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Vinblastine Suppresses Dynamics of Individual Microtubules in Living Interphase Cells

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We have characterized the effects of vinblastine on the dynamic instability behavior of individual microtubules in living BS-C-1 cells microinjected with rhodamine-labeled tubulin and have found that at low concentrations (3–64 nM), vinblastine potently suppresses dynamic instability without causing net microtubule depolymerization. Vinblastine suppressed the rates of microtubule growth and shortening, and decreased the frequency of transitions from growth or pause to shortening, also called catastrophe. In vinblastine-treated cells, both the average duration of a pause (a state of attenuated dynamics where neither growth nor shortening could be detected) and the percentage of total time spent in pause were significantly increased. Vinblastine potently decreased dynamicity, a measure of the overall dynamic activity of microtubules, reducing this parameter by 75% at 32 nM. The present work, consistent with earlier in vitro studies, demonstrates that vinblastine kinetically caps the ends of microtubules in living cells and supports the hypothesis that the potent chemotherapeutic action of vinblastine as an antitumor drug is suppression of mitotic spindle microtubule dynamics. Further, the results indicate that molecules that bind to microtubule ends can regulate microtubule dynamic behavior in living cells and suggest that endogenous regulators of microtubule dynamics that work by similar mechanisms may exist in living cells.

INTRODUCTION

It is now well established that one form of microtubule dynamics, called dynamic instability (Mitchison and Kirschner, 1984a,b), is fundamentally important in microtubule-mediated cell function. Dynamic instability is characterized by transitions between growing and shortening phases at microtubule ends. This behavior was first described in vitro (Mitchison and Kirschner, 1984a,b; Horio and Hotani, 1986; Walker *et al.*, 1988) and has subsequently been found to occur in living cells (Sammak and Borisy, 1988; Cassimeris *et al.*, 1988; Schulze and Kirschner, 1988; Shelden and Wadsworth, 1993; Dhamodharan and Wadsworth, 1995; Tanaka *et al.*, 1995). Regulation of dynamic instability appears to play an important role in determining the functions of microtubules in cells. For example, the dynamic behavior of microtubules varies throughout the cell cycle (Belmont, 1990; Saxton *et al.*, 1984; Gliksman *et al.*, 1992), with microtubules in mitotic cells exhibiting ~20-fold higher rates of exchange with tubulin in the soluble pool than microtubules in interphase cells. In addition in interphase cells, microtubule dynamics have been shown to be cell-type specific (Wadsworth and McGrail, 1990; Shelden and Wadsworth, 1993)–microtubules in epithelial cells grow and shorten at slower rates and undergo more frequent transitions from shortening to growth or pause (rescue) than microtubules in fibroblasts.

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Molecules that bind to microtubules are likely to play important roles in the regulation of microtubule dynamic behavior (Cassimeris, 1993). Recent experiments indicate that microtubule-associated proteins (MAPs),[1] which bind along the length of the microtubule, modulate microtubule dynamic behavior. For example, brain MAP-2 suppresses dynamic instability in vitro and in vivo (Pryer *et al.*, 1992; Kowalski and Williams, 1993; Toso *et al.*, 1993; Dhamodharan and Wadsworth, 1995) while XMAP, a MAP from *Xenopus* eggs, increases dynamic turnover (Vasquez *et al.*, 1994). MAP-4, a prominent MAP in tissue culture cells, has been shown to stabilize individual microtubules when it is dephosphorylated, but not when phosphorylated (Ookata *et al.*, 1995). Microtubule dynamic behavior may also be regulated by molecules that interact specifically with the microtubule ends. It has been suggested, for example, that factors that interact with the ends of microtubules could increase the frequency of transitions to rapid shortening (catastrophe factors), increasing microtubule turnover during mitosis (Belmont *et al.*, 1990). Recently, vinblastine, which is known to bind to microtubule ends (see below) has been shown to inhibit the persistent forward motion of the growth cone. This data strongly suggests that molecules that bind to the ends of microtubules contribute to the regulation of microtubule behavior in living cells (Tanaka *et al.*, 1995).

Vinblastine is a potent antitumor drug that inhibits cell proliferation at mitosis by acting on spindle microtubules (Jordan *et al.*, 1991, 1992; Wendell *et al.*, 1993). At concentrations at or above approximately 10 μM, vinblastine induces tubulin to assemble into non-microtubule polymers such as vinblastine-tubulin paracrystals or spiral protofilamentous structures (Na and Timasheff, 1982; Jordan *et al.*, 1986; Wilson and Jordan, 1994). At somewhat lower concentrations, vinblastine inhibits microtubule formation and depolymerizes existing microtubules (e.g., ~1 μM in vitro and between 100 nM and 1 μM in HeLa cells; Jordan *et al.*, 1986, 1991). For many years the antiproliferative actions of vinblastine were widely attributed to these two striking phenomena.

However, recent studies in HeLa cells have indicated that very low vinblastine concentrations ($\leq$10 nM) inhibit mitosis and cell proliferation without causing net microtubule depolymerization or inducing paracrystal formation. These studies have indicated that it is not just the presence of microtubules, but the presence of dynamic microtubules that is required for certain aspects of spindle microtubule function. Additional experiments demonstrate that microtubule dynamic behavior in vitro is exquisitely sensitive to low concentrations of vinblastine (reviewed in Wilson and Jordan, 1994). At low concentrations, very small numbers of vinblastine molecules bind directly to relatively high affinity tubulin binding sites at microtubule ends and suppress dynamics while not appreciably decreasing the microtubule polymer mass (Wilson *et al.*, 1982; Jordan and Wilson, 1990). These data indicated that the dynamics of microtubules are more sensitive to vinblastine than the quantity of microtubule polymer that assembles in the presence of the drug. Analysis of the behavior of individual microtubles in vitro further demonstrates that relatively low concentrations of vinblastine (0.2–1 μM) inhibit the growing and shortening rates and strongly increase the percentage of time that microtubules remain in an attenuated state, neither growing nor shortening detectably (Toso *et al.*, 1993). Taken together with results of studies on the action of low vinblastine concentrations on mitotic progression in HeLa cells, the data indicate that low concentrations of vinblastine inhibit mitosis by suppressing the dynamics of spindle microtubules rather than by depolymerizing them (Jordan *et al.*, 1991).

In the present study, we quantitated the actions of low vinblastine concentrations (3–64 nM) on the dynamic instability parameters of individual microtubules in live African green monkey kidney cells (BS-C-1) injected with rhodamine-labeled tubulin and imaged using low-light-level fluorescence microscopy. We also determined the intracellular vinblastine concentrations and correlated the effects of vinblastine on dynamics with its effects on the microtubule polymer mass in isolated cytoskeletons, and on microtubule organization in the vinblastine-treated cells. The results demonstrate that low concentrations of vinblastine suppress microtubule dynamics in living cells without appreciably affecting the polymer mass in a manner that is qualitatively similar to the action of vinblastine on microtubule dynamics in vitro. The results of these experiments demonstrate that vinblastine can be a useful tool to probe the function of microtubule dynamic behavior in living cells and suggest that endogenous regulators of microtubule dynamic behavior may work by mechanisms similar to vinblastine.

MATERIALS AND METHODS

Materials

The chemicals for sodium dodecyl sulfate-polyacrylamide gel electrophoresis and cell culture were purchased from Life Technologies (Bethesda, MD), except for heat-inactivated fetal bovine serum, which was purchased from JRH Biosciences (Lenexa, KA). Antibodies were purchased from Organon Teknica (Malvern, PA). Fluorescently labeled dextran and 5,6, carboxy succinimidyl ester of rhodamine were purchased from Molecular Probes (Eugene, OR). All other chemicals were purchased from Sigma Chemical (St. Louis, MO). Porcine brain tissue was obtained from Adams Farm (Athol, MA) or Outlook Farm (Westhampton, MA).

[1] Abbreviations used: G, growing phase; GDP, guanosine diphosphate; MAPs, microtubule-associated proteins; P, pause phase, state of attenuated dynamics; S, shortening phase.

