall shape (Fig. 6b). This shows that the fluorescence intensity of MTs in a particular region of wound-edge cells was predictably related to the relative distance from the nucleus. This observation was very important to our analysis; it suggested that by making measurements of fluorescence intensity in different cells at the same relative distance from the nucleus, we would be able to obtain values that accurately represent the MT levels in the cells. The curve of fluorescence intensity vs relative distance seemed to saturate at distances ≤50% of the distance from the

Fig. 5. Distribution of MTs in the leading edge of motile NRK cells. NRK cells were wounded, incubated with or without 100 nM nocodazole, extracted, fixed and immunofluorescently stained for tubulin as in the legend of Fig. 4. Shown here are the immunofluorescence images of MTs (a and c) and the corresponding phase contrast images of the leading edge (b and d) of representative cells. (a and b) Control cells; (c and d) cells treated with 100 nM nocodazole. Bar, 5 μm.

Fig. 6. Fluorescence intensity vs distance from the nucleus in wound-edge cells immunofluorescently-stained for tubulin. Cells were fixed and stained with an antibody to β-tubulin. Measurements of fluorescence intensity in individual cells were made along a line from the nucleus to the leading edge (see text). Shown here are individual measurements of 7 cells of different size, shape and length. (a) Tubulin fluorescence intensity vs the absolute distance from the nucleus to leading edge. (b) Tubulin fluorescence intensity replotted vs the relative distance from the nucleus to leading edge, with the relative distance of the nucleus edge defined as 0 and the leading edge defined as 100.
nucleus to the cell edge (Fig. 6b). Accordingly, we made all our measurements at a point 60% of the distance to the leading edge.

The results of quantitative measurements of MT fluorescence in wound-edge cells treated with various concentrations of nocodazole are shown in Fig. 7. The MT fluorescence intensity determined for cells after treatment with ≤100 nM nocodazole was indistinguishable from that of control cells (P=0.723 for 100 nM nocodazole treated cells, Student’s t-test). Cells showed reduced levels of MT fluorescence with concentrations of nocodazole at ≥150 nM, and with 200 nM nocodazole, cells showed significantly reduced levels of MTs (P<0.0001). Nonetheless, with 300 nM nocodazole, the level of nocodazole that can completely block cell locomotion (see Fig. 3), cells still retained ~40% of their MT fluorescence. With 10 μM nocodazole for 1 hour, a treatment that completely depolymerizes MTs (see below), the cells had a MT fluorescence intensity of 9.9% of that in control cells, indicating that the background fluorescence intensity for this microscopic assay is about 10%.

The above measurements were made at a set distance from the nucleus. When we checked the fluorescence intensity vs relative distance for the entire length from the nucleus to the leading edge for cells treated with nocodazole, we found that an averaged trace for cells treated with 100 nM nocodazole, was virtually indistinguishable from that for untreated cells (traces from seven cells were summed and averaged for this comparison). For cells treated with 300 nM nocodazole, the shape of the averaged trace was similar to the control curve, but the curve was shifted to the left (indicative of lower MT levels). Within the limitations of this analysis, these results confirm that our measurements made at 60% of the distance from the nucleus to the leading edge, reflected the levels of MTs throughout the leading edge for untreated cells, and cells treated with nocodazole.

To measure MT levels with a separate technique, we performed a western blot analysis of tubulin levels in polymer fractions prepared from wounded cells treated with different concentrations of nocodazole. To quantify the absolute levels of MT polymer in these cells, we compared the density of the bands in immunoblots to that of different amounts of a purified brain tubulin standard loaded on the same blot. Such a quantitative analysis of MT polymer in cells treated with different amounts of nocodazole is summarized in Table 1. This analysis confirmed quantitatively that the MT polymer level showed no detectable change with 100 nM nocodazole. For concentrations above this, decreased polymer was detected, analogous to the fluorescence intensity results obtained microscopically. At 10 μM nocodazole, a concentration that effectively depolymerizes virtually all MTs in the cells as detected by immunofluorescence (not shown), we measured no MT polymer, confirming that the assay was dependent on the presence of polymeric MTs and that complete extraction of the monomeric tubulin was achieved by the protocol we used. A point-by-point comparison of the fluorescence and western blotting assays showed that for any given nocodazole concentration, the two measurements yielded values that agreed within ~10% (Fig. 7 and Table 1).

### Relationship between the levels of MTs and the rate of NRK cell locomotion

The major goal of this study was to explore the role of MTs in cell locomotion by determining the relationship between the levels of MTs and the speed of NRK cell locomotion. We have replotted the rates of NRK cell locomotion determined at different concentrations of nocodazole against the corresponding MT fluorescence intensities determined after similar treatments with nocodazole. As shown in Fig. 8, for low nocodazole concentrations, the rate of directed locomotion of NRK cells was dramatically decreased (up to 60%) even though little MT depolymerization was detected. Yet, further decreases in the rate of locomotion (at higher nocodazole concentrations) did show a close correlation between MT levels and the remaining ~40% of the rate of locomotion (Fig. 8). These results suggest that the contribution of MTs to cell locomotion can be separated into two components: one that is not dependent on MT level and one that is.

