The Microtubule-affecting Drug Paclitaxel Has Antiangiogenic Activity

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ABSTRACT

Endothelial cell migration is a critical event during angiogenesis, and inhibitors of cell motility can affect the angiogenic process. Paclitaxel (Taxol®), a microtubule-stabilizing antineoplastic cytotoxic drug, inhibits motility and invasiveness of several cell types. The aim of this study was to investigate the effect of paclitaxel on endothelial cell functions and on angiogenesis. In vivo, paclitaxel (20–28 mg/kg i.v.) significantly inhibited the angiogenic response induced by tumor cell supernatant embedded in a pellet of reconstituted basement membrane (Matrigel) injected s.c. into C57BL/6N mice. In vitro, paclitaxel inhibited endothelial cell proliferation, motility, invasiveness, and cord formation on Matrigel in a dose-dependent manner. The antiangiogenic activity of paclitaxel was not linked to its cytotoxicity, since inhibition of endothelial cell chemotaxis and invasiveness occurred at drug concentrations which did not affect endothelial cell proliferation. Another cytotoxic drug, cisplatin, that inhibited endothelial cell proliferation in vitro, did not affect angiogenesis in vivo. These data indicate that paclitaxel has a strong antiangiogenic activity, a property that might contribute to its antineoplastic activity in vivo.

INTRODUCTION

Agents affecting the cytoskeleton are potential inhibitors of angiogenesis (1). By interfering with cell functions which require an intact cytoskeleton, such as proliferation, migration, and invasion, they can interrupt the angiogenic process at several levels. Microtubule-disrupting agents, such as colchicine and vinblastine, have been reported to inhibit cord formation and endothelial cell migration in an in vitro wound-healing assay (2, 3).

The antineoplastic drug paclitaxel affects the cytoskeleton with a unique mechanism of action: at subnanomolar concentrations, paclitaxel favors the assembly of microtubules, reducing the critical concentration of tubulin dimers and the need for cofactors such as guanosine 5'-triphosphate and microtubule-associated proteins. Paclitaxel also stabilizes formed microtubules by shifting the equilibrium between dimers and polymers in favor of the latter and by stabilizing the microtubules against dissociating conditions such as cold and CaCl2. This results in the formation of abnormal cytoskeletal structures: cells exposed to paclitaxel in vitro present new microtubules in parallel bundles, asters of mitotic spindles during mitosis, and microtubules not associated with the microtubule organizing centers (4, 5).

The cytotoxic activity of paclitaxel for tumor cells is well known, and preclinical and clinical studies showed it was effective against several tumors, including ovarian, breast, lung, and head and neck carcinomas (5). Other activities of paclitaxel related to different cell functions have been studied as well. The drug inhibits the motility of murine fibroblasts (6), ESb murine T-lymphoma (7), PC-3 prostate carcinoma (8), and A2058 melanoma cells (9). It also inhibits the production of matrix metalloproteinases by tumor cells, thus affecting their invasive behavior (7, 8).

Little is known about the effect of paclitaxel on endothelial cell functions and on angiogenesis. This study investigated the effect of paclitaxel on tumor-induced angiogenesis in in vitro and in vivo experimental models.

MATERIALS AND METHODS

Drugs and Reagents. Paclitaxel (Taxol®), provided by the Division of Cancer Treatment, National Cancer Institute, Bethesda, MD) was dissolved in 50% polyoxyethylated castor oil (Cremophor EL) and 50% ethanol and diluted in 5% glucose to the indicated concentrations for in vitro treatments. For the in vitro experiments, a 1000× stock solution of paclitaxel in absolute ethanol was prepared and then diluted in test medium. Cisplatin (Bristol-Myers Squibb, Wallingford, CT) was dissolved in water.

As a source of tumor-derived angiogenic factor, we used the supernatant of murine endothelioma eEnd.1 cells which release the angiogenic factor EDMF1 in the culture medium (10). The conditioned medium, prepared as described (10), was separated on heparin-Sepharose, and the fractions containing EDMF, corrected for molarity, were used in vitro and in vivo.

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1 The abbreviations used are: EDMF, endothelioma-derived motility factor; bFGF, basic fibroblast growth factor; MMP-2, matrix metalloproteinase 2.
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Cancer Institute, Genova, Italy).

