

Research article

Simultaneous intra/extravascular administration of antiproliferative agents as a new strategy to inhibit restenosis: The peak of reactive cell proliferation as a hallmark for the duration of the treatment

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Abstract

Background: Strictly intravascular approaches for the treatment of postangioplasty restenosis are effective in the intima and the inner parts of the media but may be insufficient to control redundant pathways in the more outer parts of the media and the adventitia. An inverse situation may occur subsequently to a strictly extravascular approach, like the recently suggested pericardial approach in pigs. We hypothesized that simultaneous intra/extravascular administration of anti-restenotic agents inhibits restenosis by blocking all stimulatory pathways in the entire arterial wall.

Methods: Fresh hearts of 25 domestic pigs were obtained from a local slaughterhouse. Left anterior descending coronary arteries (LAD) were harvested, cut into cylindrical 5 mm segments, and cultured as ex vivo porcine organ cultures (POCs). After 9 bar ballooning simultaneous intra/extravascular administration of high dose diltiazem (50 µg/mL) was carried out for a period of 1, 2, 3, 4, 5, 6, and 7 days. At day 7 and 28 proliferative activity (BrdU), neointimal thickening, and staining against smooth muscle α -actin and vWF was analysed.

Results: 7 days after ballooning administration of diltiazem for 4, 5, 6, and 7 days inhibited reactive cell proliferation by more than 50% (n.s.) as compared to control, 28 days after ballooning administration for 6 and 7 days inhibited neointimal thickening by more than 75% ($p < 0.05$). Simultaneous intra/extravascular administration of high dose diltiazem did not affect the expression of vWF in endothelial cells or smooth muscle α -actin in smooth muscle cells.

Conclusions: Simultaneous intra/extravascular administration of high dose diltiazem (50 µg/mL) has to be maintained for at least 6 days to achieve a significant inhibition of neointimal thickening. The data demonstrate the importance of the maximal reactive cell proliferation (= day 7 in the POC-model) for the calculation of the duration of the treatment period.

Introduction

Despite the outstanding success of coronary angioplasty and related techniques the problem of restenosis [1] has not yet been overcome. This is predominantly caused by the unexpected complexity of the reaction of the coronary artery wall to a relatively simple aggression with an intravascular device. In a very schematic way the main events after coronary artery ballooning are dissection, elastic recoil, thrombosis, leukocyte attack, reactive cell proliferation, and arterial remodeling [2]. Among these reactive cell proliferation remains extremely difficult to control, although many therapeutic agents have a strong antiproliferative effect [3]. This may be due to the fact that after vascular injury a huge amount of signal transduction cascades is activated in the different cell types within the entire arterial wall.

Intravascular anti-restenotic drug regimens are predominantly effective in the inner layers of the artery wall, up-regulation of redundant pathways in the outer parts of the media and the adventia are not or not entirely inhibited. Therefore we hypothesized that simultaneous intra/extravascular administration of antiproliferative agents inhibits restenosis by blocking all redundant pathways [4]. In vivo simultaneous intra/extravascular administration could be carried out by a combination of drug-eluting stents with the pericardial approach [5–7].

The domestic pig is the experimental in vivo model that mimics the characteristic features of human restenosis most closely [8]. In order to limit the number of obligatory experimental studies we have suggested previously to employ organ cultures of porcine coronary arteries (POCs) that had been obtained from a local slaughterhouse [9]. Due to the fact that internal and external surfaces of the coronary artery segments in the POC-model have full contact with the culture medium, simultaneous intra/extravascular drug therapy can be mimicked easily by adding a potential anti-restenotic agent to the culture medium [4].

In a cascade of various prescreening and complex in vitro models of human restenosis [10] we have recently demonstrated a significant antiproliferative effect of the calcium channel blocker diltiazem in a concentration of 50 µg/mL, a toxic effect could be excluded. Due to the fact that this very high concentration is settled two orders of magnitudes above the maximal plasma level subsequent to systemic administration, it can be achieved barely in a local high dose approach. In the POC-model the maximal reactive cell proliferation after ex vivo ballooning occurs at day 7, followed by a maximum of neointimal thickening at day 28 [9]. In the current study we added high dose diltiazem for 1, 2, 3, 4, 5, 6, and 7 days to the POC-model and studied, how many days the antiproliferative regime

has to be maintained in order to accomplish a significant inhibition of neointimal thickening at day 28.

Methods

Preparation of coronary organ cultures

Fresh hearts of 25 pigs (age 3–5 months, weighing 100–120 kg) were obtained from a local slaughterhouse. Left anterior descending coronary arteries (LAD) were prepared and removed carefully from the epicardial surface. Cylindric segments with a side length of 5 mm were cut from the LAD-segments. Immediately after preparation, LAD-cylinders were transferred as POC into sterile HEPES-buffered (15 mmol/L) culture medium (DMEM) without serum supplement.

Ex vivo ballooning

For ex vivo angioplasty POCs were incubated with a standard 3 mm balloon catheter (Medtronic 14K2030E, Diesseldorf, Germany) and treated with 9 bar for 60 s, the balloon/artery ratio was 1. If tearing occurred in the artery wall, the segments were not used for the study. The inflation of balloon catheters was carried out with Aqua bidest.

