Antiangiogenic Concentrations of Paclitaxel Induce an Increase in Microtubule Dynamics in Endothelial Cells but Not in Cancer Cells

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Abstract

Microtubule-targeted drugs such as paclitaxel exhibit potent antiangiogenic activity at very low concentrations, but the mechanism underlying such an effect remains unknown. To understand the involvement of microtubules in angiogenesis, we analyzed the dynamic instability behavior of microtubules in living endothelial cells [human microvascular endothelial cells (HMEC-1) and human umbilical vascular endothelial cells (HUVEC)] following 4 hours of paclitaxel treatment. Unexpectedly, antiangiogenic concentrations of paclitaxel (0.1-5 nmol/L) strongly increased microtubule overall dynamics in both HMEC-1 (86-193%) and HUVEC (54-83%). This increase was associated with increased microtubule growth and shortening rates and extents and decreased mean duration of pauses. The enhancement of microtubule dynamics by paclitaxel seemed to be specific to antiangiogenic concentrations and to endothelial cells. Indeed, cytotoxic concentration (100 nmol/L) of paclitaxel suppressed microtubule dynamics by 40% and 54% in HMEC-1 and HUVECs, respectively, as observed for all tested concentrations in A549 tumor cells. After 4 hours of drug incubation, antiangiogenic concentrations of paclitaxel that inhibited endothelial cell proliferation without apoptosis (1-5 nmol/L) induced a slight decrease in anaphase/metaphase ratio, which was more pronounced and associated with increased mitotic index after 24 hours of incubation. Interestingly, the in vitro antiangiogenic effect also occurred at 0.1 nmol/L paclitaxel, a concentration that did not alter mitotic progression and endothelial cell proliferation but was sufficient to increase interphase microtubule dynamics. Altogether, our results show that paclitaxel mediates antiangiogenesis by an increase in microtubule dynamics in living endothelial cells and suggest that the impairment of interphase microtubule functions is responsible for the inhibition of angiogenesis.

Introduction

Microtubules are highly dynamic cytoskeletal structures assembled from α/β-tubulin heterodimers. The dynamic behavior of microtubules includes dynamic instability, in which the ends of the microtubules undergo frequent episodes of slow growth and rapid shortening, as well as treadmillig, which is the net addition of tubulin at one end of a microtubule and net loss at the other end. Dynamic instability is crucial for the ability of microtubules to carry out many of their cellular functions, particularly during mitosis because they are responsible for the capture and alignment of chromosomes on the metaphase plate and their subsequent separation to the two daughter cells at anaphase (1). Microtubule dynamics are also essential for cell signaling, migration, and transport of proteins, vesicles, mitochondria, and other components throughout the cell. In living cells, microtubule dynamics are modulated by a number of factors, including the tubulin isotype composition, tubulin post-translational modifications, and microtubule regulatory proteins (2). Each cell type expresses a balance of these factors, which maintains the microtubule dynamics within a narrow range that supports their functions. We previously showed that both excessively rapid dynamics as well as suppressed dynamics correlate with impaired mitotic spindle function and inhibition of cell proliferation (3).

Microtubules are targets for anticancer drugs such as taxanes, epothilones, and Vinca alkaloids. All of these compounds potently suppress microtubule dynamics (4–8). Suppression generally results in an impairment of the metaphase-to-anaphase transition in mitosis and leads to cell death by apoptosis (4, 6–8). Suppression of microtubule dynamics also impairs interphase microtubule functions such as transport of proteins like p53 (8, 9).

Microtubule-targeted drugs (MTD) were among the first chemotherapeutics reported to have antiangiogenic properties. Angiogenesis is the formation of new blood vessels from the preexisting vasculature and is a key event in tumor growth and metastasis (10). It is a complex and dynamic process requiring activation, proliferation, migration, and differentiation of endothelial cells. Inhibition of endothelial cell proliferation blocks angiogenesis and tumor growth in animal models (11, 12). Among chemotherapeutic drugs, paclitaxel, which is widely used in the treatment of human cancer, seems to be one of the most potent inhibitors of endothelial cell proliferation and angiogenesis (13). We have recently shown that concentrations of paclitaxel >10 nmol/L inhibit endothelial cell proliferation through a G2-M arrest and induce subsequent cell death by apoptosis, similar to its effects on tumor cell lines (14). Interestingly, concentrations of paclitaxel <10 nmol/L, which are antiangiogenic in an in vitro capillary formation assay, are cytostatic with endothelial cells because their proliferation is inhibited without any accompanying apoptosis (14). This cytostasis occurs through the initiation, but not completion, of a mitochondrial apoptotic signaling pathway, without any mitotic arrest or any detectable modification of the structural organization of the microtubule network (13–15). Although it is clear that angiogenesis processes depend on changes in cytoskeletal...
dynamics, the specific involvement of microtubule dynamics in MTD-induced antiangiogenic activity has not yet been elucidated. Thus, in the current study, we have investigated, in human endothelial cells, the effects of paclitaxel on microtubule dynamics, which are highly sensitive to the action of MTDs (4). Unexpectedly, we found that paclitaxel induced an increase in microtubule dynamics in endothelial cells at drug concentrations that are antiangiogenic and cytostatic. Increased microtubule dynamics in endothelial cells may thus represent a new mechanism of action that may be responsible for the antiangiogenic activity of paclitaxel. Altogether, our results support the key role of microtubule dynamics in angiogenesis.