Cell Culture and Proliferation

African green monkey kidney cells (BS-C-1) (ATCC CCL 26) were grown in DME supplemented with 10% fetal bovine serum and 44 mM $NaHCO_3$ without antibiotics at 37°C under 5% CO_2 for most experiments and in DME with 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, antibiotics, and 10% fetal bovine sera in air for microinjection To determine the effects of vinblastine on cell proliferation, cells were seeded at 1.3×10^4 cells/ml and 2 days later the medium was replaced with medium containing the desired drug concentration. Cells were counted with a hemocytometer at the time of vinblastine addition and 20 h later. Two to four experiments were performed at each vinblastine concentration.

Determination of Microtubule Polymer Mass

For quantitation of microtubule polymer levels, cells were grown in 225 cm^2 flasks to a density of 2×10^4 cells/cm^2 (75–85% confluency). Vinblastine was added to the culture medium 4 h before measurement of the microtubule polymer mass. Cells were collected by trypsinization, lysed in a microtubule-stabilizing buffer consisting of 0.1 M piperazine-*N,N'*-bis(2-ethanesulfonic acid) (Pipes), 1 mM EGTA, 1 mM $MgSO_4$, 30% glycerol, 5% dimethyl sulfoxide, 5 mM GTP, 1 mM dithiothreitol, 1 mM tosyl arginine methyl ester, 0.05 mg/ml aprotinin, 0.02% sodium azide, and 0.125% NP-40, and then centrifuged to collect cytoskeletons. An ELISA was used to quantitate tubulin in the microtubule cytoskeletons (Thrower *et al.*, 1993). ELISA absorbance readings were collected at 30 s intervals for 15 min with a CERES UV 900 microplate reader and Kineticalc software (Biotech Instruments, Winooski, VT).

Determination of the Intracellular Vinblastine Concentration

Cells were seeded directly into polylysine-coated scintillation vials (Research Products International, Mount Prospect, IL) at a density of 8×10^3 cells/ml (2 ml/vial). After 1 day of incubation (37°C) to allow cells to adhere to the glass, the media was replaced with fresh media containing [^{3}H]vinblastine sulfate (Amersham, Arlington Heights, IL) (3–330 nM; specific activity 300–11,000 Ci/mole). Four hours later the vinblastine-containing media was removed and the cells were rapidly washed three times with 100 mM Pipes, 1 mM EGTA, 1 mM $MgSO_4$, pH 6.9 (PEM buffer). Cells were then lysed by addition of distilled water (1 ml) and scintillation fluid (Ready Protein+, Beckman Instruments, Fullerton, CA) and radioactivity was determined. Total cell volume was determined by multiplying the cell number by the mean cell volume. The cell number was determined by counting adherent cells in vials treated identically and in parallel with the vials used to determine radioactivity; cell volume was determined by measuring the diameter of trypsinized, and thus rounded, cells (mean of > 50 cells) with an ocular micrometer. Two independent vinblastine uptake experiments were performed for each vinblastine concentration. Radioactivity was determined in two separate vials for each experiment and the number of adherent cells was determined in two to four vials for each experiment.

Immunofluorescence Microscopy

Cells were fixed in 10% formalin in phosphate-buffered saline (25°C) followed by post fixation in 10% methanol (0°C). Microtubules were visualized with a mouse monoclonal antibody (E7, IG_1; a gift from Dr. Michael Klymkowsky, University of Colorado, Boulder, CO) and fluorescein-conjugated goat anti-mouse IgG as described in Jordan *et al.* (1991). Chromosomes were stained with DAPI (4,6-diamino-2-phenylindole; Sigma Chemical)

Preparation of Rhodamine-labeled Tubulin

Rhodamine-labeled tubulin was prepared by the methods of Vigers *et al.* (1988) and Hyman *et al.* (1991) with slight modifications. Phosphocellulose-purified tubulin was assembled in a buffer consisting of 4 M glycerol, 50 mM Pipes, 1.0 mM EGTA, 10 mM $MgSO_4$, 1 mM GTP at 37°C for 30 min and rhodamine-labeled by incubation for 10 min with a 40-fold molar excess of 5,6, carboxy succinimidyl ester of rhodamine to tubulin. The reaction was quenched with an excess of sodium glutamate. The rhodamine-labeled microtubules were sedimented (200,000 × *g*, 2 h, 25°C) over a 50% glycerol cushion in 50 mM Pipes, 1.0 mM EGTA, 10 mM $MgSO_4$, 1 mM GTP, and 100 mM sodium glutamate and the pellet was subjected to three rounds of temperature-dependent assembly and disassembly in glutamate buffer (1 M sodium glutamate, 0.5 mM $MgSO_4$, 1.0 mM EGTA, 1.0 mM GTP, pH 6.9; Hamel and Lin, 1981) to remove unreacted dye and inactive tubulin. The final microtubule pellet was resuspended in injection buffer (20 mM sodium glutamate, 0.5 mM $MgSO_4$, 1.0 mM EGTA, pH 7.0), centrifuged, and the clarified supernatant was drop frozen as small aliquots in liquid nitrogen and stored at −80°C until use.

Microinjection

Cells were injected with rhodamine-labeled tubulin as previously described (Shelden and Wadsworth, 1992, 1993) and incubated for an additional 3 h at 37°C to allow incorporation of the rhodamine-labeled tubulin into the microtubules. The culture medium was then replaced with fresh medium containing the appropriate concentration of vinblastine, and incubation was continued for an additional 4 h before observation.

Microscopy and Image Acquisition

Microscopy and image acquisition were performed according to the procedures of Dhamodharan and Wadsworth (1995) and Shelden and Wadsworth (1993). BS-C-1 cells were selected for these experiments because they have large, flat peripheral lamellar regions, in which many microtubules can be easily visualized. Microinjected cells were placed in a Rose chamber (Rose *et al.*, 1958) and observed using a Zeiss IM-35 inverted microscope that was maintained at 36 ± 1°C by an air curtain incubator (Nicholson Precision Instruments). Peripheral regions of cells were imaged using a Nikon 100× 1.4 NA objective lens, and images (32 frame averages) were collected at 2 s intervals using a DAGE-ISIT video camera and digitized using a Mass Comp computer. Images and the times they were acquired were stored on a Panasonic optical memory disc recorder (model TQ-2028F).

Analysis of Microtubule Dynamics

Analysis of microtubule dynamics was performed as described in detail by Dhamodharan and Wadsworth (1995). Briefly, microtubules were traced in each image using a mouse-driven cursor and the length changes between successive tracings were determined using software written by Dr. E. Shelden. The average of two tracings was used for each microtubule. The resulting data file consisting of the length changes of a particular microtubule as a function of time was then used to create a "life history" plot. Results obtained by different individuals tracking the same microtubule were similar. Changes in phase were identified by eye from the life history plots and were selected using interactive software. The computer then determined the average length, duration, and rate of the growing and shortening phases. Changes in length less than or equal to 0.5 μm were considered to be pauses (see Dhamodharan and Wadsworth, 1995). The catastrophe frequency per unit time was calculated by dividing the number of catastrophes (transitions from growing (G) to shortening (S) and from the pause state (P) to shortening (S) by the sum of the total time spent in G plus P. The rescue frequency was calculated by dividing the number of rescues (transitions from S to G and from S to P) by the total time spent in S. The catastrophe and rescue frequencies were also determined per unit length by dividing the number of transitions by the length the

microtubules grew (for the catastrophe frequency) or shortened (for the rescue frequency). Dynamicity was calculated by dividing the sum of the total length grown and shortened by the life span of the particular microtubule.

RESULTS

Effect of Vinblastine on Microtubule Polymer Mass in BS-C-1 Cells

We wanted to determine the actions of vinblastine on microtubule dynamics in living cells at vinblastine concentrations that affected microtubule function but did not appreciably affect the microtubule polymer mass. The most potent action of vinblastine in HeLa cells is inhibition of proliferation and mitosis (Jordan *et al.*, 1992). Thus, we first determined the relationship between the concentration of vinblastine added to the culture media and inhibition of BS-C-1 cell proliferation. Cells were incubated with a range of vinblastine concentrations (8 nM to 1 μM) for 20 h, the approximate duration of one cell cycle, and the number of cells was determined (MATERIALS AND METHODS). Vinblastine strongly inhibited proliferation of the BS-C-1 cells; 50% inhibition occurred at 19 nM vinblastine and complete inhibition occurred at 100 nM vinblastine.

