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ORIGINAL CONTRIBUTION

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Inhibition of smooth muscle cell proliferation after local drug delivery of the antimitotic drug paclitaxel using a porous balloon catheter

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Abstract Percutaneous transluminal coronary angioplasty is an accepted treatment for coronary artery disease. The major limitation, however, is the high incidence of restenosis which limits the long-term benefit of this intervention. Paclitaxel is a new antiproliferative agent that has generated considerable scientific interest since it was introduced in clinical trials in the early 1980s. Recent in vitro studies have shown that paclitaxel has considerable antiproliferative activity in human coculture systems. In the present study the efficacy of paclitaxel was investigated after development of an intimal plaque by electrical stimulation and additional cholesterol diet and subsequent balloon angioplasty in 63 New Zealand White rabbits. Local drug delivery of paclitaxel was accomplished in 30 rabbits with a porous balloon catheter (35 holes, hole diameter 75 μm, 2.5 mm catheter diameter). Paclitaxel was administered locally with 4 ml (solution 10^{-5} mol/L) using an injection pressure of 2 atm. To study the extent of restenosis and morphological changes, the animals were sacrificed 7, 28 or 56 days after intervention. After staining procedures quantification of SMC proliferation, intimal macrophages and morphological analyses were performed. Paclitaxel plasma concentrations were measured using HPLC technique. One week after balloon angioplasty the arteries treated with local paclitaxel delivery showed an insignificant trend towards a reduction in intimal smooth muscle cell proliferation (untreated 8.4 ± 4.9 % vs paclitaxel treated 2.4 ± 2.4 %, p = NS). However, this resulted in a significant reduction of stenosis degree of 66 % 8 weeks after intervention compared to the untreated group (untreated 41 ± 18 % vs paclitaxel treated 14 ± 11 %, p = 0.005). In conclusion, locally delivered paclitaxel prevented neointimal thickening in the rabbit carotid artery after balloon angioplasty. Local paclitaxel treatment may therefore be a clinical option for the prevention of restenosis after coronary interventions. However, further preclinical studies have to prove long-term efficacy and safety.

Key words Paclitaxel – local drug delivery – PTCA – restenosis – smooth muscle cell – rabbits

Introduction

While percutaneous transluminal coronary angioplasty has become an important tool in the management of coronary artery disease, restenosis in at least 30–50 % of cases remains the major limiting factor of long-term efficacy (22). Until now conventional systemic pharmacological therapies have failed to reduce the incidence of restenosis (12, 13). An important possible explanation for this lack of efficacy might be the low local concentration of these compounds at the site of injury. Catheter-based local drug delivery may solve this problem by ensuring higher concentration of the drug at the site of angioplasty. In addition, local drug therapy may also reduce systemic side-effects.

Paclitaxel is a clinically effective antimitotic drug in the treatment of advanced breast cancer that has a novel chemical structure and an unusual mechanism of action. The hallmark activity of the drug in vitro is its ability to enhance the assembly of stable microtubules in the absence of GTP, which is normally required for assembly (26, 27). It was demonstrated in porcine endothelial cells that microtubuli play an important role as a cytoskeletal structure in cell migration (7). Furthermore, paclitaxel interferes with PDGF-stimulated intracellular signaling, proliferation and migration (1). Paclitaxel inhibits proliferation by inducing a sustained mitotic block at the metaphase/anaphase boundary (15). Additionally, paclitaxel is highly lipophilic which promotes a rapid cellular uptake and a long-lasting effect in the cell due to structural alteration of the cytoskeleton (15, 29). The in vitro feasibility of local delivery of paclitaxel has been assessed by delivering tritium marked paclitaxel into isolated porcine coronary arteries using different delivery systems (2).

The development of a catheter-based porous balloon allows the intracoronary site-specific drug delivery of the injured vascular wall. Previous preclinical and clinical studies have demonstrated that effective drug concentrations can be achieved with the porous balloon device (3, 19–21).

Aim of the present study was to investigate the acute and long-term efficacy of intramural delivery of paclitaxel using the porous balloon catheter after experimental balloon angioplasty in an in vivo model.

Materials and methods

Paclitaxel

Paclitaxel (Sigma, Deisenhofen, Germany) was dissolved in 100 % ethanol and sterile-filtered. The stock solution was diluted to 10^{-5} mol/L in a volume of 4 ml sterile 0.9 % NaCl solution.