Materials and Methods

Cell Culture and Drug. Human umbilical vein endothelial cells (HUVEC) and human dermal microvascular endothelial cell line (HMEC-1) were obtained from the Cell Culture Laboratory in the Hôpital de la Conception, Assistance Publique-Hôpitaux de Marseille. They were routinely maintained in culture at 37°C and 5% CO2. HMEC-1 were grown in MCDB-131 medium containing 10% heat-inactivated fetal bovine serum, 2 mmol/L glutamine, 1% penicillin, and streptomycin (all from Life Technologies, Paisley, United Kingdom), 1 µg/ml hydrocortisone (Pharmacia & Upjohn, St-Quentin-Yvelines, France), and 10 ng/ml epithelial growth factor (R&D Systems, Mineapolis, MN). HUVECs were grown in standard culture RPMI 1640 (Life Technologies) containing 20% heat-inactivated fetal bovine serum, 2 mmol/L glutamine, 1% penicillin, and streptomycin, 50 IU/ml sodium heparin (Sanofi-Synthelabo, Paris, France), and 50 µg/ml endothelial cell growth supplement (BD Biosciences, Bedford, MA). HMEC-1 were used between passages 3 and 10 and HUVECs between passages 2 and 5. Human non–small cell lung carcinoma cells, A549, were maintained in RPMI 1640 containing 10% fetal bovine serum and 2 mmol/L glutamine. For supplementary control experiments, A549 culture medium was specially supplemented with 1 µg/ml hydrocortisone and 10 ng/ml epidermal growth factor. Cells were grown as monolayer cultures and passaged at intervals of 5 days.

A stock solution of paclitaxel (Alexis Co., Lausen, Switzerland) was prepared in DMSO and maintained frozen at −20°C. The different dilutions of paclitaxel were done in routine culture medium and the highest final concentration of DMSO used in cell culture was 0.1%. The ratio of drug molar concentration/cell number was identical for all experiments.

Microinjection of Rhodamine-Labeled Tubulin. Tubulin was purified from lamb brain by sulfate fractionation and ion exchange chromatography. The protein was stored in liquid nitrogen and prepared as previously described (16). The labeling by tetraethyl rhodamine succinimidyl ester (Molecular Probes, Leiden, the Netherlands) of tubulin and microinjection into cells were done as previously described (8). Briefly, exponentially growing cells were seeded 24 hours before use on glass coverslips. Injected cells were incubated 2 hours at 37°C to allow the incorporation of rhodamine-tubulin into microtubules and further incubated with paclitaxel in routine culture medium, for 4 hours to allow the achievement of an equilibrium intracellular drug concentration.

Time-Lapse Microscopy and Image Acquisition. Following drug incubation, microinjected cells were placed in RPMI 1640 culture medium lacking sodium bicarbonate and supplemented with 25 mmol/L HEPES, 4.5 g/L glucose, 30 µL Oxygen/mL (Oxyrase, Inc, Mansfield, OH) to reduce photodamage, in a double coverslip chamber maintained at 37 ± 1°C, and observed using a fluorescence microscope (Leica DM-IRBE) with a 100× objective lens, for 45 minutes as previously described (3, 6–8). Thirty-one images per cell were acquired at 4-second intervals using a digital camera (CCD camera CoolscanFX; Princeton Instruments, Trenton, NJ) driven by Metamorph software (Universal Imaging Co., Downingtown, PA) (8).

Analysis of Microtubule Dynamic Instability. Analysis of microtubule dynamic instability was done as previously described (7, 8). The positions of the plus ends of individual microtubules with time were recorded and analyzed by using Metamorph software. Changes in length ≥0.5 µm were considered as growth or shortening events. Changes in length <0.5 µm were considered as phases of attenuated dynamics or pauses. The rates of growth and shortening were determined by linear regression. Means and SE were calculated per event. The catastrophe frequency based on time was calculated by dividing the number of transitions from growth or pause to shortening by the total time growing and paused for each individual microtubule. The catastrophe frequency based on length growth was calculated by dividing the number of transitions from growth or pause to shortening by the total distance grown for each individual microtubule. The rescue frequencies based on distance or time were calculated similarly, dividing the total number of transitions from shortening to pause or growth by the length shortened or the time spent shortening, respectively, for each individual microtubule. Means and SE of transition frequencies were calculated per microtubule (n > 30) for each experimental condition from three independent experiments. Dynamicity is the total length grown and shortened divided by the life span of the population of microtubules (17).