<table>
<thead>
<tr>
<th>Nocodazole (μM)</th>
<th>Tubulin (% of control, mean ± s.d.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>0.1</td>
<td>99.1±10.6</td>
</tr>
<tr>
<td>0.2</td>
<td>62.6±10.2</td>
</tr>
<tr>
<td>0.3</td>
<td>48.1±16.7</td>
</tr>
<tr>
<td>0.4</td>
<td>45.2±2.2</td>
</tr>
<tr>
<td>10</td>
<td>(~5.9)±5.1</td>
</tr>
</tbody>
</table>

 Quantitation of MT levels in cells after treatment with indicated concentrations of nocodazole was carried out by western blot analysis as described in Materials and Methods. The same amount of protein (10 μg) from each sample was loaded on SDS-polyacrylamide gels. The calculated amount of tubulin in samples was normalized to that in the controls. Data shown is obtained from the average of 2 to 4 separate experiments.
induced by high concentrations of MT antagonists. In this on experiments in which the complete breakdown of MTs was Kupler, 1986; Vasiliev, 1991). These ideas are based in part and maintenance of cell polarity (reviewed by Singer and motion have emphasized the role of MTs in the establishment Current models for the function of MTs in directed cell loco-

**DISCUSSION**

**Relationship between MT level and cell locomotion**

Current models for the function of MTs in directed cell locomotion have emphasized the role of MTs in the establishment and maintenance of cell polarity (reviewed by Singer and Kupler, 1986; Vasiliev, 1991). These ideas are based in part on experiments in which the complete breakdown of MTs was induced by high concentrations of MT antagonists. In this study, we set out to extend these hypotheses by testing two ideas which could explain the involvement of MTs in cell locomotion, namely, whether there is a positive correlation between the MT level and the rate of cell locomotion, or whether there is a critical level of MTs required for cell locomotion. To address these questions, we established an experimental system in which we could use different concentrations of nocodazole to alter the level of MTs in the cell. With this system, we determined the relationship between the levels of MTs and the rate of locomotion in NRK fibroblasts (Fig. 8). Surprisingly, we observed that with low concentrations of nocodazole, inhibition of NRK cell locomotion (60%) occurred before significant changes in MT levels were induced. Moreover, there seemed to be a close correlation between the MT levels and the remaining 40% of the rate of cell locomotion (Fig. 8). These results do not support the notion that there is a critical level of MTs required for cell locomotion. In contrast, they suggest that MTs may be involved in more than one process related to cell locomotion (one unrelated to MT level and one related to MT level). There is reason to think that the inhibition of cell locomotion at nocodazole concentrations that do not significantly affect MT polymer levels is due to nocodazole effects on MT dynamics (see below for more discussion), so these results suggest that MT dynamics are an important element in fibroblast locomotion.

**Measurements of MT levels in cells**

The level of MTs in cells treated with nocodazole was quantitated by two independent approaches: quantitation of immuno-fluorescence images using digital video microscopy and quantitative western blot analysis. When we analyzed the cells that had been treated with 100 nM nocodazole, both the microscopic method and the biochemical method showed that the MT level in these cells did not change significantly from that in untreated control cells. We cannot, however, rule out the
possibility that there were subtle changes in the MT level in cells treated with 100 nM nocodazole, since the error in determining MT levels by either method is in the range of ±15%. Nonetheless, even if 100 nM nocodazole decreased the MT levels by 15%, the rate of locomotion was decreased by a greater proportional amount (~60%). Thus, based on these measurements, it is unlikely that the highest speed of NRK fibroblast locomotion is directly related to MT levels.

