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Monastrol, a Prototype Anti-Cancer Drug That Inhibits a Mitotic Kinesin, Induces Rapid Bursts of Axonal Outgrowth From Cultured Postmitotic Neurons

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Terminally postmitotic neurons continue to express many of the kinesin-related proteins known to configure microtubules during mitosis. Drugs that inhibit these kinesins are being developed as anti-cancer agents with the hope that they will inhibit proliferation of tumor cells without having adverse effects on the nervous system. The prototype, termed monastrol, inhibits the kinesin known as Eg5, which is essential for maintaining separation of the half-spindles. Eg5 is also highly expressed in neurons, particularly during development. Exposure of cultured sympathetic neurons to monastrol for a few hours increased both the number and the growth rate of the axons. With additional time, the overall lengths of the axons were indistinguishable from controls. Sensory neurons showed a similar short-term increase in axonal growth rate. However, prolonged exposure resulted in shorter axons, suggesting that sensory neurons may be more sensitive to toxic effects of the drug. Nevertheless, the overall health of the cultures was still far more robust than cultures treated with taxol, a drug commonly used for anti-cancer therapy. On the basis of these results, we conclude that Eg5 normally generates forces that oppose axonal growth, presumably by partially suppressing the forward advance of microtubules. We speculate that local regulation of Eg5 could be a means by which neurons coordinate rapid bursts of axonal growth with appropriate environmental cues. The comparatively modest toxic effects on the neurons over time are a hopeful sign for clinicians interested in using anti-Eg5 drugs for cancer therapy. Cell Motil. Cytoskeleton 58:10–16, 2004.

Key words: Eg5; neuron; axon; kinesin; cancer; monastrol; taxol

INTRODUCTION

During mitosis, microtubules are configured into a bipolar spindle that separates into two half-spindles to divide the duplicated chromosomes. In terminally postmitotic neurons, microtubules are configured into highly organized paraxial arrays within axons and dendrites that provide architectural support and direct organelle traffic. Changes in the configuration of microtubules during mitosis are orchestrated in part by forces generated by kinesin-related motor proteins [Scholey et al., 2003]. Because these motor-driven forces are crucial for mitosis, drugs that inhibit mitotic motors offer a novel and powerful means for inhibiting the proliferation of cancer

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cells [Yarrow et al., 2003]. In addition, there is hope that such drugs would not produce debilitating neuropathies such as those caused by treatment of cancer with taxanes [Quastoff and Hartung, 2002], which broadly obstruct microtubule functions by stimulating abnormal microtubule assembly and stabilization [He et al., 2001]. On a cautionary note, however, it is important to realize that terminally postmitotic neurons continue to express many of the so-called mitotic kinesins [Sharp et al., 1997; Ferhat et al., 1998; Buster et al., 2003]. While their precise functions in the neuron remain unclear, these motors presumably generate forces that help organize microtubules within key regions of the neuron such as axons, dendrites, and growth cones. Therefore, drugs that inhibit mitotic kinesins could, in theory, have profound effects on the nervous system as well.

In the present study, we have evaluated the effects on neurons of monastrol, the prototype anti-kinesin drug. Monastrol inhibits the mitotic motor known as Eg5 [Mayer et al., 1999; Kapoor et al., 2000; Maliga et al., 2002], which is a member of a family of kinesins crucial for maintaining separation of the half-spindles and, therefore, essential for successful mitosis [Blangy et al., 1995; Sharp et al., 1999]. Treatment of dividing cells with monastrol results in the collapse of the bipolar spindle into a non-functional monastral spindle. Monastrol is not a clinically useful drug because its association with Eg5 is weak and hence it needs to be used at rather high concentrations. Nevertheless, it is highly effective when applied to cells in culture, and provides the prototype for the design of better drugs that are more suited for clinical use. In addition, monastrol is an allosteric inhibitor, and hence likely to be selective for Eg5 over related kinesins [Maliga et al., 2002; DeBonis et al., 2003]. Our goals in this study were twofold, first to determine whether treatment of the neuronal cultures with monastrol results in any deleterious effects on neuronal health, and, second, to obtain information relevant to the normal function of Eg5 during the formation and growth of axons.