Mitosis was slowed in the presence of vinblastine. In control cells, 2.8 ± 0.2% of all cells were in mitosis. Following a 20-h incubation with 32 nM vinblastine, 11.5 ± 3.9% of cells were in mitosis, indicating that mitosis was slowed fourfold, and 40 ± 9% of cells were in mitosis at 300 nM vinblastine, indicating that mitosis was slowed 14-fold. In addition, the morphology of mitotic spindles formed in the presence of low concentrations of vinblastine was abnormal. A normal metaphase spindle in a control cell is shown in Figure 1, A and B. It consists of a bipolar spindle-shaped array of microtubules with a small tuft of astral microtubules at each pole; the chromosomes form a compact metaphase plate. Seventy-eight percent of spindles in control cells displayed this morphology, while 22% of the spindles showed abnormalities. Some control spindles categorized as abnormal may be late prometaphase spindles that will eventually form a normal metaphase plate. After incubation with low concentrations of vinblastine (8–100 nM for 20 h) most spindles were abnormal. For example, at 8 nM vinblastine, 83% of the metaphase spindles were abnormal. Most abnormal spindles were bipolar, contained a compact metaphase plate of chromosomes, and appeared to be composed of a full complement of microtubules; however, from one to several chromosomes failed to congress to the metaphase plate and appeared to be "stuck" at the spindle poles, and astral microtubules were often longer and more prominent than in control spindles (Figure 1, C and D; Type I and II as defined in Jordan *et al.*, 1991). At 8–32 nM vinblastine, some abnormal spindles were ball-shaped with no detectable bipolarity (Type III, Jordan *et al.*, 1991). At increasing concentrations of vinblastine (64–128 nM), > 96% of metaphase spindles were abnormal, and the abnormalities were more severe, that is, more chromosomes failed to congress and/or most of the spindles were ball-shaped. At 256 nM vinblastine, all spindles were abnormal with no detectable bipolarity.

We next determined the extent to which vinblastine affected microtubule polymer mass in BS-C-1 cells by incubating cells with a broad range of vinblastine concentrations (between 8 nM and 2 μM) for 4 h, isolating microtubule cytoskeletons, and then measuring the microtubule polymer mass in the cytoskeletons with a solid-phase immunoassay (ELISA) (MATERIALS AND METHODS). As shown in Figure 2, no reduction in microtubule polymer mass (i.e., no depolymerization) occurred at concentrations of vinblastine at or below 128 nM. Fifty percent depolymerization occurred at 650 nM vinblastine and depolymerization was essentially complete at ~1 μM vinblastine.

Effect of Vinblastine on Microtubule Organization in Interphase BS-C-1 Cells

Because analysis of microtubule dynamic behavior in living cells is limited to the peripheral regions of the cell, we first examined the distribution of microtubules in cells incubated with different vinblastine concentrations by immunofluorescence microscopy where the entire microtubule array could be visualized in many cells. In control cells (Figure 3A), microtubules radiated from the microtubule organizing center and extended to the cell periphery. No change in microtubule organization was detected at or below 16 nM vinblastine. At > 32 nM vinblastine, some microtubules appeared more wavy (Figure 3B), but the numbers or lengths of the microtubules seemed unaffected. At 64 nM vinblastine, little change in microtubule organization was observed (Figure 3C). Occasionally at 64 nM vinblastine, the number of microtubules in the peripheral lamellar regions of some cells was reduced slightly and the number of microtubules in the central regions of the cells appeared to increase. Higher vinblastine concentrations (128 nM and 256 nM) produced significant depolymerization in some cells. Interestingly, some cells incubated with 128 nM and 256 nM vinblastine had few remaining microtubules, whereas adjacent cells often contained a completely normal-looking complement of microtubules (Figure 3D).

The reduction in the number of microtubules in some cells as determined by immunofluorescence microscopy at 100–300 nM vinblastine was inconsistent with the lack of detectable depolymerization

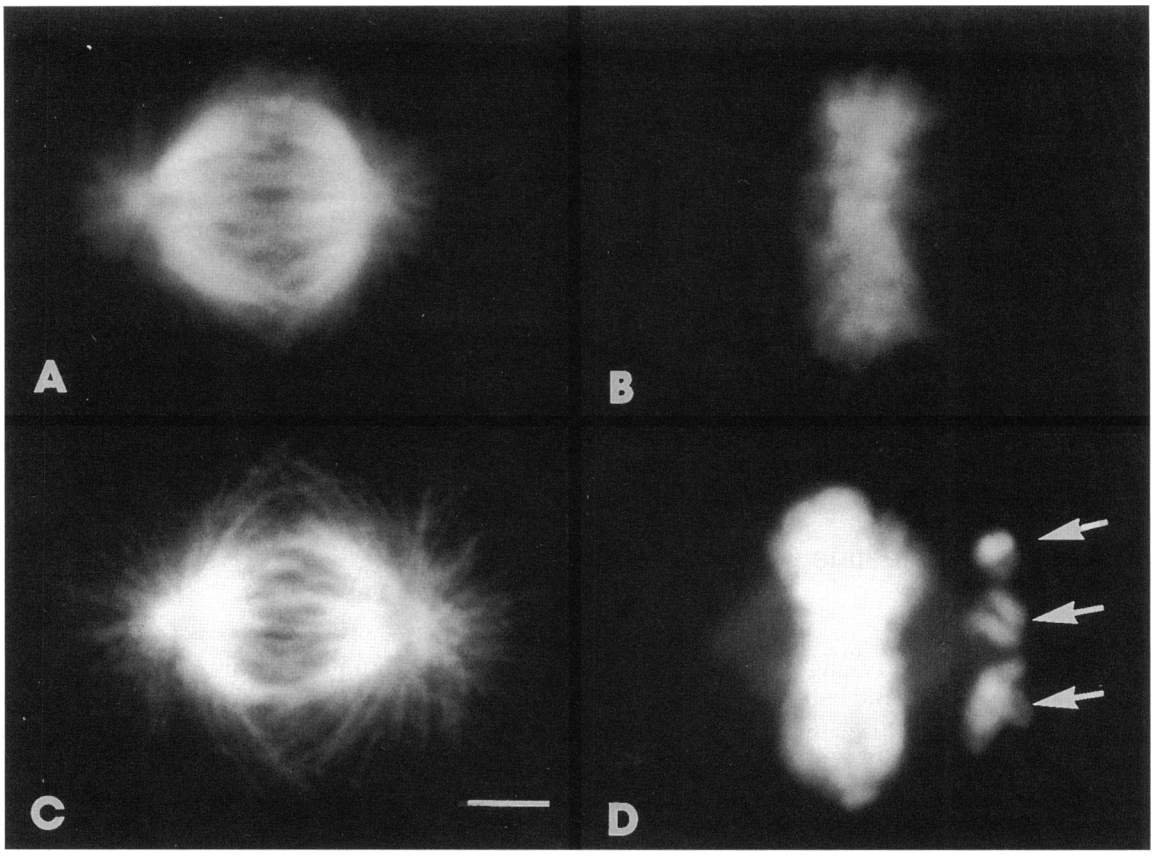

Figure 1. Effects of vinblastine on mitotic spindle organization in BS-C-1 cells. (A and C) Indirect immunofluorescence staining with an antibody to tubulin. (B and D) DAPI staining. (A and B) Metaphase spindle of a control cell. Chromosomes have congressed to form a compact metaphase plate. (C and D) Metaphase spindle following incubation with 16 nM vinblastine for 22 h. Astral microtubules (C) appear longer and more prominent than in controls; several chromosomes (arrows in D) have failed to congress and are located near one spindle pole. Bar, 5 um.

in stabilized cytoskeletons determined by ELISA (Figure 2). The loss of microtubule polymer in a minority of cells might be offset by a slight increase in polymer in other cells. Alternatively, the inability to detect depolymerization may result from clumping of microtubules in vinblastine-treated cells or from a drug-induced stabilization of the cytoskeletons during isolation. Nearly complete depolymerization of microtubules in all cells occurred at 1 μM vinblastine very high vinblastine concentrations (at or above 5 μM) induced formation of vinblastine-tubulin paracrystals (our unpublished observations).

