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Effects of local cytochalasin D delivery on smooth muscle cell migration and on collar-induced intimal hyperplasia in the rabbit carotid artery

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1 Smooth muscle cell (SMC) migration has been implicated in neointima formation after angioplasty. Therefore, we investigated whether cytochalasin D, a fungal metabolite that inhibits actin filament formation, suppressed SMC migration and collar-induced intimal hyperplasia in the rabbit carotid artery.

2 To establish effective concentrations, contractions of carotid artery rings to phenylephrine were determined after incubation with cytochalasin D (10^{-8}–10^{-6} M) for 30 min or 3 days. *In vitro* cell migration was studied using carotid artery explants and a modified Boyden chamber with SMCs isolated from the rabbit aorta. The *in vivo* effect was tested after infusion of 10^{-8}–10^{-4} M cytochalasin D into collars placed around the left carotid artery; collars placed around the right artery served as controls.

3 Contractions to phenylephrine decreased after 30 min or 3 days exposure to 10^{-7} and 10^{-6} M cytochalasin D; the effect was partly reversible. These concentrations also inhibited cellular outgrowth and SMC migration in the *in vitro* assays.

4 Immunohistochemistry showed that local delivery of 10^{-5} or 10^{-4} M cytochalasin D for 2 weeks suppressed collar-induced α-SMC actin expression in the intima by 68% and 84% respectively. However, the cross-sectional area of the intima was not reduced due to an influx of T-lymphocytes and macrophages.

5 It is concluded that cytochalasin D suppressed SMC contractility and migration *in vitro*. Although perivascular infusion of cytochalasin D inhibited collar-induced SMC migration from media to intima *in vivo* as well, the intimal hyperplasia was not reduced due to concomitant development of an inflammatory response.

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Abbreviations: ANOVA, one-way analysis of variance; DMEM, Dulbecco's modified Eagle's medium; E_{max}, maximal contraction; E_{min}, initial tension, FBS, foetal bovine serum; SMC, smooth muscle cell; TUNEL, terminal deoxynucleotide transferase-mediated deoxy-uridine-5′-triphosphate nick-end labelling; VCAM-1, vascular cell adhesion molecule-1

Introduction

Actin filaments form an important component of the cytoskeleton and are key molecules in the regulation of cell adhesion, spreading and shape and are critical for the regulation of various cell functions including muscular contraction and locomotion (Burridge & Chrzanowska, 1996; Tobacman, 1996; Lauffenburger & Horwitz, 1996; Schedlich *et al*., 1997). Migration of vascular smooth muscle cells (SMCs) is important in angiogenesis, remodelling of blood vessels and the formation of a physiological intima in conduit arteries (Diglio *et al*., 1989).

Cell migration has also been implicated in the process of late restenosis that occurs in 30–60% of cases following successful balloon angioplasty in patients with coronary disease (Bauters *et al*., 1996; Topol & Serruys, 1998). During balloon angioplasty, atherosclerotic plaque material is ruptured and the vessel wall damaged, leading to the exposure of medial SMCs to serum growth and chemotactic factors. In animal models of vessel injury, the exposure to these factors triggers vascular SMCs to migrate to the vessel lumen where they cause intimal thickening due to proliferation and deposition of large amounts of extracellular matrix (Strauss *et al*., 1994; Bauters *et al*., 1996). A plethora of mitotic and chemotactic factors is activated at the angioplasty sites and there is a redundancy of signalling mechanisms. This may explain why therapies with proven efficacy in neointima models have failed to limit restenosis in the clinic (Bult, 2000). Therefore we investigated whether a mitogen-independent inhibition of SMC migration might be achieved by targeting the cytoskeleton as a common step essential for cell locomotion.

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Cytochalasins are a group of fungal metabolites, which permeate cell membranes and cause cells to stop ruffling, stop translocating and round up (Schliwa, 1982). Functionally, cytochalasins inhibit microfilament function and polymerization by blocking actin monomer addition at the rapidly growing end of the actin filament (Cooper, 1987). In this study we first determined the effect of cytochalasin D on phenylephrine-induced contraction in the isolated rabbit carotid artery and on SMC migration *in vitro*. After having established the concentrations of cytochalasin D that decreased contractility and SMC migration, the effect of local, perivascular application of cytochalasin D on intimal hyperplasia was investigated in the rabbit carotid artery using the collar model.

