Effects of stathmin inhibition on the mitotic spindle

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SUMMARY

Stathmin is a major cytosolic phosphoprotein that plays an important role in the regulation of microtubule dynamics during cell cycle progression. It has recently been proposed that the major function of stathmin is to promote depolymerization of the microtubules that make up the mitotic spindle. In this report, we tested the prediction that a deficiency in stathmin expression would result in constitutive stabilization of microtubules and lead to abnormalities in the organization of the mitotic spindle. Our studies demonstrate that antisense inhibition of stathmin expression in K562 erythroleukemic cells results in increased ratio of polymerized to depolymerized tubulin. These changes are associated with phenotypic abnormalities of the mitotic spindle and difficulty in completing mitosis. These studies also showed that inhibition of stathmin expression results in increased susceptibility of K562 leukemic cells to the pharmacological agents, like taxol, which are known to stabilize the mitotic spindle. In contrast, stathmin inhibition results in decreased sensitivity to vinblastine, an agent that destabilizes the mitotic spindle. Thus, our experimental findings are supportive of the model that stathmin is a microtubule-destabilizing factor that plays an important role in the regulation of the mitotic spindle. We also suggest a potential therapeutic approach for cancer based on the combination of stathmin inhibition with pharmacologic agents that stabilize the mitotic spindle.

Key words: Stathmin, Mitotic spindle, Microtubule

INTRODUCTION

The mitotic spindle is a bipolar structure that plays a critical role in chromosome alignment and segregation during mitosis. It is made up of microtubules that consist of polymers of α- and β-tubulin heterodimers. Microtubules exist in a state of dynamic instability characterized by frequent transitions between phases of growth and shrinkage (Desai and Mitchison, 1997). The transition from a phase of growth to a phase of shrinkage is known as 'catastrophe' while the transition from a phase of shrinkage to a phase of growth is known as 'rescue' (Desai and Mitchison, 1997). The dynamic instability of microtubules increases markedly at the onset of mitosis when interphase microtubules have to depolymerize and then repolymerize to assemble the mitotic spindle (Hyman and Karsenti, 1996; Zhai et al., 1996). The intracellular kinetics of microtubule polymerization and depolymerization are regulated by several proteins including microtubule associated proteins (MAPs) (for review see Cassimeris, 1999) and the microtubule destabilizing proteins like stathmin and XKCM1 (Belmont and Mitchison, 1996; Walczak et al., 1996).

Stathmin (also known as p18) is a cytosolic phosphoprotein that is expressed at high levels in leukemia, lymphoma and many solid tumors including neuroblastoma, prostate, breast and ovarian cancers (Alaiya et al., 1997; Bieche et al., 1998; Brattsand et al., 1993; Friedrich et al., 1995; Ghosh et al., 1993; Hanash et al., 1988; Nylander et al., 1995; Roos et al., 1993). It is also expressed at higher levels in proliferating non-transformed cells than in non-proliferating cells (Rowlands et al., 1995). Our previous investigation of the role of this phosphoprotein in cellular proliferation demonstrated that it is a substrate for $p34^{cdc2}$ kinase, the major cyclin-dependent kinase that regulates entry into mitosis (Luo et al., 1994). These studies also demonstrated that antisense inhibition of stathmin expression results in growth inhibition and accumulation of cells in the G_2/M phases of the cell cycle (Luo et al., 1994). Marklund and his coworkers confirmed these observations and extended them by showing that over-expression of stathmin in leukemic cells also results in growth inhibition and accumulation of cells in the G_2/M phases of the cell cycle (Marklund et al., 1994; Marklund et al., 1996). These intriguing findings were later explained by the observations that were made by Belmont and Mitchison who identified stathmin as a cellular factor that promotes microtubule depolymerization by increasing the rate of catastrophe (Belmont and Mitchison, 1996). Subsequent studies suggested that stathmin may inhibit polymerization of microtubules by simple sequestration of non-polymerized tubulin (Curmi et al., 1997; Jourdain et al., 1997). More recent studies showed that stathmin may have catastrophe-promoting activity under certain conditions and tubulin-sequestering activity under other conditions (Howell et al., 1999).

Marklund et al. investigated the effects of over-expression of wild-type and mutant stathmin on microtubule dynamics in K562 erythroleukemia cells (Marklund et al., 1996). They demonstrated that microtubule polymerization is prevented in interphase in cells in which wild-type stathmin is over-expressed. However, when these same cells enter the mitotic phase of the cell cycle and stathmin is phosphorylated, tubulin can then polymerize and a mitotic spindle is formed. In

contrast, when mutant forms of stathmin that can not be phosphorylated at the cdc2 kinase target sites are expressed, the cells can not polymerize their microtubules neither in interphase nor in metaphase and the formation of the mitotic spindle is completely aborted (Marklund et al., 1996). These observations support a role for stathmin in promoting microtubule depolymerization and suggest that its phosphorylation in mitosis would result in its inactivation. This inactivation of stathmin by phosphorylation when cells enter the mitotic phase allows tubulin to polymerize and the mitotic spindle to form (Larsson et al., 1997). Recent studies from our laboratory suggest that dephosphorylation of stathmin is necessary for the disassembly of the mitotic spindle, exit from mitosis and entry into a new cell cycle (unpublished data).

