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« [Previous Article](#) | [Table of Contents](#) | [Next Article](#) »

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Articles

Paclitaxel Inhibits Arterial Smooth Muscle Cell Proliferation and Migration In Vitro and In Vivo Using Local Drug Delivery

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► Abstract

Background The antineoplastic compound paclitaxel (Taxol) causes an increased assembly of extraordinarily stable microtubules. The present study was designed to characterize the effects of paclitaxel on proliferation and migration of human arterial smooth muscle cells (haSMCs) in vitro and on neointima formation in an in vivo experimental rabbit model.

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- ▲ [Top](#)
- [Abstract](#)
- ▼ [Introduction](#)
- ▼ [Methods](#)
- ▼ [Results](#)
- ▼ [Discussion](#)
- ▼ [References](#)

Methods and Results Both monocultures of haSMCs and cocultures with human arterial endothelial cells (haECs) were used. Cell growth after 4, 8, and 14 days was determined in the absence or presence of platelet-derived growth factor-AB (PDGF-AB), basic fibroblast growth factor (bFGF), or thrombin. Nonstop paclitaxel exposure, as well as single-dose applications of paclitaxel for 24 hours or even 20 minutes (0.1 to 10.0 $\mu\text{mol/L}$), caused a complete and prolonged inhibition of haSMC growth up to day 14, with an IC_{50} of 2.0 nmol/L. Mitogens or cocultures with stimulating haECs did not significantly attenuate paclitaxel-induced effects. Immunohistochemistry showed characteristic cytoskeletal changes predominantly in the microtubule network. Additionally, in 20 male New Zealand White rabbits, intimal plaques were produced by electrical stimulation. In 10 animals, paclitaxel was locally applied by use of microporous balloons. Histologically, the intima wall area, wall thickness, and degree of stenosis were reduced significantly in paclitaxel-treated animals compared with controls.

Conclusions Our data show that paclitaxel inhibits haSMC proliferation and migration in a dose-dependent manner in monocultures and cocultures even in the presence of mitogens. Furthermore, paclitaxel prevents neointima formation in rabbits after balloon angioplasty. The long-lasting effect after just several minutes' exposure time makes this lipophilic substance a promising candidate for local antiproliferative therapy of restenosis.

Key Words: restenosis • paclitaxel • pharmacology • muscle, smooth • endothelium

► Introduction

Microtubules are important parts of the cytoskeleton, especially of the mitotic spindle. They are involved in diverse cellular functions, such as cell division, cell motility, and the maintenance of cell shape. In addition, they play a role in signal transduction (eg, from mitogen receptors), intracellular transport mechanisms, and gene activation.^{1 2} The novel diterpenoid paclitaxel (Taxol), a potent antineoplastic drug, has been shown to be a promising agent for the therapy of ovarian, breast, and other cancers.³ In contrast to the molecular mechanism of the *Vinca* alkaloids colchicine and Colcemid (demecolcine), which prevent the polymerization of tubulin into microtubules, paclitaxel stabilizes polymerized microtubules and enhances microtubule assembly.⁴ As a result, cell replication is inhibited predominantly in the G_0/G_1 and G_2/M phases of the cell cycle.⁵

Morphologically, paclitaxel-treated cells form abnormal, disorganized microtubules, leading to an epithelioid cell shape.⁶ Previous experimental in vitro studies with paclitaxel have been performed in tumor cell lines,⁷ epithelial cells,⁸ and fibroblasts, predominantly 3T3 cells.⁹

Unlike other antiproliferative agents, paclitaxel also has several properties that make it a good candidate for local drug therapy of excessive arterial smooth muscle cell proliferation in restenosis after balloon angioplasty or stent implantation; these properties have been tested in vitro,¹⁰ in animal models,¹¹ and in clinical studies¹² thus far. First, the highly lipophilic character of paclitaxel promotes a rapid cellular uptake by enabling it to easily pass through the hydrophobic barrier of cell membranes.¹³ Second, the unique mode of action supports a long-lasting antiproliferative action even after a brief, single-dose application at very low concentrations, as previously shown in tumor cells.^{1 14} An antiproliferative effect of paclitaxel on vascular cells has been shown in vitro in rat

▲	Top
▲	Abstract
■	Introduction
▼	Methods
▼	Results
▼	Discussion
▼	References

VSMCs as well as in vivo in the rat carotid artery injury model.¹⁵ Paclitaxel was found to interfere with VSMC proliferation and migration at nanomolar levels in vitro and to prevent neointimal VSMC accumulation in the carotid artery in vivo. However, the failure of nearly all human studies in the prevention of restenosis after testing a broad spectrum of antiproliferative drugs showed that promising results of animal cell cultures and in vivo studies cannot be transferred directly to the human situation.¹² Thus, the current study was designed to analyze the effects of paclitaxel on haSMC and haEC growth and structure of the cytoskeleton. Detailed experiments comparing very brief (20-minute) with nonstop incubation periods at several time points up to 14 days were designed to exclude rebound effects after drug removal between the first wave of cell proliferation and migration after injury,¹¹ eg, as described for heparins.¹² Additional studies with growth factor-stimulated haSMCs and with our previously described transfilter coculture system^{16 17} were performed to imitate the complexity of in vivo cell-cell interactions, which certainly influences the efficacy of antiproliferative drugs.

Finally, the in vitro results were substantiated by in vivo studies that used locally applied paclitaxel to prevent neointima formation after balloon dilatation of plaques that were induced by electrical stimulation of rabbit carotid arteries.