Local drug delivery device

A 135-cm long 2.5 mm SI III porous balloon catheter (Guidant, Santa Clara, CA, USA) with 35 holes in a design of 7 rows of 5 holes was used in this study, as described previously (21). Each pore has a diameter of 75 ± 15 µm. The device has an inner lumen accepting a 0.014 in guide wire and a proximal port for balloon inflation. The drug delivery catheter was prepared with a 0.9 % saline solution before it was advanced to the preformed plaque. The injection was performed using a low pressure pump

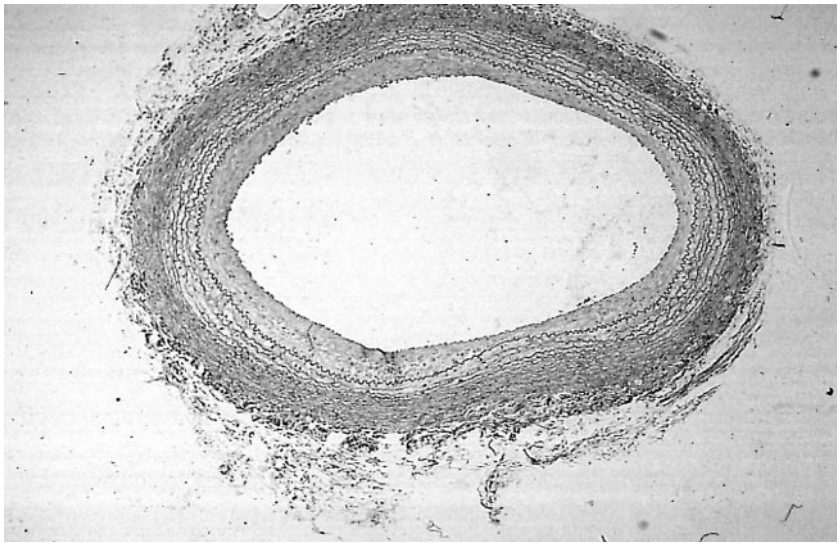

Fig. 1 Histological evaluation of morphological changes by locally delivered paclitaxel. Photomicrograph of a cross section of a rabbit carotid artery 56 days after local drug delivery. A small concentric plaque can be seen. Elastica-Van Gieson-staining. Hemalaune and eosin stain. Magnification x12.5.

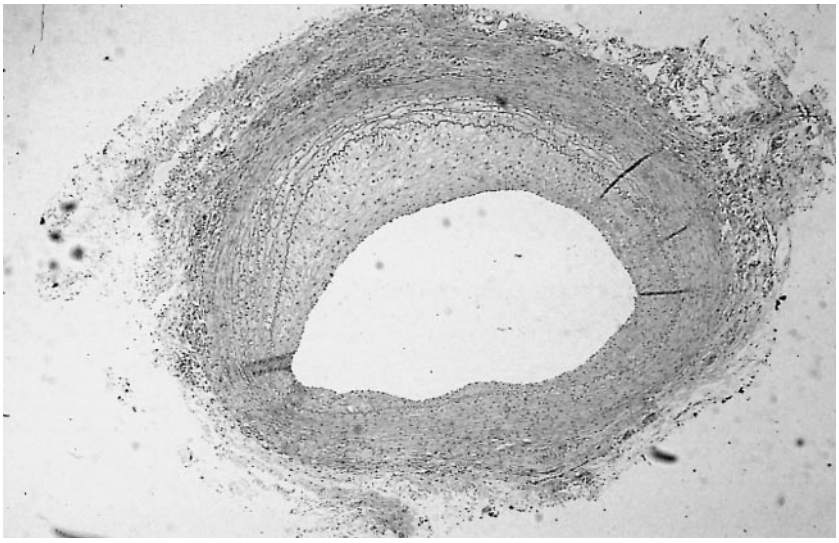

Fig. 2 Histological evaluation of morphological changes in a control animal. Photomicrograph of a cross section of a rabbit carotid artery 56 days after balloon angioplasty. A large predominant excentric plaque can be seen. Hemalaune and eosin stain. Magnification x12.5.

(DVI, CA, USA). A volume of 4.0 ml was delivered locally using an inflation pressure of 2 atm.