In this report, we describe experiments that were designed to test several predictions of the model of Belmont and Mitchison, which proposes that the major cellular function of stathmin is to promote the depolymerization of microtubules (Belmont and Mitchison, 1996). We had previously generated stable K562 erythroleukemia cell lines in which stathmin expression is inhibited to different degrees by antisense RNA (Luo et al., 1994). In experiments described below, we utilized these stable cell lines to investigate the effects of inhibition of stathmin expression on the organization of the mitotic spindle and on microtubule polymerization and depolymerization in vivo. We also used these cell lines to examine the effect of stathmin inhibition on the relative susceptibility of leukemic cells to agents like taxol and vinblastine that either stabilize or destabilize the mitotic spindle, respectively. The experiments described in this report support an important role for stathmin in promoting microtubule depolymerization which is necessary for the orderly progression through the mitotic phase of the cell cycle.

MATERIALS AND METHODS

Reagents

Taxol and vinblastine (Sigma Chemicals) were dissolved in DMSO (dimethyl sulfoxide) at 10 mg/ml and stored at –20°C as stock solutions. Hoechst 33342 (Sigma Chemicals) was dissolved in water at 1 mg/ml and stored at 4°C in the dark.

Cell lines

The two K562 erythroleukemia cell lines that were used in this study were previously generated by stable transfection with an expression vector containing the complete transcription unit for a mutant DHFR (dihydrofolate reductase) that has a low affinity for methotrexate (Luo et al., 1994). The control K562(C) cells were transfected with an expression construct without stathmin cDNA (Luo et al., 1994). The stathmin-inhibited cells were generated by transfecting the expression construct containing the full-length stathmin cDNA in the antisense orientation relative to an SV40 promoter (Luo et al., 1994). The antisense stathmin cDNA sequences were co-amplified with the DHFR sequences by exposing the transfected cells to 1 μM or 50 μM methotrexate (Luo et al., 1994). The cell lines were grown in RPMI medium supplemented with 10% FBS (fetal bovine serum) and 100 units/ml of penicillin and 100 μg/ml of streptomycin at 37°C in a humidified 5% CO_2 environment. All experiments were performed using cells in the logarithmic phase of growth.

Analysis of mitotic spindle morphology by immunofluoresence

Cells were grown in suspension in the absence and presence of taxol or vinblastine for 24 hours. The cells were washed once in PBS (phosphate buffered saline), pH 7.4, then in PBS containing 2 mM EGTA (ethylene glycol bis-N,N,N′,N′-tetraacetic acid) and cytocentrifuged on slides. The slides were fixed in 0.5% glutaraldehyde/4% paraformaldehyde for 10-15 minutes, then transferred to ice-cold methanol containing 1 mM EGTA for 20 minutes. After three 5-minute washes in PBS, the non-specific binding sites were blocked by incubation in 3% BSA (bovine serum albumin) in PBS for 15 minutes. This was followed by incubation with the anti-α-tubulin antibody (Sigma), at a dilution of 1:1500, for 1 hour at room temperature. The slides were washed three times for 5 minutes each in PBS containing 0.1% BSA and then incubated for 1 hour with the FITC-conjugated anti-mouse IgG secondary antibody (Sigma Chemicals) at a dilution of 1:200. After three washes in PBS, the cells were stained with 1.5 μM Hoechst 33342 (Sigma Chemicals), rinsed in PBS three times and mounted under coverslips using an aqueous mounting gell (Gel/Mount, Biomeda). The mitotic spindle morphology was analysed using a Zeiss fluorescence microscope. At least 1000 cells and between 80 and 120 mitoses were analyzed for each cell line in the presence or absence of drug exposure.

In vivo assessment of tubulin polymerization

The level of polymerized and depolymerized forms of tubulin in vivo were measured using a modification of the procedure that was previously described (Minotti et al., 1991; Marklund et al., 1996). Equal numbers (1×10^6) of control K562(C) and stathmin-inhibited K562(AS) cells were lysed in a microtubules stabilizing buffer containing 0.1 M Pipes (piperazine-N, N′-bis[2-ethane-sulfonic acid]), pH 6.9, 2 M glycerol, 5 mM $MgCl_2$, 2 mM EGTA, 0.5% Triton X-100 and 5 μg/ml leupeptin. Taxol was added to the lysis buffer to a final concentration of 4 μM to maintain the stability of microtubules during the course of isolation. The cell lysates were spun at 40,000 ***g*** for 20 minutes to sediment the polymerized tubulin. The supernatant containing the soluble tubulin was separated from the pellet containing the polymerized tubulin. The tubulin content in the pellets and supernatants were then analyzed by SDS-PAGE followed by immunoblotting with an anti-α-tubulin antibody (clone B-5-1-2, Sigma Chemicals). The bound antibodies were revealed by chemiluminescence. The fraction of tubulin in the polymerized state was calculated by taking the ratio of tubulin in the pellet divided by the sum of the ratios of tubulin in the pellet and its corresponding supernatant.

Proliferation assays

Cells were seeded at a density of 2×10^4 cells/ml in the presence or absence of taxol or vinblastine at the indicated concentrations. The cells were stained with trypan blue and viable cells were counted daily using a hemocytometer.

In vitro clonogenic assays

The cells were grown in the absence and presence of taxol or vinblastine at the indicated concentrations for a period of 24 hours. The cells were then washed in PBS and resuspended in 5 ml of methylcellulose-based semisolid culture medium (0.9% methylcellulose, 1% BSA, 0.1 mM β-mercaptoethanol prepared in RPMI culture medium containing 30% FBS and 100 units/ml of penicillin and 100 μg/ml streptomycin). The cells were then plated at a density of 1×10^3 cells/ml in six-well tissue culture plates and the colonies that formed were counted after 8 to 10 days.