Intracellular Vinblastine Concentrations Achieved at Various Added Vinblastine Concentrations

Vinblastine accumulates intracellularly in a number of cultured cell lines to significantly higher concentrations than the concentrations added to the media (Donoso and Himes, 1984, Jordan *et al.*, 1991); a process that requires approximately 2 h (our unpublished observations). We wanted to know the intracellular vinblastine concentration achieved for the extracellular concentrations of vinblastine used in these experiments. Vinblastine was equilibrated with the cells for 4 h before imaging the microtubules to ensure that a stable internal concentration had been achieved, and the intracellular vinblastine concentrations were determined as described in MATERIALS AND METHODS. Vinblastine concentrations added to the media ranged from 3 nM to 330 nM and vinblastine was concentrated between 230- and 280-fold over that added to the media, to intracellular levels that ranged from ~ 1 to 80 μM (Table 1).

Microtubule Dynamics in BS-C-1 Cells

BS-C-1 cells were injected with rhodamine-labeled tubulin and the derivatized tubulin was allowed to incorporate into microtubules for 3 h before addition of vinblastine. Fluorescent microtubules in the lamellar

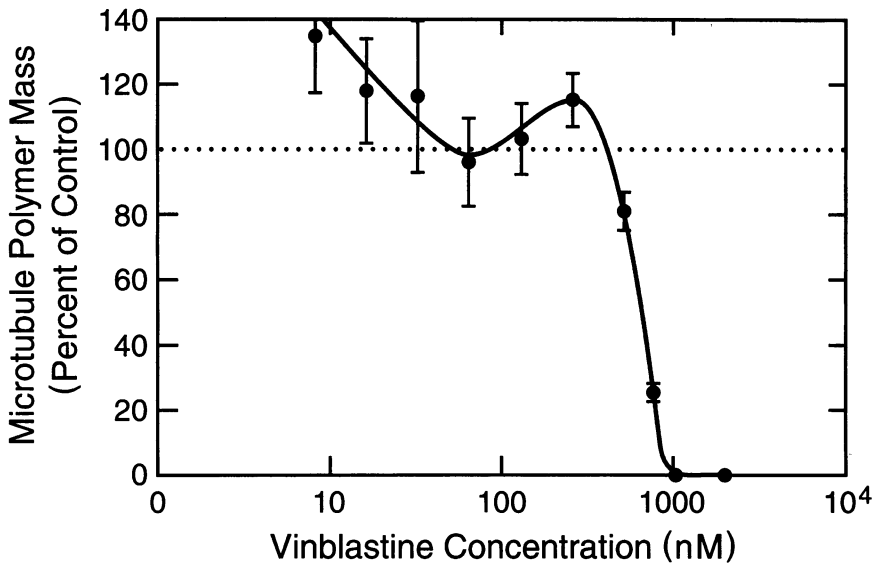

Figure 2. Effect of vinblastine on the microtubule polymer mass in BS-C-1 cell cytoskeletons. Cytoskeletons were isolated in a microtubule-stabilizing buffer after a 4-h incubation with the indicated vinblastine concentrations. Tubulin in microtubules was determined by ELISA (MATERIALS AND METHODS). The mass of microtubule polymer in control cells was 5.8 μg/mg of total cell protein.

regions of the cells were then imaged (MATERIALS AND METHODS). The dynamic behavior of microtubules in untreated control cells is shown in a video sequence (Figure 4a). Consistent with previous results (Cassimeris *et al.*, 1988; Sammak and Borisy, 1988a; Schulze and Kirschner, 1988; Shelden and Wadsworth, 1993; Dhamodharan and Wadsworth, 1995), the microtubules alternated between phases of growing, shortening, and a pause state (a state of attenuated dynamic activity). Life history plots (Figure 5) of individual microtubules clearly illustrate the dynamic behavior of the microtubules. The life history plots of a number of microtubules were used to determine the parameters of dynamic instability (Table 2). Microtubules in control cells shortened at a mean rate of 15.5 ± 10.6 μm/min, approximately twice as fast as the mean growing rate (6.9 ± 3.9 μm/min). Wide variations in the growing and shortening rates occurred, consistent with the results of Gildersleeve *et al.* (1992).

Low Concentrations of Vinblastine Strongly Suppressed Growing and Shortening Excursions

The dynamics of interphase BS-C-1 microtubules were analyzed over the vinblastine concentration range in which the drug inhibited cell proliferation but did not appreciably alter the microtubule polymer mass. As shown in the video sequence (Figure 4b), incubation of cells with 32 nM vinblastine markedly suppressed the dynamic behavior of the microtubules. Life history traces of individual microtubules (Figure 5B) also document the decrease in the dynamic behavior of individual microtubules in vinblastine and were used to determine the parameters of dynamic instability over a range of vinblastine concentrations (Table 2). Suppression of a number of parameters of dynamic instability were detected at vinblastine concentrations as low as 8 nM (Table 2; Figures 6 and 7; discussed further below), and suppressive effects on most parameters were highly significant at 32 nM vinblastine (Table 2). Microtubule behavior could not be examined at vinblastine concentrations of 128 nM or higher due to the loss of microtubules from the lamellar region and to an increase in background fluorescence that occurred at these higher concentrations. The increased background fluorescence was presumably due to a slight increase in the level of soluble fluorescent tubulin resulting from disassembly of microtubules by vinblastine that was below the level of detectability in the ELISA assays (Figure 2). In addition, changes in cellular morphology might have increased the path length through the cell, also increasing the background fluorescence.

Low concentrations of vinblastine significantly decreased the microtubule shortening rate and the length shortened during a shortening excursion. For example, a statistically significant 67% reduction in the mean shortening rate from 15.5 ± 10.6 μm/min in control cells to 5.1 ± 3.7 μm/min occurred at 32 nM vinblastine (Table 2 and Figure 6A). In addition, the rapid shortening rates typical of many of the microtubules in control cells (≥ 25 μm/min) were completely suppressed at 32 nM vinblastine and no evidence for subsets of microtubules with different shortening rates was detectable (our unpublished observations). The mean length a microtubule shortened during a shortening event was also strongly reduced (from 2.4 ± 2.4 μm in control cells to 0.7 ± 0.2 μm at 32 nM vinblastine), but the duration of the shortening events was

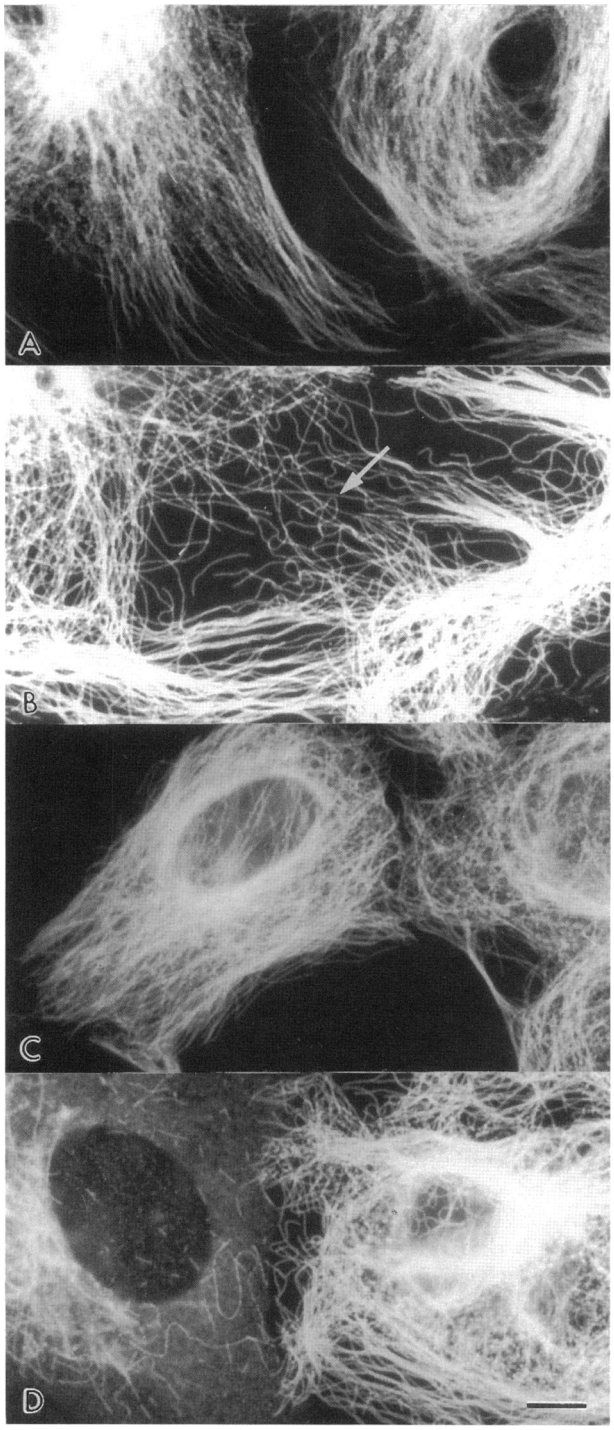

Figure 3. Microtubule organization in interphase BS-C-1 cells following incubation for 4 h with a range of vinblastine concentrations. Cells were fixed and stained with an antibody to tubulin (MATERIALS AND METHODS). (A) control cells; (B) 32 nM vinblastine, arrows indicate wavy microtubules; (C) 64 nM vinblastine, cells appear to have a normal amount of microtubules; (D) 128 nM vinblastine, most microtubules have depolymerized in the cell on the left; many microtubules remain in the cell on the right but the microtubules are clumped in the interior of the cell. Bar, 10 um.