MATERIALS AND METHODS

Cell Culture

Primary dissociated or explant cultures of rat peripheral neurons were prepared from superior cervical or dorsal root ganglia as previously described [He and Baas, 2003]. Cultures of 4T1 murine mammary carcinoma cells (ATCC) were cultured in modified RPMI 1640 in 5% CO_2 at 37°C.

Drug Treatments

Monastrol and taxol were purchased from Tocris (Ellisville, MO) and Sigma (St. Louis, MO), respectively. Both were dissolved in DMSO at concentrations that could then be added to the medium to produce the desired final concentrations with a concentration of DMSO of 0.1%. The medium for control cultures contained 0.1% DMSO only. For all experiments, monastrol was used at 100 μM, which is the highest concentration that inhibits Eg5 without inhibiting other kinesins. Taxol was used at 10 and 100 nM, which are considered high and low extremes of the "clinically relevant" range of concentrations [Morone et al., 2001].

Imaging and Analysis

The general health and appearance of the cultures were assessed by phase-contrast microscopy. For detailed morphometric analyses and studies on the microtubule array, dissociated cultures were simultaneously fixed and extracted for 10 min by replacing the medium with a solution containing 0.1% glutaraldehyde and 0.2% Triton X-100 in a standard microtubule stabilization buffer. Cultures were then treated with sodium borohydride to quench autofluorescence, exposed to a blocking solution, and then exposed overnight to a monoclonal antibody (purchased from Sigma) that recognizes a neuron-specific form of beta-tubulin known as βIII. The cultures were then rinsed extensively with PBS, exposed to an appropriate rhodamine-conjugated secondary antibody for 1 h at room temperature, rinsed extensively again, and then mounted in a medium that reduces photobleaching. Images were acquired using a Zeiss (Thornwood, NY) Axiovert 200 microscope equipped with a Hamamatsu Orca ER cooled CCD. The software controlling the camera and microscope was Axiovision 3.1 running on a Seimens Pentium 2 computer. For each experimental condition, five images were acquired, each of which encompassed 180,000 square microns of the culture dish. The regions were selected randomly except for the fact that any region was dismissed that contained fewer than three or greater than four neuronal cell bodies. Total axonal length was then measured and divided by the number of cell bodies in each image. In some cases, the total number of axons emerging from each cell body was scored. All data analysis and statistical tests were performed using Microsoft Excel.

For the explant cultures, we were interested in the average amount of axonal growth over a 4-h period of time the morning after plating. Cultures were exposed to monastrol or vehicle for these 4 h only. Phase-contrast optics was sufficient for these measurements. The images were analyzed on a Pentium 3 PC running LSM software (Zeiss). This software among other features allows drawing over images and measuring distances of the drawn lines. This feature was used to gather lengths of the 10 longest axons in two different regions of each explant. (We chose the longest axons because their tips were easier to discern; the rates of growth over 4 h were generally similar to axons of other starting lengths.) This

was done by drawing over the selected axon from the point of its origin at the explant to the tip of its growth cone.

The 4T1 carcinoma cells were seeded into 96-well plates at 1,200–1,400 cells per well, exposed to taxol, monastrol, or vehicle 24 h after plating for 96 h. Cells were then quantified using a fluorogenic cell proliferation assay kit (Oncogene, Manhasset, NY). Fluorescence data (RFU) were measured using an automated universal plate reader.