Flow cytometric analysis

PI (propidium iodide) staining of fixed whole cells was performed for cell cycle analysis (Spector et al., 1998). The cells were incubated for 24 hours in the absence or presence of taxol or vinblastine at the indicated concentrations and then washed twice in PBS and fixed in 0.5% paraformaldehyde for 30 minutes. The fixed cells were permeabilized in 0.1% Triton X-100 for 3 minutes,

washed and resuspended in 1 ml staining solution (PBS containing 0.05 mg/ml PI and 1 mg/ml RNAase). The cells were then incubated at 37°C for 30 minutes and analyzed within 2 hours using a FACstar Plus (Becton Dickinson) flow cytometer at 488 nm single laser excitation. The cell cycle distribution was determined using Lysis II software.

Morphological assessment of apoptosis

Cells were grown in the absence or presence of taxol or vinblastine for 24 hours. The cells were then washed in PBS and fixed in 3.7% paraformaldehyde for 15 minutes. The fixed cells were cytocentrifuged on glass slides, washed twice in PBS and permeabilized with 0.5% Triton X-100. The cells were then stained with Hoechst 33342 for 30 minutes at 37°C, rinsed in PBS and mounted under coverslips. The nuclear morphology of the cells was analyzed using a Zeiss fluorescence microscope.

RESULTS

Effects of stathmin inhibition on the mitotic spindle

The organization of the mitotic spindle was examined by indirect immunofluorescence using an antibody to α-tubulin and the Hoechst 33342 fluorochrome that stains chromatin. Different types of abnormalities of the mitotic spindle were seen in both the control K562(C) and the stathmin-inhibited K562(AS) cells (Fig. 1). The incidence of the different types of spindle abnormalities that were seen in the two cell lines are summarized in Table 1. In the control K562(C) cells majority of the mitotic spindles had a normal arrangement of microtubules and chromosomes. However, 23% of the mitoses in the control K562(C) cells showed some spindle abnormalities. In 4% of mitoses, bipolar spindles were seen with short tufts of astral microtubules and one or two misaligned chromosomes (i.e. type I abnormalities; Wilson and Jordan, 1994; Fig. 1B,C and b,c). In another 9% of mitoses, more chromosomes failed to align into the metaphase plate and the astral microtubules were significantly longer (i.e. type II abnormalities; Wilson and Jordan, 1994). 10% of the examined mitoses in the control K562(C) cells had type III abnormalities consisting of microtubules in a star-shaped arrangement and clusters of chromosomes that failed to form a metaphase plate (Wilson and Jordan, 1994).

Analysis of mitoses in stathmin-inhibited K562(AS) cells revealed a higher incidence of spindle abnormalities than in the control K562(C) cells (34% vs 23%, *P* value=0.002). There were about twice as many type I (9.5% vs 4%) and type II (17.5% vs 9%) abnormalities in the stathmin-inhibited cells than in the control cells. In addition, compared to the control K562(C) cells, the K562(AS) cells with type II abnormalities had a shorter interpolar distance and a larger number of 'lagging' chromosomes located near the spindle poles (Fig. 1D,d and E,e). Interestingly, the number of cells with type III spindle abnormalities was not significantly increased in the presence of stathmin inhibition. However, in a small number of mitotic K562(AS) cells, the spindle had a multipolar configuration. Interestingly, this severe spindle abnormality was never seen in the control K562(C) cells.

Effects of stathmin inhibition on tubulin polymerization

We compared the levels of polymerized and nonpolymerized tubulin in control K562(C) and stathmin-inhibited K562(AS) cells in vivo using a sensitive biochemical assay (Marklund et al., 1996; Minotti et al., 1991). This assay was based on isolation of the in vivo assembled microtubules in their polymerized state and the nonpolymerized tubulin as a soluble protein (Marklund et al., 1996; Minotti et al., 1991). The two forms of tubulin were separated by ultracentrifugation and the levels of polymerized and nonpolymerized forms of tubulin were quantified by immunoblotting with an anti-tubulin antibody. Immunoblot analyses showed a 2-fold increase in the ratio of polymerized to nonpolymerized tubulin in K562(AS) cells relative to the control K562(C) cells (Fig. 2). This suggests that inhibition of stathmin expression results in an increase in microtubule polymerization.