Simultaneous intra/extravascular application of high dose diltiazem

After ex vivo ballooning high dose diltiazem (Gödecke, Freiburg im Breisgau, Germany) was added to the culture medium in a concentration of 50 µg/mL. The agent was dissolved in distilled water. Simultaneous intra/extravascular application of high dose diltiazem was carried out by incubation of POC with diltiazem for a period of 1, 2, 3, 4, 5, 6, and 7 days. At each medium exchange the drug was renewed.

Cultivation and fixation of organ cultures

Immediately after in vitro ballooning LAD-segments were transferred to 6 well plates (Tecnomara, Fernwald, Germany) and cultured in a mixture of Waymouth's MB 752/1 and Ham F12 nutrient mixture (1:1 v/v; Bio Whittaker, Verviers, Belgium) supplemented with 15% fetal calf serum (Bio Whittaker, Verviers, Belgium) at 37°C in 5% CO₂. The culture medium was exchanged every second or third day or if diltiazem had to be administered. Culture conditions for the control groups (angioplasty without diltiazem and neither angioplasty nor diltiazem) were exactly the same as described for the angioplasty/diltiazem group.

Analysis of postangioplasty reactive cell proliferation

Paraffin sections were processed for immunohistological staining using the avidin-biotin immunoperoxidase technique [9]. Bromodeoxyuridine (BrdU), a thymidine analogue, is incorporated into the DNA of S-phase proliferating cells. 18 h foregoing fixation of the LAD organ cultures BrdU (20 µM, Serva, Heidelberg, Germany)

was added to the culture media supplemented with 20 μ M deoxycytidine (Serva, Heidelberg, Germany) in order to determine the extent of the cells undergoing DNA synthesis, as described [9]. As secondary antibody a biotinylated horse-anti-mouse antibody (Camon, Wiesbaden, Germany) was used. The number of proliferating cells in the media and in the neointima was calculated independently as: Number of BrdU-positive cells/Total cell number \times 100. Only sections cut perpendicular to the vessel wall axis were used for the analysis, five sections from different LAD segments were evaluated microscopically in each single experiment at a magnification of \times 40.

Identification of smooth muscle cells

Smooth muscle cells (SMC) were identified by positive staining of sections with antibodies against smooth muscle α -actin (Renner, Darmstadt, Germany) at a concentration of 1 μ g/mL. The detection of the primary antibody was carried out in the same way as described for the BrdU antibody (see above).

Identification of endothelial cells

Endothelial cells (EC) in sections of organ cultures were identified by positive staining with antibodies against von Willebrand factor (vWF, INCSTAR, Stillwater, USA). The detection of the primary antibody was carried out in the same way as described for the BrdU antibody (see above).

Morphometric analysis of neointimal hyperplasia

For histological examination deparaffinized sections were stained with a modified elastica-van Gieson staining (Chroma, Köngen, Germany). For calculation of the average arterial wall thickening after ex vivo ballooning five cuts from different organ culture segments were analyzed three times for each single experiment with a computerized digital image analyzer (Bioquant TM System 4 R + M Biometrics, Bilancy Consulting, Düsseldorf, Germany). Taking into account the various lumina of POC segments, the percentage of neointimal thickening was calculated as: Area of neointima/Area of neointima and media \times 100.

Statistical analysis

Data are presented as mean \pm S.D. The two-tailed U-test of Mann, and Whitney was used for determining statistical significance of differences. Statistical significance was accepted for $P < 0.05$ [11].

Results

Effect of simultaneous intra/extravascular administration of high dose diltiazem on reactive postangioplasty cell proliferation

The effect of simultaneous intra/extravascular administration of high dose diltiazem on relative reactive cell proliferation both in the neointima and the media of POC

segments was studied 7 and 28 days after ex vivo ballooning.

Seven days after ex vivo ballooning in the neointima a 39.4 fold increase of proliferative activity (n.s.) was detected, the number of BrdU-positive cells was increased from $0.1 \pm 0.34\%$ in untreated controls to $4.3 \pm 5.6\%$ (Fig. 1 and 2). After simultaneous intra/extravascular administration of diltiazem for 1 and 2 days the number of BrdU-positive cells was $2.8 \pm 6.1\%$ and $1.1 \pm 1.8\%$ corresponding to an inhibition by 36 respectively 75%. Administration of diltiazem for 3 days resulted in an increase of BrdU-positive cells by 52%, $6.6 \pm 10.4\%$ of BrdU-positive cells were detected. After incubation of POC-segments with diltiazem for 4, 5, 6, and 7 days the percentage of BrdU-positive cells was $0.8 \pm 1.1\%$, $1.9 \pm 3.6\%$, $0.3 \pm 0.7\%$, and $1.2 \pm 1.8\%$ corresponding to an inhibition by 81%, 57%, 93%, and 72%. 28 days after ballooning the number BrdU-positive cells was very small, $0.0 \pm 0.0\%$ of BrdU-positive cells were detected in untreated controls and in the POC balloon-group. Incubation with diltiazem for 1, 2, and 3 days resulted in $0.0 \pm 0.0\%$, $1.2 \pm 2.6\%$, and $0.1 \pm 0.2\%$ of BrdU-positive cells, respectively. 28 days after incubation with diltiazem for 4, 5, 6, and 7 days the number of BrdU-positive cells was $2.2 \pm 5.9\%$, $3.2 \pm 7.2\%$, $0.0 \pm 0.0\%$, and $1.1 \pm 2.4\%$.