Mitotic Index and Metaphase to Anaphase Transition. Exponentially growing HMEC-1 were seeded 24 hours before drug treatment on 8-well chamber slides (Labtek, Naperville, IL) previously coated with 0.1% gelatin (Sigma Aldrich, Steinheim, Germany). After 4 or 24 hours of treatment with paclitaxel, cells were collected by Cytospin and fixed in 3.7% formaldehyde for 30 minutes. After fixation, cells were washed in PBS and incubated for 2 minutes with 0.25 µg/ml 4’-6-diamidino-2-phenylindole (Sigma Aldrich). Finally, cells were observed using a Leica DM-IRBE microscope coupled with a digital camera (as above), and 400 mitotic and interphase cells per experimental condition were counted as previously described (6).

Statistical Analysis. For microtubule dynamic instability variables, statistical analysis was done using the Student’s t test. For mitotic index and metaphase-to-anaphase transition, an ANOVA test was used. Each experiment was done at least in triplicate.

Results

Antiangiogenic Concentrations of Paclitaxel Increased Microtubule Dynamics in Living Endothelial Cells. To elucidate the role of microtubule dynamics in paclitaxel-induced antiangiogenesis, we analyzed the dynamic instability behavior of microtubules in living HMEC-1 after 4 hours of incubation with paclitaxel. We tested several antiangiogenic concentrations of paclitaxel (0.1, 1, 5, and 100 nmol/L) that inhibit cell proliferation in a concentration-dependent manner (72 hours of incubation). They had, respectively, no effect on proliferation, or were the IC10, IC50, and IC95. In addition, 100 nmol/L induced direct endothelial cytotoxicity through apoptosis induction (14).

Figure 1A shows an image gallery of the lamellar region of an untreated HMEC-1 (control, top), a cell incubated with 5 nmol/L paclitaxel (middle), and a cell incubated with 100 nmol/L paclitaxel (bottom). In the control cell, the microtubule plus ends (e.g., microtubule, arrowheads) alternated between phases of slow growing, rapid shortening, and prolonged pause state (a state of attenuated dynamic instability). Unexpectedly, 5 nmol/L paclitaxel markedly increased microtubule dynamic instability. For example, the plus end of the microtubule indicated by the arrowheads in the middle shortened and grew again for a long distance in a short period of time (8 seconds). In sharp contrast, 100 nmol/L paclitaxel suppressed microtubule dynamic instability as indicated by the unaltered positions of their plus ends during a longer time period (96 seconds, bottom) compared with control cell (top). Figure 1B shows several life history traces of the changes in length of individual microtubules in the absence of paclitaxel (control) or in the presence of 5 and 100 nmol/L paclitaxel. The life history traces of microtubules in cells incubated with 5 nmol/L paclitaxel (middle) show extensive...
length changes as compared with control microtubules (left). However, in presence of 100 nmol/L paclitaxel, life history plots are nearly flat, indicating a marked suppression of dynamics (right).

The life history plots from at least 30 individual microtubules were used to determine the dynamic instability variables. The effects of paclitaxel on these various variables are shown in Table 1 and graphically in Fig. 2A-C. To fully characterize the mechanistic effects of paclitaxel on dynamic instability, only dynamic microtubules were included in the measurements. In control cells, 59% of microtubules were dynamic during the time of observation (Fig. 2A). Between 0.1 and 5 nmol/L paclitaxel, both the percentage of dynamic microtubules and the overall dynamicity (a variable reflecting the overall exchange of tubulin with the microtubule end) increased logarithmically ($r^2 = 0.98$ and 0.90, respectively; Fig. 2A and B). Moreover, in the same paclitaxel concentration range, the shortening rate, which was the most sensitive variable affected by paclitaxel, also increased logarithmically ($r^2 = 0.98$), whereas the growing rate increased in a linear fashion ($r^2 = 0.94$; Fig. 2C).