► Methods

Cell Isolation

Human iliac and renal arteries were obtained from organ donors (approved by the local ethics committee). haSMCs and haECs were isolated and passaged according to techniques described previously.¹⁷ haSMCs were grown in a 1:1 mixture of Waymouth MB 752/1 medium and Nutrient Mixture F12 Ham (1:1 vol/vol) supplemented with 10% FCS, 100 U/mL penicillin, and 100 µg/mL streptomycin (Gibco BRL). Postconfluence haSMCs grew in the characteristic "hill-and-valley" growth pattern. Smooth muscle origin was confirmed by immunocytochemical stainings with monoclonal antibodies against smooth muscle α -actin (Progen) and heavy-chain myosin (Sigma).

haECs were subcultured onto collagen-coated plastic culture dishes (Iwaki Glass) and were grown in an endothelial medium kit (EMK-1; Sigma) containing endothelial cell growth factor and 2% FCS. Subcultured haECs were characterized by immunocytochemical stainings with polyclonal antibodies against the von Willebrand factor (Boehringer Mannheim), and contamination by haSMCs was excluded by additional double stainings using antibodies against smooth muscle α -actin. Monolayers showed the characteristic cobblestone morphology. Cells were fed every third day and used for experiments in low passages (2 through 6).

To exclude contamination by mycoplasma, DAPI stainings (Boehringer Mannheim) were performed routinely in several passages.¹⁸

Drugs

Paclitaxel (Sigma) was dissolved in 100% ethanol and sterile filtered. Taxol was obtained from Bristol-Myers Squibb. It consisted of 7.0 mmol/L paclitaxel dissolved in a lipid vehicle, a 1:1 mixture of polyethoxylated castor oil (Cremophor EL), and absolute ethanol. To obtain different test concentrations between 0.1 nmol/L and 10.0 µmol/L, serial dilutions of a stock solution (2.5 mmol/L) were prepared with 0.9% NaCl solution. Both vehicles were also analyzed separately for

▲	Top
▲	Abstract
▲	Introduction
■	Methods
▼	Results
▼	Discussion
▼	References

antiproliferative or cytotoxic effects.

Test Assays

Cell Proliferation Assays

Cells were rinsed with PBS, trypsinized (Gibco BRL), counted with a Coulter counter (CASY I; Schärfe Systems), and seeded onto six-well plates at a density of 5×10^3 cells/cm². After cell attachment (24 hours), the number of intact cells and the mitotic index were determined and used as a baseline for day 1.

Two modes of drug application were performed: (1) nonstop incubation with the addition of paclitaxel every third day in combination with medium changes and (2) single-dose application, adding paclitaxel only once for a period of either 24 hours or 20 minutes, followed by a washout process with PBS and addition of fresh, drug-free culture medium.

After 2, 4, 6, 8, and 14 days, final cell numbers were measured by cell counting, mitotic indexes were determined by BrdU-ELISAs according to the manufacturer's instructions (colorimetric cell proliferation ELISA, Boehringer Mannheim),¹⁹ and MTT tests (Sigma) were performed according to previously described techniques.²⁰ The MTT test serves as an indirect marker for proliferation and cell viability by measuring the mitochondrial activity of cells.

Growth Factors

haSMCs were incubated with either 20.0 ng/mL PDGF-AB (R&D Systems), 15.0 ng/mL bFGF (R&D Systems), or 1.0 U/mL thrombin (Sigma) in addition to FCS. Paclitaxel was added for 24 hours. Next, drug-containing medium was rinsed with PBS and replaced by fresh culture medium (+10% FCS). Growth factors were supplemented throughout the cultivation period. The described concentrations showing maximally stimulatory effects on haSMC growth were found in pretests in which increasing concentrations (0.1, 10.0, 15.0, 20.0, and 40.0 ng/mL) of PDGF-AB and bFGF as well as thrombin (0.5 and 1.0 U/mL) were examined.

Each test consisted of six measurements for each concentration of paclitaxel (0.1, 1.0, and 10.0 nmol/L and 0.1, 1.0, and 10.0 μ mol/L) and was performed three times under identical conditions.

Transfilter Coculture System

Transfilter cocultures were prepared by coating polycarbonate filters with collagen according to techniques described previously.¹⁶ With this system, coated filters are inserted between an inner and an outer polycarbonate frame, and different cell types can be cultured in separate culture media on a growth area of 8 cm², as previously described.¹⁷ First, haECs were seeded onto the lower side of the filter at a density of 2.5×10^4 cells/cm². On the following day, haSMCs (2.5×10^4 cells/cm²) were seeded onto the opposite side of such filters, and both cell types were cocultured for 14 days.

Different concentrations of paclitaxel (1.0 nmol/L to 10.0 μ mol/L) were added either for 24 hours or for 20 minutes to the lower compartment (containing haECs) to imitate local drug application at the luminal side of the vessel. Culture medium in both compartments was then replaced by normal, paclitaxel-free culture medium, which was replaced by fresh medium every third day. After 14 days, cell numbers on both sides of the filters were determined separately by cell counting after disaggregation with trypsin/EDTA and compared with controls that were treated with equivalent concentrations of the vehicle (ethanol without paclitaxel). Each concentration was tested in a total of six cocultures.

Filter pieces were also fixed for 3 hours with 4% paraformaldehyde in PBS and embedded in araldite (Serva) as described previously.^{16 17} Semithin sections (4 μm) were taken on poly-L-lysine-coated coverslips. Cells were then stained with toluidine blue (Merck), and cell numbers on both filter sides were determined under a light microscope.

Immunocytochemical and DNA Stainings

Cytoskeletal proteins. Cells were seeded on coverslips and treated with drug as described above. After 1, 12, and 24 hours and 4 days, cells were fixed with methanol at -20°C and labeled with the following primary antibodies: monoclonal anti- β -tubulin (Sigma; $1.0\ \mu\text{g}/\text{mL}$), monoclonal anti-vimentin (Sigma; $1.25\ \mu\text{g}/\text{mL}$), monoclonal anti-smooth muscle α -actin (Progen; $2.0\ \mu\text{g}/\text{mL}$), or anti-von Willebrand factor (Boehringer Mannheim; $2.0\ \mu\text{g}/\text{mL}$). Secondary labeling was achieved with FITC-conjugated goat anti-mouse IgG ($40.0\ \mu\text{g}/\text{mL}$), Cy3-conjugated sheep anti-rabbit IgG ($10.0\ \mu\text{g}/\text{mL}$), or Cy3-conjugated sheep anti-mouse IgG ($10.0\ \mu\text{g}/\text{mL}$) (all from Sigma).