Table 1. Intracellular vinblastine concentration

Initial concentration of vinblastine in the media (nM)	Intracellular concentration of vinblastine[a] (μM)	Degree of concentration
3	0.7 ± 0.1	233 X
8	1.8 ± 0.3	225 X
16	3.6 ± 0.5	225 X
32	9.1 ± 0.8	284 X
64	18 ± 3	281 X
330	80 ± 5	242 X

[a] Values represent mean ± SE, n = 4.

not detectably affected (Table 2). Thus, vinblastine strongly decreases the extent of microtubule polymer loss during shortening excursions.

The lengths that the microtubules grew during growing events and the durations and the rates of growing were also reduced to a statistically significant degree by 32 nM vinblastine (Table 2). For example, the average length grown during a growing event was reduced by 50%, from 1.8 ± 1.6 μm in controls to 0.9 ± 0.7 μm at this vinblastine concentration. The average duration of a growing event was also reduced approximately twofold. Thirty-two nanomolar vinblastine also reduced the mean growing rate (Figure 6A). Thus, at 32 nM vinblastine, microtubules grew more slowly, for shorter distances, and for shorter periods of time than control microtubules.

Vinblastine Altered the Catastrophe and Rescue Frequencies

Microtubule dynamic instability in cells is characterized by abrupt, stochastic transitions among the phases of growing, shortening, and apparent pause (attenuation). Initially, many studies of microtubule dynamic instability in vitro were performed under conditions of net microtubule assembly in which periods of attenuated dynamic activity were rarely observed. However, in vivo (Shelden and Wadsworth, 1993; Dhamodharan and Wadsworth, 1995) and under steady-state conditions in vitro (Toso *et al.*, 1993; Panda *et al.*, 1994; Derry *et al.*, 1995) microtubules spend a considerable fraction of total time in an attenuated state, neither growing nor shortening detectably by video microscopy. Thus, microtubule dynamic behavior in cells and in vitro at steady state is characterized by transitions among six different states. We are defining catastrophe as a transition either from the growing state or from the apparent pause state to the shortening state and we define rescue as a transition from the shortening state either to the growing state or to the apparent pause state. The catastrophe or rescue

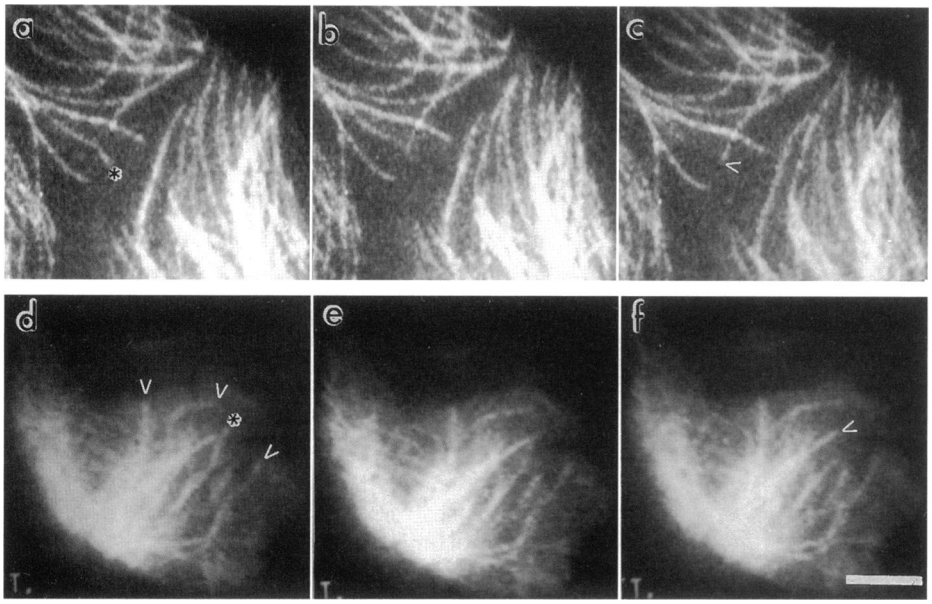

Figure 4. Microtubule dynamic behavior in living BS-C-1 cells. Cells were injected with rhodamine labeled tubulin and individual microtubules were imaged as described in MATERIALS AND METHODS. Images were acquired at 2 s intervals and are shown at 10 s intervals. A control cell (panels a–c) and a cell incubated with 32 nM vinblastine (panels d–f) are shown. The microtubule marked with an asterisk in panel a subsequently disassembles; the microtubule marked with the arrowhead in panel c grew (compare with panel a). In panels d–f, microtubules marked with arrowheads do not detectably change length; of the two microtubule ends marked with an asterisk in panel d, one disassembles and the other remains unchanged in length (arrowhead in panel f). Bar, 5 um.

frequencies were calculated in two different ways: by determining the number of transitions per unit time and by determining the number of transitions per unit length grown or shortened during a growing or shortening event (Kowalski and Williams, 1993; Dhamodharan and Wadsworth, 1995).

Low concentrations of vinblastine significantly reduced the catastrophe frequency, both when analyzed in terms of the number of catastrophes per unit time and the number of catastrophes per unit length grown (Table 2 and Figure 7). As the concentration of vinblastine was increased from 8 nM to 32 nM, microtubules underwent fewer transitions to shortening per unit time (Figure 7A). For example, when cells were incubated with 32 nM vinblastine, the catastrophe frequency/unit time was decreased by 53% (from 0.030/s in controls to 0.014/s) (Figure 7A). Similarly, when calculated in terms of the number of catastrophes per length grown, the data indicate that as the vinblastine concentration was increased from 8 nM to 32 nM, a greater number of tubulin subunits was added to the microtubule end before a transition into shortening (catastrophe). For example, the catastrophe frequency/length grown decreased by 77% (from 0.87/μm in control cells to 0.20/μm at 32 nM vinblastine) (Figure 7B). Interestingly, as the vinblastine concentration was increased from 32 nM to 64 nM, the catastrophe frequency increased rather than decreased (Figure 7, A and B). The increase in catastrophe frequency at 64 nM vinblastine may have occurred as a result of vinblastine binding to low affinity vinblastine-binding sites along the microtubule surface, which induces the peeling of protofilaments at microtubule ends and depolymerization (see DISCUSSION).

In contrast to the ability of vinblastine to reduce the catastrophe frequency, vinblastine did not detectably affect the time-based rescue frequency (Table 2; Figure 7A). However, measurement of the rescue frequency as a function of the length of microtubule depolymerized during a shortening event indicated that vinblastine induced a significant concentration-dependent increase in the rescue frequency (the number of rescues/μm increased approximately twofold from 0.33/μm in controls to 0.77/μm at 32 nM vinblastine; Table 2, Figure 7B).

Low Concentrations of Vinblastine Increased the Duration of the Pause State and the Percentage of Time the Microtubules Spent in the Pause State

Vinblastine significantly increased the mean duration of a pause event. For example, 32 nM vinblastine increased the duration of a pause event by 66% from a mean of 11.6 ± 7.4 s in control cells to 19.2 ± 16.8 s (Figure 6 B, Table 2). Vinblastine also increased the number of pause events of very long duration (our unpublished observations). In addition, 32 nM vinblastine significantly increased the percentage of total time the microtubules spent in the pause state (from 36% to 59%) and correspondingly decreased the percentage of total time that the microtubules spent growing and shortening (Figure 6C).