RESULTS

Effects of Monastrol on Dissociated Cultures of Sympathetic Neurons

We initially focused on dissociated cultures of rat sympathetic neurons because they have been utilized for most of our previous work on the mechanisms and molecules that organize neuronal microtubules. When plated on a substrate of polylysine/laminin, the neuronal cell bodies adhere within 30 min and then begin to extend broad lamellae that rapidly collapse into cylindrical axons tipped by growth cones. Within a few hours, virtually all of the neurons have multiple short axons that continue to grow rapidly. By overnight, the axons have formed a dense meshwork, with individual axonal lengths often exceeding 1,000 μm. Monastrol (or vehicle alone) was added to the cultures 30 min after plating, after the adhesion of the cell bodies but before the genesis of axons. Axonal lengths were calculated per cell body at 4, 24, 48, and 72 h after plating. Quantification of axonal lengths was performed on cultures that had been fixed at each of the time-points, and then prepared for immunofluorescence visualization of microtubules. In this way, we could quantify axonal length from the same cultures that we used to examine for potential abnormalities in the microtubule array. In addition, we could unequivocally distinguish neurons from process-bearing non-neuronal cells because the antibody used for these experiments recognizes only the βIII-tubulin isoform, which is neuron-specific.

At the 4-h time-point (Fig. 1A–C), control neurons ($n = 50$) had 2.33 ± 0.34 axons per cell body, whereas the monastrol-treated neurons ($n = 50$) had 4.82 ± 0.22 axons per cell body. At 24 h, control neurons ($n = 50$) had 3.95 ± 0.24 axons per cell body, whereas the monastrol-treated neurons ($n = 50$) had 3.72 ± 0.32 axons per cell body. By 4 h, control and monastrol-treated neurons had extended 71.84 ± 10.13 and 342.56 ± 39.10 total microns of axonal length per cell body, indicating an increase in length of 377% in the presence of the drug (Fig. 1D). By 24 h (Fig. 1E,F), control and monastrol-treated neurons had extended $5{,}177.32 \pm 225.57$ and $6{,}100.16 \pm 667.24$ total microns of axonal length per cell body, respectively. Thus, there were significant quantifiable differences in both the number and length of axons extended by the neurons at 4 h (t-test, $P < 0.01$), but these differences were no longer detectable by 24 h. At the 48- and 72-h time points, it was impossible to quantify the number of axons emerging from individual cell bodies, due to the density of the cultures. As with the 24-h time point, there was no significant difference in axonal length compared to control cultures at these later time points. Other than the increases in axonal number and length at the 4-h time-point, the appearance of the neurons in the monastrol-treated and control cultures was indistinguishable from one another. Both the control and the monastrol-treated neurons were healthy and robust in appearance both at the phase-contrast level and in terms of microtubule distribution observed in the immunofluorescence preparations. Confirming its efficacy at inhibiting proliferation of cells, monastrol drastically reduced the appearance of non-neuronal cells in the cultures. Data for later time points are shown in Figure 1G.

Effects of Monastrol Vs. Taxol on Dissociated Cultures of Rat Sensory Neurons

Because taxol is the most common microtubule-related drug used to treat cancer, and because peripheral neuropathies induced by taxol most drastically affect the long sensory axons extended by dorsal root ganglia, we decided to perform a side-by-side analysis of the effects of monastrol and taxol on dissociated cultures of rat sensory neurons. When cultured on polylysine/laminin, sensory neurons are more delayed in the onset of axogenesis compared to the sympathetic neurons. Therefore, we were unable to quantify a 4-h time-point, because there were essentially no axons to score in either control or drug-treated cultures. However, to address the issue of axonal number, we examined cultures a few hours after axons emerged from the cell body (roughly 10 h after plating). Unlike the case with the sympathetic neurons, there was no significant difference between control (1.75 ± 0.22 axons/cell body; $n = 15$) and monastrol-treated (1.66 axons/cell body ± 0.33; $n = 15$) neurons with regard to this point.