DNA staining. For additional nucleus staining, cells were rinsed and incubated for 30 minutes with DAPI dissolved in methanol ($250\ \text{ng}/\text{mL}$) before incubation with the described primary antibodies.

Detection of apoptosis. Apoptosis was detected by one of two methods: (1) DNA staining by DAPI.¹⁸ Cells were rinsed with PBS, fixed with methanol, incubated for 30 minutes with the DNA dye, and rinsed again several times with PBS. (2) TUNEL method.²¹ Proliferating cells were seeded on coverslips (5×10^4 cells/mL), incubated for 1 day routinely, and treated with paclitaxel for 6 hours. Then, the In Situ Cell Death Detection Kit, AP (Boehringer Mannheim) was used according to the manufacturer's instructions. The total cell number, TUNEL-stained cells, and cells with ≥ 5 DAPI-stained nucleus fragments were determined microscopically: 1000 total cells/cover slide were counted in six different sets of cultures of paclitaxel-treated haSMCs and haECs. The apoptotic index was determined as the number of positively stained cells divided by the total cell number counted $\times 100$.

Rabbit Carotid Artery Injury Model

Animal model. Intimal plaques were produced in 20 male New Zealand White rabbits (Thomae; Biberach, Germany) by use of the electrostimulation model as described previously.²² In brief, this model is based on the local and transmural electrical stimulation of the carotid artery under standardized conditions to produce plaques of comparable size before intervention.

Drug preparation. For local delivery, the described paclitaxel stock solution was diluted to a final concentration of $10.0\ \mu\text{mol}/\text{L}$ in a volume of 4 mL sterile 0.9% NaCl solution.

Local drug-delivery device. A 2.1-mm microporous infusion catheter (Cordis) consisting of a porous membrane with both 0.4- and 0.8- μm pore diameters was used. The device has a proximal port for balloon inflation and a shaft diameter of 3.5F.²³ The balloon was inflated with a low-pressure pump (DVI). A volume of 4.0 mL was delivered locally with the use of an inflation pressure of 2 atm.

Study protocol. After the stimulation period, transluminal balloon angioplasty of the preformed plaque was performed in a total of 20 male rabbits (2.6 to 3.2 kg body weight). For angioplasty, a 2.0-mm balloon catheter (Micro-Hartzler, ACS) was introduced into the exposed vessel by direct arteriotomy and then advanced into the region of plaque formation. The balloon was inflated to 5 atm for 60 seconds. The rabbits were then randomized in a pharmacologically nontreated control group in which balloon angioplasty was performed alone (BA group, $n=10$) and a local drug-delivery group

(LDD group, n=10). In the LDD group, the porous balloon was advanced into the region of the predilated plaque, and 4 mL of the paclitaxel solution was delivered with a pressure of 2 atm over a time period of ≈ 30 seconds. The rabbits were killed 28 days after balloon treatment. The carotid arteries were perfused in situ with 500 mL of a 0.1 mol/L cacodylate-buffered 2% paraformaldehyde solution under physiological pressure. A 3- to 4-cm segment of the treated carotid artery with a polytetrafluoroethylene cuff on it was excised, and the proximal and distal ends were marked with sutures.

Immunohistochemical stainings. The technique of the immunohistological examination of vessel sections has been described previously²² and is based on stainings with monoclonal antibodies against BrdU and smooth muscle α -actin using an avidin-biotin-peroxidase complex. To confirm the endothelial origin of the luminal cell lining, sections were additionally stained with a polyclonal antibody against von Willebrand factor (goat anti-human factor VIII-related antigen, Atlantic Antibodies).²⁴

Quantitative histopathology. All sections were quantitatively analyzed by computerized morphometry, as described previously,²⁵ by an independent investigator who was blinded to the type of treatment protocol. In sections stained with the elastica van Gieson stain, luminal perimeter (equal to the length of the endothelial lining), intimal area, and intimal and medial thicknesses were traced manually under stereoscopic control by use of standard software (Bioquant, Bilany Consulting). To obtain the percentage of proliferative cells, the number of BrdU-positive cells was determined and related to the absolute number of intimal and medial smooth muscle cells. For the quantification of endothelial regeneration, the number of endothelial cells was counted and related to the length of the endothelial layer.

Statistical Analysis

Results of the experiments are expressed as mean \pm SD. The significance of differences between BA and LDD vessels of the in vivo study, as well as the in vitro results of single treatment groups that were related to controls, were proved by the Student unpaired two-tailed *t* test unless otherwise stated. In vitro results in which two or more treatment groups were compared (ie, Fig 2) were tested by means of two-way ANOVA. By both the Student *t* test and the Tukey test/Scheffé's F test, probability values of $<.05$ were considered to be significant; values of $P<.001$ were considered to be highly significant.²⁶

► Results

Effects of Paclitaxel on haSMCs

Nonstop Incubation

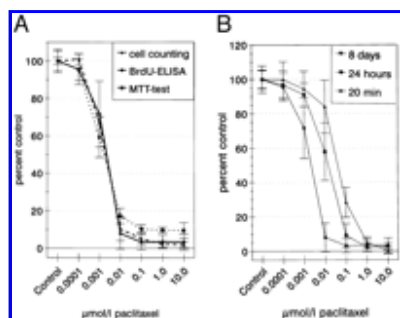
Paclitaxel caused a dose-dependent inhibition of haSMC proliferation after 1, 2, 4, 6 (data not shown), and 8 days of incubation time (Fig 1A). All three assays yielded comparable results with respect to the IC_{50} and the IC_{max} . The cell-counting assay resulted in an IC_{50} of 2.1 nmol/L and an IC_{max} of 1.0 μ mol/L after

8 days' incubation, the BrdU-ELISA resulted in an IC_{50} of 2.3 nmol/L and an IC_{max} of 0.1 to 1.0 μ mol/L, and the MTT test resulted in an IC_{50} of 1.6 nmol/L and an IC_{max} of 0.1 to 1.0 μ mol/L.