Dynamicity is a parameter that reflects the overall dynamics of the microtubules (the total detectable microtubule growing and shortening per unit time; Toso *et al.*, 1993). As shown in Table 2, dynamicity was strongly reduced by vinblastine. At 32 nM vinblastine, for example, the dynamicity was reduced by 75% from 7.2 ± 1.2 to 1.8 ± 0.2 μm/min.

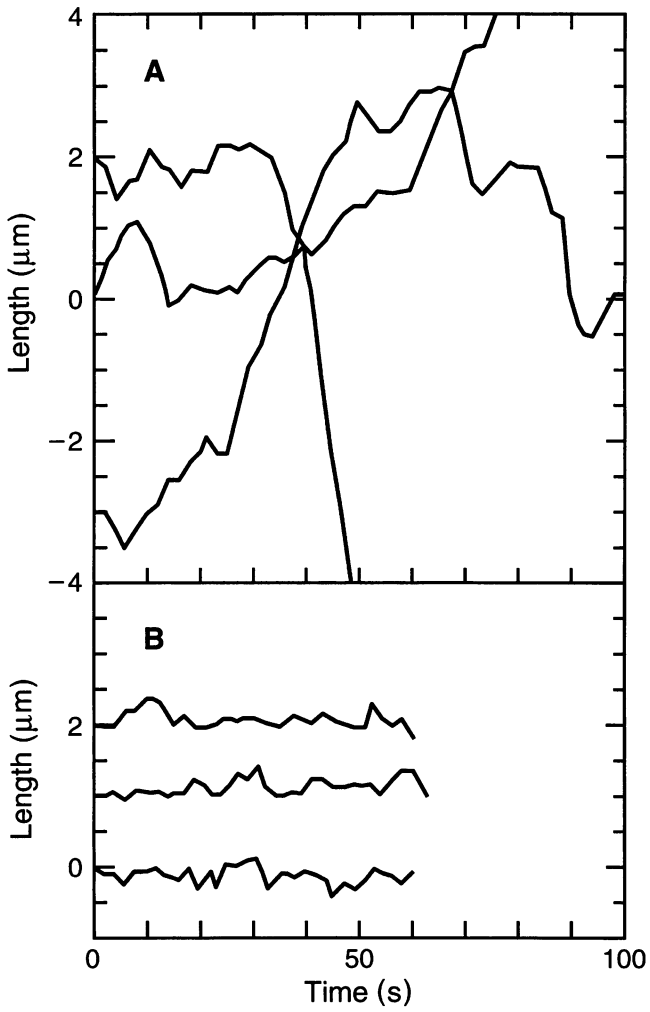

Figure 5. Changes in length with time of three control microtubules in peripheral lamellae of living BS-C-1 cells (A) and three microtubules in cells incubated with 32 nM vinblastine (B). Cells were microinjected with rhodamine-labeled tubulin, equilibrated for 3 h, then incubated ± vinblastine for an additional 4 h. Microtubules were imaged and the data was analyzed as described in MATERIALS AND METHODS.

DISCUSSION

Suppression of Dynamics at Low Vinblastine Concentrations in the Absence of Significant Microtubule Depolymerization

The results presented here demonstrate that the dynamic instability behavior of microtubules in interphase BS-C-1 cells is strongly suppressed by low concentrations of vinblastine and that suppression of dynamics occurs in the absence of microtubule depolymerization or major reorganization of the interphase microtubule array. The actions of vinblastine on microtubule dynamics in BS-C-1 cells occur at the same vinblastine concentrations that strongly inhibit proliferation of these cells, slow mitosis, and induce subtle abnormalities in the organization of the mitotic spindle. The changes in spindle organization induced in BS-C-1 cells resemble abnormalities induced in HeLa cells following treatment with low concentrations of several antimitotic drugs including vinblastine and taxol (Jordan *et al.*, 1991–1993). The results strongly support the hypothesis that mitotic slowing and mitotic abnormalities induced by these drugs are a direct result of suppression of dynamics of mitotic spindle microtubules.

No microtubule depolymerization was detectable at vinblastine concentrations below 128 nM as determined by immunofluorescence microscopy, and the microtubule polymer level was not reduced in the cells below 260 nM vinblastine as determined by ELISA of cytoskeletons isolated from the cells (Figures 2 and 3). Thus, the results confirm the hypothesis that the most sensitive detectable action of vinblastine in cells is suppression of microtubule dynamics, and they strongly support the idea that suppression of microtubule dynamics by vinblastine is its most potent chemotherapeutic mechanism (Jordan *et al.*, 1991). Interestingly, microtubule polymer levels in cells incubated with very low concentrations of vinblastine (< 32 nM) increased over levels in control cells (Figure 2). A similar increase in microtubule polymer levels at low vinblastine concentrations was observed in HeLa cells (Jordan *et al.*, 1991) and may reflect a vinblastine-induced increase in microtubule stability during isolation of cytoskeletons.

Mechanism of Kinetic Stabilization of Microtubule Ends by Vinblastine

As a result of a large number of studies on the interactions of vinblastine with tubulin and microtubules in vitro, we now have a relatively clear understanding of the mechanism of action of this drug (reviewed in Wilson and Jordan, 1994). At low vinblastine concentrations, addition and loss of tubulin at microtubule ends is inhibited by the rapid and reversible binding of one or a few vinblastine molecules to high affinity binding sites (K_d ~2 μM) in tubulin at the microtubule ends (Wilson *et al.*, 1982). It appears that when tubulin is bound at the microtubule end, its affinity for vinblastine increases substantially as compared with the affinity of soluble tubulin dimers for vinblastine (Na and Timasheff, 1980a,b; Wilson *et al.*, 1982; discussed in Toso *et al.*, 1993). Importantly, Na and Timasheff (1980a,b) found that the binding of vinblastine to tubulin increases the affinity of tubulin for itself. Thus binding of vinblastine to tubulin at a microtubule end may induce a conformational change in the molecule of tubulin to which it is bound, which increases the affinity of the tubulin for adjacent tubulin molecules in the microtubule lattice. In support of this idea, vin-

Table 2. Effects of vinblastine on parameters of microtubule dynamic instability in BS-C-1 cells

	Vinblastine concentration				
Parameter	Control	8 nM	16 nM	32 nM	64 nM
Number of microtubules	24	17	19	24	18
g-duration (s)	17.1 ± 14.4	13.9 ± 11.4	12.4 ± 10.7*	11.3 ± 8.5*	7.9 ± 5.1**
g-length (μm)	1.8 ± 1.6	1.6 ± 1.1	1.4 ± 0.7*	0.9 ± 0.7**	0.7 ± 0.2**
g-rate (μm/min)	6.9 ± 3.9	9.5 ± 7.6*	8.0 ± 5.0	5.5 ± 3.4*	6.4 ± 2.8
s-duration (s)	8.9 ± 4.8	8.4 ± 4.6	8.3 ± 4.5	10.6 ± 6.0	8.3 ± 4.3
s-length (μm)	2.4 ± 2.4	1.5 ± 1.1*	1.5 ± 1.5	0.7 ± 0.2**	0.9 ± 0.5**
s-rate (μm/min)	15.5 ± 10.6	12.6 ± 11.4	10.9 ± 8.2*	5.1 ± 3.7**	7.2 ± 4.0**
Catastrophe frequency (per s)	0.03 ± 0.01	0.03 ± 0.004	0.01 ± 0.004**	0.01 ± 0.003**	0.03 ± 0.009
Rescue frequency (per s)	0.07 ± 0.01	0.1 ± 0.02	0.09 ± 0.03	0.07 ± 0.018	0.11 ± 0.031
Catastrophe frequency (per μm)	0.87 ± 0.30	0.63 ± 0.15	0.22 ± 0.08*	0.2 ± 0.067*	0.76 ± 0.156
Rescue frequency (per μm)	0.33 ± 0.07	0.55 ± 0.090*	0.54 ± 0.15	0.77 ± 0.158**	0.84 ± 0.171**
p-duration (s)	11.6 ± 7.4	10.7 ± 9.1	14.1 ± 17.8	19.2 ± 16.8**	24.0 ± 19.7**
dynamicity (μm/min)	7.2 ± 1.2	5.0 ± 0.6	4.0 ± 0.6**	1.8 ± 0.2**	2.4 ± 0.3**

Note: g = growth; s = shortening; p = pause or attenuation. The values are mean ± SD, with the exception of catastrophe and rescue frequency which are ± SE.