The general appearance of the monastrol-treated neurons was similar to that of control neurons except for a significant difference in axonal length at all time points. In contrast to the results on the sympathetic neurons, the total levels of axonal outgrowth in monastrol-treated cultures compared to control cultures were diminished at each of the three time points. Total axonal lengths per cell body were less than half the control levels at 24 (see Fig. 2A,B) and 48 h, and slightly above half at 72 h. As with the sympathetic cultures, the non-neuronal cells were depleted by the monastrol, but the effect was more dramatic because sensory cultures normally contain more non-neuronal cells than sympathetic cultures. We suspect

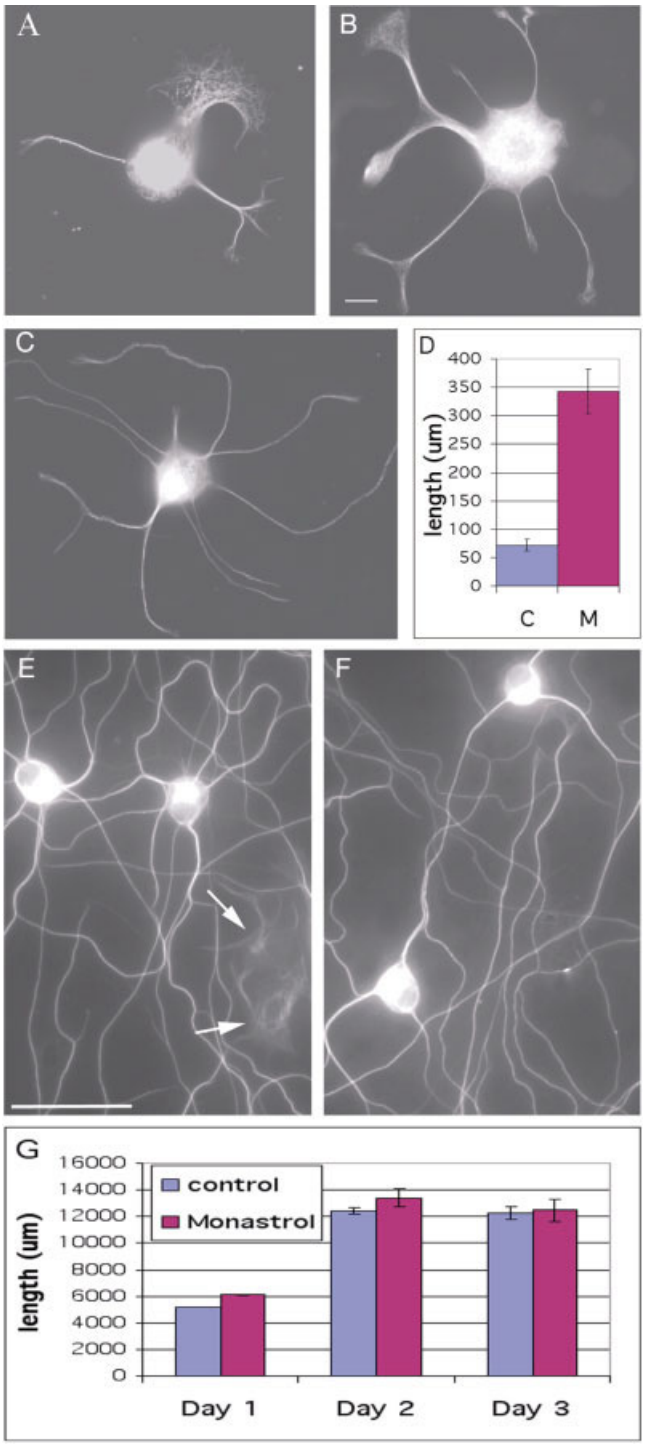

Fig. 1. Effects of monastrol on dissociated cultures of rat sympathetic neurons. Cultures are immunostained for βIII-tubulin. **A:** Neurons 4 h after plating in vehicle alone. **B,C:** Neurons 4 h after plating in monastrol. Note the increase in axonal number and length in the presence of monastrol. **D:** Summary of data for 4-h time-point. **E,F:** Neurons after 24 h of exposure to vehicle (E) or monastrol (F); cultures are indistinguishable in appearance. *Arrows* in E indicate non-neuronal cells (slightly fluorescent due to nonspecific labeling with the secondary antibody) that are significantly diminished after monastrol treatment. No alterations in microtubules are apparent as a result of monastrol. **G:** Summary of data for later time points. Bar in B (A–C) = 10 μm; bar in E (E–F) = 50 μm.