Effect of paclitaxel on angiogenesis in vivo. Pellets of Matrigel containing a tumor-derived angiogenic factor (EDMF) were implanted s.c. in C57BL/6N mice and treated i.v. on days 0 and 2 with the indicated dose of paclitaxel or vehicle. After 4 days, the angiogenic response was evaluated. Data are expressed as hemoglobin content of the pellet (g/dl) and means of the data from at least two independent experiments (n = 10–18). Hemoglobin content of pellets containing Matrigel alone was 0.29 ± 0.07 g/dl (n = 16). *, P ≤ 0.05; **, P ≤ 0.005 (Mann-Whitney U test). Bars, SE.

Matrigel was kindly provided by Dr. A. Albini (National Cancer Institute, Genova, Italy).

In Vivo Angiogenesis Assay. To study angiogenesis in vivo, the method described by Passaniti et al. (11) and previously utilized to evaluate inhibitors of angiogenesis (12) was used. Briefly, tumor cell supernatant, prepared as described above, was embedded in a pellet of Matrigel (10 mg/ml, 0.5 ml) along with heparin (16 units/ml) and injected s.c. into male C57BL/6N mice (Charles River, Calco, Italy). Mice received paclitaxel (28 mg/kg, unless otherwise indicated) or cisplatin (4 mg/kg) i.v. immediately before and 2 days after the injection of the pellet of Matrigel. Control mice received the same volume of vehicle.

After 4 days, the angiogenic response was evaluated at autopsy by two blinded observers. Macroscopic evaluation of hemorrhage within and around the Matrigel pellet was graded from 0 to 4+, depending on the extent and intensity of hemorrhage. The pellet was then removed, and the hemoglobin content was measured with the Drabkin procedure as described (12).

For histological analysis, the Matrigel pellets in combination with surrounding skin and soft tissue were fixed in 10% neutral buffered formalin for 24 h and embedded in paraffin. Five-μm sections were stained with H&E, and the following histological features were independently evaluated and graded by two blinded observers: (a) cellularity associated with the surface of the pellet (scored as: 1, scanty; 2, moderate; and 3, abundant); (b) cells invading the pellet (scored as above); (c) presence of cords and tubes within the pellet (scored as: 0, none; 1, occasional; 2, moderate; and 3, prominent); and (d) presence of blood-filled channels and/or lacunae (scored as above). For each sample, the resulting sum was recorded as the final score.

In Vitro Assays. Endothelial cells were obtained from human umbilical vein and cultivated as described (12). A colorimetric assay was used to assay proliferation (12). Briefly, 4 × 10⁵ cells were plated in each well of a 96-well plate. After 24 h, paclitaxel or cisplatin was added at the concentration indicated in “Results”; the drugs were either removed after 4 h or left for the duration of the assay (72 h). After 3 days, the plate was stained with 0.5% crystal violet in 20% methanol, rinsed with water, and air dried. The stain was eluted with a 1:1 solution of ethanol:0.1 M sodium citrate, and absorbance at 540 nm was measured with a Multiscan MC (Titertek, Flow Laboratories, Milan, Italy; Ref. 12). Data are expressed as the percentage of control proliferation (vehicle-treated cells); the IC₅₀ (drug concentration causing 50% inhibition) was calculated from the plotted data.

Chemoattraction was assessed using the Boyden chambers and 8-μm pore size, gelatin-coated polycarbonate Nucleopore filters, as described previously (12). To study chemoattraction, the filter was coated with an even layer of Matrigel (0.5 mg/ml), and the assay was conducted as described elsewhere (12). Endothelial cells were detached by a brief exposure to 0.25% trypsin-0.02% EDTA, washed in DMEM with 0.1% BSA, resuspended in this medium at the concentration of 1–2 × 10⁵ cells/ml, and added to the upper compartment of the Boyden chamber. Paclitaxel or cisplatin was added to the endothelial cells at the indicated concentration and incubated throughout the assay (4 h for chemotaxis, 6 h for chemoinvasion). Data are expressed as the percentage of control migration (vehicle-treated cells); the IC₅₀ (drug concentration causing 50% inhibition) was calculated from the plotted data.

To analyze the production of matrix metalloproteinases, subconfluent cultures of endothelial cells were washed three times with serum-free medium and incubated for 20 h in the same medium containing vehicle or paclitaxel. The conditioned medium was then collected and processed for gelatin zymography; the remaining cells were counted. Gelatin zymography was performed essentially as described (13), with 1 mg/ml gelatin in a 10% polyacrylamide-SDS gel. The sample volume was adjusted according to the number of cells. The experiment was repeated twice.

