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Drug-eluting stents: Sirolimus and paclitaxel differentially affect cultured cells and injured arteries

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Abstract

Sirolimus and paclitaxel eluted from stents inhibit cell proliferation and other cellular processes by dramatically different mechanisms. In this study, the effects of sirolimus and paclitaxel on cultured human coronary artery smooth muscle and endothelial cell function or cell cycle changes in balloon-injured arteries were directly compared. Both sirolimus and paclitaxel inhibited smooth muscle and endothelial cell proliferation. However, only paclitaxel inhibited smooth muscle and endothelial cell migration at low (nM) concentrations. Sirolimus arrested smooth muscle and endothelial cells in the G0/G1 phase of the cell cycle without inducing apoptosis while paclitaxel produced apoptosis in both cell types at low nanomolar concentrations. Although both agents blocked neointimal formation, sirolimus applied locally to injured rat carotid arteries increased the percentage of cycling vascular cells in G0/G1 without inducing apoptosis while paclitaxel increased the percentage of cycling cells in S and G2/M phases while inducing apoptosis. These results suggest that sirolimus reduces neointimal hyperplasia through a cytostatic mechanism while paclitaxel produces apoptotic cell death.

Keywords: Sirolimus; Paclitaxel; Restenosis; Cell proliferation; Cell cycle

1. Introduction

The long-term success of percutaneous translumenal revascularization of diseased coronary arteries has been limited by restenosis. Some determinants of restenosis, namely vascular recoil and thrombosis, have been addressed by the use of stents and the development of highly effective antiplatelet regimens (e.g. clopidogrel and aspirin), respectively. However, reocclusion of stented vessels secondary to neointimal hyperplasia has only recently been addressed by the introduction of drug-eluting stents that provide controlled release of antiproliferative agents into the vessel wall.

In the normal coronary artery, proliferative and migratory processes of vascular smooth muscle and endothelial cells exist at low basal levels. Vascular trauma resulting from balloon injury and stent deployment can lead to proliferative, migratory and secretory changes within cells of the vessel wall. These changes alter the normal vascular architecture by increasing vessel wall thickness and reducing lumen size. Upon injury, cells within the vessel wall proliferate and subsequently migrate through the internal elastic lamina forming the neointima. The neointimal cells, which also proliferate, exhibit smooth muscle cell markers and are of a secretory phenotype (Campbell et al., 1988; Clowes et al., 1983a). Although there is a debate over whether neointimal smooth muscle-like cells originate within the media (Clowes et al., 1983a), adventitia (Li et al., 2000; Scott et al., 1996; Shi et al., 1996) and/or the bone marrow (Sata et al., 2002; Han et al., 2001), the neointimal cells subsequently increase neointimal mass by secreting matrix proteins (Clowes et al., 1983b).

Drug-eluting stents that release either sirolimus or paclitaxel are now being used clinically to block the process of arterial neointimal hyperplasia following revascularization procedures

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involving stents. As antiproliferative agents, sirolimus and paclitaxel differ dramatically in their antiproliferative mechanisms. Sirolimus, a macrocyclic antibiotic immunosuppressant produced by *Streptomyces hygroscopicus*, arrests cell cycle progression at the G1/S transition point. Paclitaxel, a naturally occurring antineoplastic agent extracted from *Taxus brevifolia* bark (Wani et al., 1971), interferes with the cell cycle predominantly at mitosis (Marx et al., 1995; Manfredi and Horwitz, 1984).

The molecular mechanisms by which sirolimus or paclitaxel affects smooth muscle cell proliferation are now being elucidated. Sirolimus binds to the immunophilin FK-506 binding protein-12 (FKBP-12, Koltin et al., 1991; Marx et al., 1995). The resulting sirolimus/FKBP-12 complex inhibits the kinase activity of mammalian target of rapamycin (mTOR or FRAP), subsequently reducing the activity of multiple kinases associated with mitogen-induced cell proliferation ($p70^{s6k}$, cyclin E/CDK-2). This reduction in kinase activity is thought to be mediated by increased levels of the cyclin-dependent kinase inhibitor $p27^{Kip1}$ (Marx et al., 1995; Luo et al., 1996; Nourse et al., 1994). The resultant effects of the sirolimus/FKBP-12 complex on mTOR-associated pathways lead to cell cycle arrest at the G1/S transition point (Jayaraman and Marks, 1993; Marx et al., 1995). In contrast, paclitaxel achieves its antiproliferative effects by disrupting microtubule dynamics (Wilson et al., 1974) through binding to the amino-terminus of β-tubulin (Rao et al., 1994), preventing normal microtubule depolymerization and promoting microtubule stabilization (Manfredi and Horwitz, 1984). The effect on β-tubulin by paclitaxel results in the arrest of cells in the M phase of the cell cycle leading to cell death (Rowinsky and Donehower, 1995).

As microtubules are involved in a host of cellular processes ranging from cell migration, cytokinesis and a variety of metabolic processes, it is notable that the antiproliferative, antimigratory and metabolic effects of paclitaxel are concentration dependent, as various cellular processes involving microtubules may have differential sensitivities to paclitaxel depending upon the state of microtubules involved in each process (Dumontet and Sikic, 1999). Extracellular paclitaxel concentrations greater than 20 nM typically arrest cells in G2/M (mitotic arrest), while lower concentrations can arrest cells in G1, S, G2 and/or M phases while undergoing rapid or even delayed pro-apoptotic changes (Allman et al., 2003; Au et al., 1998; Blagosklonny et al., 2002; Das et al., 2001; Giannakakou et al., 2001; Lieu et al., 1997; Shu et al., 1997). In contrast to the well-established antiproliferative mechanisms of paclitaxel, it has been suggested that the antiproliferative activity of paclitaxel in vascular smooth muscle cells is due to primary and post-mitotic G1 arrest without an induction of apoptosis (Blagosklonny et al., 2004). Taken together, the precise effects of paclitaxel on arterial cells remain unclear.

Cell proliferation and migration may also share similar molecular mechanisms (Boehm and Nabel, 2001). Thus, agents whose primary mechanism of action is to block cell proliferation may also impact cell migration, a process involved in neointimal formation (Casscells, 1992). Sirolimus acts primarily to reduce proliferation of vascular smooth muscle cells while not affecting smooth muscle cell migration acutely (Poon et al., 1996). However, migratory pathways sharing common molecular machinery with proliferative pathways can be affected by sirolimus. Specifically, sirolimus can reduce vascular smooth muscle cell migration following prolonged exposure through FKBP-12 interaction (Poon et al., 1996) and $p27^{Kip1}$ expression (Sun et al., 2001). In addition to its antiproliferative activity, paclitaxel acts both acutely and chronically as a potent inhibitor of vascular smooth muscle cell migration by virtue of its effects on microtubules, which are critical for both cell migration and proliferation (Axel et al., 1997).