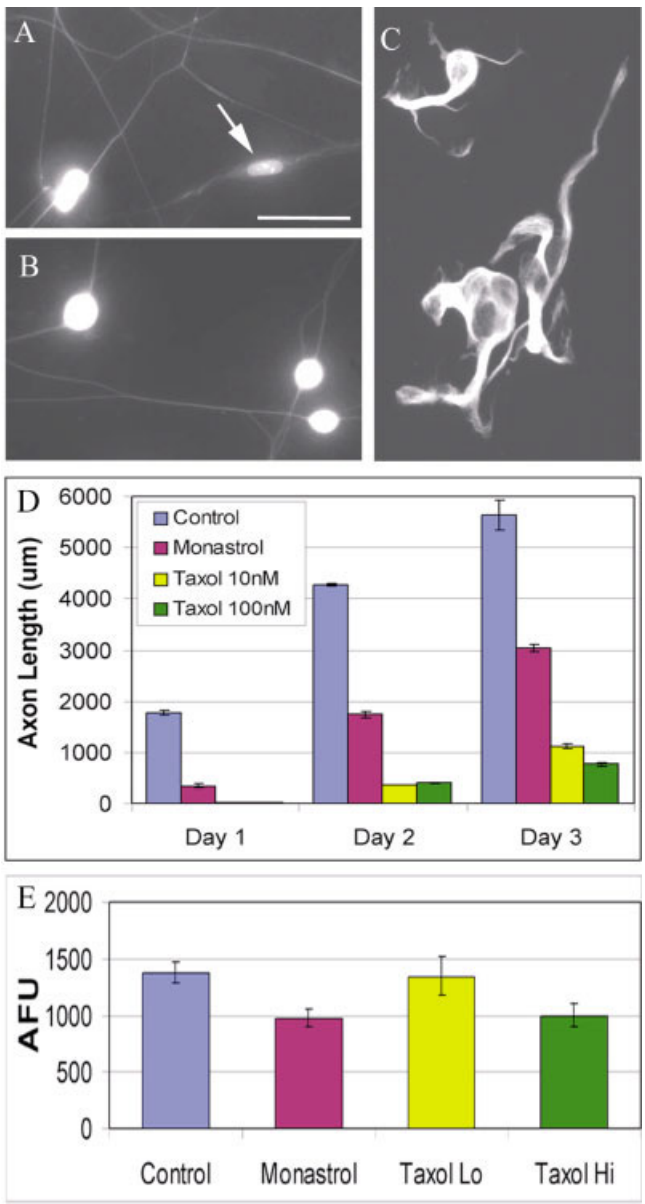

Fig. 2. Effects of monastrol and taxol on dissociated cultures of rat sensory neurons and a murine carcinoma cell line. Cultures are immunostained for βIII-tubulin. **A–C:** Neurons after 24 h of exposure to vehicle alone (A), monastrol (B), or the lower dose of taxol (C). Axons are about half as long in the presence of monastrol compared to controls, but neuronal morphology is otherwise similar to controls. *Arrow* in A indicates a non-neuronal cell (slightly fluorescent due to nonspecific labeling with the secondary antibody), of which there are fewer after monastrol treatment. Axonal growth is severely stunted in the presence of taxol, and neuronal morphology is dramatically abnormal. Microtubules are also abnormal in taxol-treated cultures (see text). **D,E:** Summary of data from neuronal cultures and the carcinoma cell line. Note that monastrol and the higher dose of taxol both produce a noticeable inhibition of cell proliferation while the lower dose of taxol does not. Bar in A (A–C) = 10 μm.