In control cells, the plus ends of dynamic microtubules grew at a mean rate of 4.9 ± 0.6 μm/min, slower than their mean shortening rate of 7.5 ± 0.6 μm/min. The mean growing and shortening lengths were 1.2 ± 0.1 and 1.6 ± 0.1 μm, respectively and microtubules spent 65% of their time in a paused state, neither growing nor shortening to a detectable extent (Table 1). After incubation with paclitaxel, the growth rate and length increased in a concentration-dependent manner (Table 1). Interestingly, other variables were also altered significantly over the entire paclitaxel concentration range of 0.1–5 nmol/L, but not in a paclitaxel concentration–dependent manner. These included reductions in the duration of shortening events, in the duration of attenuated states, and in the distance-based catastrophe frequency as well as increases in the shortening rate and length, and in the time-based rescue frequency. These effects were almost maximal at the lowest concentration tested (0.1 nmol/L). Thus, at 0.1 nmol/L paclitaxel, the mean duration of pause and shortening was decreased by 45% and 36%, respectively, and the shortening rate, shortening length, and time-based rescue frequency was increased by 94%, 25%, and 50%, respectively, and these percentages did not change greatly at 5 nmol/L paclitaxel. Together, both the concentration-dependent and the concentration-independent changes led to a vast increase in microtubule dynamicity ranging from an 86% increase at 0.1 nmol/L paclitaxel to a 193% increase at 5 nmol/L paclitaxel.

In sharp contrast, at a relatively high concentration (100 nmol/L), paclitaxel suppressed microtubule dynamic instability, very similar to its effects on microtubule dynamics in tumor cells at concentrations that block mitosis and inhibit proliferation (7, 18). It decreased the percentage of dynamic...
Overall dynamicity was increased by 54% and 70% in living events and decreased the mean duration of pause. Finally, the increased the rates and extents of both growth and shortening microtubules from 52% to 83% and 76%, respectively. They also paclitaxel (0.1 and 2 nmol/L) increased the percentage of dynamic with our observations on HMEC-1 cells, low concentrations of (100 nmol/L) for cell proliferation (ref. 14; Table 2). Consistent proliferation (0.1 nmol/L), or with the IC50 (2 nmol/L) or the IC95 a concentration that was antiangiogenic without inhibiting cell HUVECs. They were incubated 4 hours without paclitaxel or with extended our examination to another endothelial cell type, living instability at low paclitaxel concentrations in HMEC-1 cells, we To confirm the unusual observation of increased dynamic microtubules to only 33% and suppressed microtubule dynamics by 40%. The main variables affected by this drug concentration were the shortening rate and length, which were decreased by 32% and 38% respectively, and the distance-based rescue frequency which was increased by 61%.

To confirm the unusual observation of increased dynamic instability at low paclitaxel concentrations in HMEC-1 cells, we extended our examination to another endothelial cell type, living HUVECs. They were incubated 4 hours without paclitaxel or with a concentration that was antiangiogenic without inhibiting cell proliferation (0.1 nmol/L), or with the IC50 (2 nmol/L) or the IC95 (100 nmol/L) for cell proliferation (ref. 14; Table 2). Consistent with our observations on HMEC-1 cells, low concentrations of paclitaxel (0.1 and 2 nmol/L) increased the percentage of dynamic microtubules from 52% to 83% and 76%, respectively. They also increased the rates and extents of both growth and shortening events and decreased the mean duration of pause. Finally, the overall dynamicity was increased by 54% and 70% in living HUVECs treated with 0.1 and 2 nmol/L paclitaxel, respectively. The only relevant difference observed in paclitaxel effects in the two cell types was that the time-based rescue frequency was increased by 50% ($P < 0.05$) in HMEC-1 whereas this variable was not significantly altered in HUVECs (Tables 1 and 2).

As shown with HMEC-1, the cytotoxic concentration of paclitaxel (100 nmol/L) suppressed microtubule dynamics. It decreased the overall dynamicity by 28% in HUVECs, similar to the 40% decrease in HMEC-1. The shortening rate and length were decreased by 40% and 24%, respectively, and the distance-based rescue frequency was increased by 31%.

Altogether these results indicate that paclitaxel acts on human endothelial cells by a novel mechanism, which involves an increase in microtubule dynamics rather than a suppression.

**The Paclitaxel-Induced Increase in Microtubule Dynamics Seemed to Be Specific to Endothelial Cells.** To determine whether the increase in microtubule dynamics at low paclitaxel concentrations is specific to endothelial cells, we also investigated the effect of paclitaxel in A549 tumor cells at the IC50 (2 nmol/L/paclitaxel) and at a low concentration that did not affect cell proliferation (0.1 nmol/L), under the same experimental conditions (Table 3).

Similar to our previous observations with paclitaxel in A549 cells (7), at the IC50 (2 nmol/L), the rates and extents of both growth and shortening events and the dynamicity were significantly decreased. The duration of pauses and the distance-based rescue frequency were increased. Moreover, in contrast with its effects in endothelial cells, 0.1 nmol/L paclitaxel had no significant effect on the variables of microtubule dynamic instability in A549 cells except for slightly suppressing the microtubule shortening length by 19% and the overall dynamicity by 11%.

To ensure that the paclitaxel-induced increase in microtubule dynamics, observed in endothelial cells, was not related to cell culture conditions, we also measured microtubule dynamics in A549 cells cultivated in presence of hydrocortisone and epidermal growth factor and treated with 0.1 nmol/L paclitaxel. In such conditions, we found that 0.1 nmol/L paclitaxel induced a decrease in microtubule overall dynamicity (data not shown), confirming the specificity of increased microtubule dynamics to endothelial cells.