Although sirolimus and paclitaxel share in their ability to inhibit vascular smooth muscle cell proliferation and differentially affect migration, previous studies have not directly compared the effects of these two agents on cell cycle and apoptosis in proliferating human coronary artery smooth muscle and endothelial cells in culture. Moreover, the effects of locally applied sirolimus or paclitaxel on cell cycle and apoptotic changes within balloon-injured arteries have not been directly examined. This study compares, in a head-to-head fashion, the effects of sirolimus or paclitaxel on human coronary artery smooth muscle and endothelial cell proliferation, migration and cell cycle changes. In addition, the effects of local perivascular application of sirolimus or paclitaxel on cell cycle and apoptotic changes in balloon-injured rat carotid arteries are described.

2. Methods and materials

2.1. Reagents

Sirolimus was obtained from Wyeth (Madison, NJ). Paclitaxel was purchased from Sigma-Aldrich (St. Louis, MO). Sirolimus and paclitaxel stock solutions were prepared in neat dimethylsulfoxide (DMSO) and serially diluted in serum-free medium prior to application to cells. CycleTEST™ PLUS DNA Reagent Kits were purchased from Becton Dickinson (Mansfield, MA) and used according to the manufacturer's instructions. Falcon brand tissue culture products were obtained from Becton Dickinson (Franklin Lakes, NJ). Human coronary artery smooth muscle and endothelial cells, culture media, smooth muscle cell SingleQuots® media supplements, endothelial cell SingleQuots® media supplements, trypsin/ethylene diamine tetracetic acid (EDTA) and trypsin neutralizing solution were obtained from Cambrex (Walkersville, MD). BD Falcon™ FluoroBlock™ 24-multiwell insert system and Type I rat tail collagen were purchased from Becton Dickinson (Mansfield, MA). Calcein AM dye was purchased from Molecular Probes (Eugene, OR).

2.2. Human coronary artery smooth muscle and endothelial cell culture

Coronary artery smooth muscle cells (passages 3–8) were cultured at 37 °C in a humidified CO_2 (5%) incubator in smooth

muscle cell basal medium supplemented with 5% fetal bovine serum (FBS) and SingleQuots® (referred to as complete medium). Coronary artery endothelial cells were maintained at 37 °C, 5% CO_2 in endothelial cell basal medium supplemented with 5% FBS and SingleQuots® (complete medium). For subculturing, smooth muscle and endothelial cells were rinsed with Hanks' balanced salt solution, treated with trypsin/EDTA until detached and then neutralized with trypsin neutralizing solution. Both smooth muscle and endothelial cell cultures were split at ~70–90% confluence.

2.3. Cell proliferation assay

Cell proliferation was assessed using [^{3}H]-thymidine incorporation. Coronary artery smooth muscle and endothelial cells (passages 5–8) were rinsed with Hanks' balanced salt solution and trypsinized as described above. Cells were then counted and seeded onto a 96-well flat-bottomed plate in complete medium at a density of 5000 cells/well/200 μl. Cells were allowed to adhere for 24 h at 37 °C, 5% CO_2 and then incubated in basal medium for 24 h to allow for cell synchronization. Sirolimus or paclitaxel was then added to the cells in basal medium and allowed to incubate for 1 h at 37 °C, 5% CO_2. Sirolimus was tested at concentrations ranging from 0.044 to 200 nM, while paclitaxel was tested at concentrations ranging from 0.22 to 3000 nM. To obtain the various test concentrations, stock solutions were serially diluted in basal medium containing 2% DMSO. Cells were then incubated for 48 h in the presence of 2% FBS as stimulant, vehicle or drug, and [^{3}H]-thymdine. Following the 48 h incubation, cells were treated with trypsin/EDTA and then harvested. Inhibition of proliferation was calculated from [^{3}H]-thymidine incorporation as a percentage of the FBS-stimulated control. The inhibitory concentration (IC_{50}) was determined using nonlinear regression (4-parameter fit, Prism V.3.03).

2.4. Cell migration assay

Cells that reached 60–70% confluency from passages 5–9 were removed from the flasks by first rinsing with Hanks' balanced salt solution, then incubating with trypsin/EDTA until detached. Detached cells were then treated with trypsin neutralizing solution. The 24-multiwell plate inserts were coated with 50 μg/ml type I collagen (rat tail) for 1–2 h followed by two washes with Hanks' balanced salt solution. Coronary artery smooth muscle cells were resuspended at 0.040×10^6 cells/ml in basal medium and pipetted into the 24-multiwell plate inserts. Coronary artery endothelial cells were resuspended at 0.160×10^6 cells/ml in basal medium and pipetted into the 24-multiwell plate inserts. Vehicle, sirolimus or paclitaxel was added to the cells 1 h prior to the addition of basal medium/5% FBS beneath the inserts. After a 22 h incubation at 37 °C, 5% CO_2, the inserts were placed in 24-multiwell plates containing Hanks' balanced salt solution and washed. The inserts were then transferred to 24-multiwell plates containing Calcein AM at 4 μg/ml and incubated for 90 min at 37 °C, 5% CO_2. The inserts were transferred again to 24-multiwell plates containing Hanks' balanced salt solution, and the fluorescence of cells that migrated through the membrane inserts was measured using a fluorescence plate reader (Tecan Saphire). Migration data were expressed as percent inhibition of the maximum number of migrated cells.

2.5. Assessment of DNA content by flow cytometry

Cells that reached 60–70% confluency from passages 5–9 were removed from the flasks by first rinsing with Hanks' balanced salt solution, and then incubating with trypsin/EDTA until detached, followed by the addition of trypsin neutralizing solution. Cells were resuspended at 0.033×10^6 cells/ml in complete medium and cultured for 24 h at 37 °C, 5% CO_2. The complete medium was replaced with basal medium, following a Hanks' balanced salt solution rinse, to allow for cell synchronization by serum starvation for 24 h. Vehicle, sirolimus or paclitaxel was added in basal medium 1 h prior to the addition of complete medium containing 2% FBS. At the appropriate time point (24–48 h), cells were incubated with trypsin/EDTA until detached followed by the addition of trypsin neutralizing solution. Viability was assessed by trypan blue exclusion. The cells were washed and resuspended in the buffer solution of the CycleTEST™ PLUS DNA Reagent Kit according to the manufacturer's instructions, flash frozen in a 100% methanol–dry ice bath and stored at −80 °C until thawed. For flow cytometric analysis, thawed cells were stained with propidium iodide. DNA histograms were acquired using a Becton Dickenson FACSort flow cytometer. DNA histogram analysis was performed using ModFit LT 3.0 software (Verity Software Houses, Inc., Topsham, ME).