In the media the number of BrdU-positive cells was always very low, no matter whether or not cells were incubated with diltiazem or treated with the balloon or both. 7 days after ballooning $0.0 \pm 0.0\%$ of BrdU-positive cells were detected in untreated controls, 0.2 ± 0.2 after 9 bar ballooning. Incubation with diltiazem for 1, 2, and 3 days resulted in $0.1 \pm 0.0\%$, $0.0 \pm 0.0\%$, and $0.7 \pm 1.3\%$ of BrdU-positive cells. After incubation with diltiazem for 4, 5, 6, and 7 days the number of BrdU-positive cells was $0.0 \pm 0.0\%$, $0.1 \pm 0.2\%$, $0.1 \pm 0.1\%$, and $0.0 \pm 0.0\%$. 28 days after ballooning $0.0 \pm 0.0\%$ were found in untreated controls and after 9-bar ballooning. Incubation of POC-segments with diltiazem for 1, 2, and 3 days resulted in $0.1 \pm 0.1\%$, $0.1 \pm 0.2\%$, and $0.0 \pm 0.0\%$ of BrdU-positive cells, after incubation for 4, 5, 6, and 7 days $0.1 \pm 1.4\%$, $0.1 \pm 0.3\%$, $0.1 \pm 0.1\%$, and $0.2 \pm 0.4\%$.

No statistical significance was reached seven and 28 days after ballooning between the number of BrdU-positive cells in the balloon-group in comparison to the balloon/diltiazem group.

Effect of simultaneous intra/extravascular administration of high dose diltiazem on neointimal thickening

The effect of simultaneous intra/extravascular administration of high dose diltiazem on relative neointimal thickening was analysed 7 and 28 days after 9 bar ballooning (Fig. 3 and 4).