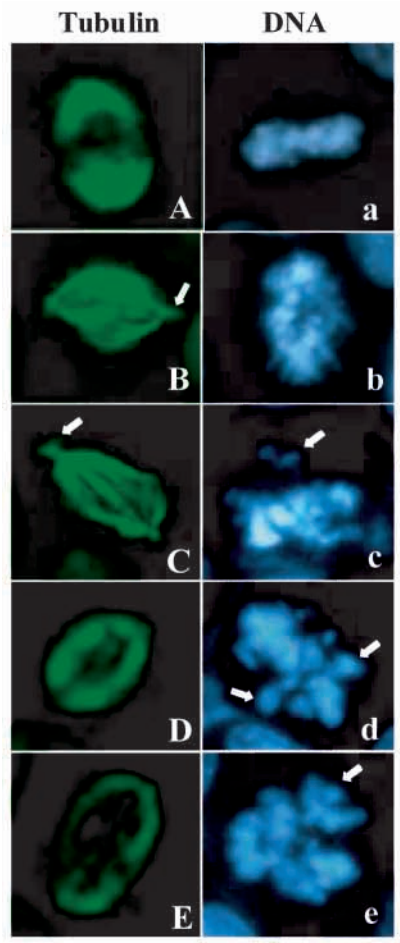

Fig. 1. Microtubule and chromosome organization in K562 cells in the presence or absence of stathmin inhibition. (A,B,C,D,E) Indirect immunofluorescence staining for α-tubulin; (a,b,c,d,e) Hoechst 33342 staining of chromatin in the same cells. The selected panels illustrate the types of mitotic abnormalities that were seen in the control K562(C) and stathmin-inhibited K562(AS) cells. (A and a) A normal spindle and metaphase plate. Spindles with type I abnormalities show small tufts of astral microtubules (arrows in B,C), a normal metaphase plate (b) or a metaphase plate with one or two misaligned chromosomes (arrow in c). Spindles with type II abnormalities show a shorter interpolar distance (D) or a partial loss of polar microtubules (E) with two or more misaligned chromosomes (arrows in d and e).

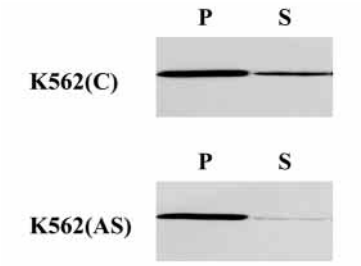

Fig. 2. Effect of stathmin inhibition on tubulin polymerization. The autoradiographs represent quantitative western blot analyses of nonpolymerized and polymerized forms of tubulin derived from K562 cells. The top panel shows the total pellet (P) and soluble (S) fractions of tubulin derived from control K562(C) cells. The bottom panel shows the total pellet (P) and soluble (S) fractions of tubulin derived from K562(AS) cells.

Effects of stathmin inhibition on the susceptibility to pharmacologic agents that interfere with the mitotic spindle

Effects on cell proliferation

We compared the effects of exposure to taxol or vinblastine on the proliferation of control K562(C) and stathmin-inhibited K562(AS) cells. Exposure to taxol at a concentration of 1 nM had essentially no effect on the proliferation of the control K562(C) cells (Fig. 3A). In contrast, the same concentration resulted in a reproducible decrease in the proliferation of the stathmin-inhibited K562(AS) cells (Fig. 3A). Exposure to taxol at a concentration of 2 nM resulted in a moderate decrease in the proliferation of the control K562(C) cells whereas the stathmin-inhibited K562(AS) stopped proliferating completely when exposed to the same concentration of the drug (Fig. 3A). Thus, inhibition of stathmin expression results in increased sensitivity of K562 leukemic cells to the antiproliferative effects of taxol.

When the control K562(C) cells were exposed to vinblastine at a concentration of 2 nM, a modest reduction in their rate of proliferation was observed (Fig. 3B). In contrast, the proliferation of the stathmin-inhibited K562(AS) cells was not affected by the same drug concentration (Fig. 3B). When the two cell lines were exposed to vinblastine at a concentration of 4 nM, both the control K562(C) and the stathmin-inhibited K562(AS) cells showed markedly reduced proliferative rates. Proliferation could not be assessed in the presence of 6 nM vinblastine since exposure to this concentration for more than 24 hours was highly toxic to both cell lines. Thus, inhibition of stathmin expression in the K562(AS) cells is associated with relative resistance to the antiproliferative effects of vinblastine.

Table 1. Mitotic spindle abnormalities in control K562(C) and stathmin-inhibited K562(AS) cells

	K562(C)	K562(AS)	*P* value*
Type I abnormalities	4%	9.5%	0.025
Type II abnormalities	9%	17.5%	0.012
Type III abnormalities	10%	7%	0.71
All mitotic abnormalities	23%	34%	0.002

**P* values for a 2×2 table based on 2442 K562(C) cell and 1321 K562(AS) cells.

Similarly, in experiments not shown, inhibition of stathmin expression also resulted in relative resistance to the antiproliferative effects of nocadozole, another microtubule destabilizing agent.

Effects on cell cycle progression

Cell cycle progression in the absence and presence of taxol or vinblastine was investigated by flow cytometric analysis of the DNA content of PI stained cells. This analysis was performed on both control K562(C) and stathmin-inhibited K562(AS) cells that were exposed to taxol at a concentration of 2 nM or to Vinblastine at concentrations of 4 nM and 6 nM. The DNA histograms shown in Fig. 4 show the cell cycle profiles of control K562(C) and stathmin-inhibited K562(AS) cells before and after 24 hours of drug exposure. As previously described, inhibition of stathmin expression in K562(AS) cells results in accumulation of cells in the G_2/M phases of the cell cycle (Luo et al., 1994). Interestingly, exposure of the two cell lines to taxol resulted in a striking perturbation in cell cycle progression characterized by accumulation of cells in the hypodiploid region of the histograms. However, a larger fraction of hypodiploid cells was seen following taxol exposure of the stathmin-inhibited K562(AS) cells (27%) than the control K562(C) cells (13%) (Fig. 4A). A hypodiploid fraction in a DNA histogram is generally indicative of apoptosis (Darzynkiewicz et al., 1992). Thus, we analyzed the morphology of the taxol-exposed cells by DNA staining with Hoechst 33342 to identify apoptotic cells that have undergone nuclear fragmentation (Allen et al., 1997). A significantly larger