Animal model

In male New Zealand White rabbits an intimal plaque was produced using the electrostimulation model as previously described (4). In brief, this model is based on the implantation of two graphite-coated gold electrodes in the adventitia of the common carotid artery under general anesthesia. The electrodes were held in position by a Teflon cuff on either side of the artery. Thin, subcutaneously placed leads from the electrodes were connected to a small plastic socket attached to the skull. With an external stimulation unit, this arrangement allowed the local and transmural electrical stimulation of the right carotid artery under standardized conditions in order to produce plaques of comparable sizes before intervention. Constant-current DC impulses (15 ms/impulse, 0.1 mA, 10 Hz) were applied twice daily for 30 minutes with a time interval of 8–10 hours between the stimulation cycles for a period of 28 days in each animal. To induce a fibromuscular cholesterol-rich plaque, all animals received a 0.5 % cholesterol diet (Altromin, Lage, Germany) during the stimulation period.

Study protocol

A total of 63 male New Zealand White rabbits (2.1–2.5 kg BW) were used in this study. After the stimulation period transluminal balloon angioplasty of the preformed plaque was performed in 58 rabbits. After adequate anesthesia with intramuscular metomidate-HCl (8 mg/kg BW) and fentanyl-base (0.1 mg/kg BW) angioplasty was performed with a 2.0 mm balloon catheter (Micro-Hartzler, Guidant, CA, USA) introduced by direct arteriotomy into the exposed vessel and then advanced into the plaque region. The balloon was inflated to 5 atm for 60 s. The rabbits were open randomised in an untreated control group (n = 28) and a local paclitaxel delivery (LPD) group (n = 30). Five rabbits were electrically stimulated and served as a sham group without intervention.

In the LPD group the porous balloon was advanced into the region of the predilated plaque and 4 ml of paclitaxel was delivered with a pressure of 2 atm during a time period of approximately 30 s. After the catheter device was removed, the incision of the arteriotomy was closed by 7–0 polypropylene sutures. To avoid bacterial infections, all rabbits were on antibiotic therapy during the following 3 days.

Application of bromodeoxyuridine

To determine the extent of cells undergoing DNA synthesis, 5'-bromo-2'-deoxyuridine (Brdu), a thymidine analogue, was given to each animal before excision of the vessels as described previously (9). Eighteen hours before the animals were sacrificed, each rabbit received a subcutaneous neck depot consisting of 100 mg Brdu/kg BW and 75 mg 2'-deoxycytidine (d-cyt)/kg BW (both from Sigma Inc., Deisenhofen, FRG). Intramuscular injections (30 mg Brdu/kg BW, 25 mg d-cyt/kg BW) were given 18 and 12 hours before the vessels were excised.

Tissue analysis

The animals were sacrificed with an overdose of intravenous metomidate-HCl. The carotid arteries were per-

fused in situ with 500 ml 0.1 M cacodylate-buffered 2 % paraformaldehyde solution under physiological pressure. A 3 to 4 cm segment of the treated carotid artery with the Teflon cuff was excised, and the proximal and distal ends were marked with sutures. Staining of the sections was performed (HE, van Gieson, BrdU, RAM 11, α-Actin) as described elsewhere (8, 9). Staining for factor VIII-related antigen was performed to confirm the endothelial origin of the cells using an immunoperoxidase technique with rabbit anti-goat immunoglobulin G and avidin-biotin complex (21).

Quantitative histopathology

All sections were quantitatively analyzed by computerized morphometry. The sections were projected by a side tubus affixed to the stereoscope (Olympus Inc., Hamburg, FRG) onto a digitizing pad (Summagraphics Inc., Seymour, CT). The luminal perimeter (length of endothelial layer), and internal and external elastic lamina were traced manually under stereoscopic control. The area of the intima and media, the residual lumen and the stenosis degree were calculated using standard software (Bioquant, Bilany Consulting, Düsseldorf, FRG). To provide a quantitative parameter of endothelial proliferation, the absolute number of endothelial cells was counted and related to the length of the endothelial layer. To obtain the number of proliferating SMC, the number of BrdU-positive cells in the intimal and medial layer were determined and related to the total cell number. In addition the number of intimal and medial macrophages were counted and also related to the total cell number. In order to evaluate cytotoxicity the absolute number of cell nuclei in the intimal and medial layer was counted and related to the intimal and medial area.

Measurement of paclitaxel concentration

Plasma was obtained from the rabbits immediately after paclitaxel application (n = 7), after 5 (n = 9) and 30 (n = 5) min. Plasma paclitaxel concentrations were measured at the Klinische Pharmakologie, Freiburg i.B., Germany by HPLC using a modification of the method described by Willey (32).