that the slowing of axonal growth may relate (at least in part) to the diminution in non-neuronal cells, which provide factors that support axonal growth. Treatment with taxol, at either the lower or higher dose, killed roughly half the neurons after 24 h, and markedly altered the morphology of the surviving neurons. The total length of the axons of the surviving neurons was diminished to less than 2% of control levels at 24 h, less than 10% at 48 h, and roughly 10 or 20% at 72 h (depending on the taxol concentration). With regard to morphology, the axons of taxol-treated neurons were extremely abnormal in appearance, displaying a very thick diameter and dense bundles of microtubules that often curved (Fig. 2C). As expected, fluorescence intensity for microtubules was at least double in the taxol-treated cultures compared to control, and there were disorganized knots of microtubules in their axons. Similar observations have been reported in previous studies on cultured neurons treated with taxol [Letourneau and Ressler, 1984]. Data are summarized in Figure 2D.

Figure 2E is a graph displaying the effects of monastrol and taxol on cell proliferation in a mammary carcinoma cell line. Monastrol and the higher dose of taxol reduced cell proliferation (no significant difference between them), while the lower dose of taxol is indistinguishable from control. Thus, a concentration of taxol that produces severe effects on neuronal morphology is not noticeably effective at reducing cancer cell proliferation, while a dose of monastrol that produces far less deleterious effects on neurons is equally as effective as the higher dose of taxol at reducing cancer cell proliferation.

Short-Term Effects of Monastrol on Explant Cultures of Sympathetic and Sensory Neurons

The experiments on the dissociated cultures of sensory neurons failed to reveal any increase in axonal growth rates in response to monastrol, as observed with the sympathetic neurons at the 4-h time-point. However, this may simply relate to the fact that the onset of axogenesis is slower in cultures of sensory neurons. In addition, it is unclear from the studies on the dissociated sympathetic cultures whether the burst of axonal growth induced by monastrol is peculiar to axogenesis, or whether pre-formed axons would respond in a similar fashion. To address these issues, we wished to study the acute effects of monastrol on axons that had already formed. Toward this end, we prepared explant cultures of sensory and sympathetic ganglia, permitted them to grow axons overnight, and then exposed the cultures to monastrol or vehicle for 4 h. Figure 3A shows an example of an explant culture. The ten longest axons in selected regions were measured before and after treatment. In the case of the sympathetic neurons, the average axonal length grew 177.33 ± 12.91 μm in control dishes, and

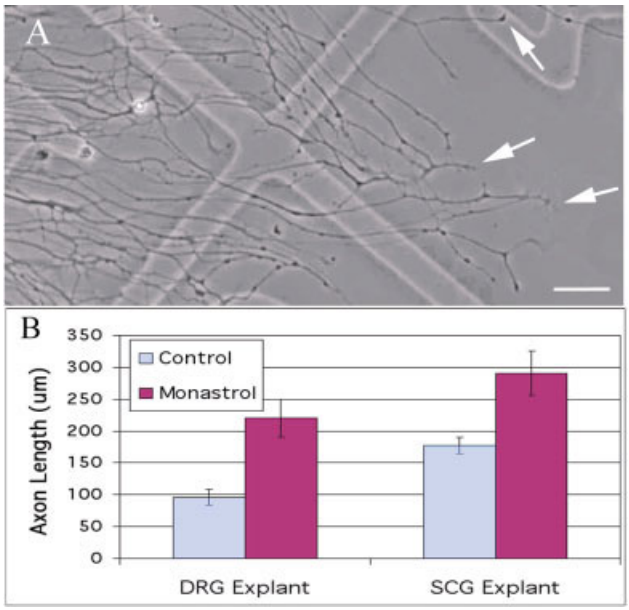

Fig. 3. Effects of 4-h monastrol treatment on already-grown axons from explant cultures. **A:** Portion of an explant culture from superior cervical ganglia showing axon tips (*arrows* show examples) that were monitored for growth. **B:** Summary of data. Axonal growth is increased relative to controls in explant cultures treated with monastrol in the case of both sensory (DRG) and sympathetic (SCG) neurons. Bar in A = 20 μm.