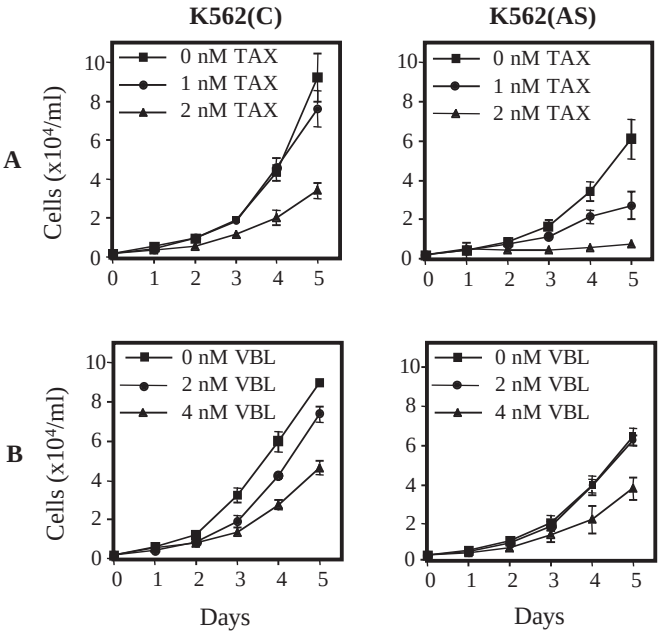

Fig. 3. Effects of taxol and vinblastine on the rate of proliferation control and stathmin-inhibited cells. Exponentially growing control K562(C) and stathmin-inhibited (K562.AS) cells were plated at a density of 0.2×10^5 cells/ml in the absence and presence of taxol (A) and vinblastine (B) at specified concentrations. Viable cells were counted daily in a hemocytometer for 5 consecutive days. The data plotted are the mean ± s.d. of three different experiments.

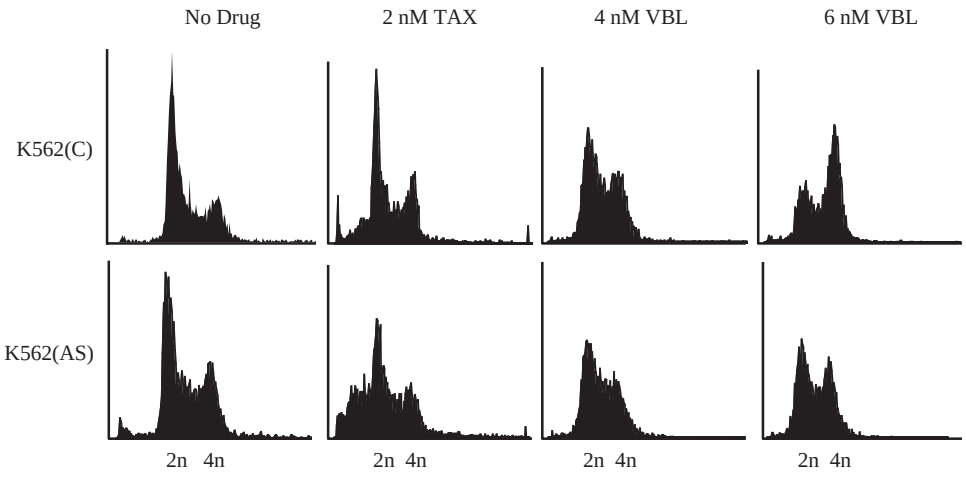

Fig. 4. Effects of taxol and vinblastine on the cell cycle profile of control and stathmin-inhibited cells. Control K562(C) and stathmin-inhibited K562(AS) cells were grown in the presence and absence of the specified concentrations of taxol (TAX) or vinblastine (VBL). After 24 hours, the cells were harvested and DNA content was determined by flow cytometry.

fraction of the stathmin-inhibited K562(AS) cells were apoptotic following exposure to taxol than the control K562(C) cells (Fig. 5). After 24 hours of exposure to taxol, both cell lines showed a mild degree of apoptosis (4.2% in the control K562(C) cells and 4.1% in stathmin-inhibited K562(AS) cells). However, after 72 hours exposure to taxol, the fraction of apoptotic cells increased to 7.2±2.4% in the absence of stathmin inhibition and to 20±4.2% in the presence of stathmin inhibition. Thus, inhibition of stathmin expression results in increased sensitivity of K562 cells to the apoptotic effects of taxol. Cell cycle progression was also evaluated in the two cell lines following exposure to vinblastine. As expected, control K562(C) cells accumulated in the G_2/M phase of the cell cycle in a concentration-dependent manner (Fig. 4). The vast majority of control K562(C) cells were found in the G_2/M phase of the cell cycle after exposure to 6 nM vinblastine. In contrast, a much smaller fraction of the stathmin-inhibited K562(AS) cells accumulated in the G_2/M phases following exposure to vinblastine (Fig. 4). Thus, inhibition of stathmin expression protects K562 cells from the mitotic arrest that is induced by vinblastine.