*values significant at 95% confidence level.

**values significant at 99% confidence level.

blastine decreases the dilution-induced off-rate constant at microtubule net assembly ends (Jordan and Wilson, 1990). Thus, the results of the present study can be understood in terms of the known mechanism of action of vinblastine. Vinblastine binding to the microtubule ends stabilizes the microtubule, i.e., vinblastine reduces the shortening rate and the catastrophe frequency.

In contrast to colchicine, which forms a complex with a soluble tubulin dimer that then binds to microtubule ends (Margolis and Wilson, 1977; Sternlicht and Ringel, 1979; Farrell and Wilson, 1980; Skoufias and Wilson, 1992), vinblastine appears to inhibit tubulin exchange at microtubule ends by binding directly to the ends (Wilson *et al.*, 1982; Jordan and Wilson, 1990). Also unlike the action of colchicine, vinblastine does not become buried in the core of the microtubule along with tubulin as a co-polymer but rather, at low concentrations, it remains strictly at the microtubule ends (Jordan and Wilson, 1990). Vinblastine inhibits the rate and extent of microtubule growth (Table 2; see also Toso *et al.*, 1993). Thus, microtubule growing events that occur in the presence of vinblastine must require previous dissociation of vinblastine from the microtubule end. Vinblastine may inhibit the growing rate by sterically preventing tubulin addition to the microtubule end. Alternatively, vinblastine may inhibit tubulin addition by inducing a conformational change in tubulin at the microtubule end that decreases the affinity of incoming molecules of tubulin for the end.

Vinblastine strongly increased the percentage of total time that BS-C-1 cell microtubules spent in the pause state, where length changes, if they occurred, were below the resolution of the video microscope (length changes less than 0.5 μm). A 0.5-μm length of a microtubule consists of ~850 tubulin dimers and, thus, a significant number of tubulin addition or loss events could occur that would not be detected by video microscopy. Vinblastine (32 nM) suppressed the mean growing and shortening rates to 5.5 μm/min and 5.1 μm/min, respectively. Thus, the dramatic increase in the percentage of time the microtubules spent in the pause state (an increase of 68% as compared with controls) may result simply from vinblastine-induced suppression of the growing and shortening rates to such a degree that during a large fraction of the time, the extent of growing or shortening was below the resolution of the video microscope.

Vinblastine significantly altered the transition frequencies between the growing and shortening phases. These transitions are thought to be due to the gain and loss of a small stabilizing "cap" or terminal layer consisting of GTP- or GDP-P_i-tubulin subunits at the microtubule ends (Kirschner and Mitchison, 1986; Carlier, 1989; Melki *et al.*, 1990; Stewart *et al.*, 1990; Walker *et al.*, 1991; Caplow, 1992). The presence of the cap is thought to stabilize the microtubules and promote their elongation. Loss of the cap by dissociation of GTP- or GDP-P_i-tubulin is thought to result in rapid shortening (Walker *et al.*, 1991; Erickson and O'Brien, 1992; Hyman *et al.*, 1995). Low concentrations of vinblastine markedly reduced the catastrophe frequency (frequency of transition to rapid shortening), indicating that vinblastine might act directly on the mechanism responsible for cap gain. Vinblastine suppressed the

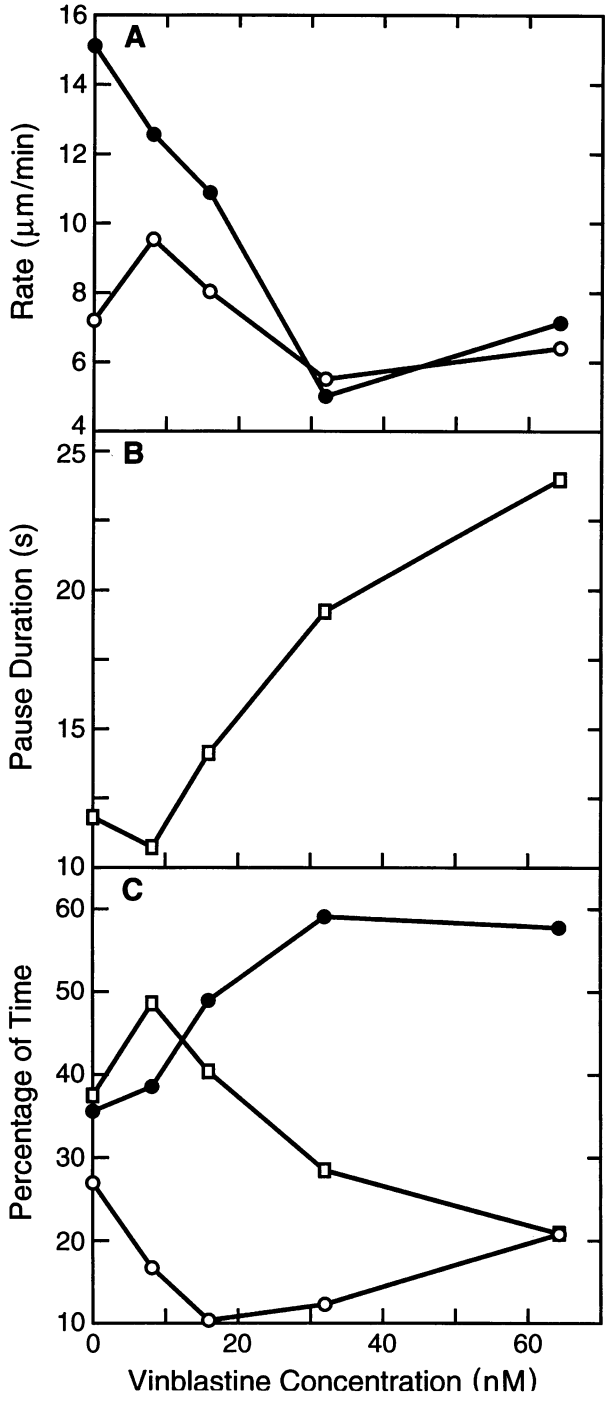

Figure 6. Effect of vinblastine at concentrations ranging from 8 nM to 64 nM on dynamic instability parameters in living BS-C-1 cells. (A) Mean rate of shortening (closed circles), mean rate of growing (open circles); (B) mean duration of the pause state; (C) percentage of total time that microtubules spent in the growing phase (open squares), the shortening phase (open circles), or in the pause state (closed circles).

catastrophe frequency both when calculated as events/unit time and as events/unit length. Thus, binding of vinblastine to tubulin at the microtubule ends may maintain the tubulin in a conformation that stabilizes the GTP- or GDP·P_i tubulin cap. The results of Na and Timasheff (1980a,b), indicating that binding of vinblastine to tubulin increases the affinity of tubulin for itself, support the idea that vinblastine might stabilize the associations between GTP-tubulin or GDP·P_i-tubulin dimers at the microtubule end. Vinblastine inhibits the GTPase activity of soluble tubulin (David-Pfeuty *et al.*, 1979) and thus vinblastine might act by affecting the size or chemical nature of the stabilizing cap by slowing the GTP hydrolysis rate by or slowing the release of P_i at microtubule ends required for transition to rapid shortening. An intriguing idea is that in cells, vinblastine binding to microtubule ends might inhibit binding of catastrophe-promoting factors to the ends, thus blocking transitions to the shortening state.

In contrast to the suppressive effects of vinblastine on the catastrophe frequency, vinblastine did not significantly alter the rescue frequency when determined as events/unit time, suggesting that vinblastine does not directly affect regain of a lost cap. Vinblastine did increase the rescue frequency when determined as events/unit length. From the results of Na and Timasheff (1980a,b), binding of vinblastine even briefly to the end of a shortening microtubule might be expected to transiently stabilize the end, slowing tubulin dissociation. As a result of shortening more slowly, a recapping event that occurs at a given time frequency will occur following a shorter loss of microtubule length in the presence of vinblastine than in control microtubules. That transitory binding of vinblastine does occur at the ends of depolymerizing microtubules can be inferred because at any specific concentration, vinblastine decreases the shortening rate of individual microtubules to approximately the same extent regardless of the length shortened during any single shortening event (Figure 6). The inability of vinblastine to increase the rescue frequency/unit time may be due to inhibition of GTP-tubulin addition by steric hindrance during the transitory residence of vinblastine at a microtubule end, thus preventing cap regain while vinblastine is present.

