Synergistic Antiangiogenic Effects of Stathmin Inhibition and Taxol Exposure

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Abstract

Stathmin is one of the key regulators of the microtubule cytoskeleton and the mitotic spindle in eukaryotic cells. It is expressed at high levels in a wide variety of human cancers and may provide an attractive target for cancer therapy. We had previously shown that stathmin inhibition results in the abrogation of the malignant phenotype. The microtubule-interfering drug, taxol, has both antitumorogenic and antiangiogenic properties. We had also shown that the antitumor activities of taxol and stathmin inhibition are synergistic. We hypothesized that taxol and stathmin inhibition may also have synergistic antiangiogenic activities. A replication-deficient bicistronic adenoviral vector that coexpresses green fluorescent protein and an anti-stathmin ribozyme was used to target stathmin mRNA. Exposure of endothelial cells to anti-stathmin adenovirus alone resulted in a dose-dependent inhibition of proliferation, migration, and differentiation into capillary-like structures. This inhibition was markedly enhanced by exposure of transduced endothelial cells to very low concentrations of taxol, which resulted in a virtually complete loss of proliferation, migration, and differentiation of endothelial cells. In contrast, exposure of nontransduced endothelial cells to taxol alone resulted in a modest inhibition of proliferation, migration, and differentiation. Our detailed analysis showed that the antiangiogenic effects of the combination of stathmin inhibition and taxol exposure are synergistic. Our studies also showed that the mechanism of this synergistic interaction is likely to be mediated through the stabilization of microtubules. Thus, this novel combination may provide an attractive therapeutic strategy that combines a synergistic antitumor activity with a synergistic antiangiogenic activity. (Mol Cancer Res 2007;5(8):773–82)

Introduction

Angiogenesis, the development of new blood vessels from pre-existing vasculature, is essential for the growth of primary tumors and metastasis (1). The cellular cytoskeleton plays a critically important role in the angiogenic process (2). The cytoskeleton undergoes dramatic structural rearrangements during the various stages of angiogenesis, including proliferation, migration, and differentiation of endothelial cells into vascular structures (2). Although the actin cytoskeleton is known to play a pivotal role in the regulation of changes in endothelial cell shape and endothelial cell migration, there is mounting recent evidence in support of the involvement of the microtubule cytoskeleton in angiogenesis (2, 3). Microtubules are essential for cell signaling, protein and organelle trafficking, cell migration, and many other cellular functions. The importance of microtubule dynamics in the process of angiogenesis is supported by the observation that microtubule-interfering agents perturb endothelial cell functions (4, 5). Taxol is a chemotherapeutic agent, which has antitumor activity against a wide range of human cancers. It is also one of the most potent inhibitors of angiogenesis (4, 5). Exposure of endothelial cells to taxol was shown to inhibit their proliferation, migration, and formation of a vascular network on Matrigel (4, 5). Similarly, exposure to taxol in vivo was shown to result in a marked inhibition of the angiogenic response (4).

Stathmin was originally identified as a major cellular protein that is expressed at high levels in a wide variety of human malignancies (6-9). Stathmin was later shown to be a microtubule-destabilizing factor that plays a critically important role in the control of cellular proliferation by promoting microtubule depolymerization (10, 11). Thus, stathmin became the founding member of a family of microtubule-depolymerizing proteins that regulate the assembly and disassembly of the mitotic spindle through cell cycle–specific changes in its state of phosphorylation (10-13). Our previous studies had shown that the inhibition of stathmin expression interferes with mitotic progression (14) and is associated with a marked increase in the content of polymerized microtubules and an increase in spindle abnormalities (12, 15). We also recently showed synergy in the antitumor activities of taxol and stathmin inhibition in prostate cancer cells (16).

In this report, we examined the hypothesis that taxol and stathmin inhibition may also have synergistic antiangiogenic activities. We used an anti-stathmin ribozyme-based strategy for inhibiting stathmin expression in endothelial cells. We had previously designed three anti-stathmin hammerhead ribozymes that were shown to cleave stathmin mRNA catalytically (17). More recently, we incorporated these ribozymes into adenoviral gene transfer vectors and showed that they could suppress the...
malignant phenotype of prostate cancer cells (18). In this study, we have explored the potential utility of anti-stathmin adenovirus as an antiangiogenic agent both as a single agent and in combination with taxol.

**Results**

We used human umbilical vascular endothelial cells (HUVEC) as an in vitro model to test the potential of the anti-stathmin adenovirus as an antiangiogenic agent. We used a bicistronic adenoviral vector that coexpresses the anti-stathmin ribozyme along with the green fluorescent protein (GFP) as a selectable marker for targeting stathmin in HUVECs (18). As controls, cells were either mock infected with PBS or infected with the control Ad.GFP adenovirus without anti-stathmin ribozyme sequences (18). HUVECs were efficiently transduced by these recombinant adenoviruses as determined by flow-cytometric analysis of GFP fluorescence. Table 1 represents quantitative data of GFP fluorescence after infection of HUVECs with the control Ad.GFP or Ad.Rz.GFP adenoviruses at different multiplicities of infection (MOI).