Cord formation on Matrigel was assessed by plating endothelial cells (6 × 10⁶/0.5 ml) on a layer or polymerized Matrigel (270 μl) essentially as described (14). Paclitaxel (1 nm) or vehicle (absolute ethanol) was added to the medium. Photographs were taken with an inverted microscope 48 h after plating.

RESULTS

Paclitaxel Affects Angiogenesis in Vivo. s.c. injection of a tumor cell supernatant embedded in a pellet of Matrigel induced a strong angiogenic response in 4 days, with macroscopic hemorrhages around the pellet and a 2.8–3.3-fold in-
Significant increase in the hemoglobin content compared to pellets containing Matrigel alone. Systemic treatment of mice with paclitaxel resulted in dose-dependent inhibition of the angiogenic response compared to vehicle-treated animals. Inhibition of hemoglobin content was significant at 20 mg/kg ($P < 0.05$) and was maximal at 28 mg/kg, with no effect at 10 mg/kg (Fig. 1). In the same assay, batimastat and tissue inhibitor of metalloproteinase 2, used as reference drugs, caused up to 38 and 75% inhibition of the hemoglobin content of the pellets, respectively (12).

Macroscopic evaluation of the area revealed markedly less hemorrhage surrounding the pellet in the animals treated with 20 and 28 mg/kg paclitaxel: 55% of the vehicle-treated mice presented hemorrhage compared to 22% and 20% of the mice treated with 28 and 20 mg/kg paclitaxel, respectively. Moreover, the mean intensity of hemorrhage scored 1.03 ± 0.3, 0.17 ± 0.08, and 0.25 ± 0.2 for vehicle, 28 and 20 mg/kg paclitaxel, respectively.

Histological analysis of pellets containing the angiogenic factors showed a variable degree of cellularity surrounding and invading the pellets of Matrigel. Cells were scattered singly...
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Fig. 3  Effect of paclitaxel on in vitro endothelial cell proliferation, motility, and chemoinvasion. The antiproliferative effect of paclitaxel was assessed by treating endothelial cell with the drug for 4 h (•) or 72 h (---). Chemotaxis and chemoinvasion were tested in a Boyden chamber using tumor cell-conditioned medium (○) or bFGF (□) as attractants. In these assays, paclitaxel was added to the cells at the concentration indicated and left throughout the assay (4 and 6 h, respectively). Data are expressed as the percentage of proliferation, migration, and invasion compared to control response (ethanol alone), and the means of triplicates from one experiment are representative of at least three. Bars, SE.

Fig. 4  Zymographic analysis of MMP-2. Endothelial cells were exposed to vehicle or different concentrations of paclitaxel, and volumes of the supernatant corresponding to the same number of cells were electrophoresed on gelatin-containing SDS-polyacrylamide gels. After processing, MMP-2 appears as a clear band of gelatinolytic activity at 72 kDa.

Order of figure captions:
- Fig. 3
- Fig. 4

Throughout the Matrigel or organized in thin cords and tubules without a clearly defined lumen, in blood vessels with a recognizable lining of flattened endothelial cells, and in larger blood lacunae, without any apparent cell lining (Fig. 2a). A significant reduction in the angiogenic response was observed in the pellets of mice treated with paclitaxel compared to vehicle. The cellularity surrounding and infiltrating the pellets was reduced, and cords, tubules, vessels, and lacunae were less frequent (Fig. 2b). The histological score of the cases under study, evaluated as described in “Materials and Methods,” ranged from 3 to 11. A strong response (histological score ≥6) was observed in 7 of 10 vehicle-treated mice, but only in 1 of 8 paclitaxel-treated mice (P = 0.024, Fisher’s exact test).

Paclitaxel Affects Endothelial Cell Functions in Vitro. In vitro experiments were then performed to study the possible mechanisms of the antiangiogenic activity of paclitaxel. The effect was investigated on endothelial cell functions related to the angiogenic process: proliferation, migration, invasiveness, and cord formation.

The effect of paclitaxel on endothelial cell proliferation was assessed with a colorimetric assay, and endothelial cells were exposed to the drug for either 4 h or 3 days. The short time exposure (4 h) was chosen to compare the effect of paclitaxel on proliferation and motility in the same experimental conditions (see below). When endothelial cells were exposed to paclitaxel for 4 h, only a marginal inhibitory effect was observed (up to 17.3% inhibition; Fig. 3). A strong inhibition of endothelial cell proliferation (up to 60.5% inhibition) was instead observed when cells were exposed to paclitaxel for 3 days (Fig. 3, dashed line).