2.6. Rat carotid injury model

2.6.1. Surgical and other procedures

All animal procedures were conducted in accordance with Institutional Animal Care and Use policies, USDA regulations and AAALAC-International guidelines. All surgical procedures were conducted using aseptic technique. Male Lewis rats (250–450 g, Charles River Laboratories) were anesthetized with a mixture of ketamine, xylazine, acepromazine (50,10, and 1 mg/kg, respectively) and the ventral neck region was shaved and disinfected. Rats were warmed on a 37 °C warming pad throughout surgery and post-operatively until ambulatory. Through a midline incision, the left common and external carotid arteries were exposed. Upon controlling blood flow in the external carotid artery, a 2F Fogarty embolectomy catheter was introduced through an arteriotomy. The catheter was advanced proximally through the external carotid into the common carotid artery to the first mark (approximately 3 cm) on the catheter shaft. The balloon was inflated such that the balloon was distended to a diameter of approximately 2 mm using a saline-filled 1 ml gas-tight Hamilton® syringe with a threaded plunger. The balloon was gently pulled distally through the common carotid artery using a slight twisting motion. Once the common carotid artery was balloon-injured, the balloon was deflated and the balloon injury procedure was

repeated for a total of three passes. Following balloon injury, the catheter was withdrawn through the arteriotomy and bleeding was controlled by permanently ligating the proximal external carotid artery ligature. Blood flow through the common and internal carotid arteries was confirmed by the presence of a strong pulse in both arteries.

Following balloon injury, the left common carotid artery within the neck region was gently dissected away from the surrounding connective tissue and vagus nerve. A silastic tube (1.5 mm ID) was filled with either pluronic F127 or a suspension of either sirolimus or paclitaxel (200 μg/vessel) in pluronic F127. The tube was then gently introduced around the adventitial side of the exposed common carotid artery, thereby initiating perivascular drug or vehicle delivery. The overlying musculature and connective tissue were repaired with suture and the skin wound closed using stainless steel surgical staples. The closed wound was swabbed with povidone iodine and 5 ml of sterile saline were given subcutaneously for fluid replacement. Rats were allowed to recover for up to 14 days, depending upon the desired endpoint.

2.6.2. Rat carotid artery injury model study design

The effects of perivascular application of sirolimus or paclitaxel on neointimal hyperplasia, vascular cell cycle as well as proliferation and apoptosis in the rat carotid artery injury model were assessed as described in Table 1. For assessment of neointimal hyperplasia (at 14 days post-injury, $n=5$ per treatment group) or immunohistochemistry ($n=4$ per treatment group) 7 days post-injury, rats were euthanized by overexposure to CO_2 (Beaver et al., 2000). Following the cessation of breathing, rats were exsanguinated by transcardial saline perfusion and vessels were fixed in situ using transcardial perfusion of 10% neutral buffered formalin. The treated region of the injured common carotid artery was then harvested and the perivascular silastic tube was removed. The contralateral vessel of the vehicle-treated group served as the untreated control (normal). Vessels were stored in neutral buffered formalin for subsequent histologic preparation and histomorphometric analysis.

Fixed vessels were then embedded in paraffin and sectioned at three levels. Vessel sections to be analyzed for neointimal hyperplasia were mounted on coated glass slides and stained with either hematoxylin/eosin or Verhoff van Geisson elastic stain. Lumenal, neointimal and medial areas were then assessed using computerized morphometric analysis programs calibrated using a stage micrometer. Vessel sections used for the assessment of cell proliferation and apoptosis were subjected to anti-Proliferating Cell Nuclear Antigen (PCNA) and Terminal deoxynucleotide transferase-mediated dUTP nick-End Labeling (TUNEL) immunohistochemistry using commercially available reagents.

For assessment of vascular cell cycle ($n=5$ per treatment group), treated and normal control vessels were harvested post-euthanasia at 7 days post-injury from non-perfused rats (see above). Vessels were then thoroughly minced using crossed scalpels and placed in a DMSO/citrate/sucrose cryoprotective solution (BD CycleTEST™ PLUS DNA Reagent Kit). The mincate was snap-frozen and stored at −80 °C for subsequent analysis. Upon analysis, mincates were treated with a trypsin/detergent solution (CycleTest kit, Becton Dickinson), which effectively extrudes cell nuclei from the minced tissue. The digestion was terminated, the suspension centrifuged, and the resulting nuclear suspension was treated with propidium iodide, forming a fluorescent complex with nuclear DNA. DNA histograms of vascular tissue nuclear suspensions were obtained as described above. Approximately 12,000–20,000 events were obtained for subsequent analysis of cell cycle histograms. Cell cycle analysis was performed following acquisition of the DNA histogram using the MODFIT cell cycle analysis algorithm.

Table 1
Histomorphometry, cell cycle (FACS) and immunohistochemistry study design in rat carotid injury model

Group	Treatment	Time post-injury (days)	Sample size Histo/FACs/Immunohisto
1	Uninjured	7 or 14	5/5/4
2	Injured+pluronic	7 or 14	5/5/4
3	Injured+pluronic+200 μg sirolimus	7 or 14	5/5/4
4	Injured+pluronic+200 μg paclitaxel	7 or 14	5/5/4

7 days for immunohistochemistry or FACs, 14 days for histomorphometry.

2.7. Statistical analysis

Where appropriate, data were subjected to one-way analysis of variance (ANOVA). When a significant difference across experimental group means was determined from ANOVA ($P<0.05$), group means were compared to the vehicle control mean by the Dunnett's post hoc test. Means were determined to be significantly different when $P<0.05$.

3. Results

3.1. Effects of sirolimus and paclitaxel on coronary artery smooth muscle and endothelial cell proliferation and viability

Sirolimus and paclitaxel inhibited coronary artery smooth muscle and endothelial cell proliferation in a dose-dependent manner (Fig. 1) whether cells were exposed to the respective drug for 48 h or 5 days (smooth muscle cells only). With 48 h exposure, sirolimus incompletely inhibited smooth muscle and endothelial cell proliferation, with a maximum level of inhibition of approximately 70% of maximally stimulated controls. The antiproliferative IC_{50} sirolimus for smooth muscle (48 h or 5 days) and endothelial cells was approximately 2.5–14 nM. The antiproliferative effect of paclitaxel on smooth muscle cell proliferation was less at 48 h than at 5 days, when paclitaxel produced almost complete inhibition of smooth muscle cell proliferation. It is interesting that in endothelial cells, paclitaxel inhibited proliferation ($IC_{50}=28$ nM) by greater than 90% after only 48 h of exposure (Fig. 2). The effects of sirolimus or paclitaxel on endothelial cell proliferation were not evaluated at