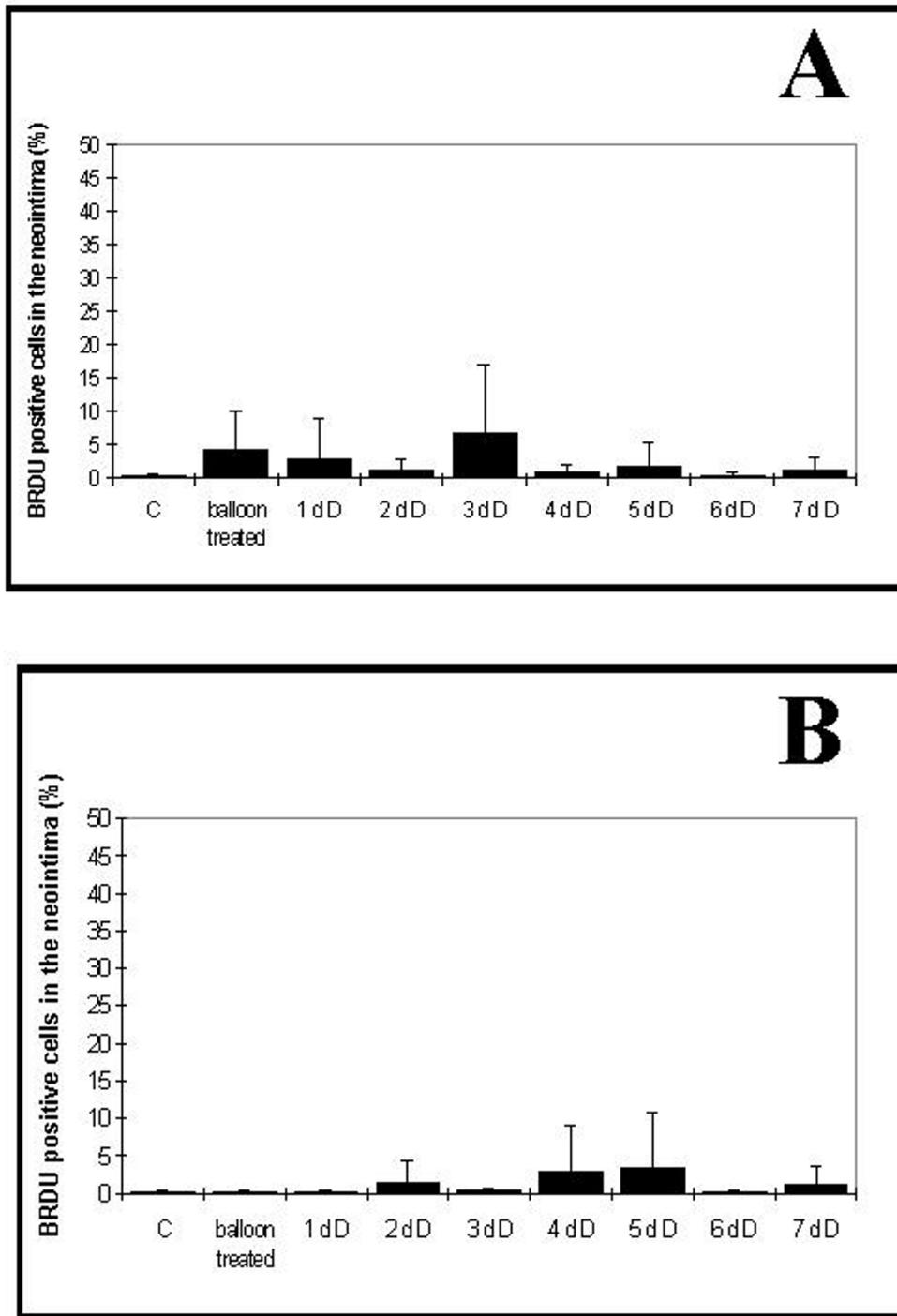


Figure 1
 Effect of simultaneous intra/extravascular administration of high dose diltiazem (50 µg/mL) for 1 – 7 days on time course of cells undergoing DNA synthesis in coronary organ cultures 7 (A) and 28 days (B) after ex vivo ballooning. The number of cells in the neointima with positive bromodeoxyuridine incorporation is depicted in relation to the total cell number.

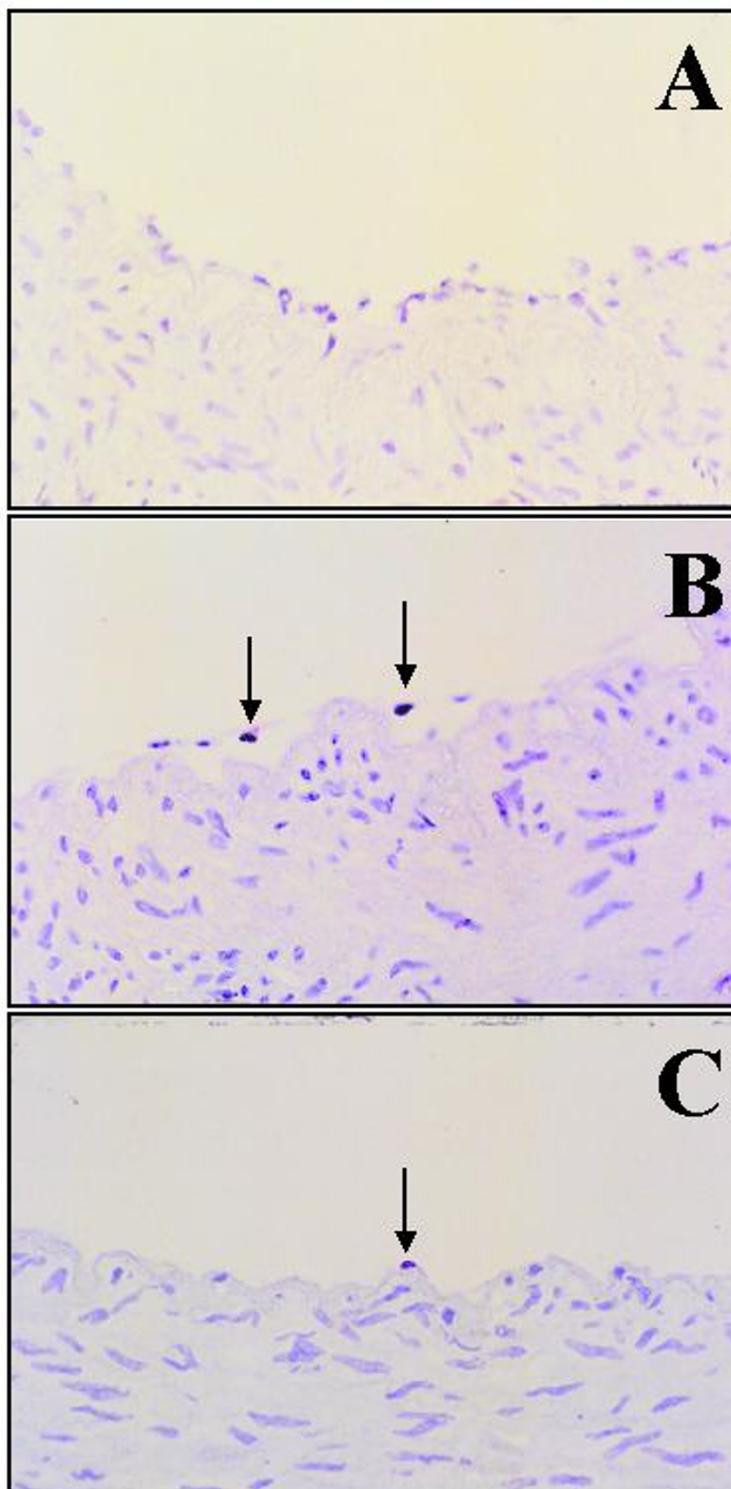


Figure 2
Micrographs showing serial sections of porcine coronary organ cultures at day 7, staining with BrdU (avidin /biotin method): untreated control (A), ex vivo ballooning (B) and ex vivo ballooning with simultaneous intra/extravascular antiproliferative drug therapy (C). arrows = cells with positive staining against BrdU.