Paclitaxel also inhibited endothelial cell motility response to tumor-derived angiogenic factors. Inhibition was dose dependent, occurring at concentrations as low as 10 nm and reaching 86.2% at 100 nm (Fig. 3). The IC50 was 0.4 nm. In one experiment, the angiogenic factor bFGF was used as a chemoattractant, and in this case paclitaxel also inhibited the endothelial cell chemotactic response (Fig. 3).

An important event during angiogenesis is the degradation of the basement membrane by endothelial cells to allow their migration into the underlying interstitial matrix. Matrix-degrading enzymes, whose production is stimulated by several angiogenic factors, play an important role in matrix digestion by invading endothelial cells (15). As a model to study endothelial cell invasiveness, we used the chemoinvasion assay in which endothelial cells are induced to digest a layer of reconstituted basement membrane (Matrigel) and to migrate through the filter. Paclitaxel inhibited endothelial cell chemoinvasion, with maximum inhibition (72%) at 10 nm and an IC50 of 0.5 nm (Fig. 3). We also found that production of the matrix metalloproteinase MMP-2, evaluated by zymography, was reduced in paclitaxel-treated endothelial cells (up to 37% inhibition at 10 μM, Fig. 4).

The final event during angiogenesis is the organization of endothelial cells in a three-dimensional network of tubes. In vitro, endothelial cells plated on Matrigel align themselves, forming cords, already evident a few hours after plating. The
Fig. 5 Cord formation by endothelial cells in Matrigel. Endothelial cells (6 × 10⁶) were seeded on a layer of Matrigel in the presence of vehicle (a) or paclitaxel (1 nM, b). After 48 h, formed cords were photographed under an inverted microscope (×63). The experiment was repeated three times.

addition of paclitaxel (1 nM) to the assay resulted in complete inhibition of endothelial cell alignment and cord formation (Fig. 5).

Lack of Antiangiogenic Activity of Cisplatin in Matrigel. To rule out a direct association between antiangiogenic and antiproliferative activity, we have studied the effect on endothelial cell functions in vitro and on angiogenesis in vivo of another cytotoxic drug, cisplatin, known to have a strong antiproliferative effect on different cell types. As expected, cisplatin was very effective in inhibiting endothelial cell proliferation in vitro: already after a 4-h exposure IC₅₀ was 200 μM (Fig. 6). Inhibition of endothelial cell motility was observed only at toxic concentrations of cisplatin (IC₅₀, 290 μM). Notwithstanding its antiproliferative activity, cisplatin (4 mg/kg) did not inhibit the angiogenic response in Matrigel in vivo (Fig. 6).

DISCUSSION
Tumor-induced angiogenesis, the formation of neovessels from preexisting ones, is critical for supporting tumor growth
and progression not only by providing the necessary blood supply but also by allowing metastastic cells into the circulation (16). Promising preclinical studies have shown the antineoplastic activity of angiostatic agents, and several antiangiogenic drugs are currently under investigation in clinical trials. The present study reports that paclitaxel inhibits angiogenesis in vivo by tumor cell supernatants. This indicates that the antiangiogenic activity of the drug might contribute to its antineoplastic activity, thus tumor growth might be affected not only by direct cytotoxicity for tumor cells but also by inhibition of neovessel formation. This might be particularly important in the case of vascular tumors and tumors characterized by a strong angiogenic activity. Indeed, paclitaxel has been recently described to be active in a Phase II clinical trial in patients with HIV-associated Kaposi's sarcoma, a vascular tumor characterized by a strong angiogenic activity (14, 17).

Paclitaxel inhibited angiogenesis at doses (20–28 mg/kg) that have shown antitumor activity in preclinical studies in mice. Indeed, these doses inhibit the growth of different murine tumors in syngeneic mice and human tumors transplanted in nude mice (18, 19).