290.50 ± 35.50 μm in monastrol-treated dishes. In the case of the sensory neurons, the average axonal length grew 96.00 ± 12.00 μm in control dishes, and 220.00 ± 30.00 μm in monastrol-treated dishes. Thus, in the sympathetic and sensory cultures, there was a 96 and 129%, respectively, increase in axonal growth rate in the presence of monastrol compared to controls (*t*-test, $P < 0.01$). On this basis, we conclude that in both sensory and sympathetic neurons, acute exposure to monastrol induces a clear and dramatic increase in the rate of axonal growth. Observations after longer exposure times were consistent with those from the dissociated cultures (data not shown). Data are summarized in Figure 3B.

DISCUSSION

While we cannot completely dismiss the possibility that monastrol might affect other kinesins in neurons, available evidence suggests that the drug is highly specific to Eg5 in mitotic cells and in vitro [Mayer et al., 1999; Kapoor et al., 2000; Maliga et al., 2002; DeBonis et al., 2003]. Thus, we believe that the results obtained from these studies most likely reflect an inhibition in the functions of this motor within neurons. Eg5 is the vertebrate member of the BimC family of kinesin-related proteins. This family of motors is probably the most extensively studied of any of the force-generating mitotic

kinesins and yet there is still some mystery surrounding its mode of action. BimC motors are known to form homotetrameric complexes, with four motor domains projected outward [Kashina et al., 1996]. Therefore, in theory, they can drive apart microtubules of opposite orientation and zipper together microtubules of the same orientation, and this has been shown in vitro [Walczak et al., 1998]. It also makes sense that the heterotetramers could generate forces that move individual microtubules with their minus ends leading, if the motor complex is tethered to a structure with greater resistance to movement. Early studies with mutants and function-blocking antibodies showed that inhibition of BimC motors results in a monastral spindle, which prompted the conclusion that these motors are essential for separation of the duplicated centrosomes during prophase [Blangy et al., 1995; Heck et al., 1993]. Subsequent studies on the fly homologue, termed KLP61F, showed that the motor is not required for centrosome separation but that it is required to prevent other motor-forces from driving the half-spindles back together after their separation [Sharp et al., 1999]. This is presumably due to forces generated by the motor between oppositely oriented microtubules in the midzone. Studies on monoasters of microtubules in vitro have shown that Eg5 opposes the outward movement of microtubules by cytoplasmic dynein, presumably by generating forces that drag the microtubules inward [Gaglio et al., 1996]. Related studies in vitro and also in *Drosophila* early embryos have shown that BimC motors also oppose the activity of a class of unusual kinesins (which includes homologues called HSET, CHO2, or NCD), which generate movement in the same direction as cytoplasmic dynein relative to the microtubule [Sharp et al., 1999; Mountain et al., 1999]. Finally, recent studies suggest that Eg5 may be part of a stationary spindle matrix that helps mediate mitotic movements by other motors [Kapoor and Mitchison, 2001].