Within the IC_{max} range of 0.1 to 10.0 μ mol/L, an almost complete growth inhibition was observed

▲	Top
▲	Abstract
▲	Introduction
▲	Methods
■	Results
▼	Discussion
▼	References

compared with controls ($100.0 \pm 5.1\%$) (cell counting: $0.3 \pm 2.1\%$, $P < .001$; BrdU-ELISA: $1.6 \pm 4.9\%$, $P < .001$; and MTT tests: $9.5 \pm 4.2\%$, $P < .001$ versus controls).



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Figure 1. Paclitaxel-induced growth inhibition of haSMCs.

A, Comparison of percent cell proliferation after 8 days' nonstop incubation measured by cell-counting assays, BrdU-ELISAs, or MTT tests. All three test assays showed nearly identical results concerning IC_{50} and IC_{max} values, with significant growth-inhibitory effects in a concentration range between 0.01 and 10.0 $\mu\text{mol/L}$ paclitaxel ($P < .001$ versus controls). B, BrdU-ELISA after 8 days' cultivation time: comparison of nonstop incubation for 8 days with single-dose application for 24 hours or 20 minutes. All data are shown as mean \pm SD values.

Single-Dose Application

When exposure time was restricted from 8 days nonstop to 24 hours or 20-minute single-dose treatment, the dose-dependent growth-inhibitory effect of paclitaxel persisted after 4, 8, and 14 days' incubation (Fig 1B). Almost complete growth inhibition was again observed by 1.0 to 10.0 $\mu\text{mol/L}$ paclitaxel with nearly identical IC_{max} . Shortening of application times, however, resulted in a slight shift of the inhibition curves toward higher concentrations (Fig 1B), reflected by an increased IC_{50} (IC_{50} by BrdU-ELISAs after 8 days: nonstop, 2.3 nmol/L; 24 hours, 13.0 nmol/L; 20 minutes, 41.2 nmol/L). No significant rebound phenomena were observed after drug removal.

To differentiate the specific mechanism of the effect of paclitaxel on microtubules from other unspecific effects, cell toxicity and apoptosis were studied. Cell counting at day 2 (after 24 hours' paclitaxel treatment) showed that even at 10.0 $\mu\text{mol/L}$, cell numbers did not fall below those measured before the addition of paclitaxel. Viability tests (MTT tests, trypan blue stainings) confirmed that no cell loss occurred and that haSMCs remained viable within the first 24 hours after drug addition as well as after 2, 4, 6, and 8 days' incubation (data not shown). Microscopically, no cell rounding or cell detachment was observed. Remarkable unspecific cell toxicity was seen only at very high concentrations ($\geq 100.0 \mu\text{mol/L}$ paclitaxel), when even the vehicle ethanol exerts toxic effects. Fluorescence microscopic examinations of cells stained with the DNA dye DAPI and stainings by the TUNEL technique showed that paclitaxel in concentrations between 0.1 nmol/L and 10.0 $\mu\text{mol/L}$, which may be a potentially favorable range for local delivery, did not significantly induce apoptosis. A remarkable number of disrupted, fragmented cell nuclei in the cytoplasm of paclitaxel-treated haSMCs, indicating an apoptotic process,²⁷ was observed only at very high doses (50.0 to 100.0 $\mu\text{mol/L}$). At 50.0 $\mu\text{mol/L}$, an apoptotic index of 80% haSMCs positively stained by the TUNEL method and 25% DAPI-stained, totally fragmented cell nuclei were found, whereas lower doses (0.1 nmol/L to 10.0 $\mu\text{mol/L}$) showed apoptotic indexes $< 5\%$ (cell numbers are not shown). Ethanol showed no apoptotic effects. In haEC cultures, fragmented cell nuclei were found only sporadically. Assays performed with Taxol (paclitaxel dissolved in 50% ethanol/50% Cremophor EL) yielded results comparable to those described for paclitaxel (data not shown).

The vehicle ethanol, used in concentrations equivalent to 0.1 nmol/L to 10.0 $\mu\text{mol/L}$ paclitaxel,

exerted no significant growth-inhibitory effects on haSMCs and haECs. In contrast, high doses of Cremophor EL, as used in Taxol preparations, exhibited independent inhibitory (1.0 to 10.0 $\mu\text{mol/L}$) and even toxic (10.0 to 100.0 $\mu\text{mol/L}$) effects on haSMCs and haECs (data not shown).

Effect of Paclitaxel on Growth Factor–Stimulated haSMCs

Continuous incubation of haSMCs with PDGF-AB (20.0 ng/mL) in the presence of 10% FCS led to a significant growth stimulation after 4 days' incubation ($162.3 \pm 7.9\%$ versus $100.0 \pm 6.9\%$ for controls; $P < .001$). bFGF (15 ng/mL) showed similar growth-stimulatory effects ($141.2 \pm 9.7\%$ versus $100.0 \pm 6.9\%$ for controls; $P < .001$). The results correspond to those obtained in pretests. With the addition of paclitaxel for 24 hours, cell proliferation was inhibited dose dependently (BrdU-ELISA after 4 days: IC_{50} of 9.4 nmol/L with PDGF-AB and 6.9 nmol/L with bFGF). In contrast to PDGF-AB and bFGF, thrombin (1.0 U/mL) showed only moderate growth-stimulatory effects on haSMCs ($105.3 \pm 4.0\%$). Cell growth of thrombin-treated cells was also inhibited by paclitaxel in a dose-dependent fashion (BrdU-ELISA after 4 days: IC_{50} of 21.9 nmol/L). Comparable data were measured by use of the BrdU-ELISA (Fig 2) or the MTT test (data not shown). Statistical analysis showed highly significant effects within and between all three different growth factor treatment groups, all paclitaxel concentrations, and each paclitaxel concentration (between 0.01 and 10.0 $\mu\text{mol/L}$) compared with the control group that was also treated with the corresponding growth factor [C(+GF) in Fig 2].