Paclitaxel inhibited the proliferation of endothelial cells. The drug did not affect endothelial cell attachment to tissue culture plastic (data not shown), thus ruling out that the inhibition of cell proliferation is indirectly mediated by an effect of paclitaxel on cell adhesion. Given the effect of paclitaxel on endothelial cell proliferation, it was therefore important to study the relationship between antiproliferative and antiangiogenic activity. To this purpose, two approaches were followed. Studying the effect of the drug on in vitro endothelial cell functions relevant to the angiogenic process, we have found that paclitaxel inhibited endothelial cell motility in response to tumor-derived chemotactic factors and to bFGF. Interestingly, endothelial cell motility was inhibited to a greater extent and at lower drug concentrations than was proliferation. Moreover, the antiproliferative effect of paclitaxel was greatly reduced when endothelial cell proliferation was assessed in treatment conditions similar to those used for the chemotaxis assay (4-h exposure to the drug), and no relevant antiproliferative effect was observed at the same doses that inhibited chemotaxis. These findings suggest that the effect of paclitaxel on endothelial cell motility might be more important than that on proliferation in the overall inhibition of angiogenesis in vivo. Paclitaxel might conceivably affect tumor-induced angiogenesis in vivo at local concentrations lower than those necessary to cause a cytotoxic effect on tumor cells. The cytotoxic properties of paclitaxel make it difficult to recognize whether its antineoplastic activity is due to inhibition of angiogenesis or to a direct toxic effect on tumor cells. More studies are thus needed to clarify the therapeutic relevance of these findings and to develop treatment strategies.

The second indication that the antiangiogenic effect of paclitaxel is not merely dependent on inhibition of proliferation is the lack of antiangiogenic activity of cisplatin, another cytotoxic antineoplastic drug. Cisplatin inhibited endothelial cell proliferation in vitro but not angiogenesis in vivo at doses that are related to the maximum tolerated dose and that have shown antineoplastic activity in murine systems (19, 20). This finding is in agreement with other studies describing the lack of antiangiogenic activity of cisplatin (21, 22). Both paclitaxel and cisplatin inhibited endothelial cell motility; however, in contrast to paclitaxel, the inhibition of cell motility by cisplatin appears to be a direct consequence of its high antiproliferative activity, since it occurred only at doses which inhibited cell proliferation.

Fig. 6  Effect of cisplatin on endothelial cell functions in vitro and angiogenesis in vivo. The effect of cisplatin on cell proliferation was assessed by treating endothelial cells with the drug for 4 h (○) or 72 h (– - -). Chemotaxis was tested as described in the legend to Fig. 3. Data are expressed as the percentage of proliferation and migration compared to control response, and the means for data from two independent experiments are represented. Angiogenesis was assayed as described in the legend to Fig. 1. Mice received cisplatin (4 mg/kg) or vehicle i.v. on days 0 and 2. Data are expressed as hemoglobin content of the pellet (g/dl), and the means for data from two independent experiments (n = 12–17) are represented. Hemoglobin content of pellets containing Matrigel alone was 0.31 ± 0.18 g/dl (n = 17). Bars, SE.
Altogether, our findings indicate that the antiproliferative effect of paclitaxel is not the main mechanism for its antiangiogenic activity and that drugs which affect endothelial cell proliferation are not necessarily antiangiogenic. Our study also indicates that the in vivo Matrigel model of angiogenesis represents a good model to study true antiangiogenic drugs independently on their effect on endothelial cell growth.

The first evidence of an inhibitory activity of microtubule-affecting drugs on endothelial cell migration was reported in studies on an in vitro wound-healing assay using paclitaxel, colchicine, and vinblastine (2, 3, 23). Interestingly, in this assay, colchicine and vinblastine inhibited cell motility without affecting proliferation however (2). Recently, the inhibitory activity of paclitaxel on angiogenesis in the chick chorioallantoic membrane model has been reported, although no mechanism for this inhibition has been studied (24).

Paclitaxel also prevented two other functions related to angiogenesis involving the interaction of endothelial cells with the extracellular matrix: cord formation in a three-dimensional matrix and endothelial cell invasiveness through a layer of Matrigel. These processes require both motility and the production of matrix-degrading enzymes by invading cells. Paclitaxel affected both activities: besides its effect on motility, it also reduced the production of the matrix metalloproteinase MMP-2 by endothelial cells, as already reported for other cell types (8).

Antiangiogenic factors can act by different mechanisms: they can either act directly on endothelial cell functions or block angiogenesis indirectly, e.g., by blocking the production of angiogenesis stimulating factors or by inhibiting the recruitment and activation of inflammatory cells known to release angiogenic factors (1, 16). The finding that paclitaxel inhibited several endothelial cell functions in vitro indicates that its antiangiogenic activity in vivo is not indirect, but that it acts directly on the endothelial cell response to angiogenic factors.

This study demonstrated that paclitaxel is a potent antiangiogenic agent. The effect does not appear to be a mere consequence of its cytotoxicity, since it inhibits other endothelial cell functions with even greater potency. We propose that the antiangiogenic property of paclitaxel might contribute to its antineoplastic activity.

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