Effects on clonogenic potential

We also investigated the effects of taxol and vinblastine on the clonogenic potential of K562 leukemic cells in the presence or absence of stathmin inhibition. Control K562(C) and stathmin-inhibited K562(AS) cells were exposed to either taxol or vinblastine for 24 hours and then plated in a methylcellulose-based semisolid medium. The results of the clonogenic assays in the presence or absence of drug exposure are presented in Fig. 6. When the control K562(C) cells were exposed to taxol or vinblastine, their clonogenicity decreased to 56% and 70% respectively. As we previously reported, inhibition of stathmin expression results in a marked decrease in the clonogenic potential of the K562(AS) cells compared to the control K562(C) cells (Jeha et al., 1996). Interestingly, exposure of the stathmin-inhibited K562(AS) cells to taxol resulted in a very marked reduction of their clonogenic potential (13%) relative to the clonogenic potential of the untreated control K562(C) cells. In marked contrast, exposure of the stathmin-inhibited K562(AS) cells to vinblastine had essentially no effect on the clonogenic potential of those cells. Similarly, when nocodazole was used instead of vinblastine, the clonogenicity of the control K562(C) cells decreased while the clonogenicity of the stathmin-inhibited K562(AS) cells was not affected (data not shown). Thus, inhibition of stathmin expression results in increased susceptibility to the anti-clonogenic effects of taxol and decreased susceptibility to the anti-clonogenic effects of vinblastine and nocodazole.

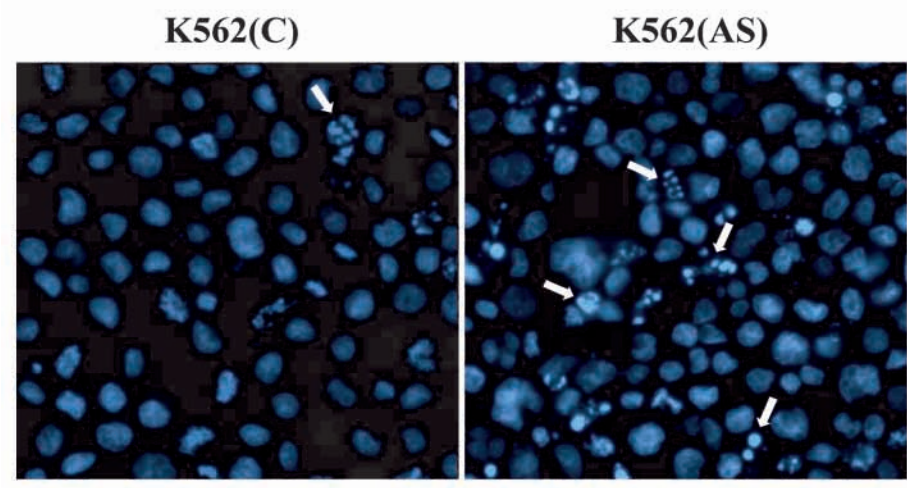

Fig. 5. Effect of taxol on apoptosis in control K562(C) and stathmin-inhibited K562(AS) cells. Brightly fluorescent apoptotic cells (arrows) show characteristic condensation and/or marginalization of chromatin and nuclear fragmentation.

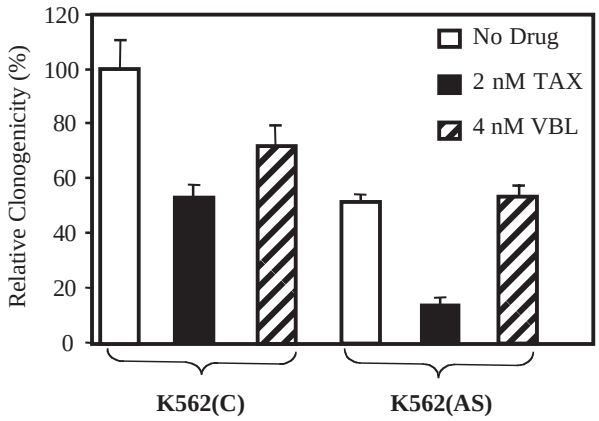

Fig. 6. Effects of taxol and vinblastine on the clonogenic potential of control and stathmin inhibited K562 leukemic cells. Control K562(C) and stathmin-inhibited K562(AS) cells were grown in the absence (open bars) and presence of 2 nM taxol (filled bars) or 4 nM vinblastine (hatched bars). After 24 hours, 10^3 cells were washed and plated in drug-free semisolid medium and the colonies formed were counted after 8 days. The relative clonogenicity of control K562(C) and stathmin-inhibited K562(AS) cells after drugs exposure was calculated considering the relative clonogenicity of untreated control cells as 100%. The data presented are the mean ± s.d. of three different experiments.

Effects on the morphology of mitotic spindle

We analyzed the effects of stathmin inhibition on the susceptibility of the mitotic spindles of leukemic cells to the effect of taxol and vinblastine. The results of this analysis are summarized in Table 2. In the absence of drug exposure, slightly more mitotic cells were seen in the antisense-inhibited K562(AS) cells than in the control K562(C) cells (56 vs 34). The fraction of cells in mitosis essentially doubled in both K562(C) and K562(AS) cells following exposure to taxol. In contrast, upon exposure to vinblastine, the fraction of control K562(C) undergoing mitosis almost doubled (34 to 65) while the fraction of K562(AS) cells undergoing mitosis was essentially unchanged (56 to 54).