Statistical evaluation

All values are expressed as mean ± 1 SD. Differences between the number of thrombus formations were evaluated with the Fisher's Exact Test. Differences between balloon angioplasty and local paclitaxel delivery treated vessels were evaluated with the Wilcoxon test. P-values of $p < 0.05$ were considered to show significant differences (25).

Results

Intimal wall area and stenosis degree

The results of the morphometric and histologic evaluation are summarized in Table 1. After 28 days of electrical stimulation, determination of mean intimal wall area

Table 1 Results of morphometric evaluation of intimal and medial area, intimal and medial cell density and proliferation, number of intimal and medial macrophages and endothelial cells. The data are given as mean ± 1SD. $^{\#}p < 0.05$ vs. untreated control group

	28 days ES (n=5)	7 days after intervention		28 days after intervention		56 days after intervention	
		untreated group (n = 7)	LPD group (n = 11)	untreated group (n = 11)	LPD group (n = 12)	untreated group (n = 10)	LPD group (n = 7)
Thrombus	0	3	3	1	4	0	0
Intimal wall area (mm^2)	0.13 ± 0.07	0.22 ± 0.07	0.11 ± 0.04$^{\#}$	0.35 ± 0.29	0.18 ± 0.14$^{\#}$	0.29 ± 0.18	0.13 ± 0.11$^{\#}$
Medial wall area (mm^2)	0.43 ± 0.11	0.25 ± 0.05	0.41 ± 0.10	0.83 ± 0.41	0.46 ± 0.13	0.67 ± 0.28	0.56 ± 0.12
Stenosis degree (%)	20 ± 6	27 ± 10	14 ± 6$^{\#}$	32 ± 21	25 ± 13	41 ± 18	14 ± 11$^{\#}$
Intimal cell density (mm^2)	5432 ± 2354	6363 ± 1245	9112 ± 3202	5057 ± 2202	7186 ± 1516$^{\#}$	4647 ± 2472	9591 ± 4268$^{\#}$
Medial cell density (mm^2)	2355 ± 546	3604 ± 431	3345 ± 1229	1741 ± 1093	2839 ± 1157	1836 ± 1075	2831 ± 560
Intimal SMC proliferation (%)	0.5 ± 0.8	8.4 ± 4.9	2.4 ± 2.4	0.6 ± 0.3	0.5 ± 0.6	0.3 ± 0.3	0.4 ± 0.5
Medial SMC proliferation (%)	0.3 ± 0.2	5.0 ± 2.0	2.2 ± 3.2	1.1 ± 1.0	0.9 ± 1.2	0.6 ± 0.6	0.5 ± 0.6
Intimal macrophages (%)	1.0 ± 1.5	3.9 ± 0.6	11.5 ± 10.5	6.7 ± 11.3	4.3 ± 3.8	1.8 ± 1.6	4.1 ± 8.8
Medial macrophages (%)	0.5 ± 0.6	3.2 ± 2.2	3.0 ± 3.1	1.5 ± 1.9	1.1 ± 1.4	0.8 ± 1.6	4.0 ± 6.8
Luminal perimeter (mm)	3.51 ± 0.52	2.94 ± 0.38	2.66 ± 0.55	2.59 ± 0.81	2.35 ± 0.5	1.91 ± 0.64	2.79 ± 0.64
Endothelial cells (1/mm)	38 ± 11	25 ± 15	60 ± 14$^{\#}$	41 ± 18	75 ± 21$^{\#}$	45 ± 21	66 ± 19

in the control group was 0.13 ± 0.07 mm². After 28 days, a continuous increase in mean intimal wall area in the untreated group occurred with 0.35 ± 0.29 mm². In contrast, roughly no increase in the mean intimal wall area was observed in the LPD treated animals with 0.18 ± 0.14 mm² (p = 0.015). This significant difference was further maintained during the following four weeks. Mean intimal wall area was 0.29 ± 0.18 mm² after 56 days in the untreated group. In contrast, the mean intimal wall area in the LPD treated animals showed only a moderate increase during this time period with 0.13 ± 0.11 mm² (p = 0.018). Morphometric analysis showed a mean stenosis degree in the control group of 20 ± 6 %. One week after intervention there was a significant difference of stenosis degree between the untreated (27 ± 10 %) and the LPD group (14 ± 6 %; p = 0.042). Two months after BA there was still a statistically significant difference of stenosis degree between the untreated group (41 ± 18 %) compared to the LPD group (14 ± 11 %; p = 0.005).