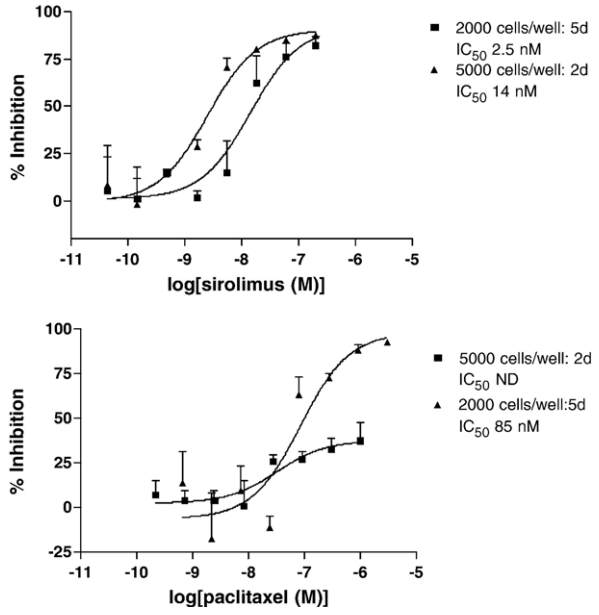

Fig. 1. Effects of sirolimus (top) or paclitaxel (bottom) on cultured human coronary artery smooth muscle cell proliferation at two conditions. Data are presented as mean percentage inhibition (of maximal proliferation)+S.D. IC_{50} values were calculated using nonlinear sigmoidal regression analysis (Prism v.3.03).

5 days. In a separate study, sirolimus-treated smooth muscle cells were able to proliferate following a washout period of 120 h. In contrast, proliferation of smooth muscle cells treated with paclitaxel was inhibited with similar potency following the same washout period (data not shown), suggesting that paclitaxel produces an irreversible antiproliferative effect in coronary artery smooth muscle cells.

It should be noted that over the linear range of their respective antiproliferative dose–response functions, neither sirolimus nor paclitaxel reduced the viability of coronary artery smooth muscle or endothelial cells stimulated with FBS as determined in a routine cell viability assay (MTS-based CellTiter 96, Promega, WI, data not shown) under conditions similar to those used for the determination of cell proliferation. This suggests that the antiproliferative effects of either agent on either cell type were not due to non-specific necrotic cell death.

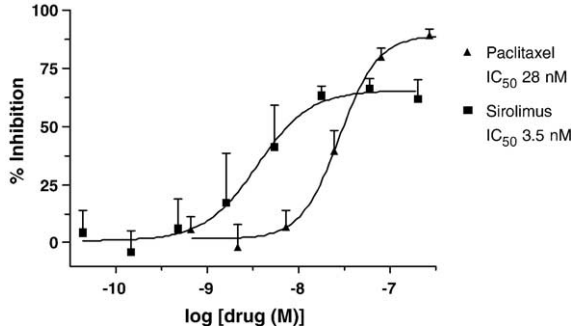

Fig. 2. Effects of sirolimus or paclitaxel on cultured human coronary endothelial cell proliferation. Data are presented as mean percentage inhibition (of maximal proliferation)+S.D. IC_{50} values were calculated using nonlinear sigmoidal regression analysis (Prism v.3.03).

3.2. Effects of sirolimus and paclitaxel on coronary artery smooth muscle and endothelial cell migration

The effects of sirolimus and paclitaxel on FBS-stimulated coronary artery smooth muscle cell migration following a 24 h exposure are shown in Fig. 3. Paclitaxel inhibited smooth muscle cell migration with an IC_{50} of 56 nM and produced complete inhibition at higher concentrations compared to vehicle alone. In contrast, sirolimus inhibited FBS-induced migration at a concentration of 10 μM by only ~50%. Paclitaxel also completely inhibited FBS-stimulated endothelial cell migration over a concentration range similar to that which inhibited smooth muscle cell migration (IC_{50}=56 nM), suggesting that paclitaxel is equipotent at inhibiting coronary artery smooth muscle and endothelial cell migration. In contrast, sirolimus had no effect on endothelial cell migration, even at concentrations as high as 10 μM.

3.3. Effects of sirolimus and paclitaxel on coronary artery smooth muscle and endothelial cell cycle

In order to evaluate the effects of sirolimus and paclitaxel on the relative percentages of coronary artery smooth muscle and endothelial cells in each phase of the cell cycle, DNA content was quantitated in extruded nuclei from each cell type using flow cytometry. Representative effects (from three separate experiments) of 24 or 48 h sirolimus or paclitaxel exposure on the relative percentages of nuclei from smooth muscle and endothelial cells in G0/G1, S and G2/M at various concentrations are illustrated in Fig. 4 (smooth muscle cells) and Fig. 5 (endothelial cells). As seen in Figs. 4 and 5 (top), 24 h FBS stimulation increased the relative percentage of smooth

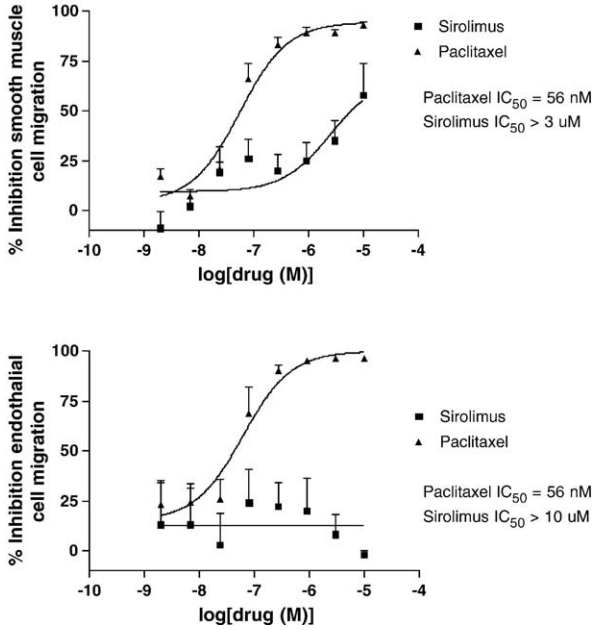

Fig. 3. Effects of sirolimus or paclitaxel on cultured human coronary smooth muscle and endothelial cell migration. Data are presented as mean percentage inhibition (of maximal migration)+S.D. IC_{50} values were calculated using nonlinear sigmoidal regression analysis (Prism v.3.03).