Several actions of 64 nM vinblastine on BS-C-1 microtubules and their dynamics were distinct from the actions of lower concentrations of vinblastine. Similar to the actions of lower vinblastine concentrations, 64 nM vinblastine reduced the mean rate and length of the shortening events, the duration and lengths of the growing events, and increased the duration of the pause times and the percentage of time the microtubules spent in the pause state

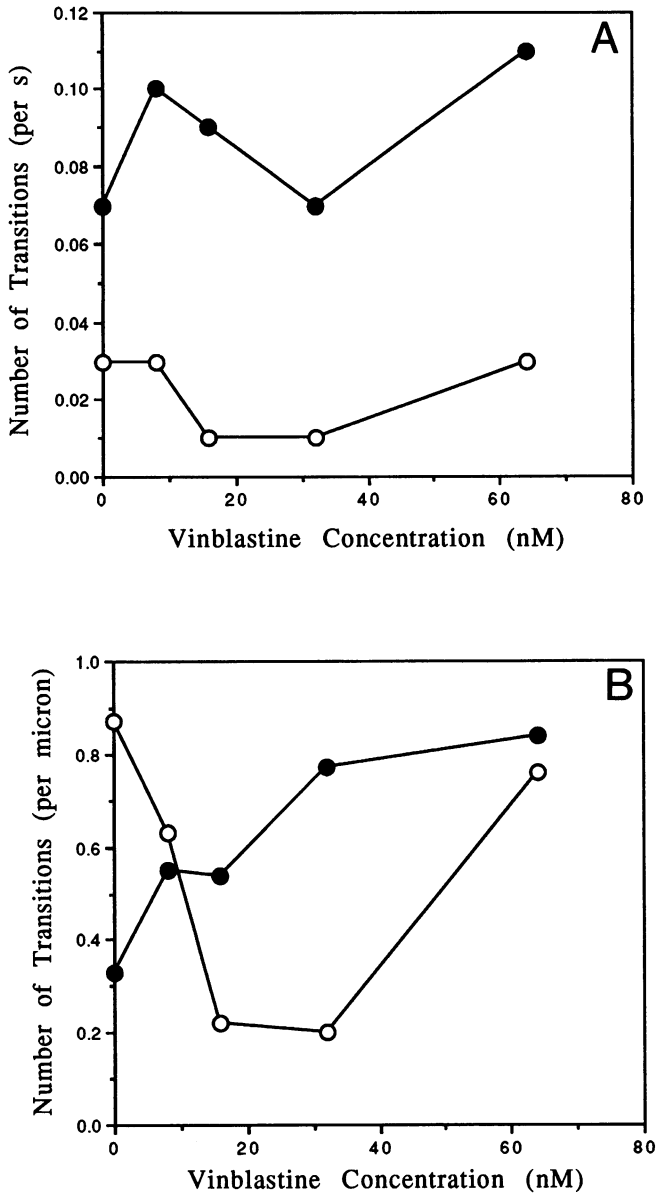

Figure 7. Effect of vinblastine on the catastrophe and rescue frequencies of microtubules in peripheral lamellae of living BS-C-1 cells. (A) Number of rescues per second of time in the shortening phase (closed circles), number of catastrophes per second of time in the growing phase and the pause state (open circles). (B) Number of rescues per length of microtubule lost during shortening phase (open circles), number of catastrophes per length of microtubule grown during growing phase (closed circles).

(Table 2, Figure 6). However, 64 nM vinblastine sharply increased the catastrophe frequency as compared with lower vinblastine concentrations. In addition there were fewer microtubules present in the peripheral lamellae and an increased background fluorescence, presumably due to a slight increase in the soluble tubulin level in the cells. At relatively high vinblastine concentrations, vinblastine binds to a low affinity class of tubulin binding sites on microtubules that is located along the surface of the microtubule. Stoichiometric binding of vinblastine to these microtubule binding sites is associated with fraying of the microtubule ends and microtubule depolymerization (Jordan *et al.*, 1986; Singer *et al.*, 1989). The finding that 64 nM vinblastine increased the catastrophe frequency of BS-C-1 microtubules in cells indicates that a concentration of 64 nM vinblastine added to the culture medium is sufficient to achieve an intracellular concentration that permits vinblastine to bind to the low affinity sites. Such binding may exert a destabilizing action at the microtubule ends that counteracts, to some degree, the stabilizing effect of vinblastine binding to the high affinity sites.

Apparent Reduced Sensitivity to Vinblastine of Microtubule Dynamics in BS-C-1 Cells as Compared with the Sensitivity of Bovine Brain Microtubules In Vitro

The intracellular concentrations of vinblastine achieved in BS-C-1 cells when 8–64 nM vinblastine was added to the culture media were between 1.8 and 17.5 μM; approximately 250-fold higher. These intracellular vinblastine concentrations are higher than those required to suppress dynamics of bovine brain microtubules in vitro (0.1–1 μM; Toso *et al.*, 1993; Jordan and Wilson, 1990) and higher than the intracellular vinblastine concentrations required to inhibit mitosis in HeLa cells (0.04–0.15 μM; Jordan *et al.*, 1991; Wendell *et al.*, 1992). The apparent insensitivity of BS-C-1 interphase microtubules to vinblastine could result from a decreased binding affinity of vinblastine for the ends of the BS-C-1 microtubules as compared with HeLa cell microtubules or bovine brain microtubules. This might occur as a consequence of differences in the tubulin isotype composition. For example, the tubulin isotype composition in CVI monkey kidney cells differs significantly from the composition of bovine brain tubulin (Lopata and Cleveland, 1987; Banerjee *et al.*, 1988), and the isotype composition of microtubules clearly affects their dynamic instability behavior (Panda *et al.*, 1994). Further, the effects of vinblastine on microtubule polymerization in bulk solution are known to be modulated by the isotype composition of the microtubules (Khan and Luduena, 1994). An alternative possibility is that vinblastine might be bound to other components in BS-C-1 cells, leading to a decrease in the amount of vinblastine available to bind to the microtubules.

Comparison of the Action of Vinblastine in BS-C-1 Cells with the Effects of Vinblastine in Other Cells

Vinblastine has been used recently as a tool to investigate the roles of microtubule dynamics in cells (e.g., Baas and Ahmad, 1993; Tanaka *et al.*, 1995). In *Xenopus* fibroblasts, where the dynamics of individual microtubules could be readily measured, incubation with 10 nM vinblastine for periods ranging from less than one minute to several minutes reduced the growing and shortening rates by approximately 50–60% and increased the percentage of time the microtubules spent in the paused state from 43–81% (Tanaka *et al.*, 1995). The experimental design differed from that used in the present study in that the intracellular vinblastine concentration was not determined, but was likely to be increasing rapidly during the short time course of the experiment. Although the effects of vinblastine on the transition frequencies and on the microtubule polymer mass were not determined, the ability of vinblastine to suppress dynamics was similar to that observed in this study. When similar concentrations of vinblastine were applied to *Xenopus* growth cones, a dramatic reduction in axonal elongation was observed (Tanaka *et al.*, 1995). The observations that low doses of vinblastine suppress individual microtubule dynamics similarly in BS-C-1 cells and in *Xenopus* fibroblasts strongly suggest that vinblastine will be an important probe with which to examine the contribution of microtubule dynamics to cell motion and mitosis in diverse cells.

Conclusions

The results presented here demonstrate that microtubule dynamic behavior in cells is suppressed by low concentrations of vinblastine in the absence of microtubule depolymerization. Thus, drugs that act like vinblastine may be used as powerful tools under carefully defined conditions to investigate the role of microtubule dynamic behavior in living cells (Baas and Ahmad, 1993; Tanaka *et al.*, 1995). These results also demonstrate that endogenous molecules acting like vinblastine may bind in small numbers to microtubule ends (from one to a few molecules per microtubule) and potently regulate cellular microtubule dynamic behavior. These data also further support the idea (Jordan *et al.*, 1991–1993; Wendell *et al.*, 1993; Derry *et al.*, 1995) that the most potent antitumor activity of vinblastine, taxol, and a number of other antimitotic drugs may result from suppression of microtubule dynamics.

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