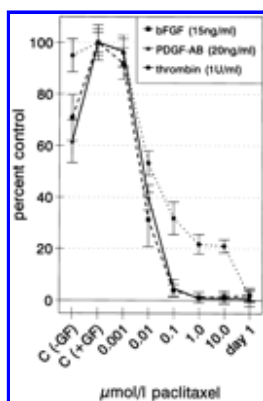


Figure 2. Mitotic index measured by BrdU-ELISAs in haSMC cultures [C(+GF)] stimulated by 15 ng/mL bFGF, 20 ng/mL PDGF-AB, and 1 U/mL thrombin versus controls [C(-GF)]. The induced growth-stimulatory effects were also blocked dose dependently by single-dose (24-hour) incubation with paclitaxel. Highly significant effects ($P < .001$, tested by two-way ANOVA) were observed within and between all three different growth factor treatment groups, all paclitaxel concentrations, and each different paclitaxel concentration (between 0.01 and 10.0 $\mu\text{mol/L}$) compared with the growth factor–stimulated control group [C(+GF)]. All data are shown as mean \pm SD values.

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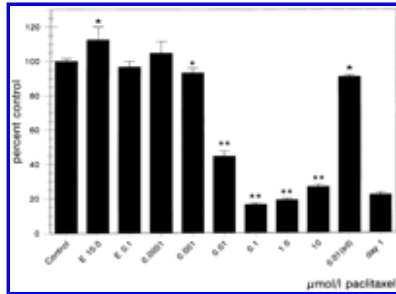
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Thus, growth stimulation by the addition of PDGF-AB, bFGF, or thrombin did not significantly attenuate growth inhibition of paclitaxel.

Effects of Paclitaxel on haECs

Similar studies were performed to test the influence of paclitaxel on haEC growth. Paclitaxel was added to proliferative haEC cultures in the log phase of cell growth. Nonstop and single-dose (24-hour) applications were performed, and cell proliferation was determined after 6 days by use of cell counting, BrdU-ELISA, and MTT tests. A dose-dependent, significant growth inhibition occurred at high concentrations (0.01 to 1.0 $\mu\text{mol/L}$; $P < .001$ versus controls), whereas lower paclitaxel doses (0.1 to 1.0 nmol/L) did not inhibit haEC growth significantly (Fig 3). Furthermore, no unspecific cytotoxic effects were observed within this concentration range. Immunocytochemistry showed

normal expression of the von Willebrand factor (data not shown). In control experiments, ethanol concentrations used in the 10.0- $\mu\text{mol/L}$ paclitaxel preparations (concentration of ethanol corresponds to $\approx 0.4\%$ [vol/vol]) stimulated haEC proliferation significantly ($P < .05$ versus controls) and partially counterbalanced the effects of paclitaxel.



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Figure 3. Effect of paclitaxel on haEC growth.

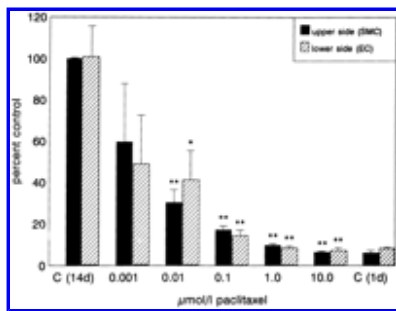
Determination of cell numbers after 6 days by cell counting. Day 1 values show basal cell numbers seeded before paclitaxel was added and control values show final cell numbers at day 6 serving as controls for normal cell growth. Paclitaxel caused a dose-dependent inhibition of haEC growth at higher concentrations (0.01 to 10.0 $\mu\text{mol/L}$). These effects were partially counterbalanced by growth-stimulatory effects of the vehicle ethanol (E 10.0 and E 0.1). In contrast to the inhibitory effects of nonstop applications, single doses (sd) of 0.01 $\mu\text{mol/L}$ paclitaxel did not inhibit haEC proliferation significantly. All data are shown as mean \pm SD values. * $P < .05$, ** $P < .001$ versus controls.

Effects of Paclitaxel on Transfilter Cocultures

The transfilter coculture system is a cell culture model that imitates the architecture of a normal vessel wall. We have previously shown that haECs can influence haSMC growth in transfilter cocultures.¹⁶ Stimulated by proliferating but not confluent haECs, haSMCs migrate into the opposite (haEC) compartment, forming a cell-rich, neointima-like structure.¹⁷

On the basis of these data, the effect of paclitaxel on smooth muscle cell proliferation and migration in coculture with proliferative haECs was evaluated (Fig 4). Neither cell type was confluent immediately after seeding (day 1). haSMCs of controls reached confluence after 5 to 7 days. Single-dose applications of paclitaxel into the lower (haEC) compartment for 24 hours or even 20 minutes dose dependently reduced total cell numbers per dish (sum of the lower and upper compartments), reaching significance at 0.01 to 10.0 $\mu\text{mol/L}$ ($P < .001$ versus controls). Cell numbers, however, did not fall below those measured on day 1 (Fig 4), indicating that paclitaxel exerted no cytotoxic effects on cocultures during the entire cultivation period. By microscopic examination, no cell rounding and no cell detachment characteristic of cell death was observed during the entire cultivation period (not shown). Separate analysis of cell numbers on the upper and lower sides of the filter showed that single doses of 0.01 to 10.0 $\mu\text{mol/L}$ paclitaxel in the lower compartment (haEC side) significantly inhibited haSMC proliferation in the upper compartment. Furthermore, paclitaxel induced a dose-dependent inhibition of cell numbers in the lower compartment compared with controls (Fig 4). The total cell number on the lower side of the filter represents the number of haECs and the result of haSMC migration from the upper to the lower filter side, followed by proliferation of subendothelial haSMCs. Because the number of haECs remains stable after reaching confluence,¹⁶ our results show that haSMC migration through the filter pores and after proliferation was inhibited significantly by a brief, single dose of paclitaxel ($P < .001$ versus controls).