We first examined the effects of adenovirus-mediated gene transfer of anti-stathmin ribozyme on the level of stathmin expression in HUVECs. Figure 1 shows an autoradiograph of a Northern blotting experiment using RNA isolated from uninfected cells or cells infected with either control Ad.GFP or Ad.Rz.GFP adenoviruses. The level of stathmin mRNA was normalized against 18S rRNA as an internal control. As expected, the cells infected with the control Ad.GFP adenovirus did not show significant changes in the level of stathmin mRNA compared with uninfected cells (Fig. 1, lanes 1 and 2). In contrast, the level of stathmin mRNA in cells infected with Ad.Rz.GFP was decreased by 69.9% relative to the uninfected cells (Fig. 1, lane 3). Similarly, Western blot analysis showed a 72.6% reduction in the level of stathmin protein in Ad.Rz.GFP-infected cells relative to control Ad.GFP-infected cells. Thus, the anti-stathmin ribozyme is capable of down-regulating stathmin expression in endothelial cells.

**Effects of Anti-Stathmin Ribozyme and Taxol on Endothelial Cell Proliferation**

Proliferation of quiescent endothelial cells is one of the key steps required for angiogenesis. Thus, we tested the effects of adenovirus-mediated gene transfer of anti-stathmin ribozyme on the proliferation of endothelial cells (Fig. 2). Infection of HUVECs with the control Ad.GFP adenovirus at different MOIs did not show a significant inhibition of growth compared with uninfected cells (Fig. 2A). In contrast, cells infected with Ad.Rz.GFP showed a dose-dependent increase in growth inhibition at increasing MOI, with complete suppression of growth at MOI of 100 (Fig. 2B). We also assessed the effects of combination of anti-stathmin ribozyme and taxol on the proliferation of HUVECs (Fig. 3). To determine whether taxol would enhance the growth inhibitory effects of the anti-stathmin ribozyme, we used low concentrations of the recombinant adenoviruses (MOI of 25) and picomolar concentrations of taxol. Exposure of uninfected cells or cells infected with the control Ad.GFP adenovirus to increasing concentrations of taxol showed a modest dose-dependent decrease in proliferation (Fig. 3A and B). In contrast, exposure of Ad.Rz.GFP-infected cells to the same concentrations of taxol markedly potentiated the growth inhibitory effects of the anti-stathmin ribozyme, leading to a complete suppression of endothelial cell proliferation at a concentration of 6 pMol/L (Fig. 3C). These data show that the anti-stathmin ribozyme inhibits endothelial cell proliferation, and that inhibition of stathmin markedly sensitizes endothelial cells to the growth inhibitory effects of taxol.

**Effects of Anti-Stathmin Ribozyme and Taxol on Endothelial Cell Migration**

Endothelial cell migration is another critically important process in the development of new blood vessels that support tumor growth (1). Based on the important role of stathmin in the regulation of microtubule dynamics, we postulated that the anti-stathmin ribozyme may interfere with the ability of endothelial cells to migrate in response to a chemotactic gradient. Thus, we examined the effects of the recombinant adenoviruses on endothelial cell migration in a Boyden chamber assay (Fig. 4). This assay is widely used to evaluate chemotactic migration as an in vitro model of tumor-induced migration.

### Table 1. Quantitative Analysis of GFP Fluorescence of Infected HUVECs

<table>
<thead>
<tr>
<th>MOI</th>
<th>Ad.GFP (%)</th>
<th>Ad.Rz.GFP (%)</th>
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<tr>
<td>25</td>
<td>36.6</td>
<td>34.5</td>
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<tr>
<td>50</td>
<td>56.1</td>
<td>53.4</td>
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<tr>
<td>100</td>
<td>78.9</td>
<td>81.7</td>
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*Percentage of fluorescent cells was determined by flow cytometry in cells infected with either Ad.GFP or Ad.Rz.GFP adenoviruses at different MOIs.