Intimal and medial cell density

After 28 days of electrostimulation 5432 ± 2354 cells/mm² were counted in the intimal layer. Seven days after intervention there was a non-significant difference between the untreated group and LPD treated animals. However after 28 and 56 days a relevant increase in intimal cells per mm² was found in the paclitaxel treated group compared to the untreated group (28 days: p = 0.02, 56 days: p = 0.025). Quantification of medial cell density showed no significant difference 7, 28 and 56 days after intervention.

Quantification of smooth muscle cell proliferation

The percentage of proliferating cells in the intimal layer was 0.5 ± 0.8 % 28 days after electrostimulation. An insignificant trend towards a reduced number of proliferating cells was found 7 days after intervention with 8.4 ± 4.9 % in the untreated group and with 2.4 ± 2.4 % after LPD. After 28 and 56 days the percentage of proliferating cells had returned to preinterventional levels and showed no difference between the groups.

Quantification of medial SMC proliferation was found to be 0.3 ± 0.2 cells/mm² in the group 28 days after electrostimulation without intervention. Seven days after treatment a non-significant increase in the number of BrdU-positive cells was observed in the untreated group compared to the LPD treated animals. One and two months after intervention the extent of cells undergoing DNA synthesis was comparable to the baseline level before intervention in both groups.

Time course of macrophage appearance in the treated vessels

In the preinterventional control group, 28 days after electrical stimulation, the percentage of macrophages was 1.0 ± 1.5 % in the intimal layer. Quantification of macrophages 7 days after intervention showed an increase in the number of macrophages in both groups with no significant difference. One and two months after intervention there was no significant difference in the number of intimal macrophages between the untreated group and the animals after LPD treatment. The number of medial macrophages was not significantly affected by the treatment during the observation period.

Quantification of endothelial cells

The electrostimulation control-group revealed a luminal perimeter of 3.51 ± 0.52 mm and 38 ± 11 ECs/mm. Seven days after intervention the arteries still revealed large areas of endothelial cell loss. Seven and 28 days after intervention the LPD treated animals showed a significant increase in the number of endothelial cells per mm.

Measurement of paclitaxel plasma concentration

Within one minute after application the mean plasma concentration was 26.2 ± 14.6 ng/ml (3 animals were below the detection limit of 5 ng/ml). Five minutes after application the concentration was 35.7 ± 49.4 ng/ml (one animal below the detection limit) and 30 minutes after treatment it was below the detection limit in all 5 evaluated animals.

Morphological changes due to local drug delivery

On histological examination 7 days after intervention 3 animals in the LPD group and 3 animals in the untreated group showed mural thrombus formation or a subtotal occlusion (p = ns). One month after intervention 4 thrombi were observed in the LPD treated animals, in contrast to one thrombus which was found in the untreated animals (p < 0.05). Two months after intervention no thrombus formation was found in either group. The animals with thrombi were excluded from the immunohistological quantification.

Discussion

The ideal drug for local drug delivery should theoretically combine several properties. It should have a rapid cellu-

lar uptake (therefore it should be lipophilic), a prolonged cellular binding, a powerful action preferably against several targets of cell activation and no severe cytotoxic local or systemic side effects. Paclitaxel is a potent inhibitor of the replication of various neoplastic cells. Paclitaxel promotes microtubule assembly and stabilizes microtubules by shifting the dynamic equilibrium toward microtubule assembly (26, 30). In essence, paclitaxel decreases the critical concentration of tubulin required for microtubule assembly in the presence or absence of factors that are usually essential for this function, such as exogenous GTP or microtubule-associated proteins (27).

In the human coculture system it was demonstrated that just a short (20 min) and single addition of paclitaxel at a concentration of 10^{-5} mol/L resulted in an inhibition of SMC proliferation and migration for a period up to 14 days (1). This may be due to the very lipophilic structure of paclitaxel which facilitates a rapid cellular uptake and onset of action, while a subsequent strong binding to the beta-subunit of tubulin leads to a long-lasting effect on the arrangement of the cytoskeleton (15, 29). In vitro paclitaxel effects were observed after a few minutes and the effects were reversible and concentration/time dependent (17). Paclitaxel was found to interfere both with platelet-derived growth factor-stimulated SMC migration and proliferation in rat and human cell cultures (1, 28). In a previous in vivo study using immunohistological staining and electron microscopy microtubule bundles could be observed in the paclitaxel treated animals (10). Microtubules modulate the response of cells to several mitogenes and cytokines by affecting the transmembrane signal pathways and other surface processes (23). Activation of protein kinases, e.g., mitogen-activated protein kinases (MAPK), by growth factors is associated with microtubule depolymerization and is inhibited by paclitaxel (18).