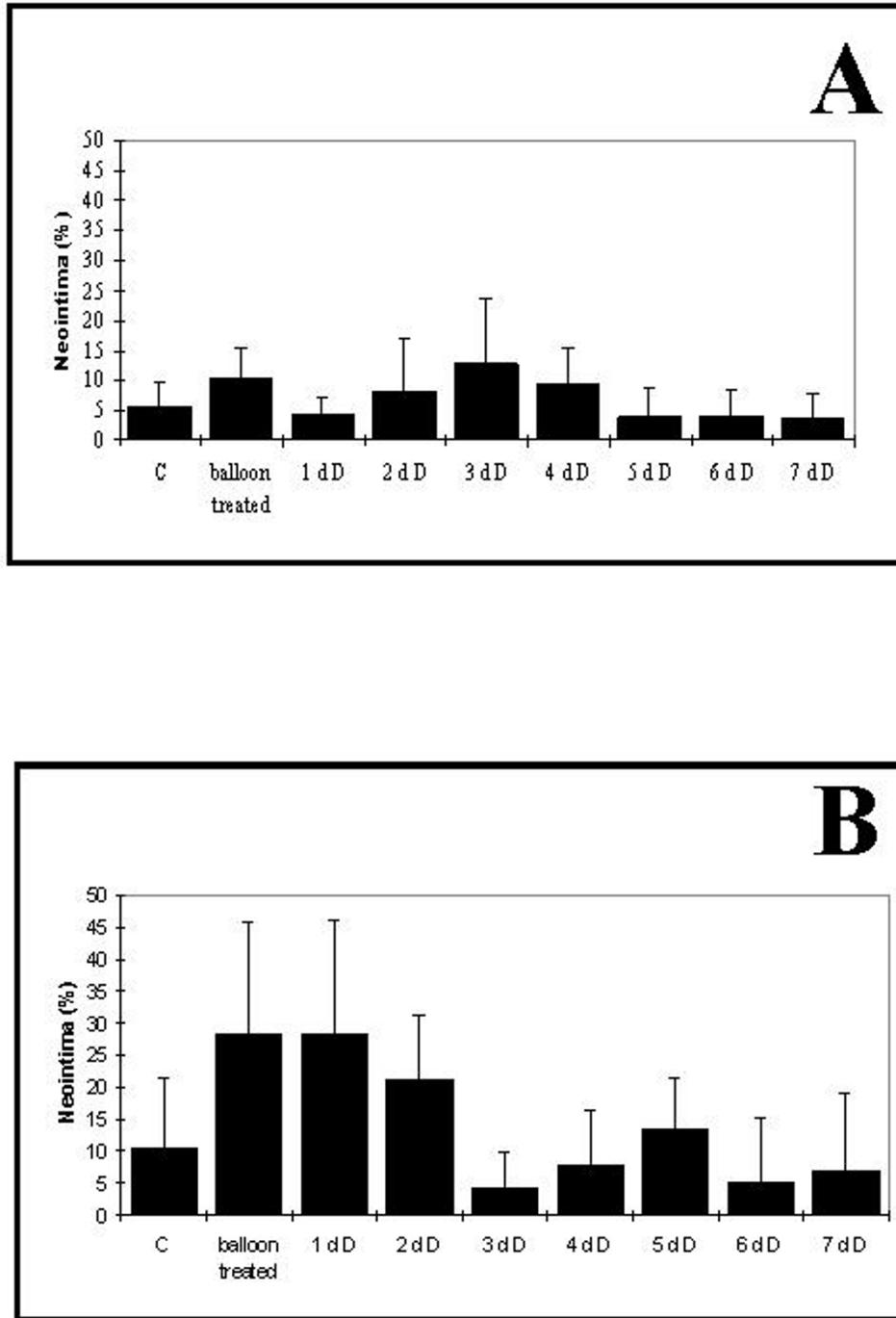


Figure 3
Effect of simultaneous intra/extravascular administration administration of high dose diltiazem (50 $\mu\text{g}/\text{mL}$) for 1 – 7 days on neointimal thickening in porcine organ cultures 7 (A) and 28 days (B) after ballooning. Each column represents five different vessels, * = $p < 0.05$.