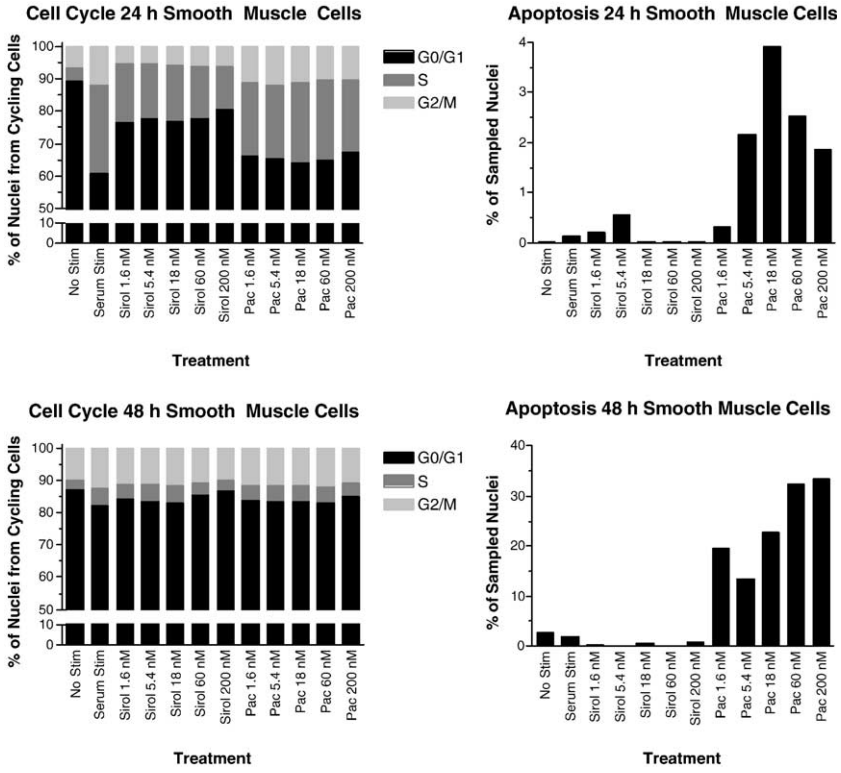

Fig. 4. Effects of sirolimus (Sirol) or paclitaxel (Pac) on cultured human coronary artery smooth muscle cell cycle phases and sub-G0/G1 nuclei (apoptotic index) after a 24 or 48 h exposure (representative experiment). Data were derived from flow cytometric analysis of cultured human coronary artery smooth muscle nuclei labeled with propidium iodide analyzed using MODFIT. Data are expressed as the percentage of nuclei in cycle (G0/G1, S and G2/M phases). Apoptotic nuclei are expressed as a percentage of the total number of nuclei analyzed.

muscle and endothelial cell nuclei in S and G2/M phases and decreased the percentage of cells in G0/G1 compared to unstimulated (serum-starved) cells, an effect consistent with an increase in the number of cycling cells (cell proliferation). With an exposure period of 24 h, sirolimus increased the relative percentage of smooth muscle and endothelial cell nuclei in G0/G1 with a concomitant decrease in the percentage of smooth muscle and endothelial cell nuclei in both S and G2/M phases, even at concentrations as low as 1.6 nM, when compared to vehicle-treated, FBS-stimulated cells. At 24 h, sirolimus did not substantially increase the percentage of sub-G0/G1 nuclei of either cell type.

In contrast to sirolimus, the effects of 24 h paclitaxel exposure on the relative percentage of nuclei in G0/G1, S or G2/M did not differ from the percentage of nuclei in each phase of FBS-stimulated smooth muscle or endothelial cells. However, there was evidence of paclitaxel-induced apoptosis (an increase in the percentage of sub-G0/G1 nuclei) in each cell type with concentrations as low as 5.4 and 18 nM for smooth muscle and endothelial cells, respectively. Moreover, the pro-apoptotic paclitaxel concentrations are below the IC_{50}'s for paclitaxel-induced inhibition of smooth muscle or endothelial cell proliferation and migration.

At 48 h (Figs. 4 and 5), the effects of FBS stimulation on the relative increase in the percentage of smooth muscle or endothelial cell nuclei in S and G2/M phases typically seen at 24 h were no longer evident. A lack of serum stimulation dramatically increased the relative percentage of the number of sub-G0/G1 or fragmented endothelial cell nuclei (Fig. 5), indicating substantial apoptosis (hence, the inability to assess the relative percentage of endothelial cells in G0/G1, S or G2/M at 48 h vs. unstimulated cells). The relative changes in G0/G1, S and G2/M observed with sirolimus treatment in serum-stimulated cells at 24 h could not be observed at 48 h in either cell type. Paclitaxel either produced no apparent changes in G0/G1, S and G2/M phases in smooth muscle or endothelial cells after a 48 h exposure period in some experiments, or increased the relative percentage of cells of each cell type in both S and G2/M phases when compared to serum-stimulated controls (shown in Fig. 5). Moreover, paclitaxel dramatically increased the percentage of sub-G0/G1 or apoptotic smooth muscle and endothelial cell nuclei at 48 h, an effect not observed with sirolimus treatment (see above).

3.4. Effects of perivascular application of sirolimus and paclitaxel on neointimal hyperplasia following vascular injury

The effects of local perivascular application of sirolimus or paclitaxel were evaluated in balloon-injured rat carotid arteries. A 200 μg suspension of sirolimus or paclitaxel formulated in pluronic gel was chosen for perivascular delivery based upon the dose that was shown to be safe and effective at reducing neointimal hyperplasia in pilot studies. This dose level, although slightly higher than those found on drug-eluting stents,

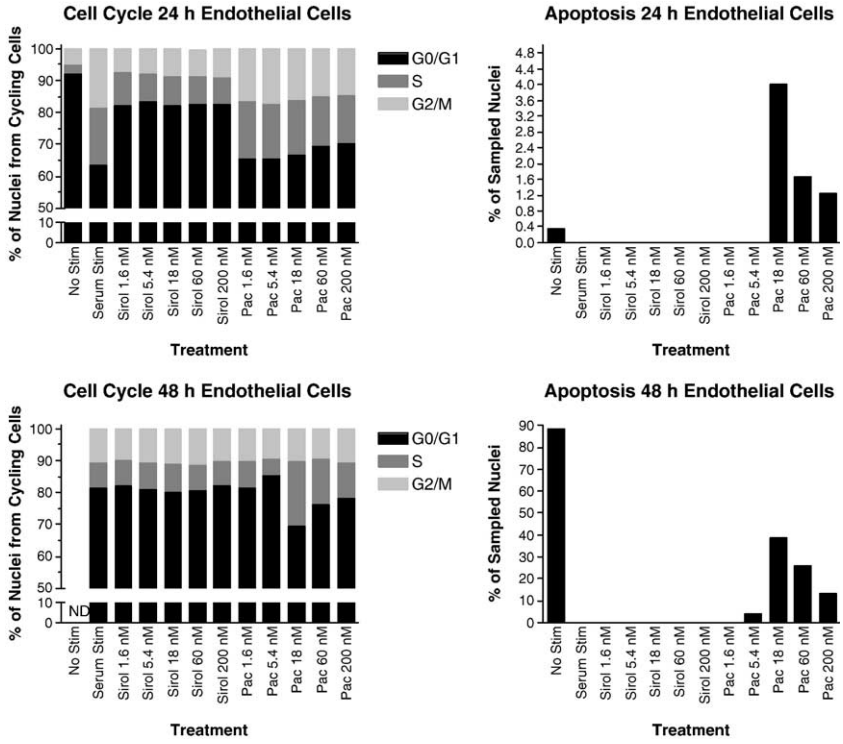

Fig. 5. Effects of sirolimus (Sirol) or paclitaxel (Pac) on human coronary artery endothelial cells cell cycle phases and sub-G0/G1 nuclei (apoptotic index) after a 24 or 48 h exposure (representative experiment). Data were derived from flow cytometric analysis of cultured human coronary artery smooth muscle cell nuclei labeled with propidium iodide analyzed using MODFIT. Data are expressed as the percentage of nuclei in cycle (G0/G1, S and G2/M phases). Apoptotic nuclei are expressed as a percentage of the total number of nuclei analyzed.

was selected since the drugs had to overcome additional diffusion barriers (e.g. adventitia) not encountered with stent-based delivery to gain access to the vascular media.