These results confirm previous observations that inhibition of tumor cell proliferation is associated with suppression of microtubule dynamic instability (7, 18). Furthermore, they suggest that the paclitaxel-induced increase in microtubule dynamics is specific to endothelial cells.

**Slowed Mitotic Progression Was Dispensable for Antiangiogenesis.** To understand the mechanism by which increased microtubule dynamics could be responsible for inhibiting angiogenesis, we investigated the effects of antiangiogenic and cytotoxic concentrations of paclitaxel on the mitotic index and the transition from metaphase to anaphase in HMEC-1 cells. Fig. 3 shows the concentration-dependent effect of paclitaxel on the mitotic index, measured after both 4 and 24 hours of drug incubation. Interestingly, low antiangiogenic concentrations of paclitaxel (0.1-5 nmol/L) did not significantly altered the mitotic index, except for 5 nmol/L paclitaxel (IC90) after 24 hours of incubation, that slightly increased it from 2.5% in control cells to 6.3% ($P < 0.05$). In contrast, cytotoxic concentrations of paclitaxel (10-100 nmol/L) increased the mitotic index after 4 hours of drug incubation and even more after 24 hours of incubation. Thus, the mitotic index reached 51% after 24 hours of incubation with 100 nmol/L paclitaxel.

The ratio of cells in anaphase to cells in metaphase is inversely proportional to the time required to pass through metaphase to...
Increased microtubule dynamics. Proliferation is not necessary for antiangiogenesis related to tumor cells at a wide range of concentrations, starting at 0.1 nmol/L, paclitaxel increased both the percentage of dynamic microtubules and their overall dynamicity in very similar fashions. This increase in microtubule dynamics associated with its antiangiogenic activity, has never been described. The anaphase/metaphase ratio decreased from 0.68 in control cells to 0.12 after 4 hours of incubation with 5 nmol/L paclitaxel, and from 0.4 in control cells to 0.26 (P < 0.05) at 1 nmol/L paclitaxel and to 0.08 (P < 0.05) at 5 nmol/L paclitaxel after 24 hours of incubation. These results indicate that the metaphase-to-anaphase transition was slowed down by cytostatic concentrations of paclitaxel (1-5 nmol/L). Cytotoxic concentrations of paclitaxel (10-25 nmol/L) more strongly decreased the metaphase-to-anaphase transition at both time treatment, and no anaphase was observed after 24 hours of incubation with 100 nmol/L paclitaxel.

Interestingly, the antiangiogenic concentration of paclitaxel that did not inhibit endothelial cell proliferation (0.1 nmol/L) but that increased microtubule dynamics, did not statistically alter the metaphase-to-anaphase transition (−24%, P = 0.31, after 24 hours of drug incubation) or the mitotic index. Moreover, at this concentration mitotic spindles were normal bipolar spindles similar to those observed in untreated cells (data not shown). Mitosis occurred normally and no aneuploid or multinucleated cells were formed after cell division (data not shown). These results suggest that the increase in interphase microtubule dynamics must reach a minimal level to impair mitotic progression and inhibit endothelial cell proliferation. Moreover, this result shows that inhibition of endothelial cell proliferation is not necessary for antiangiogenesis related to increased microtubule dynamics.

**Discussion**

**Microtubule Dynamics Are Increased by Paclitaxel in Endothelial Cells.** In this study, we found that paclitaxel exerted a biphasic effect on microtubule dynamics in endothelial cells depending on the drug concentration. At cytotoxic concentrations that caused mitotic arrest and subsequent cell death by apoptosis (100 nmol/L), paclitaxel suppressed microtubule dynamics in a way that was very similar to that observed in tumor cells (7, 18). However, we found that noncytotoxic lower concentrations of paclitaxel (<10 nmol/L) increased microtubule dynamics in endothelial cells. This mechanism of action of paclitaxel, which is associated with its antiangiogenic activity, has never been described.