![Figure 1](image-url)
endothelial migration. When HUVECs were infected with the control Ad.GFP adenovirus at different MOIs, the cells fully retained their ability to migrate through the filter compared with uninfected cells (Fig. 4A). In contrast, HUVECs infected with Ad.Rz.GFP showed a dose-dependent inhibition of chemotactic migration (Fig. 4A and B). Ad.Rz.GFP inhibited migration by 5.7-, 7.5-, and 10.7-fold at MOIs of 25, 50, and 100, respectively (Fig. 4B). We also evaluated the effects of the combination of anti-stathmin adenovirus and taxol on endothelial cell migration (Fig. 5). We used low concentrations of taxol (2–6 pmol/L) for relatively shorter periods (24 h). These low concentrations of taxol had essentially no cytotoxic or growth inhibitory effects on HUVECs (data not shown). Uninfected cells or cells infected with the control Ad.GFP adenovirus showed a modest dose-dependent inhibition of migration following exposure to these low concentrations of taxol (Fig. 5A and B). In contrast, cells infected with Ad.Rz.GFP adenovirus at a low MOI of 25 showed a 5.7-fold inhibition of migration in the absence of taxol (Fig. 5A). Upon exposure of Ad.Rz.GFP-infected cells to the same concentrations of taxol, migration was markedly inhibited by 8.6- and 10-fold at concentrations of 2 and 4 pmol/L, respectively. A virtually complete impairment of the ability of the endothelial cells to migrate was observed at a 6-pmol/L concentration of taxol (Fig. 5C). Thus, stathmin inhibition interferes with the ability of endothelial cells to migrate, and its antimigratory effects can be markedly enhanced by low noncytotoxic concentrations of taxol.

**Effects of Anti-Stathmin Ribozyme and Taxol on Endothelial Cell Differentiation**

The sprouting of new blood vessels in vivo can be recapitulated in vitro in a Matrigel-based system in which endothelial cells can migrate and organize in a manner similar to the early steps of angiogenesis (19). Matrigel is an artificial source of basement membrane matrix that consists primarily of proteins such as laminin, proteoglycan, and collagens (19). When HUVECs are plated on Matrigel, they differentiate to form tubular branching structures that resemble capillaries that infiltrate the tumor in vivo (19). Thus, we designed experiments to determine if stathmin inhibition would interfere with the ability of HUVECs to differentiate into capillary-like structures on Matrigel (Fig. 6). Figure 6A shows representative photomicrographs of tube formation in uninfected cells and in cells infected with either control Ad.GFP or Ad.Rz.GFP adenovirus. Microscopic examination 2 to 3 h after the cells were plated showed end-to-end alignment and elongation of endothelial cells that formed branching structures on Matrigel. After 20 to 22 h on Matrigel, uninfected HUVECs and HUVECs infected with control Ad.GFP formed complex networks of long anastomosing tubes (Fig. 6A). In contrast, HUVECs infected with Ad.Rz.GFP formed abortive capillary networks with short distorted tubes and fewer anastomoses (Fig. 6A). Ad.Rz.GFP inhibited tube formation by 35%, 50%, and 87% at MOIs of 25, 50, and 100, respectively (Fig. 6B). Thus, the anti-stathmin adenovirus shows significant antiangiogenic effects in an in vitro Matrigel assay.

We also analyzed the effects of the combination of anti-stathmin ribozyme with taxol on tube formation in the same in vitro assay (Fig. 7). Here also, we used short exposure and low concentrations of taxol that had no antiproliferative or cytotoxic effects (data not shown). Exposure of uninfected cells or cells infected with Ad.GFP adenovirus to 2 pmol/L taxol did not result in the inhibition of tube formation, and the endothelial cells were able to form a complex network of long anastomosing structures (Fig. 7A). When the same cells were exposed to 4 and 6 pmol/L taxol, tube formation was modestly reduced by 16% and 27%, respectively (Fig. 7A). In contrast, tube formation of cells infected with the Ad.Rz.GFP adenovirus at a low MOI of 25 was reduced by 35% in the absence of taxol. Exposure of Ad.Rz.GFP-infected cells to 4 pmol/L taxol reduced tube formation by 75%, whereas exposure to 6 pmol/L taxol completely aborted tube formation (Fig. 7A). Thus, the anti-stathmin ribozyme significantly enhances the antiangiogenic effects of taxol in an in vitro Matrigel assay.

To determine whether the antiangiogenic effects of anti-stathmin ribozyme and taxol are additive or synergistic, we used the median effect analysis method of Chou and Talalay (20). This method identifies an interaction as synergistic, additive, or antagonistic by comparing the observed combination effects to
the expected additive effects (20). We used the Calcusyn software suite (Biosoft) that models the equations of the Chou and Talalay method to assess the therapeutic interaction between the anti-stathmin adenovirus and taxol. This software takes into account both the potency ($D_m$ values) and the shapes of the dose-effect curves ($m$ values) to precisely analyze the combination effect of two agents. The combination index (CI) values and the fraction affected ($F_a$) at each dose were used to generate the $F_a$–CI plot. As shown in Fig. 7B, when the anti-stathmin ribozyme was combined with taxol, the CI values at

FIGURE 3. Effects of combination of anti-stathmin ribozyme and taxol on the rate of proliferation of HUVECs. A, Growth curves of uninfected cells in the absence and presence of different concentrations of taxol as indicated. B, Growth curves of cells infected with the control Ad.GFP adenovirus at a low MOI of 25 in the absence and presence of different concentrations of taxol as indicated. C, Growth curves of cells infected with Ad.Rz.GFP at a low MOI of 25 in the absence and presence of different concentrations of taxol as indicated. The data are presented as a mean of the triplicate alternate-day cell counts.