In the absence of drug exposure, the incidence of mitotic abnormalities was higher in the K562(AS) cells than in the K562(C) cells (19 vs 8). Moreover, exposure to taxol increased the fraction of mitotic cells with morphological abnormalities by 2.8-fold in the control K562(C) cells (9 to 23) and by 3.3-fold in the stathmin-inhibited K562(AS) cells (19 to 63). Interestingly, although the number of cells with abnormal spindles increased to a similar extent after taxol exposure, the severity of those abnormalities in the two cell lines was significantly different. Thus, the number of mild spindle abnormalities (type I and II) in control K562(C) was essentially unchanged after taxol exposure (4 to 5). In contrast, in stathmin-inhibited K562(AS) cells, the number of type I and II abnormalities increased (15 to 25) following taxol exposure. Moreover, the number of cells with severe spindle abnormalities after taxol exposure increased by 4.5-fold in control K562(C) cells (4 to 18) and by almost 9.5-fold in stathmin-inhibited K562(AS) cells (4 to 38). The vast majority of the stathmin-inhibited cells with severe type III abnormalities had microtubules particularly organized in multipolar spindles (up to 5-6 poles) and chromosomes distributed in a corresponding number of metaphase-like plates (Fig. 7). In contrast, exposure to vinblastine increased the fraction of mitotic cells with morphological spindle abnormalities by 3.8-fold in the control K562(C) cells (8 to 31) and by less than 1.2-fold in the stathmin-inhibited K562(AS) (19 to 22). Essentially identical observations were made when the two cell lines were exposed to nocodazole (data not shown). Thus, inhibition of stathmin expression makes the mitotic spindles of K562 cells more susceptible to the effects of taxol and less susceptible to the effects of vinblastine and nocodazole.

DISCUSSION

The ability to perturb gene expression selectively in a target cell provides a powerful tool for probing the function of a protein of interest. The initial clues that stathmin may have a role in mitosis came from studies from several laboratories, including our own, that demonstrated that inhibition of stathmin expression results in growth inhibition and accumulation of cells in mitosis (Luo et al., 1994; Marklund et al., 1994). Surprisingly, over-expression of the same gene in leukemic cells also resulted in a mitotic arrest (Marklund et al., 1996). This apparent paradox was resolved by the identification of stathmin as a protein that regulates the dynamics of the mitotic spindle by promoting microtubules depolymerization (Belmont and Mitchison, 1996). Thus, it is not surprising that any significant perturbation in the level of expression of stathmin would interfere with the normal progression through the mitotic phase of the cell cycle. Marklund et al. tested this model by analyzing the effects of over-expression of stathmin on the morphology of the mitotic spindle. They also investigated the effects of over-expression of stathmin on the relative susceptibility of leukemic cells to pharmacologic agents that stabilize or destabilize the spindle (Marklund et al., 1996). Their findings were generally

Table 2. Mitotic abnormalities in control K562(C) and stathmin-inhibited K562(AS) cells in the presence or absence of taxol (TAX) and vinblastine (VBL)

	K562(C)			K562(AS)		
	No drug	TAX	VBL	No drug	TAX	VBL
Cell in mitosis	34	70	65	56	120	54
Total number of cells with mitotic spindle abnormalities	8	23	31	19	63	22
Number of cells with type I and II spindle abnormalities	4	5	14	15	25	12
Number of cells with type III spindle abnormalities	4	18	17	4	38	10

The numbers in the table refer to the number of mitoses or the number of mitotic abnormalities per 1000 cells.