Figure 4. Growth inhibition of haSMCs in coculture with haECs in the transfilter coculture system after a single-dose application (20 minutes) of paclitaxel applied into the endothelial compartment. The graphs show cell numbers 14



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days after drug replacement by culture medium. Solid bars show haSMC numbers on the upper filter side and hatched bars the total of haECs and migrated haSMCs on the lower side. Cell numbers at day 1 (C 1d) and at day 14 (C 14d) served as controls for normal cell growth of haSMCs. Data are shown as mean±SD. * P <.05, ** P <.001 versus controls.

These findings were confirmed by histological observations on semithin sections of several filter segments in which haECs and haSMCs were identified with the use of specific antibodies against von Willebrand factor as well as smooth muscle α -actin, respectively. Stainings with toluidine blue showed the formation of three to five cell layers on both sides of the filter in untreated controls, indicating migration and proliferation of haSMCs on the lower filter side. These processes were nearly totally inhibited by high paclitaxel concentrations (0.1 to 10.0 μ mol/L), resulting in a single subconfluent cell layer on both filter sides.

Cytoskeletal Changes

Microscopic observations were performed to analyze morphological alterations of treated haSMCs that confirmed the specific mode and extent of paclitaxel effects. Even short-term treatment with 0.1 to 10.0 μ mol/L paclitaxel for 1, 12, or 24 hours, as well as continuous treatment for 8 days, led to typical alterations of cell morphology. The cell shape was round instead of elongated, and cell size was smaller (Fig 5A and 5B). Immunofluorescence stainings were used to characterize paclitaxel-induced changes in the intracellular distribution of three of the main cytoskeletal protein classes: β -tubulin, the intermediate filament vimentin, and the contractile filament smooth muscle α -actin.

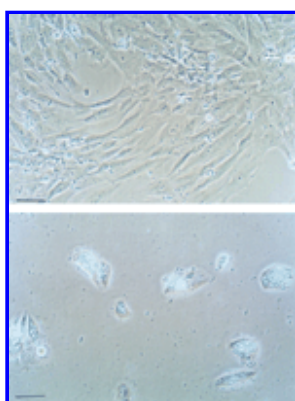


Figure 5. The effect of nonstop incubation with 1.0 μ mol/L paclitaxel on haSMC morphology. Phase-contrast reflection micrographs. A, Control culture after 8 days' cultivation. Elongated haSMCs with defined processes form a confluent monolayer with the typical "hill-and-valley" growth pattern. There are still some dividing cells (arrow). B, In paclitaxel-treated cultures, cell numbers are reduced dramatically. haSMCs are smaller and discoid with a loss of tail processes. There is no evidence of dividing cells. Scale bars represent 20 μ m.

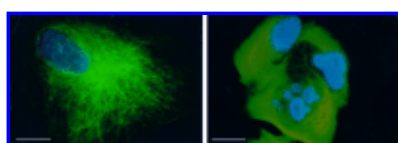
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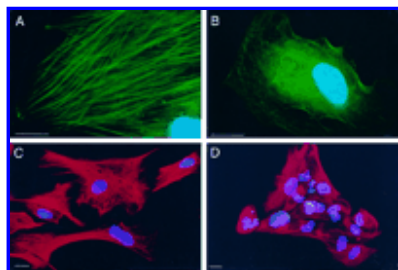
Immunocytochemical stainings for β -tubulin showed numerous densely packed groups of short and

decentralized microtubules that were not connected to any perinuclear center (Fig 6B) as observed in controls (Fig 6A) according to results described by others.^{9 28} The distribution of α -actin filaments was heterogeneous: some treated cells showed a disarrangement of α -actin fibers with circumferential bundles, whereas the majority showed the normal formation of straight filaments extending throughout the entire cytoplasm (Fig 7A and 7B).^{6 8 29} Vimentin structures showed the normal molecular structure^{8 30} (Fig 7C), but the distribution within the cytoplasm was altered similar to the β -tubulin organization (Fig 7D).⁶



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Figure 6. Immunofluorescence micrographs showing the effect of 1.0 $\mu\text{mol/L}$ paclitaxel on cytoplasmic microtubule distribution. A, Nontreated haSMCs stained with a monoclonal anti- β -tubulin antibody (nuclear counterstaining with DAPI). Microtubules are densely packed in a microtubule organizing center near the nucleus and form a network through the entire cytoplasm that reaches to the cell periphery. B, Paclitaxel-treated haSMCs are smaller, ellipsoid, and show an unorganized, decentralized tubulin distribution with densely packed tubulin rings in the cell periphery. Scale bars represent 5 μm .



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Figure 7. Immunofluorescence micrographs demonstrating the effect of 1.0 $\mu\text{mol/L}$ paclitaxel on the distribution of the contractile filament smooth muscle α -actin and the intermediate filament vimentin in haSMCs. A, Smooth muscle α -actin staining of control cultures showing typical straight α -actin filaments predominantly orientated along the cell axis. B, Paclitaxel causes a disarrangement of α -actin bundles with a partial circumferential orientation of the filaments. C, The vimentin network of untreated control cells is expanded over the entire cytoplasm. D, Paclitaxel treatment has no remarkable effects on vimentin assembly. Only the arrangement within the cytoplasm is altered according to the changes of cell shape and size. Scale bars represent 5 μm .

Results of the In Vivo Study

Quantitative Histopathology

Mean intimal wall area 28 days after balloon angioplasty (Fig 8) was significantly smaller in the LDD group ($0.21 \pm 0.11 \text{ mm}^2$) than in the BA group ($0.36 \pm 0.29 \text{ mm}^2$; $P = .01$). Histological examination (Fig 9A and 9B) also revealed a significant difference in intimal wall thickness 28 days after angioplasty, with a thickness of $110 \pm 39 \mu\text{m}$ in the LDD group compared with $138 \pm 85 \mu\text{m}$ in the BA group ($P = .04$; Fig 8). The degree of stenosis was $26 \pm 8\%$ in the LDD group compared with $34 \pm 20\%$ in the BA group ($P = .01$; Fig 8).