The results of the present study show that inactivation of Eg5 with monastrol negatively affects the proliferation of cancerous cells and other mitotic non-neuronal cells as expected, but actually has a positive short-term effect on axonal growth. When applied to either sensory or sympathetic neurons for a few hours, monastrol results in an enhanced rate of axonal growth. In sympathetic neurons, which initiate axogenesis much faster than sensory neurons, there is also an increase in the total number of axons produced by each cell body. Given that Eg5 is specialized to generate forces on microtubules, we would conclude that Eg5 normally functions to oppose the forward advance of microtubules required for axons to grow. Available evidence suggests that microtubules are transported down axons by cytoplasmic dynein [Dillman et al., 1996; Ahmad et al., 1998]. This suggests that the manner by which Eg5 antagonizes axonal growth might be comparable to the manner by which it opposes dynein-driven forces in microtubule asters. If so, Eg5 would tend to drag microtubules backwards, which would partially attenuate their forward movement by cytoplasmic dynein. If postmitotic neurons express a kinesin such as HSET/CHO2/NCD, this motor and Eg5 might generate a push and pull on the microtubules that can be regulated by modulating the activity of either motor. Another idea, which is quite different, is that Eg5 might promote the looping of the microtubules back on themselves to generate overlapped regions of non-uniform orientation, similar to the spindle midzone. Looped bundles are known to exist in growth cones that are stalled in their advance [Dent et al., 1999].

We suspect that Eg5 may help regulate axonal development in response to signal cascades affected by environmental cues. Signals that slow axonal growth would increase the activity of Eg5 whereas signals that speed axonal growth would decrease the activity of Eg5. In mitotic cells, it has been established that Eg5 is phosphorylated by p34cdc2, and that this phosphorylation is necessary for association of Eg5 with microtubules [Blangy et al., 1995; Sawin and Mitchison, 1995]. Neurons do not express p34cdc2, but perhaps other kinases and phosphatases regulate Eg5 activity by modulating the levels of Eg5 that are associated with microtubules in discrete regions of the neuron. For example, neurons express CDK5, which shares many of the same substrates as p34cdc2, and Eg5 has also been shown to have a putative phosphorylation site for STK6 [Giet et al., 1999]. We previously used an Eg5 antibody that showed enrichments of immunoreactivity along microtubules in the most distal regions of growing axons [Ferhat et al., 1998]. Other more recent antibodies fail to show this enrichment (Yu et al., unpublished data), leading us to believe that the earlier antibody may have recognized a phosphovariant of Eg5. Interestingly, we originally speculated that Eg5 might antagonize rather than augment axonal growth because this variant was also observed to be localized in dendritic sprouts with very limited growth potential [Ferhat et al., 1998]. Of course, regulation of microtubule-related motors is complex, and there are several other possibilities for how Eg5 activity might be regulated in neurons and coordinated with the growth needs of developing axons.

One of the key purposes of this study was to evaluate the prototype anti-kinesin drug for its effects on neuronal health, given the potential for anti-kinesin drugs to be used for cancer therapy. The fact that short-term treatment with monastrol actually enhances axonal growth contrasts with anti-cancer drugs such as the taxanes, which are highly deleterious to axonal formation and growth. Over longer exposure times, there is no indication of any kind of toxicity of monastrol to cultures of sympathetic neurons. Axonal growth rates are slowed to about half their normal rates in sensory cultures ex-

posed to monastrol, but the neurons otherwise appear to be normal in terms of morphology and microtubule organization. This is profoundly different from the results of treatment with taxol, which severely stunts axonal growth, alters neuronal morphology, causes abnormal microtubule accumulations, and kills many of the neurons. We suspect that the diminution in axonal growth rate in sensory cultures is partially but not completely the result of the loss of non-neuronal support cells from the cultures, as other methods for knocking down non-neuronal cells are less deleterious to axonal outgrowth over a similar time-frame (unpublished observations). We are hopeful that newer anti-kinesin drugs (which can be used at much lower concentrations) will produce even better results when applied to sensory neurons as well as other types of neurons that are exposed to drugs used to treat cancer. It is also provocative to consider the possibility that anti-kinesin drugs with less toxicity might cause a sustained increase in axonal growth rates, and hence might be useful for augmenting axonal regeneration after injury.

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