In both primary endothelial cells (HUVEC) and an established endothelial cell line (HMEC-1), antiangiogenic concentrations of paclitaxel increased both the percentage of dynamic microtubules and their overall dynamicity in very similar fashions. This increase in microtubule dynamics seems to be specific to endothelial cells as it has not been reported in tumor cells with any MTDs (4). However, the effect of low concentrations of MTDs on tumor cell is still poorly examined. In the current study, we investigated paclitaxel effects on tumor cells at a wide range of concentrations, starting at 0.1 nmol/L, a concentration that did not inhibit cell proliferation. Importantly, these ineffective concentrations of paclitaxel did not significantly alter microtubule dynamics in A549 cells. Moreover, even when

<table>
<thead>
<tr>
<th>Variables</th>
<th>Control</th>
<th>IC₀ 0.1 nmol/L (%)</th>
<th>IC₁₀ 1 nmol/L (%)</th>
<th>IC₅₀ 5 nmol/L (%)</th>
<th>IC₉₅ 100 nmol/L (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of Dynamic microtubules</td>
<td>59</td>
<td>79</td>
<td>91</td>
<td>93</td>
<td>33</td>
</tr>
<tr>
<td>Mean rates (μm/min)</td>
<td>4.9 ± 0.6</td>
<td>5.4 ± 0.5</td>
<td>7.0 ± 0.4⁺ (±42)</td>
<td>9.7 ± 0.6¹ (±98)</td>
<td>5.7 ± 0.7</td>
</tr>
<tr>
<td>Mean duration (min)</td>
<td>7.5 ± 0.6</td>
<td>14.6 ± 1.5⁺ (±113)</td>
<td>16.0 ± 1.4⁺ (±113)</td>
<td>5.1 ± 0.4 (±32)</td>
<td></td>
</tr>
<tr>
<td>Mean length (μm)</td>
<td>0.24 ± 0.03</td>
<td>0.25 ± 0.04</td>
<td>0.22 ± 0.02 (±62)</td>
<td>0.18 ± 0.02 (±32)</td>
<td>0.18 ± 0.02 (±32)</td>
</tr>
<tr>
<td>% Time spent</td>
<td>1.2 ± 0.1</td>
<td>1.3 ± 0.1</td>
<td>1.5 ± 0.1⁺ (±25)</td>
<td>1.7 ± 0.2⁺ (±42)</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>Time-based catastrophe frequency (min⁻¹ ± SE)</td>
<td>9</td>
<td>26</td>
<td>39</td>
<td>33</td>
<td>10</td>
</tr>
<tr>
<td>Time-based rescue frequency (min⁻¹ ± SE)</td>
<td>1.6 ± 0.1</td>
<td>2.0 ± 0.2⁺ (±25)</td>
<td>2.4 ± 0.2⁺ (±50)</td>
<td>2.4 ± 0.2⁺ (±50)</td>
<td>1.0 ± 0.1⁺ (±38)</td>
</tr>
</tbody>
</table>

NOTE: Variables of microtubule dynamic instability in living HMEC-1 incubated 4 hours with 0.1, 1, 5, or 100 nmol/L paclitaxel. The effect of these paclitaxel concentrations on cell proliferation after 72 hours of incubation is noted. The percentage change from control values is shown for the variables that were significantly different from controls.

*P < 0.05 (Student’s t test).

1P < 0.001 (Student’s t test).

1P < 0.01 (Student’s t test).
cultivated in the presence of hydrocortisone and epidermal growth factor, no increase in microtubule dynamics was observed in A549 cells. Epothilone B, another drug that suppresses microtubule dynamics in association with mitotic block in tumor cells, had no effect at low concentrations on microtubule dynamics in MCF-7 cells (5). Altogether, these results suggest that the paclitaxel-induced increase in microtubule dynamics is specific to endothelial cells. They raise the question of the regulation of microtubule dynamics in endothelial cells and the mechanism underlying this novel effect of paclitaxel.

### How Are Microtubule Dynamics Modulated in Endothelial Cells?
Analysis of microtubule dynamics showed that overall dynamicity was very low in endothelial cells (i.e., 2.4 and 4.3 μm/min for HMEC-1 and HUVECs, respectively) compared with 9.9 μm/min for A549 cells. The basal level of microtubule dynamics that is compatible with their function may thus depend upon the cell type and also upon signaling pathways downstream of microenvironment signals. The low level of microtubule dynamics in endothelial cells together with the observed novel effect of paclitaxel on microtubule dynamics may result from interactions between several factors, including the tubulin isotype composition, tubulin post-translational modifications, and microtubule regulatory proteins.

A partial proteomic analysis in HUVECs suggested that these cells express class I Va and IVb β-tubulin isotypes (19). The detection of the brain-specific βIVA tubulin isotype at the protein level in endothelial cells is very interesting as it has not been found in A549 cells (20, 21), suggesting that the tubulin isotype composition in endothelial cells may differ significantly from that of tumor cells.

Another interesting result of our experiments was that the percentage of microtubule dynamic suppression, in endothelial cells, by 100 nmol/L paclitaxel (IC50) corresponded to the suppression by 2 nmol/L paclitaxel (IC95) in A549 cells. This result suggests that the enhancing effect of low concentrations of paclitaxel on microtubule dynamics coexists with the suppressive effect of high concentrations. In other words, it takes more paclitaxel to overcome the increase in dynamics induced by low concentrations of paclitaxel, thus indicating two distinct mechanisms of action of the drug in endothelial cells.