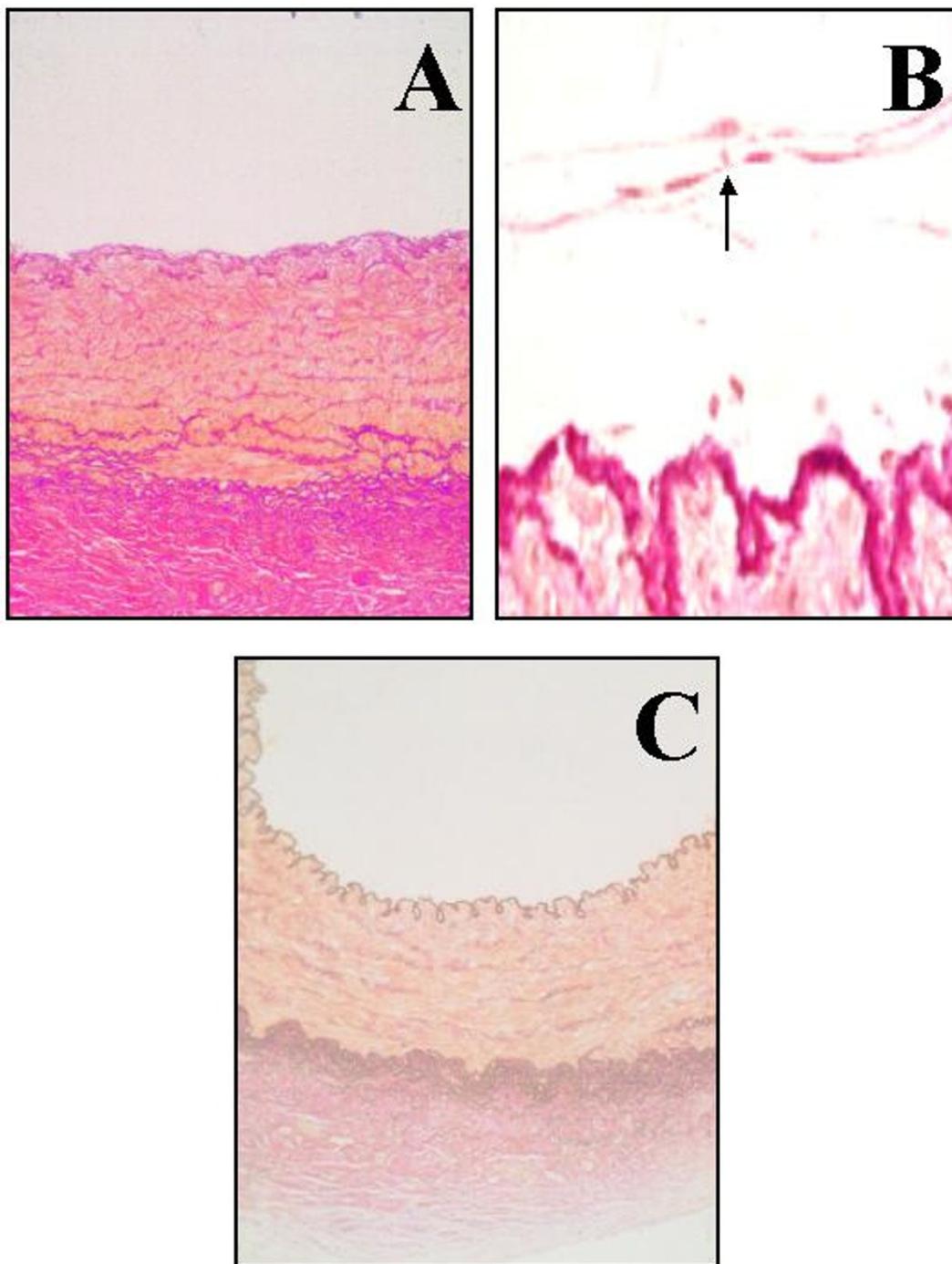


Figure 4
Micrographs of sections of porcine coronary organ cultures at day 28, staining with elastica van gieson: untreated control (A), ex vivo ballooning (B) and ex vivo ballooning with simultaneous intra/extravascular antiproliferative drug therapy (C). arrow = area of neointimal thickening.

7 days after ballooning neointimal thickening in the balloon treated group was significantly increased 1.8 fold ($p < 0.05$) from $5.5 \pm 4.0\%$ in the untreated group to $10.3 \pm 4.8\%$. simultaneous intra/extravascular incubation with diltiazem for 1 and 2 days caused an inhibition of neointimal thickening by 59% (n.s.) and 21% (n.s.), corresponding to $4.2 \pm 3.0\%$ and $8.1 \pm 8.8\%$. After simultaneous intra/extravascular administration of diltiazem for 3 days neointimal thickening was increased (n.s.) by 24% ($12.8 \pm 11\%$). Incubation of POC-segments for 4, 5, 6, and 7 days resulted in a neointimal thickening of $9.1 \pm 6.3\%$, $4.0 \pm 5.0\%$, $4.0 \pm 4.5\%$, and $3.7 \pm 4.1\%$, corresponding to an inhibition (n.s.) by 12%, 61%, 61%, and 64%.