FIGURE 4. Effects of anti-stathmin ribozyme on chemotactic migration of HUVECs. A, Representative photomicrographs illustrate chemotactic migration in uninfected cells and cells infected with either control Ad.GFP or Ad.Rz.GFP adenoviruses at a MOI of 100 as indicated. B, Graph represents quantitative data of chemotactic migration in uninfected cells and cells infected with either Ad.GFP or Ad.Rz.GFP adenoviruses at different MOIs as indicated. Hatched column, fold migration in uninfected cells that was arbitrarily assigned as 100% at baseline. Filled columns, fold migration in cells infected with Ad.GFP at different MOIs as indicated. Open columns, fold migration in cells infected with Ad.Rz.GFP at different MOIs as indicated.
2, 4, and 6 pmol/L taxol were 0.12, 0.08, and 0.02, respectively. Similarly, when the anti-stathmin ribozyme was combined with taxol, the CI values in the chemotaxis assay at 2, 4, and 6 pmol/L taxol were 0.24, 0.20, and 0.11, respectively. The CI values in the proliferation assay at 2, 4, and 6 pmol/L taxol were 0.20, 0.19, and 0.14, respectively. All CI values were significantly <1, which is indicative of a marked synergistic interaction between taxol and anti-stathmin adenovirus.

Because stathmin inhibition and taxol exposure are known to result in stabilization of microtubules, we designed an experiment to assess the effect of combination of anti-stathmin ribozyme and taxol on tubulin polymerization in endothelial cells. Thus, we compared the effects of stathmin inhibition on tubulin polymerization in HUVECs in the presence and absence of taxol exposure. We used a biochemical assay that allows the separation of the in vivo assembled microtubules in their polymerized state and unpolymerized soluble tubulin (12, 15). The two forms of tubulin were separated by ultracentrifugation, and the levels of polymerized and unpolymerized tubulin were quantified by immunoblotting with an antitubulin antibody (Fig. 8). The ratio of polymerized to soluble (P/S) tubulin in cells infected with the control Ad.GFP was 0.17, whereas the ratio of P/S tubulin in cells infected with Ad.Rz.GFP was 0.37 (Fig. 8A). This represents a 2.2-fold increase in the ratio of P/S tubulin following infection of cells with anti-stathmin adenovirus (Fig. 8C). When Ad.GFP-infected cells were exposed taxol, the P/S ratio was 0.4 (Fig. 8D). Thus, exposure of Ad.GFP-infected cells to taxol resulted in a 2.3-fold increase in the P/S ratio. Finally, when Ad.Rz.GFP-infected cells were exposed to taxol, the P/S ratio was 1.0 (Fig. 8D). Thus, taxol exposure of anti-stathmin adenovirus-infected cells resulted in a further 2.7-fold increase in the P/S ratio. Hence, the combined effect of taxol and anti-stathmin ribozyme resulted in a 5.9-fold increase in the P/S ratio. This suggests that stathmin inhibition results in increased polymerization of microtubules that is further enhanced by exposure to taxol.

**Discussion**

For angiogenesis to take place, the cytoskeleton has to undergo dramatic structural reorganization during the processes of endothelial cell proliferation, migration, and differentiation to form new blood vessels. The microtubule-disrupting drug taxol is known to inhibit several endothelial cell functions and interfere with the angiogenic process. Thus, taxol has an antiangiogenic effect in addition to its direct antitumor cell effect (4). As in taxol exposure, inhibition of stathmin is known to result in the stabilization of microtubules and antitumor effects (12, 15, 18, 21, 22). Thus, microtubule-directed chemotherapy that consists of taxol in combination with anti-stathmin therapy is attractive because both interventions target the same microtubule pathway and may provide an effective form of antitumor and antiangiogenic therapy.