The effects of perivascularly applied sirolimus or paclitaxel on neointimal hyperplasia compared to pluronic vehicle treatment in injured rat carotid arteries are shown in Figs. 6 and 7. Both sirolimus and paclitaxel reduced neointimal formation by at least 80% compared to vehicle-treated vessels ($P<0.05$). Aside from the reduction in the size of the neointima, the vessels treated with either sirolimus or paclitaxel had similar morphologic characteristics as untreated vessels (Fig. 7). It should be noted that upon gross observation, the skeletal muscle layers directly surrounding the carotid arteries treated with perivascularly applied paclitaxel exhibited evidence of patchy necrosis, an effect not observed with perivascular sirolimus administration.

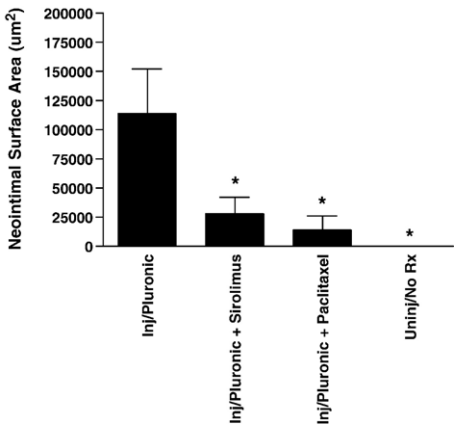

Fig. 6. Effects of perivascular sirolimus or paclitaxel application on neointimal hyperplasia in balloon-injured rat carotid arteries. Injured=Inj, Rx=treated, Uninj.=uninjured. Data are presented as mean neointimal surface area+S.E.M., $n=5$ per treatment group. *$P<0.05$.

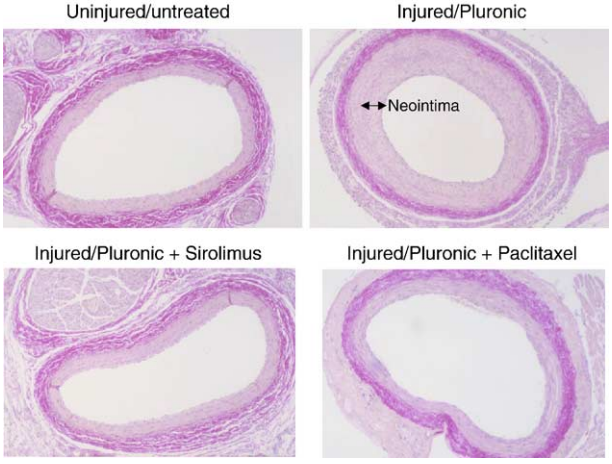

Fig. 7. Histologic effects of perivascular sirolimus or paclitaxel application on neointimal hyperplasia in balloon-injured rat carotid arteries. 40× magnification, H&E stain.

3.5. Effects of perivascular application of sirolimus and paclitaxel on vascular cell cycle following vascular injury

The effects of locally applied sirolimus or paclitaxel on phases of the cell cycle of injured vascular cells were evaluated by quantitating fluorescently labeled DNA by flow cytometry from nuclei extruded from minced vascular tissue (Fig. 8). In the uninjured state, greater than 90% of the vascular cells were found to be in G0/G1, typical for non-proliferating tissue. Balloon injury induced a marked rise in the percentage of vascular cell nuclei in S and G2/M phases and a concomitant reduction in the percentage of nuclei in G0/G1, indicating an elevation in cell proliferation within the vessel wall.

Relative to vehicle treatment of injured vessels, perivascular sirolimus treatment significantly reduced ($P<0.05$) the percentage of cells in S and G2/M while significantly increasing ($P<0.05$) the percentage of cells in G0/G1. In contrast, paclitaxel significantly increased ($P<0.05$) the percentage of nuclei from the injured vascular wall in G2/M and S phases while significantly reducing ($P<0.05$) the percentage of cells in G0/G1 when compared to vehicle-treated injured vessels. In addition, paclitaxel significantly increased ($P<0.05$) the percentage of apoptotic (sub-G0/G1) nuclei within the entire pool of sampled nuclei compared to vehicle treatment (Fig. 8, right). Perivascularly applied sirolimus produced a modest but not statistically significant reduction in the percentage of apoptotic nuclei compared to vehicle-treated controls.

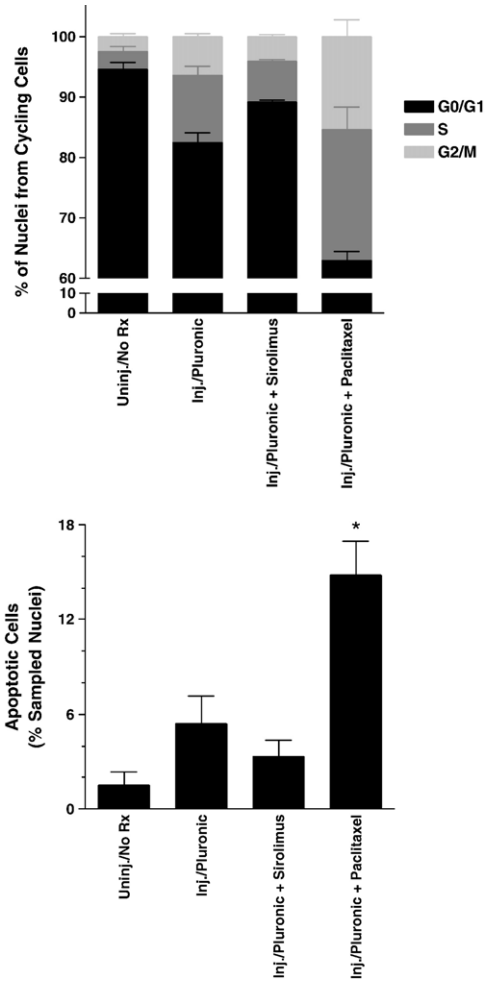

Fig. 8. Effects of perivascularly applied sirolimus or paclitaxel on cell cycle phases (top) and apoptosis (bottom) within cells of the vascular wall. $n=5$ per treatment group. Injured=Inj, Rx=treated, Uninj.=uninjured. Data were derived from flow cytometric analysis of vascular nuclei labeled with propidium iodide analyzed using MODFIT. Data are represented as the mean percentage of cells in cycle (G0/G1, S and G2/M)+S.E.M. Apoptotic data are expressed as a percentage of total nuclei analyzed. *$P<0.05$.