One plausible explanation for the novel enhancement of microtubule dynamics by extremely low concentrations of paclitaxel in endothelial cells may be the activation of a specific signaling pathway by paclitaxel, which could lead to the alteration of post-translational modifications of tubulin that modify microtubule-associated protein (MAP) binding to microtubules (22) or which could regulate the activity of MAPs by phosphorylation/dephosphorylation. For example, inhibition of phosphatases by okadaic acid, which prevents dephosphorylation of stabilizing MAPs, thus could regulate the activity of MAPs by phosphorylation/dephosphorylation.
In addition, the highly divergent COOH-terminal sequences among tubulin isotypes may provide a mechanism responsible for the binding of isotype-specific microtubule regulatory proteins. There are several groups of such MAPs that either bind to the microtubules along their entire length, such as the stabilizing MAP 4 and Tau, or that regulate microtubule dynamics through their interaction with microtubules, such as CLIP-170, EB1, and Tau, or that regulate microtubule dynamics through their association with microtubule ends, such as the destabilizing MAPs, including TAU, TACC, and TPX2. Interestingly, we found that the effect of antiangiogenic concentrations of paclitaxel on microtubule dynamics was logarithmic, possibly reflecting a modulating effect of antiangiogenic concentrations of paclitaxel on microtubule dynamics. Microtubules are extremely important for cell proliferation since their dynamic properties are required at all stages of mitosis. Here, we found that cytostatic concentrations of paclitaxel (1-5 nmol/L) that increased the dynamic instability of interphase microtubules in endothelial cells also caused a slowing of the metaphase-to-anaphase transition, suggesting that the dynamic properties of microtubules within the mitotic spindle might also be altered. These results are consistent with our previous results (3) demonstrating in A549-T12 cells, a paclitaxel-resistant and dependent cell line, that the privation of paclitaxel caused an increase in interphase microtubule dynamics which correlated with delayed metaphase-to-anaphase transition and mitotic abnormalities. Delayed mitotic progression may thus represent the mechanism responsible for the inhibition of endothelial cell proliferation in the absence of apoptosis that we previously observed in endothelial cells (14). However, the fact that the metaphase-to-anaphase transition was slowed down in mitotic endothelial cells treated with low concentrations of paclitaxel does not indicate whether the dynamics of mitotic microtubules were decreased or increased. It is conceivable that a disturbance with the interphase functions of microtubules including cell signaling and transport of vesicles, mitochondria and proteins throughout the cell might also be involved in the inhibition of cell proliferation.

### Table 3. Effects of paclitaxel on the variables of microtubule dynamic instability in living A549 cells

<table>
<thead>
<tr>
<th>Variables</th>
<th>Control</th>
<th>IC₀ 0.1 nmol/L (%)</th>
<th>IC₅₀ 2 nmol/L (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of Dynamic microtubules</td>
<td>72</td>
<td>69</td>
<td>52</td>
</tr>
<tr>
<td>Mean rates (μm/min)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>12 ± 0.6</td>
<td>12.7 ± 0.7</td>
<td>9.4 ± 0.7* (−22)</td>
</tr>
<tr>
<td>S</td>
<td>21.3 ± 1.4</td>
<td>21.2 ± 1.5</td>
<td>12.5 ± 1.1* (−41)</td>
</tr>
<tr>
<td>Mean duration (min)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>0.17 ± 0.01</td>
<td>0.15 ± 0.01</td>
<td>0.16 ± 0.01</td>
</tr>
<tr>
<td>S</td>
<td>0.14 ± 0.01</td>
<td>0.12 ± 0.01</td>
<td>0.15 ± 0.02</td>
</tr>
<tr>
<td>P</td>
<td>0.17 ± 0.02</td>
<td>0.18 ± 0.01</td>
<td>0.26 ± 0.02* (+53)</td>
</tr>
<tr>
<td>Mean length (μm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>2.1 ± 0.2</td>
<td>2.0 ± 0.1</td>
<td>1.5 ± 0.2* (−29)</td>
</tr>
<tr>
<td>S</td>
<td>3.1 ± 0.2</td>
<td>2.5 ± 0.2* (−19)</td>
<td>1.8 ± 0.2* (−42)</td>
</tr>
<tr>
<td>% Time spent</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>33</td>
<td>33</td>
<td>25</td>
</tr>
<tr>
<td>S</td>
<td>28</td>
<td>22</td>
<td>23</td>
</tr>
<tr>
<td>P</td>
<td>39</td>
<td>45</td>
<td>52</td>
</tr>
<tr>
<td>Time-based catastrophe frequency (min⁻¹ ± SE)</td>
<td>2.6 ± 0.3</td>
<td>2.5 ± 0.3</td>
<td>2.0 ± 0.3</td>
</tr>
<tr>
<td>Time-based rescue frequency (min⁻¹ ± SE)</td>
<td>5.5 ± 0.7</td>
<td>7.5 ± 0.9</td>
<td>6.2 ± 0.8</td>
</tr>
<tr>
<td>Distance-based catastrophe frequency (μm⁻¹ ± SE)</td>
<td>0.52 ± 0.06</td>
<td>0.50 ± 0.06</td>
<td>0.70 ± 0.09</td>
</tr>
<tr>
<td>Distance-based rescue frequency (μm⁻¹ ± SE)</td>
<td>0.30 ± 0.04</td>
<td>0.35 ± 0.04</td>
<td>0.57 ± 0.08* (+90)</td>
</tr>
<tr>
<td>Overall dynamicity (μm/min)</td>
<td>9.9</td>
<td>8.8 (−11)</td>
<td>5.1 (−48)</td>
</tr>
</tbody>
</table>