In this study, we evaluated the effects of anti-stathmin adenovirus on angiogenesis as a single agent and in combination with taxol using different in vitro assays of angiogenesis. Our studies show that adenovirus-mediated gene transfer of anti-stathmin ribozyme can effectively inhibit the proliferation and migration of endothelial cells and their ability to form tubular structures, a process that mimics angiogenesis in vivo. It is theoretically possible that the impaired ability of endothelial cells to differentiate into capillary-like structures upon stathmin inhibition may be a result of inhibition of their proliferation. Several studies have shown that stathmin inhibition interferes with the process of cell migration (23-25). One of the early events that occurs during directed cell migration is the reorientation of the centrosome (26). This is preceded by the extension of a new pseudopod in the migrating cell in the intended direction of movement (26). Centrosome reorientation toward the leading edge has been described in migrating endothelial cells and other cell types (27). This phenomenon is thought to be mediated by microtubule arrays that become polarized by selective stabilization of a subset of unusually stable microtubules at the leading edge (28). Because stathmin...
is necessary for microtubule depolymerization, stathmin inhibition may result in excessive stability of the microtubule network and interfere with the ability of the cells to reorient the centrosome and to extend a new pseudopod, thereby mediating an antimigratory effect. Because taxol is also known to have an antiproliferative and antimitotic effect, we wanted to exclude the possibility that the activity of taxol in the different assays would reflect its antimitotic or cytotoxic effects. Thus, we exposed the endothelial cells to low concentrations of taxol for relatively short periods. Under these conditions, taxol had essentially no cytotoxic or growth inhibitory effects on HUVECs. These concentrations and duration of taxol exposure that we used are consistent with published reports that showed antiangiogenic properties at low subnanomolar concentrations that do not inhibit endothelial cell proliferation (4, 29). Under these conditions, taxol manifested some antiangiogenic activity that was markedly enhanced in the presence of stathmin inhibition. The observed antiangiogenic effects seemed to be much greater than the effects of either agent alone, suggesting the possibility of a synergistic interaction. The synergy was confirmed by the combination index method of Chou and Talalay (20). Because both taxol and stathmin inhibition are known to stabilize microtubules, it is not surprising that the combination of the two interventions would result in synergistic antiangiogenic effects. These findings are consistent with our recent study that showed synergistic inhibition of growth of prostate cancer cells upon stathmin inhibition and taxol exposure (16). Nonetheless, the molecular mechanism(s) responsible for the observed synergistic interaction between stathmin inhibition and taxol.

**FIGURE 6.** Effects of anti-stathmin ribozyme on differentiation of HUVECs into capillary-like structures. **A.** Representative photomicrographs illustrate tube formation in uninfected cells and cells infected with either control Ad.GFP or Ad.Rz.GFP at a MOI of 100 as indicated. All images were taken 24 h after plating on Matrigel. Left, photomicrographs illustrate tube formation as seen under light microscope; right, photomicrographs illustrate tube formation as seen under fluorescence microscope. **B.** Graph represents quantitative data of tube formation in uninfected cells and cells infected with either Ad.GFP or Ad.Rz.GFP adenoviruses at different MOIs as indicated. Hatched column, percentage of tube formation at baseline in uninfected cells, which was arbitrarily assigned as 100%. Filled columns, percentage of tube formation in cells infected with Ad.GFP at different MOIs as indicated. Open columns, percentage of tube formation in cells infected with Ad.Rz.GFP at different MOIs as indicated.
is not clear. Stathmin is known to promote microtubule depolymerization, and its deficiency has been shown to shift the equilibrium between polymerized and unpolymerized tubulin in favor of polymerized tubulin (10, 12, 15, 21). Taxol, on the other hand, is known to stabilize microtubules by binding to polymerized tubulin (22). Our studies on the effect of combination of taxol and stathmin inhibition in HUVECs showed that the combination results in a larger increase in the ratio of P/S tubulin than either single agent alone. This may explain, at least in part, the observed synergy between stathmin inhibition and taxol exposure.

Terminal nucleotidyl transferase–mediated nick end labeling analysis of Ad.Rz.GFP-infected HUVECs after taxol exposure showed very little apoptosis (<3%; data not shown). This suggests that the observed antiangiogenic effects resulting from the combination of taxol and anti-stathmin adenovirus are not a result of cytotoxicity. In contrast to primary endothelial cells, when stathmin inhibition was combined with taxol in cancer cells, we observed a marked increase in apoptosis (16). Normal cells have functional checkpoints that would arrest progression through the cell cycle until the spindle abnormalities are corrected. In contrast, cancer cells generally lack functional mitotic checkpoints and can exit mitosis and complete the cell cycle in spite of spindle abnormalities. This would be expected to lead to apoptosis during subsequent cycles of cell division.

A number of cellular functions have been attributed to stathmin, in addition to its well-documented role in cell motility and tumor cell metastasis. A role of stathmin in migration was first suggested by Niethammer et al. (25), who showed that stathmin may be important in cell motility and inhibition (30). Interestingly, several other studies have supported a recent report by Miyashita et al. (30), which suggested that stathmin expression might be regulated by a specific transcription factor. When these authors used antisense oligonucleotides to inhibit Vezf1 expression in endothelial cells, they observed the same antiangiogenic effects as stathmin inhibition (30).