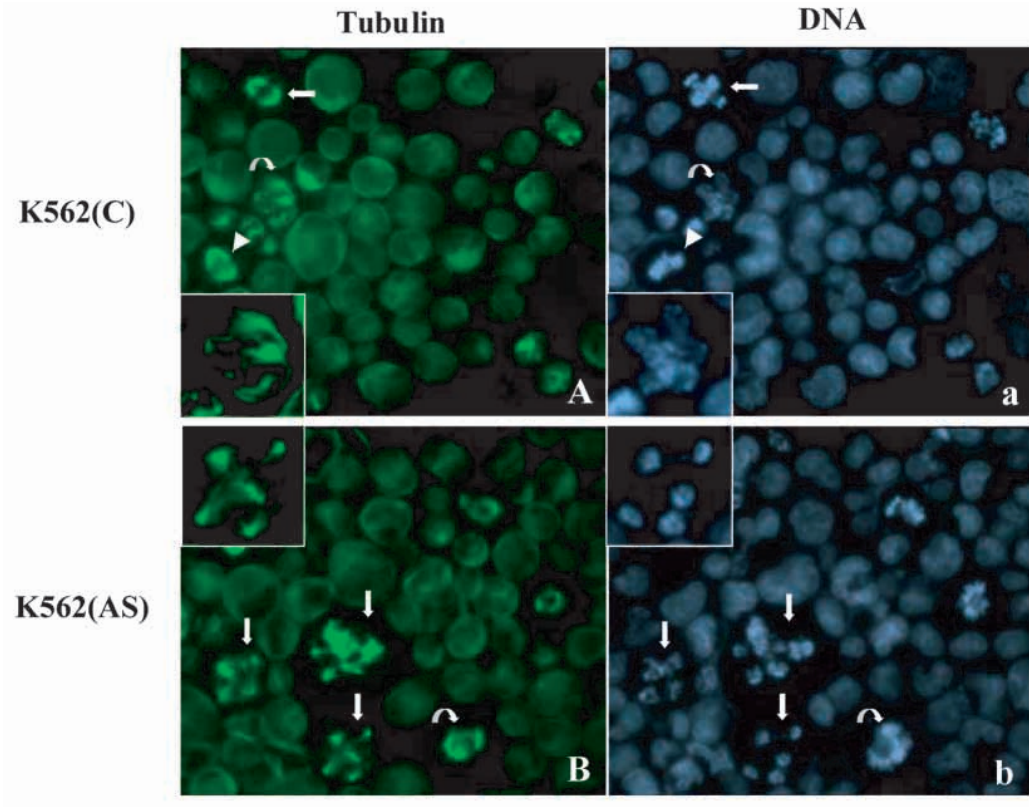

Fig. 7. Microtubule and chromosome organization in the presence or absence of Taxol exposure. The two panels on the left show α-tubulin staining by indirect immunofluorescence while the two panels on the right show Hoechst 33342 staining of chromatin in the same cells. (A and a) Representative of control K562(C) cells; (B and b) representative of stathmin-inhibited K562(AS) cells. A mitotic cell with normal metaphase is marked by arrow heads. The horizontal arrows mark a mitotic cell with type II spindle abnormality with a few chromosomes located near the spindle poles. The curved arrows mark mitotic cells with type III spindle abnormalities with disorganized arrays of microtubules and randomly distributed chromosomes (shown at a higher magnification as insets in A, a). The vertical arrows mark mitotic cells with type III spindle abnormalities with multipolar spindles and chromosomes distributed in multiple metaphase-like plates (shown at a higher magnification as insets in B, b).

supportive of the model proposed by Belmont et al. (Belmont et al., 1996), although a different conclusion was reached about the role of phosphorylation of stathmin in the regulation of its activity (Marklund et al., 1996).

The availability of stably-transfected K562 cell lines in which stathmin expression is inhibited allowed us to test several predictions of the model that was proposed by Belmont and Mitchison (Belmont and Mitchison, 1996). The first prediction is that under conditions where stathmin expression may be limiting, the equilibrium between the polymerized and depolymerized tubulin would be shifted in favor of polymerized tubulin. This prediction was verified experimentally by demonstrating a higher ratio of polymerized to nonpolymerized tubulin in stathmin-inhibited K562(AS) cells than in control K562(C) cells. A second prediction is that cells in which stathmin expression is inhibited should manifest more abnormalities of the mitotic spindle than the control cells. This was also verified by the detection of a significantly larger number of abnormal spindles in stathmin-inhibited K562(AS) than in the control K562(C) cells. The presence of spindle abnormalities in K562(C) cell in the absence of stathmin inhibition should not be surprinsing since these are leukemic cells. Genetic instability, absence of mitotic checkpoints and spindle failure are commonly associated with neoplastic transformation (Hartwell and Kastan, 1994). K562 erytroleukemic cells are known to be nearly triploid (Lozzio and Lozzio, 1975), probably a result of disrupted segregation of chromosomes and/or abnormal mitotic exit. Normal chromosome segregation and mitotic exit are processes that are dependent on the formation of a normal mitotic spindle (Andreassen et al., 1996; Sorger et al., 1997; Sluder et al., 1997). A third prediction of the model is that inhibition of stathmin expression should result in differential susceptibility of leukemic cells to pharmacologic agents that interfere with the mitotic spindle. If the major role of stathmin in mitosis is to promote microtubule depolymerization, a deficiency of stathmin would be expected to result in increased microtubule polymerization. Since taxol is known to stabilize the polymerized form of tubulin (Jordan et al., 1993; Jordan and Wilson, 1999), stathmin inhibition should result in increased susceptibility to the mitotic effects of taxol. Moreover, since vinblastine is known to destabilize microtubules (Jordan et al., 1991; Jordan and Wilson, 1999), stathmin inhibition should result in decreased susceptibility to the mitotic effects of vinblastine. The experiments described above demonstrate that inhibition of stathmin results in increased susceptibility of leukemic cells to taxol as demonstrated by greater growth inhibition, cell cycle perturbations and mitotic spindle abnormalities than in the absence of stathmin inhibition. In contrast, inhibition of stathmin expression results in decreased susceptibility to vinblastine when examined by the same assays. Thus, the effects of inhibition of stathmin expression on the susceptibility to taxol and vinblastine that we describe in this report are fully compatible with the predictions of the model that was proposed by Belmont and Mitchison (Belmont and Mitchison, 1966).

Interestingly, the mitotic spindle abnormalities that are induced by taxol exposure are qualitatively and quantitatively similar to those induced by stathmin inhibition. However, when cells are exposed to taxol in the presence of stathmin inhibition,

the increase in mitotic spindle abnormalities is more than additive (i.e. 63>23+19). Thus, the effects of the combination of taxol and stathmin inhibition on the mitotic spindle appear to be synergistic. This suggests that it may be possible to design a therapeutic strategy to take advantage of this synergy. For example, it may be possible to combine antisense or ribozyme strategies that inhibit stathmin expression with pharmacologic therapy with taxol to result in a more effective abrogation of the malignant phenotype. It may also be possible to develop pharmacologic agents that block the interaction between stathmin and tubulin resulting in inhibition of its activity. Combinations of such pharmacological agents with taxol may also result in a synergistic anti-tumor effect. Since stathmin is expressed at very high levels in essentially all types of transformed cells (Alaiya et al., 1997; Bieche et al., 1998; Brattsand et al., 1993; Friedrich et al., 1995; Ghosh et al., 1993; Hanash et al., 1988; Nylander et al., 1995; Roos et al., 1993) such therapeutic approaches may be useful in the treatment of a wide range of human cancers.

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