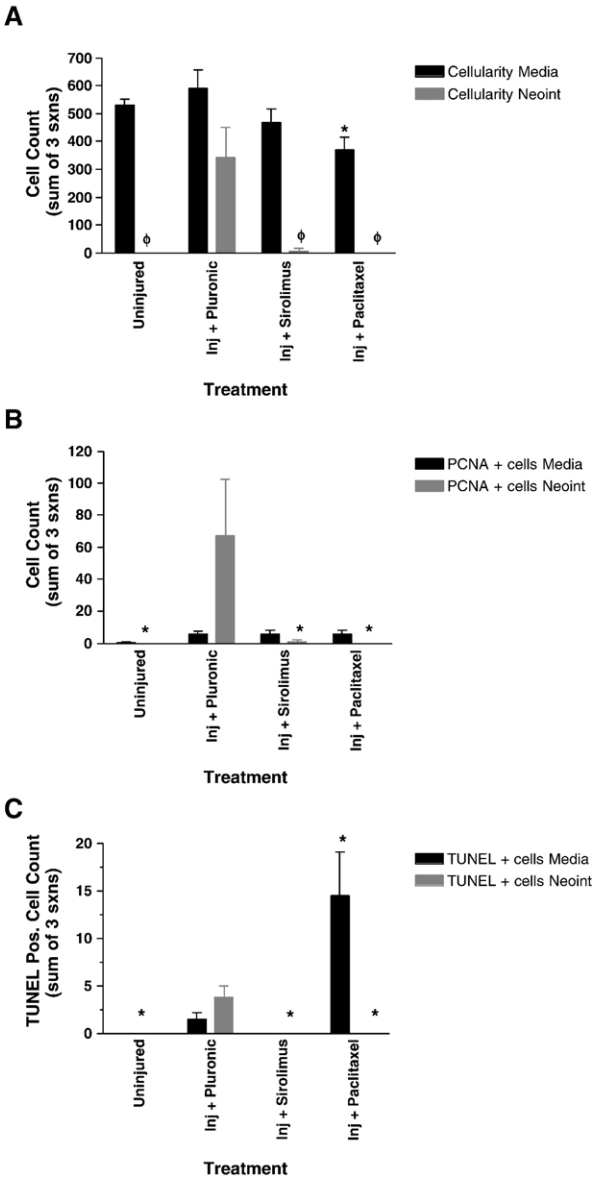

Fig. 9. Effects of perivascular sirolimus or paclitaxel treatment on total cell count (A) proliferating (PCNA positivity) (B) and apoptotic (TUNEL positivity) cells (C) in the rat carotid artery 7 days after injury. Inj=injured, PCNA=proliferating cell nuclear antigen. Data are presented as mean cell number per field+S.E.M. *$P<0.05$ vs. Inj. Pluronic or Uninj. by Dunnett's. $^{\phi}P<0.05$ vs. Inj. Pluronic by Dunnett's.

3.6. Effects of perivascular application of sirolimus and paclitaxel on cell proliferation and apoptosis following vascular injury

To confirm the above findings with locally applied sirolimus and paclitaxel in the injured rat carotid artery, the effects of perivascular administration of sirolimus and paclitaxel on cell proliferation (anti-PCNA immunohistochemistry) and apoptosis (TUNEL immunohistochemistry) were evaluated 7 days after balloon injury and local treatment (Fig. 9). Light microscopic examination of hematoxylin/eosin stained sections revealed that balloon injury produced a cellular neointima by 7 days (Fig. 9A) post-injury. Both local sirolimus and paclitaxel treatment significantly reduced ($P<0.05$) neointimal cellularity 7 days after injury compared to vehicle-treated controls (Fig. 9A). In addition, paclitaxel significantly reduced ($P<0.05$) the cellularity of the media when compared to the medial cellularity of either the vehicle-treated or uninjured controls. Balloon injury substantially increased cell proliferation as indexed by the number of PCNA positive cells within the neointima of vehicle-treated controls compared to uninjured vessels at 7 days following surgery. There was a mild increase in cell proliferation within the media of vehicle-treated vessels compared to uninjured controls (Fig. 9B). Neointimal cell proliferation was significantly reduced ($P<0.05$) by perivascular sirolimus or paclitaxel administration compared to vehicle-treated controls (Fig. 9B). Balloon injury also increased the number of apoptotic cells within the media compared to uninjured vessels as indexed by TUNEL positivity. Since both sirolimus and paclitaxel dramatically reduced neointimal cellularity at day 7, the apoptotic index within the neointima was significantly lower following treatment with either agent compared to vehicle-treated controls. Interestingly, paclitaxel significantly increased ($P<0.05$) the number of apoptotic cells within the media compared to vehicle-treated controls (Fig. 9C). No apoptotic cells in the vascular media were detectable in the sirolimus treatment group (Fig. 9C).

4. Discussion

Until recently, neointimal hyperplasia due to arterial injury has limited percutaneous coronary artery revascularization procedures. The use of drug-coated stents that elute sirolimus or paclitaxel into the vessel wall has limited neointimal hyperplasia and improved the rate of restenosis following vascular dilation and stenting. Although sirolimus and paclitaxel are effective antirestenotic agents when applied lumenally via stent delivery, their effects on cultured human coronary artery smooth muscle and endothelial cells have not been addressed by direct comparison. Further, the impact of locally delivered sirolimus or paclitaxel on injured vascular wall cell cycle, cell proliferative and apoptotic changes has received relatively little attention. The present study examined the activity of sirolimus and paclitaxel on coronary artery smooth muscle and endothelial cell proliferation, migration, cell cycle and apoptosis. In addition, the effects of perivascular application of therapeutically relevant doses of sirolimus or paclitaxel on balloon-injured vascular cell cycle, proliferation and apoptosis were evaluated.

In cultured coronary artery smooth muscle and endothelial cells, both sirolimus and paclitaxel inhibited cell proliferation in a dose-dependent manner. Sirolimus inhibited proliferation of both smooth muscle and endothelial cells with low nanomolar potency and maximal antiproliferative activity of approximately 70% with FBS stimulation, regardless of the duration of exposure (2 or 5 days). The antiproliferative activity and potency of sirolimus in both cell types were similar to that observed in other vascular smooth muscle cell types (Marx et al., 1995). Further, the effects of sirolimus on smooth muscle cell proliferation were reversible since cells resumed proliferation several days after washout of sirolimus.