NOTE: Variables of microtubule dynamic instability in living A549 cells incubated 4 hours with 0.1 or 2 nmol/L paclitaxel. The effect of these paclitaxel concentrations on cell proliferation after 72 hours of incubation is noted. The percentage change from control values is shown for the variables that were significantly different from controls.

*P < 0.01 (Student’s t test).

1P < 0.001 (Student’s t test).

2P < 0.05 (Student’s t test).
Interestingly, paclitaxel-induced antiangiogenesis also occurs at concentrations that do not inhibit cell proliferation (15, 25, 26). In the current study, we showed that very low antiangiogenic concentration of paclitaxel (0.1 nmol/L) increased microtubule dynamics without significantly altering mitotic progression. This result suggests that the slowing of mitotic progression was not necessary to antiangiogenesis and that the increase in microtubule dynamics may be responsible for antiangiogenesis through the impairment of microtubule interphase functions that may regulate cell migration or other processes. Dynamic properties of interphase microtubules play an important role in cell polarization and migration (26), which are key events in angiogenesis. Several studies indicate that low concentrations of paclitaxel (≤5 nmol/L) inhibit human endothelial cell migration (15, 25). However, the mechanism responsible for this effect remains to be determined.

Finally, our study shows that, besides being useful as tumor agents, MTDs represent valuable tools to decipher the different roles of microtubule dynamics.

Acknowledgments


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References

Announcements

MEETING OF THE RADIATION RESEARCH SOCIETY

The annual meeting of the Radiation Research Society will be held at the State University of Iowa, Iowa City, on June 22–24, 1953. The Society will be the guest of the University, and all meetings will be held on the campus. The program will consist of: (1) Two symposia, one on "The Effects of Radiation on Aqueous Solutions," which includes the following speakers: E. S. G. Barron, Edwin J. Hart, Warren Garrison, J. L. Magee, and A. O. Allen. The second is "Physical Measurements for Radiobiology" and companion talks by Ugo Fano, Burton J. Moyer, G. Failla, L. D. Marinelli, and Payne S. Harris. (2) On Monday night, June 22, a lecture by Dr. L. W. Alvarez on meson physics has been tentatively scheduled. On Tuesday night, June 23, Dr. L. H. Gray of the Hammersmith Hospital, London, will speak on a topic to be announced. Dr. Gray's lecture is sponsored by the Iowa Branch of the American Cancer Society. Those desiring to report original research in radiation effects, or interested in attending or desiring additional information, please contact the Secretary of the Society, Dr. A. Edelmann, Biology Department, Brookhaven National Laboratory, Upton, L.I., New York.

ERRATUM

The following correction should be made in the article by Beck and Valentine, "The Aerobic Carbohydrate Metabolism of Leukocytes in Health and Leukemia. I. Glycolysis and Respiration," November, 1952, page 891; substitute for the last paragraph:

The data in Table 8 permit several interesting calculations. If one compares the amount of glucose actually disappearing with the sum of the amount equivalent to lactic acid produced plus that equivalent to O2 consumption, it is seen that the amount of glucose "cleavage products" exceeds the amount of glucose utilized by 12 per cent in N and 27 per cent in CML and is exceeded by the glucose utilized by 16 per cent in CLL. If the assumption is made that, in this respect, the myeloid and lymphoid cells of leukemia are similar to those of normal blood, it may be that the computed normal figure represents a summation of the myeloid (M) and lymphoid (L) cells that make up the normal leukocyte population. Thus, if $M = +0.27$ and $L = -0.16$ and the normal differential is 65 per cent M and 35 per cent L, then

$$0.65 (+0.27) + 0.35 (-0.16) = +0.12$$

a figure identical to the observed +0.12 for normal leukocytes.
Antiangiogenic Concentrations of Paclitaxel Induce an Increase in Microtubule Dynamics in Endothelial Cells but Not in Cancer Cells

Eddy Pasquier, Stéphane Honore, Bertrand Pourroy, et al.


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