In rat carotid arteries it could be demonstrated that neointimal SMC accumulation after balloon dilatation and endothelial denudation could be prevented by plasma levels of 50–60 nM (28). In a previous study using the double-balloon catheter in the same animal model local paclitaxel delivery resulted in significantly reduced neointima formation (10). A significant difference in intimal wall thickness and stenosis degree between untreated and LPD treated animals was found during the whole observation period. These results demonstrate that the antiproliferative effect starts very early after delivery. Quantitative analysis showed that paclitaxel treatment reduced the neointimal area after 8 weeks by 66 % compared to untreated animals. Several of the paclitaxel treated animals showed no increase in intimal wall thickness at all.

In the present study, the evaluation of the intimal cell density displayed unexpected results. One and two months after intervention a significantly higher cell density was observed in the paclitaxel treated animals. These results are explained by the relationship between a very small intimal area and a constant intimal cell number. The very small intimal area is most probably the result of a reduced production of extracellular matrix, which was shown to occur in other cell types after paclitaxel treatment (5, 16). During the follow-up period an additional decrease in intimal area was observed. Since the number of cells was found to be unchanged an increase of cell density in the intima was observed. In contrast to the effects seen in the intimal layer, the cell density in the medial layer showed in the paclitaxel treated animals a constant level compared to a trend towards a reduction in the untreated animals.

Local delivery of paclitaxel in vivo inhibits the proliferation of neointimal smooth muscle cells in the early stage after delivery. However the difference seven days after balloon angioplasty showed a trend towards a reduction but was not statistically significant. The reason might be the evaluation of a rather late time point after intervention in this study. It has been previously shown in the same animal model that SMC proliferation after vascular injury has its maximum three days after intervention and can be increased as late as two weeks after intervention (9). Our results indicate that the antiproliferative action of paclitaxel occurs within the first week after injury and cannot be observed later by BrdU-staining. However, this early although not prolonged effect has resulted in a reduction of intimal and medial thickness late after intervention.

Quantification of intimal macrophages displayed an insignificant increase in the LPD treated animals seven days after balloon angioplasty. This observation is most probably due to the additional vessel wall injury resulting from local delivery. It is noteworthy that obviously local paclitaxel delivery in this study did not result in a local reduction of monocyte invasion or macrophage proliferation. Compared to a previous study which investigated the accumulation of macrophages after balloon angioplasty (8) there was a nonsignificant trend towards an increase of macrophages after local paclitaxel delivery.

Mural thrombus formation was observed in seven rabbits in the LPD treated group compared to four animals in the untreated group. Thrombus formation after coronary angioplasty is a common phenomenon and is correlated with the kind and extent of the vessel wall injury. In this study an additional antithrombotic treatment was avoided to allow analysis of paclitaxel effects alone. It goes without question that local drug delivery using a porous balloon catheter after balloon angioplasty presents an additional vessel injury. As demonstrated previously, jet injuries are the predominant histological finding after fluid delivery using porous balloon catheters (11). Aside from the cellular response within the arterial wall additional and increased thrombus for-

mation has to be expected. Although the numbers of vessels with thrombus formation was not dramatically increased in the LPD animals vs. control animals (7 vs. 4), it is strongly suggested to use a rigid anticoagulant regimen when application of paclitaxel might be performed by intracoronary delivery in humans with coronary artery disease.

Limitations of the study

Although the rabbit electrostimulation technique is a very elegant and reproducible animal model to produce intimal plaques, results from these studies are not necessarily predictive of therapeutic success in humans. Larger series of animal experiments as well as studies investigating the effects of paclitaxel in coronary arteries and different species are necessary. The principal toxicity of paclitaxel occurs only when paclitaxel plasma levels are maintained above an apparent threshold of 50–100 nM for durations beyond 5 h (6, 14). In a phase I clinical trial peak plasma concentration with a dose of 275 mg/m^2 was approximately 8 μM (31). These concentrations are 100-fold higher than those found in the present experiments. The cardiotoxicity of paclitaxel observed in anticancer trials is usually mild and reversible (24). Initial experimental in vivo results in porcine coronary arteries using a double balloon catheter design showed no adverse events due to local paclitaxel delivery (unpublished data).

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