In contrast, the antiproliferative activity of paclitaxel in coronary artery smooth muscle cells was weak with 2 days of exposure, but paclitaxel exposure of 5 days elicited near complete inhibition of smooth muscle cell proliferation, with an IC_{50} in the low nanomolar range. The antiproliferative activity observed with paclitaxel in the present study was similar to that reported by Axel et al. (1997) in aortic smooth muscle cells. Coronary artery endothelial cells were more sensitive to the antiproliferative effects of paclitaxel, exhibiting near complete inhibition of proliferation with just two days of continuous exposure. In contrast to sirolimus, inhibition of cell proliferation by paclitaxel was not reversible following drug washout. Although both sirolimus and paclitaxel inhibited smooth muscle and endothelial cell proliferation, the latency to the maximum antiproliferative activity seen with paclitaxel in cultured coronary artery smooth muscle cells was longer than that observed with sirolimus. While the antiproliferative effects of sirolimus and paclitaxel in smooth muscle cells were durable, only sirolimus showed reversibility of its antiproliferative effect upon drug washout.

Smooth muscle cell migration is a factor in the formation of neointima following vascular injury (Casscells, 1992). However, repopulation of the injured intima with functional endothelial cells is necessary for reducing the propensity of thrombus formation in the revascularized and stented region of the coronary artery. Endothelial cell migration is critical for the reestablishment of a functional endothelium following coronary artery stenting. Sirolimus had only weak antimigratory activity in cultured coronary artery smooth muscle cells and had no measurable antimigratory effect in coronary artery endothelial cells. The antimigratory activity of sirolimus in smooth muscle cells in the present study was less than that reported in other studies (Poon et al., 1996; Sun et al., 2001) where a longer duration of incubation of smooth muscle cells with sirolimus (22 vs. 48 h) was used.

In contrast, paclitaxel was found to be a potent and highly efficacious antimigratory agent against both coronary artery smooth muscle and endothelial cells. Relative to sirolimus, it is likely that the reestablishment of a functional endothelium is delayed following deployment of a paclitaxel-eluting stent and, for this reason the risk of stent thrombosis may be increased with the use of paclitaxel relative to sirolimus.

Sirolimus and paclitaxel affect cell cycle by distinctly different mechanisms. The binding of sirolimus to FKBP12 is thought to arrest proliferating smooth muscle cells at G1 by blocking mTOR activity, increasing $p27^{kip1}$ activity and reducing downstream cyclin protein function (Jayaraman and Marks, 1993; Marks, 2003; Marx et al., 1995). Paclitaxel, at higher concentrations, blocks cell division at mitosis, while at lower concentrations paclitaxel affects other cellular processes that can lead to arrest in S phase or at the G1/S interface or apoptosis (Fan, 1999; Giannakakou et al., 2001). The effects of high dose mitotic arrest or low dose effects on a variety of cellular metabolic processes result in either immediate or delayed apoptotic cell death in a number of cell types (Allman et al., 2003; Au et al., 1998; Blagosklonny et al., 2002; Das et al., 2001; Giannakakou et al., 2001; Lieu et al., 1997; Shu et al., 1997). Mitotic arrest induced by paclitaxel has been shown to result in a large, multinucleated cell phenotype (e.g. mitotic slippage) with subsequent cell death by apoptotic or non-apoptotic mechanisms in a number of cell types (Abal et al., 2003; Blagosklonny et al., 2002; Bottone et al., 2003; Jung et al., 2004; Wan et al., 2004). This suggests that the observations of Blagosklonny et al. (2004) on the effects of paclitaxel in cultured smooth muscle cells are actually consistent with paclitaxel-induced cell death and not G1 arrest.

The present work highlights the mechanistic differences between sirolimus and paclitaxel on proliferating cultured vascular cells and injured vessels. Our findings suggest that sirolimus arrests proliferating coronary artery smooth muscle and endothelial cells in G1, consistent with the proposed cytostatic mechanism of action. Further, in contrast to the findings of Blagosklonny et al. (2004), paclitaxel induced apoptotic cell death within 24–48 h in both cell types at concentrations below 10 nM, suggesting that paclitaxel acts on coronary artery smooth muscle and endothelial cells through a cytotoxic mechanism. Cell cycle analysis of injured rat carotid arteries treated with locally applied sirolimus or paclitaxel shed further light on the differences between these agents with regard to their ability to reduce neointimal cell proliferation and hyperplasia. It is clear that both agents administered perivascularly reduce neointimal hyperplasia in balloon-injured rat carotid arteries. However, only locally applied sirolimus reduced the percentage of cells in S and G2/M phases by arresting cells in G1 without inducing apoptosis. In contrast, the effects of local paclitaxel treatment of injured carotid arteries increased the percentage of cells in both S and G2/M phases, consistent with mitotic arrest. Further, paclitaxel significantly increased the percentage of sub-G0/G1 cells in injured carotid arteries compared to vehicle treatment, indicating the presence apoptotic cell death. This increase in apoptotic cells induced by local paclitaxel treatment was confirmed using TUNEL immunohistochemistry. The effects of paclitaxel-induced vascular wall apoptosis were predominantly found to be within the media 7 days after balloon injury. Paclitaxel-induced apoptotic cell death could also explain the concomitant loss of medial cellularity in the present study and medial vasotoxicity findings (medial thinning, wall hemorrhage and necrosis) observed in other studies (Heldman et al., 2001).

Taken together, sirolimus and paclitaxel inhibit neointimal hyperplasia by profoundly different mechanisms. Sirolimus appears to exert a cytostatic effect on cells within the injured vessel wall by arresting them in the G1 phase of the cell cycle without inducing apoptosis. In contrast, the predominant mechanism by which paclitaxel reduces neointimal hyperplasia is cytotoxicity. The relative differences of each agent on cultured vascular endothelial cells suggest that the process of reendothelialization may be relatively impaired in vessels treated with paclitaxel compared to those treated with sirolimus. This lends support to the need for relatively extended clinical exposure to antiplatelet agents in patients receiving paclitaxel-coated stents. Although the use of drug-eluting stents has been shown to be relatively safe, of particular concern is the cytotoxicity of paclitaxel on smooth muscle cells, an effect not observed with sirolimus. Paclitaxel-induced smooth muscle cytotoxicity and potential abnormalities in vessel wall integrity should be considered when considering the use of this agent to prevent restenosis following stenting of relatively thin-walled vessels. Further insight into the mechanistic differences between sirolimus and paclitaxel in the prevention of restenosis should emerge as data become available from widespread clinical use of sirolimus- or paclitaxel-eluting stents.

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