FIGURE 7. Effects of combination of anti-stathmin ribozyme and taxol on differentiation of HUVECs. A, Graph represents quantitative data of tube formation in uninfected cells and cells infected with either Ad.GFP or Ad.Rz.GFP adenoviruses at a low MOI of 25 and exposed to different concentrations of taxol as indicated. Filled columns, percentage of tube formation in uninfected cells in the absence and presence of different concentrations of taxol as indicated. Open columns, percentage of tube formation in cells infected with the control Ad.GFP adenovirus in the absence and presence of different concentrations of taxol as indicated. Hatched gray columns, percentage of tube formation in cells infected with Ad.Rz.GFP adenovirus in the absence and presence of different concentrations of taxol as indicated. B, Evaluation of therapeutic interactions between anti-stathmin ribozyme (Rz) and different concentrations of taxol on tube formation. The Chou and Talalay combination index method was used to determine whether the interaction is synergistic. The Fa-CI plot was constructed using the Calcusyn software. The additive effect of the combination of anti-stathmin adenovirus and taxol is represented at CI = 1 (dashed line). A CI < 1 denotes a synergistic interaction, a CI of 1 denotes an additive interaction, and a CI >1 indicates an antagonistic interaction.
Baldassarre et al. proposed that stathmin might play a role in the migration and metastasis of sarcoma cells. Their studies suggested that p27Kip1 binds to stathmin and impairs its function, thus abrogating the ability of sarcoma cells to migrate and invade (23). In the same study, they showed that high levels of stathmin expression and low levels of p27Kip1 in vivo in human sarcoma correlate with the metastatic phenotype (23). Thus, stathmin expression seems to be critically important in cancer as reflected by its distinct roles in cell division, angiogenesis, and tumor cell metastasis.

Targeting the vascular endothelial cells that line the blood vessels and sprout to form vascular channels to feed the tumor may provide a promising strategy for cancer therapy. In the treatment of many of the very common solid tumors, new combinations of drugs are being used that target both the transformed epithelial cells (i.e., antitumor therapy) and the endothelial cells (i.e., antiangiogenic therapy). There is increasing evidence that these combinations may be superior to traditional chemotherapy. Neither of these strategies are curative in patients with advanced disease. Several lines of evidence suggest that the effectiveness of these therapies could be enhanced by designing therapeutic strategies that provide both antitumor as well as antiangiogenic effects. For instance, combination of anti-vascular endothelial growth factor (VEGF) monoclonal antibody (bevacizumab) with cytotoxic therapy resulted in better response rates and overall survival in several cancers, including non–small lung cancer, myeloma, and pancreatic cancer (31, 32). Our previous studies had shown that stathmin inhibition can suppress the malignant phenotype of different cancer cell types (18, 33). We had also shown a synergy in the antitumor activities mediated by stathmin ribozyme and taxol in prostate cancer cells (16). The present study suggests that stathmin inhibition can also have an antiangiogenic effect that is markedly enhanced by its combination with taxol. Thus, this novel combination of stathmin inhibition with a drug that targets the mitotic spindle-like taxol may provide an active therapeutic strategy that combines antitumor activity with antiangiogenic activity. This hypothesis needs to be tested in the future in preclinical and clinical models of human cancer.

Materials and Methods

Reagents

Taxol (paclitaxel) was purchased from Sigma. It was dissolved in DMSO as a 10 mmol/L stock solution and stored at −20°C in aliquots.

Cell Culture

Early passage HUVECs were purchased from Cambrex Bio Science Walkersville, Inc. These cells were grown in complete endothelial growth medium (EGM-2; Clonetics) at 37°C in a humidified 5% CO2 environment. All experiments were done using cells between passages 2 and 4. The 293 human embryonic kidney cells were used to propagate the recombinant adeno viruses. The 293 packaging cell line was maintained using cells between passages 2 and 4. The 293 human embryonic kidney cells were used to propagate the recombinant adenoviruses. The 293 packaging cell line was maintained using cells between passages 2 and 4.

Production of Recombinant Adenoviruses

The recombinant adenoviruses that we used in this study were previously described in detail (18). Briefly, a replication-deficient bicistronic adeno viral vector that coexpresses GFP and an anti-stathmin ribozyme (Rz305) was constructed for targeting human stathmin mRNA (18). The control vector contained the GFP reporter gene under the control of the CMV5 promotor without anti-stathmin ribozyme sequences (18). Each recombinant transfer plasmid was cotransfected with part of the Ad5 genome selected to promote in vivo homologous recombination between the two DNA molecules, resulting in infectious adenoviruses (18). The recombinant adenoviruses were propagated in 293 cells, purified by cesium chloride gradient ultracentrifugation, dialyzed, and stored at −80°C (18, 34). The infectious viral titers were determined by plaque assays in 293 cells as described (18, 34).