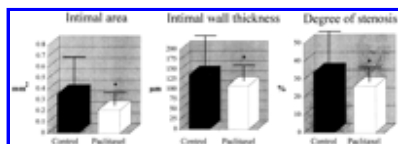
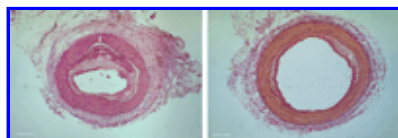


Figure 8. Results of the quantitative histopathological examinations of the in vivo study. The intimal area ($P = .01$), intimal wall thickness ($P = .04$), and degree of stenosis ($P = .01$) show significant differences between the control (BA) group and the paclitaxel-treated (LDD) group.

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Figure 9. Photomicrographs of cross sections showing the effect of paclitaxel on the vessel lumen 28 days after balloon angioplasty. Elastica van Gieson staining. Scale bars represent 300 μm . A, Vessel segment of an untreated control animal (BA group) showing a characteristic eccentric plaque (p) and a significant intimal thickening (i) with a lumen reduction $>50\%$. The disruption (arrow) is due to the histological preparation. B, Vessel segment of a paclitaxel-treated animal (LDD group) showing a wide vessel lumen and a moderate intimal thickening.

On histological examination, one thrombus formation was found in each of the two groups and excluded from further quantitative histological evaluation.

Quantification of Smooth Muscle Cell Proliferation

The percentage of proliferative cells in the intimal layer was $0.67 \pm 0.6\%$ 28 days after local drug delivery and $0.58 \pm 0.34\%$ after balloon angioplasty alone ($P=\text{NS}$). Quantification of the proliferative activity in the media also did not show any significant differences between the LDD and BA groups ($0.81 \pm 0.5\%$ versus $0.7 \pm 0.46\%$; $P=\text{NS}$).

Examination of Endothelial Regeneration

In all animals studied, regenerated endothelium was present in the balloon-injured areas. In the animals that underwent angioplasty alone (BA group), immunohistological quantification showed 45 ± 17 endothelial cells/ mm^2 compared with 47 ± 12 endothelial cells/ mm^2 in the LDD group after local paclitaxel delivery ($P=\text{NS}$).

► Discussion

In the present study, it could be shown that paclitaxel, the first representative of a new class of microtubule-affecting drugs, exerts very potent, dose-dependent, inhibitory effects on haSMC proliferation and migration. We found that paclitaxel dissolved in ethanol inhibits haSMC growth at least as potently as described for other cell types, such as tumor cells, fibroblasts, or rat smooth muscle cells.^{15 31 32}

This favors paclitaxel as a promising drug for local application to prevent restenosis after percutaneous transluminal coronary angioplasty. The antiproliferative potential of this very lipophilic compound is characterized by a sustained effect over a period of 14 days after a brief (20-minute), single-dose treatment of haSMCs in a wide concentration range without showing rebound or cytotoxic effects. The IC_{50} in monocultures of haSMCs continuously incubated with paclitaxel for a period of 8 days was 1.6 to 0.3 nmol/L, as determined by three different test assays. In regard to the growth-inhibitory potency of paclitaxel, the IC_{50} in haSMCs was half as much as described for rat smooth muscle cells (5.8 nmol/L).¹⁵ haSMCs respond 2- to 50-fold more sensitively to paclitaxel than several tumor cell lines.^{13 31 33} IC_{50} in colon cancer, gliosarcoma, leukemia, or ovarian tumor

▲	Top
▲	Abstract
▲	Introduction
▲	Methods
▲	Results
■	Discussion
▼	References

cells, for example, has been shown to vary between 4.0 and 100.0 nmol/L.¹³

Cell counting 24 hours after paclitaxel exposure **did** not reveal a significant decrease in cell numbers due to acute toxicity or induction of apoptotic cell death. Only at very high concentrations (≥ 50.0 $\mu\text{mol/L}$) **did** we find evidence of apoptosis. Approximately 80% of haSMCs showed DNA breaks, and 25% of haSMCs demonstrated significant cell nucleus fragmentation, both of which might be indicators of apoptosis.^{27 33 34} Thus, the specific effect of paclitaxel on microtubules is not overlapped by apoptosis at therapeutically relevant doses and thus does not have any important consequence for further in vivo studies.

The effect of paclitaxel on haEC growth was also studied because reendothelialization plays an important role after vascular injury in vivo. At low doses of paclitaxel (< 0.1 μmol), growth of haEC monocultures was less inhibited than haSMC growth, whereas high doses of paclitaxel (0.1 to 10.0 $\mu\text{mol/L}$) exerted comparable effects in both haECs and haSMCs. These results are in concordance with results achieved in an endothelial wound repair model using porcine aortic endothelial cell cultures in which lower doses, such as 1.2 nmol/L paclitaxel, **did** not affect endothelial cell growth. Only higher doses resulted in a remarkable reduction of endothelial cell proliferation and migration.³⁵ Comparable results were obtained with the use of the commercially available preparation Taxol instead of paclitaxel dissolved in ethanol. The use of Taxol, however, carries concerns because the lipoid vehicle and stabilizer Cremophor EL causes independent effects on vascular cell growth. Furthermore, it may cause hypersensitive reactions in humans and may be incompatible with catheter materials.^{1 13 36}