28 days after ballooning neointimal thickening of POC segments was significantly ($p < 0.05$) increased 2.6 fold from $10.5 \pm 11.0\%$ in untreated controls to $28.4 \pm 17.8\%$. No effect was found after incubation with diltiazem for 1 day. Incubation with diltiazem for 2 and 3 days resulted in an inhibition of neointimal thickening by 25% (n.s.) and 86% ($p < 0.05$), corresponding to $21.2 \pm 10.0\%$ and $4.1 \pm 5.9\%$. After Incubation of POC-segments for 4, 5, 6, and 7 days with diltiazem neointimal thickening was inhibited by 73%, 53%, 82% ($p < 0.05$) and 76% ($p < 0.05$), corresponding to $7.7 \pm 8.8\%$, $13.3 \pm 8.0\%$, $5.1 \pm 10.2\%$, $6.8 \pm 12.1\%$.

Identification of smooth muscle and endothelial cells

In the media of POC segments staining with antibodies against smooth muscle α -actin was always positive, no matter whether or not organ cultures were treated with ballooning or diltiazem or both. The number of smooth muscle α -actin positive cells in the media decreased with increased time in culture, only few POC-segments stained positive after 28 days in culture. In the neointima of POC segments staining against smooth muscle α -actin was negative after ballooning and negative after ballooning and EP diltiazem incubation.

Staining of POC-segments with antibodies against vWF was always positive in untreated controls, but decreased after ballooning, simultaneous intra/extravascular incubation with high dose diltiazem did not further decrease the number of cells with positive staining against vWF.

Discussion

Simultaneous intra/extravascular administration of antiproliferative agents is in a way the maximal strategy to inhibit all pathways that might have been activated after angioplasty. This ex vivo study demonstrates two major findings: 1) the POC-model is a suitable model to study this maximal antiproliferative strategy, and 2) the simultaneous intra/extravascular antiproliferative approach has to be maintained for 6 or 7 days, if a significant inhibition of neointimal thickening shall be achieved. This period of

time is almost identical with the peak of reactive cell proliferation in the POC-model [9].

Activation of SMC proliferation and migration after angioplasty via abundant stimulatory pathways is not restrained to inner layers of the vessel wall. Recently Ciezki et al. [12] have reviewed autopsy records of patients who received PTCA prior to death. The authors located sites of cellular proliferation by immunohistochemical staining and demonstrated that reactive cell proliferation after PTCA was found throughout the treated arterial section's axial plane. An anti-restenotic approach that is administered selectively from the intravascular surface of a dilated artery will inhibit mainly stimulatory pathways that activate SMC proliferation and migration in the inner layers of the artery wall but will fail to inhibit stimulatory pathways in the outer parts of the media and in the adventitia. This may explain why local antiproliferative strategies that are administered via selective endoluminal delivery systems as e.g. the Scimed Dispatch Coronary Infusion Catheter failed to inhibit experimental restenosis [13].

A new dimension of antiproliferative drug therapy has been opened by suggestions to use the pericardium as an perivascular drug depot [5-7]. Verrier reported of a successful transatrial access to the normal pericardial space in 6 dogs and 13 pigs [5], Laham et al. [6] demonstrated in 49 yorkshire pigs a safe subxyphoid access. First clinical experiences with the subxyphoid access have been reported lately by Macris et al. [7]. Although the pericardial approach is a very encouraging technique that will acquire its place in the treatment of restenosis within the next years it should be kept in mind that a strictly extravascular approach may produce the inverse situation that stimulatory pathways of reactive cell proliferation are inhibited in the adventitia and the more distal parts of the media but not in the neointima and the inner parts of the media. The simultaneous intra/extravascular administration of antiproliferative agents is likely to inhibit consequently all stimulatory pathways in the entire injured artery wall.

Coronary arteries of domestic pigs respond in a similar way to vascular injury as do human coronary arteries [8]. In order to reduce the number of indispensable animal studies we characterized in a previous study a model of restenosis with coronary organ cultures of domestic pigs obtained from a local slaughterhouse [9]. In these POCs the maximal reactive proliferation of SMC occurred at day 7 after ex vivo ballooning, the maximal neointimal thickening was detected at day 28. By adding of antiproliferative agents to the culture medium POCs progress to an elegant ex vivo model for studies of simultaneous intra/extravascular drug administration [4], because both intravascular and extravascular surfaces of the 5 mm cylindrical

organ culture segments have full contact with the agents administered to the culture medium.

In POCs reactive cell proliferation after ex vivo ballooning was reduced by more than 50% after simultaneous intra/extravascular administration of diltiazem for 4, 5, 6 or 7 days (n.s.), neointimal thickening was significantly inhibited after high dose diltiazem incubation for 6 or 7 days. A strong inhibitory effect of diltiazem is in accordance with prior reports of our laboratory that diltiazem in a concentration of 50 µg/mL significantly inhibited reactive cell migration and proliferation in various in vitro models of human coronary restenosis [10]. Clinical studies applying diltiazem as anti-restenotic agent after peroral administration have not been successful [14,15], with the exception of one study [16] that has only been reported as an abstract. Peak plasma levels of diltiazem are approximately 0.12 µg/mL after a 90 mg dose and 0.15 µg/mL after a 120 mg/dose [17], the administered dosage of diltiazem in these clinical studies varied between 90 mg twice a day [16] and 120 mg three times a day [15]. The negative results of systemically administered diltiazem in these clinical studies was not surprising, because plasma levels were settled more than two orders of magnitudes below the concentrations required for a significant anti-proliferative effect [10].