**Mechanism of inhibition of fibroblast locomotion by low concentrations of nocodazole**

There are several mechanisms that could account for the inhibitory effect of low concentrations of nocodazole on cell locomotion in the absence of detectable effects on MT levels. These include: (1) non-specific effects of nocodazole; (2) a subpopulation of MTs sensitive to nocodazole and important for cell locomotion; (3) subtle changes in MT length; or (4) altered MT dynamics. The first possibility is unlikely since low concentrations of vinblastine, another MT depolymerizing agent, also inhibited NRK cell locomotion, implying that nocodazole affects cell locomotion most probably through its action on MTs rather than through some unique, unknown effect of nocodazole. This idea is also made unlikely by our results with taxol. The second possibility can be envisaged if there were a subset of MTs that was particularly sensitive to nocodazole and was particularly important for cell locomotion. However, this mechanism cannot explain the observation that low concentrations of taxol, an agent with well-characterized MT-stabilizing effects (Schiff et al., 1979), also inhibited locomotion. The possibility that low concentrations of nocodazole decreased MT lengths without causing reduction in MT levels is a viable possibility. However, any shortening of MTs would have to be subtle, otherwise we would have detected it as an obvious decrease in the approach of MTs to the leading edge; in both control and 100 nM nocodazole-treated cells, we observed MTs to contact the leading edge of the cell (see Fig. 5). These results suggest that the fourth mechanism, i.e. dampened MT dynamics, is the most likely explanation for the decreased locomotion by low concentrations of nocodazole. This mechanism is supported by recent studies which have shown that MT drugs such as nocodazole, vinblastine and taxol decrease the dynamic instability of MTs in vitro (Jordan et al., 1993; Wilson et al., 1993; Wilson and Jordan, 1994; Toso et al., 1993; Derry et al., 1995) and that vinblastine affects MT dynamics in vivo (Iyengar et al., 1993; Tanaka and Kirschner, 1995). Moreover, when used at low concentrations in vivo, these MT drugs interfere with the mitotic spindle in HeLa cells without affecting the MT polymer level (Jordan et al., 1992, 1993). Tanaka et al. (1995), showed that growth cone motility was decreased with concentrations of vinblastine that were confirmed to decrease MT dynamics, although they did not measure the effect of vinblastine on MT levels. We are currently measuring the effects of low concentrations of nocodazole on the parameters of dynamic instability of MTs in vivo.

**How can MTs regulate cell locomotion rate?**

Our data suggest that the dynamics of MTs could regulate the locomotion of NRK cells. We showed that low concentrations of nocodazole inhibited NRK cell locomotion without significantly affecting the level of MTs. The inhibition of locomotion by low concentrations of another MT-depolymerizing drug (vinblastine) and a MT-stabilizing drug (taxol) is consistent with the nocodazole data. The idea that MT dynamics are critical for cell locomotion is not adequately accounted for by models which propose that MTs serve as tracks for delivering membrane components to the leading edge or that MTs polarize actin activity (see Introduction). We hypothesize that dynamic MTs contribute to cell locomotion by interacting with transient structures or assemblies (‘targets’), whose stabilization or maturation is rate-limiting for cell locomotion. Without interaction with MTs, the transient structures are not stabilized or matured and cannot contribute to the advancement of the cell. By dampening MT dynamics, nocodazole (and taxol) would decrease the chance of MTs hitting their targets and would thus inhibit locomotion. At higher concentrations, nocodazole may also limit access of the MTs to their putative targets by decreasing the length of the MTs.

While it is hard to know what targets the dynamic MTs might interact with without further experiments, three possibilities are worth considering: (1) lamellipodial protrusions that advance the front of the cell; (2) nascent focal adhesion plaques that provide traction for forward movement; and (3) putative MT-stabilizing sites. Both lamellipodial protrusions and focal adhesion plaques form transiently at the front of the cell and are necessary for fibroblast locomotion. The maturation and/or maintenance of these structures might be essential for cell locomotion and MTs might provide the necessary maturing or stabilizing effect on them. Another possible target for dynamic MTs are putative factors involved in MT stabilization. According to this idea, it is not the structures that the dynamic MTs interact with that are important for locomotion, but the effect this interaction has on the MTs themselves. Once stable MTs are formed by interacting with the target, they might serve an important function for locomotion, e.g. as preferred tracks for the transport of vesicles to the leading edge. Stable, detyrosinated MTs in motile fibroblasts have been shown to be preferentially oriented towards the direction of locomotion (Gundersen and Bulinski, 1988; Nagasaki et al., 1992). In other studies, we have found that stable, detyrosinated MTs are the preferred sites of interaction for other cellular organelles such as intermediate filaments (G. Gurland and G. G. Gundersen, unpublished). While these data support a role for stable MTs in cell locomotion, this idea still needs to be tested directly.

Our results suggest that dynamic MTs are necessary for fibroblast locomotion; nonetheless, we also observed a strong correlation between the MT level and a portion of the rate of cell locomotion (Fig. 8). This result is consistent with the idea that part of the contribution of MTs to cell locomotion is due to the transport of rate-limiting materials to the leading edge during cell locomotion. However, with the higher concentrations of nocodazole that affect MT polymer levels, it is also conceivable that MT dynamics were further dampened. Even if no further reduction in individual MT dynamics resulted from higher nocodazole concentrations, according to our ‘transient target’ model, a reduction in MT number would lead to a further reduction in MTs interacting with the transient target, resulting in a further decrease in locomotion. Thus, the correlation in MT levels and locomotion rate, which we observed for nocodazole concentrations ≥150 nM, is consistent with a role for MTs in delivering vesicles to the leading edge or in interacting with transient targets.

In summary, we have provided evidence that MTs play an important role in determining the rate of fibroblast locomotion.
Our data establish the relationship between MT levels and locomotion rate and suggest that MT dynamics is a rate-limiting factor in the regulation of fibroblast locomotion. These data point to the notion that MTs may act to interact with transient targets in the leading edge of motile cells and we are currently examining this idea.

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REFERENCES


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