In contrast to other compounds of the colchicine type, which inhibit microtubule assembly,^{32 37} paclitaxel shifts the balance of microtubule assembly and disassembly toward microtubule assembly, forming numerous unorganized and decentralized microtubules inside the cytoplasm.^{4 9 38} Although both paclitaxel and colchicine inhibit cell division in the M phase, the opposite biological mechanisms involved seem to be of functional relevance. It has been shown previously that microtubules modulate the response of cells to several mitogens and cytokines by affecting the transmembrane signal pathways and other surface processes.^{39 40 41} Activation of protein kinases such as mitogen-activated protein kinases by growth factors is associated with microtubule depolymerization⁴² and is inhibited by paclitaxel.⁴³ In addition to inhibiting mitogen-activated protein kinase activity, paclitaxel reduces growth factor-stimulated release of transcription factors such as nuclear factor- κB ⁴⁴ and thus influences the expression of proto-oncogenes such as *c-jun* and *c-myc* at different stages.⁴⁵ Microtubule disassembly induced by colchicine in combination with cytokines such as insulin, epidermal growth factor, fibroblast growth factor, or serum can result in synergistic growth-stimulatory effects and increased DNA synthesis.^{32 46} This might be one reason why colchicine failed in preventing in vivo smooth muscle cell proliferation in restenosis⁴⁷ despite the fact that initial results in vitro showed growth-inhibitory effects of colchicine on unstimulated smooth muscle cells.⁴⁸ In contrast, stimulation with bFGF, PDGF, or thrombin as performed in the present study **did** not significantly attenuate the antiproliferative effects of paclitaxel. The growth factor doses used in the present study were found to exert optimal growth-stimulatory effects, and our results are in accordance with those described for other cells.^{49 50} Our results with growth-stimulated haSMCs also confirm observations by others, eg, in mouse embryonic cells, that paclitaxel inhibits initiation of DNA synthesis induced by several growth factors.⁵¹

In addition to the experiments in monocultures, the effect of paclitaxel on our previously described

transfilter coculture system consisting of haSMCs and haECs was studied.¹⁷ This model extends the findings in monocultures and the chemoinvasion studies of Sollott et al¹⁵ in several ways. First, proliferative haECs exert a stimulatory effect on haSMC proliferation, as previously shown.¹⁶ These interactions are transmitted directly by cell-cell contacts and by the secretion of growth factors.⁵² Second, the transfilter coculture model provides information about cell migration, because untreated haSMCs regularly migrate across the filter membrane into the haEC compartment when stimulated by proliferative haECs, forming a cell-rich in vitro neointima.^{17 53} Third, the transfilter coculture imitates normal arterial vascular architecture,^{16 54} enabling experiments that simulate local drug delivery, eg, by applying the substance during a short period onto the endothelial side.

In the transfilter coculture system, a brief (20-minute) addition of paclitaxel applied solely to the endothelial cell compartment resulted in an inhibition of haSMC proliferation and migration for a period ≤ 14 days. This may be due to the very lipophilic structure of paclitaxel, which facilitates a rapid cellular uptake and onset of action, whereas a subsequent strong binding to the β -subunit of tubulin leads to a long-lasting effect on the arrangement of the cytoskeleton.^{55 56} Initial effects of paclitaxel on spindle organization have been observed in several cell types within 3 to 5 minutes at concentrations of 5.0 to 50.0 $\mu\text{mol/L}$ and in ≈ 10 minutes at 0.01 to 0.1 $\mu\text{mol/L}$.⁵⁷ When complete disassembly of microtubules is induced by nocodazole, the microtubule reassembly starts just 2 minutes after addition of paclitaxel.³⁷ The antimigratory effect of paclitaxel can most likely be attributed to the observed changes in the cytoplasmic arrangements of β -tubulin and smooth muscle α -actin, which affect cell shape, size, and motility.^{6 8 28 29 58} Changes of the cytoplasmic organization of vimentin might also influence cell shape and size. Controversial results were described by others with various cell types, such as fibroblasts,⁶ granulosa cells,³⁰ or epithelial cells.⁸ Immunofluorescence stainings of haSMCs alone showed no clear evidence of a direct effect of paclitaxel on the vimentin assembly. However, no defined parameters or methods are available that characterize the "turnover" of vimentin filaments, as described for actin filaments or microtubules. Vimentin filaments are also found to colocalize with microtubules and stabilize actin structures.⁶ Thus, the observed alterations of the vimentin distribution might be simply a consequence of changes in cell shape and size induced by altered microtubules.

In rat VSMCs, the inhibition of cell migration occurred at concentrations that were ≈ 10 -fold lower than those needed to inhibit cell proliferation.¹⁵ In the study by Sollott et al,¹⁵ neointima formation in balloon-denuded rat carotid arteries was significantly prevented by systemic administration of paclitaxel, using plasma levels 100-fold below those achieved in tumor patients.^{1 3}

Both its rapid cellular uptake and long-lasting action over a broad concentration range as well as its efficacy in rat and human cells make paclitaxel a very promising candidate for local drug delivery to reduce the proliferative and migratory components that are involved in restenosis after angioplasty or stent implantation. In recent years, several catheter-based devices have been developed to deliver high doses of pharmacological or molecular agents locally to the site of angioplasty while keeping the risk of systemic side effects to a minimum.⁵⁹

In preliminary animal experiments of this study using the microporous balloon for local paclitaxel delivery in the rabbit carotid artery, this compound also demonstrated its potential to inhibit neointima formation 4 weeks after intervention. No differences in the process of reendothelialization could be observed between paclitaxel-treated animals and control animals. Toxic or allergic side effects did not occur at any time point of the study. These results, however, only represent a very

limited evaluation of paclitaxel therapy in vivo but substantiate in vitro findings in monocultures and cocultures with human vascular cells.⁶⁰ Further experimental studies in larger animal models are required to evaluate the benefits and risks of local delivery of paclitaxel in coronary arteries.

▶ Selected Abbreviations and Acronyms

BA group	= pharmacologically nontreated control group in which only balloon angioplasty was performed
bFGF	= basic fibroblast growth factor
BrdU	= bromodeoxyuridine
DAPI	= 4',6-diamidino-2-phenylindole dihydrochloride
ELISA	= enzyme-linked immunosorbent assay
FCS	= fetal calf serum
haEC	= human arterial endothelial cell
haSMC	= human arterial smooth muscle cell
IC _{max}	= maximal inhibitory concentration
LDD group	= local drug delivery group
PDGF	= platelet-derived growth factor
PDGF-AB	= platelet-derived growth factor (isoform AB)
TUNEL	= terminal deoxynucleotidyl transferase-mediated nick end labeling
VSMC	= vascular smooth muscle cells

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▲ Top
▲ Abstract
▲ Introduction
▲ Methods
▲ Results
▲ Discussion
• References

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