In accordance with prior reports of Koo and Gotlieb [18] and our laboratory [9] staining against smooth muscle α -actin in the media was constantly clearly positive. Simultaneous intra/extravascular incubation of POCs with diltiazem in a concentration of 50 µg/mL did not convert the α -actin structures of SMC in the media. These data are supported by the fact that diltiazem in concentration of 50 µg/mL did not alter the cytoskeletal structures of smooth muscle α -actin, vimentin, and α -tubulin structures in human coronary SMC in vitro [10]. Staining against smooth α -actin in the neointima was negative at day 7 and day 28. Kocher et al. [19] have suggested that one possible explanation for this phenomenon may be that actively proliferating SMC express a different F-actin phenotype from the α -isoform recognized by the smooth muscle α -actin antibody. This explanation is confirmed by the fact that reactive cell proliferation in the POC was very low in the media but increased in the neointima.

Staining of POC-segments with antibodies against vWF was always positive in untreated controls, but decreased after ballooning. This result is in accordance with the hypothesis that endothelial injury is an important stimulus for reactive cell proliferation after angioplasty. EC did not recover after being damaged during the ballooning, the number of vWF factor positive cells was identical 7 and 28 days after ballooning. The missing recovery of EC in POCs was probably predominantly caused by the fact that we

did not use EC-specific culture medium. Simultaneous intra/extravascular administration of high dose diltiazem for up to seven days did not further decrease the number of vWF positive cells. This may be substantial for a sufficient re-endothelialization of the inner vessel wall after angioplasty in vivo.

The uncertain correlation between data of in vitro and in vivo studies on one side and animal and human species on the other side is a fundamental problem of restenosis research. We have recently demonstrated in 3D human coronary in vitro model [20] that the positive effects of aspirin in early atherosclerosis and restenosis can only be achieved if a concentration of 5 mM is administered, more than 10-times the commonly administered oral dosage. Although the ex vivo POC-model is very close to the in vivo situation in the domestic pig [21], potential problems may arise by the absence of blood flow, blood pressure and shear stress, kidney or liver activities. Therefore the significant inhibition of neointimal hyperplasia in the POC-model after simultaneous intra/extravascular administration of 50 µg/mL of diltiazem has to be considered as an absolute minimum. It may be necessary to increase both the period of administration and the concentration of diltiazem in order to reproduce an antiproliferative effect in vivo.

Although the domestic pig mimics the human situation very closely major differences endure between porcine and human tissue. Lately our laboratory has demonstrated [22] that the maximal proliferative response after ex vivo ballooning in human organ cultures of renal arteries (HOCs) occurred at day 21 after ballooning, followed by a maximum of neointimal thickening at day 56. These POC/HOC species differences with a maximum of anti-proliferative activity at day 7/21 and a maximum of neointimal hyperplasia at day 28/56 will be of extreme importance if the time period of simultaneous intra/extravascular administration of diltiazem in humans shall be estimated in the future.

The ex vivo POC-model [9] has the unique advantage that it can be compared easily to an in vivo model of the same species, the domestic pig [21]. Both in the ex vivo POC-model [9] and the in vivo domestic pig model [21] the maximal reactive cell proliferation and the maximal neointimal thickening after vascular injury occurs at the same days: day 7 respectively day 28. It might be speculated that the similarities between the data of the ex vivo POC-model and the in vivo domestic pig model can be discovered as well between the ex vivo HOC-model and the situation of the clinical patient after angioplasty. The unknown in vivo situation of the clinical patient might therefore be calculated or at least approached by the three understood variables. If that hypothesis is confirmed by

other groups and by our own future research the combination of the in vivo domestic pig model with the ex vivo POC- and HOC-models may offer the closest approach to human coronary artery restenosis so far described.

The ex vivo/in vivo analogous conclusion can be applied to approach the reaction of human coronary arteries in the clinical patient after simultaneous intra/extravascular administration of high dose diltiazem. Data of the study presented would correspond to variable 1, data of variable 2 and 3 are not yet available. Therefore the following studies should be carried out in the near future: 1) simultaneous intra/extravascular administration of diltiazem in the domestic pig for a period of 7 days, detection of reactive cell proliferation and neointimal thickening at day 7 and day 28, and 2) simultaneous intra/extravascular administration of diltiazem in the ex vivo HOC-model for a period of 21 days and detection of reactive cell proliferation and neointimal thickening at day 21 and day 56.

Analogous conclusions based on the relation between ex vivo/in vivo domestic pig models and the ex vivo HOC-model have the potential to abridge porcine/human species differences. Due to the significant importance of this hypothesis we would like to encourage other groups in the field to focus human restenosis by applying ex vivo POC-[9] and HOC-models [22] in combination with the in vivo domestic pig model [21].

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