Adenoviral Infections In vitro

Cells were seeded in six-well culture plates 24 h before virus infection. Cells were infected with recombinant adenoviruses at different MOIs of 25, 50, or 100 in a 2% reduced serum medium for 3 h. After infection, the virus was removed, and the cells were further incubated in complete endothelial growth medium. The efficiency of transduction by the recombinant adenoviruses was assessed at 48 or 72 h after infection by measuring the fraction of cells that expressed GFP by flow cytometry (18). The fraction of GFP-positive cells was determined using the WinList software.

Northern Analysis

Uninfected cells and cells infected with either control Ad.GFP or Ad.Rz.GFP adenoviruses at MOI of 100 were harvested 72 h after infection. Total RNA was isolated from these cells by guanidinium thiocyanate-phenol-chloroform extraction (35). About 20 μg of each RNA sample were denatured in glyoxal and separated by agarose gel electrophoresis as described (35). A 1.5-kb XbaI fragment from the stathmin cDNA (36) was labeled by random priming and used as a probe in hybridization experiments. The northern filter was stripped and rehybridized to ribosome and 18S rRNA probes.

Western Analysis

Uninfected cells and cells infected with either control Ad.GFP or Ad.Rz.GFP adenoviruses at MOI of 100 were harvested 72 h after infection. The cell pellets were lysed for 30 min on ice in a buffer consisting of 50 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, and 1% Triton X-100 (18). The cell extracts were clarified by centrifugation, and the protein concentrations were determined by using a Bio-Rad protein assay kit. About 25 μg of each protein extract were electrophoresed in a 15% SDS-polyacrylamide gel, transferred to membrane, and blocked in PBS containing 5% nonfat milk powder and 0.2% Tween 20 for 1 h at room temperature. The filter was incubated overnight at 4°C with an anti-stathmin antibody (BD PharMingen) in PBS containing 0.1%Tween 20, followed by incubation with horseradish peroxidase–conjugated anti-mouse immunoglobulin G (IgG, Sigma) for 2 h. The filter was then washed several times with PBS containing 0.2%...
Tween 20. As an internal control, the same filter was hybridized to anti-actin antibody (Oncogene Research Products), followed by incubation with horseradish peroxidase–conjugated anti-mouse IgM (Calbiochem) as above. The bands were visualized by chemiluminescence using an enhanced chemiluminescence kit (Amersham Biosciences).

Endothelial Cell Proliferation Assay

To assess the effects of anti-stathmin ribozyme on the rate of proliferation of endothelial cells, equal numbers of HUVECs (2 × 10^5 cells) were infected in triplicates with either control or anti-stathmin adenoviruses at different MOIs (25, 50, or 100). The cells were harvested and stained with trypan blue to determine cell viability. Cells were counted on a hemocytometer on alternate days, and the data were plotted in graphical form to generate growth curves.

To assess the effects of combination of anti-stathmin ribozyme and taxol on the proliferation of HUVECs, equal numbers of cells (2 × 10^5 cells) were infected in triplicates with either control or anti-stathmin adenoviruses at a low MOI of 25 as above. Uninfected cells or cells infected with either Ad.GFP or Ad.Rz.GFP were cultured in the absence or presence of different concentrations of taxol (2–6 pmol/L). Cells were counted on alternate days, and the growth curves were generated as above.

Boyden Chamber Assay of Chemotactic Endothelial Cell Migration

Cell migration was assessed in a Boyden chamber. HUVECs were infected in triplicates in six-well dishes with either the control or the anti-stathmin ribozyme carrying adenovirus at different MOIs (25, 50, or 100). After 48 h, the cells were trypsinized, and equal number (2 × 10^5 cells) of uninfected cells or cells infected with either Ad.GFP or Ad.Rz.GFP were plated in the upper wells of a Boyden chamber (BD Biosciences). The lower wells of the chamber contained either basal medium (without chemotactant) or conditioned medium that was supplemented with VEGF (20 ng/mL) or 10% FBS as chemotactants to stimulate migration. Cell migration was determined after 18 to 20 h as the fraction of cells transmigrated through the filter toward the conditioned medium. The cells that remained on the upper surface of the filter were removed by wiping with cotton swabs. The cells on the lower surface were stained with Diff-Quick stain and quantified under a light microscope by counting the number of cells that migrated in five randomly selected low-power fields. The assay was done in triplicates. Fold migration was determined by dividing the mean number of cells migrating through the membrane toward the chemotactant by the mean number of cells migrating through the membrane without chemotactant.

To assess the effects of the combination of anti-stathmin ribozyme with taxol on endothelial migration, HUVECs were infected in triplicates with either control or anti-stathmin adenoviruses at a low MOI of 25. After 48 h, cells were trypsinized and washed in PBS, and an equal number (2 × 10^5 cells) of uninfected cells and cells infected with either control Ad.GFP or Ad.Rz.GFP adenoviruses were added to the upper chamber of the inserts in triplicates. The appropriate concentration of taxol (0, 2, 4, and 6 pmol/L) was added to each well, and the cells were further incubated for 20 h. Migration of HUVECs in the absence and presence of different concentrations of taxol was determined in triplicates as above.

In vitro Tube Formation Assay of Endothelial Cell Differentiation

HUVECs were infected in triplicates with either control Ad.GFP or Ad.Rz.GFP adenoviruses at different MOIs (25, 50, or 100). After 48 h, the cells were trypsinized, and equal numbers of cells (1 × 10^5 cells per well) were added in a 500-μL volume in 24-well plates precoated with Matrigel (300 μL per well). As an additional control, a similar number of uninfected HUVECs were also plated on Matrigel. After 18 to 20 h, the plated cells were observed under a light microscope to assess tube formation. The tube formation was quantified by considering a three-branch point event as one tube as previously described (37). Tube formation was scored in uninfected, Ad.Rz.GFP and control Ad.GFP-infected cells by light microscopy under ×4 magnification in six randomly chosen fields. Mean tube formation was plotted relative to the control, which was arbitrarily assigned a value of 100%.

To assess the effects of the combination of anti-stathmin ribozyme with taxol on differentiation, HUVECs were infected in triplicates with either the control or anti-stathmin adenoviruses at a low MOI of 25. After 48 h, cells were trypsinized and washed in PBS, and equal numbers (1 × 10^5 cells) of uninfected cells and cells infected with either control Ad.GFP or Ad.Rz.GFP adenoviruses were plated onto Matrigel as above. The appropriate concentrations of taxol (2, 4, and 6 pmol/L) were added to each well in triplicates, and the cells were further incubated for 20 h. Tube formation in the absence and presence of different concentrations of taxol was assessed as above.

Statistical Analysis and Evaluation of Combination Effects

The data are expressed as mean ± SD. The data were analyzed for statistical significance using the two-tailed Student's t test. P values <0.05 were considered statistically significant.

To evaluate the potential synergy between the anti-stathmin adenovirus and taxol, we used the Calculyn software suite (Biosoft), which applies the method of Chou and Talalay (20). Dose-response curves were generated for anti-stathmin adenovirus and taxol alone to determine the median effect plots. The CI values for each dose and the corresponding effect [i.e., the fraction affected (Fa)] were used to generate the Fa-CI plot. According to Chou and Talalay, a CI < 1 indicates a synergistic interaction, a CI of 1 indicates an additive interaction, and a CI > 1 indicates an antagonistic interaction.

Biochemical Analysis of Microtubule Polymerization

In vivo

Equal numbers of cells (1 × 10^6) were infected with control Ad.GFP or Ad.Rz.GFP adenoviruses at MOI of 100 for 3 h. The virus was removed, and the cells were then exposed to 6 pmol/L taxol for 72 h. The amounts of polymerized and soluble tubulin in Ad.GFP or Ad.Rz.GFP-infected cells were
were subjected to high-speed centrifugation at 40,000 g for 30 min at 22°C to sediment the polymeric tubulin. Supernatants containing soluble tubulin were separated from the pellets containing the polymerized tubulin. The pellets containing the polymerized tubulin were solubilized in microtubule-stabilizing buffer and subjected to ultrasonic waves for 10 min to ensure complete disruption of the microtubules. The tubulin content in the pellets and supernatants was then analyzed by SDS-PAGE quantified in the presence and absence of taxol using a procedure we described previously (12, 15). Briefly, the cells were lysed in microtubule stabilizing lysis buffer containing 0.1 mol/L PIPES (pH 6.9), 2 mol/L glycerol, 5 mmol/L MgCl₂, 2 mmol/L EGTA, 0.5% Triton X-100, 4 mol/L taxol, and 5 μg/mL leupeptin. Taxol was included in the lysis buffer to stabilize the microtubules during extraction and to maintain their stability during subsequent processing. The cell lysates were subjected to high-speed centrifugation at 40,000 × g for 30 min at 22°C to sediment the polymeric tubulin. Supernatants containing soluble tubulin were separated from the pellets containing the polymerized tubulin. The pellets containing the polymerized tubulin were solubilized in microtubule-stabilizing buffer and subjected to ultrasonic waves for 10 min to ensure complete disruption of the microtubules. The tubulin content in the pellets and supernatants was then analyzed by SDS-PAGE followed by immunoblotting with an anti–α-tubulin antibody (clone B512; Sigma). The band abundances were visualized by chemiluminescence. The data were quantified using Scion image software.

References

Synergistic Antiangiogenic Effects of Stathmin Inhibition and Taxol Exposure

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