Methods

Animals and isolation of carotid arteries

The ethical committee of the University of Antwerp approved the animal studies. Male New Zealand White rabbits (2.2–3.5 kg) were housed individually and fed standard rabbit chow. For the isolation of the carotid artery the animals were anaesthetized with sodium pentobarbital (30 mg kg^{-1} body weight, i.v.) and both carotid arteries were clamped, excised and placed in cold Krebs–Ringer solution. Thereafter the rabbits were sacrificed by a sodium pentobarbital overdose (60 mg kg^{-1}, i.v.).

Acute contraction studies

Segments (2 mm width) from naïve rabbit carotid arteries were mounted in isolated organ baths filled with 5 ml of Krebs–Ringer solution (37°C, continuously gassed with 95% O_2–5% CO_2) for force measurements at 6 g loading tension as described (Geerts *et al*., 1999). Tension was measured isometrically with a Statham UC_2 force transducer (Gould, Cleveland, U.S.A.) connected to a data acquisition system (Moise 3, EMKA Technologies, Paris, France). After an equilibration period of 45 min the segments were exposed to 50 mM KCl. After washout, cumulative concentration response curves of phenylephrine were constructed after 30 min incubation with 0, 10^{-8}, 10^{-7} or 10^{-6} M cytochalasin D ($n=7$). At the end of the experiment, the segments were either fixed in a 4% formaldehyde solution and embedded in paraffin for immunohistochemistry or snap-frozen in liquid nitrogen for microfilament staining.

To determine whether the effects of cytochalasin D were reversible responses to 50 mM KCl were measured before and immediately after 30 min incubation with 0 ($n=4$, time control) or 10^{-6} M ($n=4$) cytochalasin D. After washout (3×10 min) the segments were incubated in Krebs–Ringer solution without cytochalasin D for 30 min before measuring the contraction to 50 mM KCl. This procedure was repeated four times.

Organ culture of rabbit carotid artery segments

Carotid artery segments (2 mm width) from naïve rabbits were placed individually in 12 wells cell culture plates (Corning, Cambridge, MA, U.S.A.) containing 2 ml Dulbecco's modified Eagle's medium (DMEM, Gibco Ltd, Paisley, U.K.) and antibiotics (penicillin 100 u ml^{-1}, streptomycin 100 μg ml^{-1}, gentamycin 100 μg ml^{-1} and polymixin B sulphate 100 u ml^{-1}). Segments were separately supplemented with either 10^{-8} M ($n=6$), 10^{-7} M ($n=6$) or 10^{-6} M ($n=6$) cytochalasin D and placed in a humidified 5% CO_2 incubator at 37°C. Control segments ($n=6$) were incubated without cytochalasin D. After 3 days of incubation the vascular reactivity of the segments was determined as described above.

Evaluation of cellular outgrowth from rabbit carotid artery explants

Carotid artery segments (2 mm width) were incubated as described above, but the DMEM was supplemented with 10% heat inactivated foetal bovine serum (FBS, Gibco Ltd, Paisley, U.K.). Segments were separately exposed to 0 ($n=13$), 10^{-8} ($n=13$), 10^{-7} ($n=13$) or 10^{-6} ($n=14$) M cytochalasin D. After 10 days of incubation in a humidified 5% CO_2 incubator at 37°C the carotid segments were removed and the cells migrated from the explant were harvested by trypsinization (Fluka, Switzerland). The number of cells was determined after staining of the nuclei with propidium iodide (Sigma, St. Louis, MO, U.S.A.) and evaluated by flow cytometry (FACSort, Becton-Dickinson) (Vindelov & Christensen, 1990). In each experiment the number of nuclei detected for 5 min by flow cytometry was also expressed as percentage of the events counted in the control incubation. In a separate experiment, cells migrated out of carotid artery explants were grown on culture slides (Falcon, U.S.A.), fixed with either methanol for immunohistochemistry or with acetone for microfilament staining.

Migration of aortic SMCs

Migration of SMCs was assessed by a modified Boyden Chamber method using microchemotaxis chambers separated by a polycarbonate filter with 8 μm pore size (Transwell, Costar, Cambridge, MA, U.S.A.). Briefly, SMCs were isolated from explants of the aorta by collagenase and elastase digestion (Seye *et al*., 1997) and cultured in Ham F10 medium supplemented with 10% foetal bovine serum. More than 95% of cells in the primary culture were α-SMC actin positive. Cells between passage 3 and 5 were growth arrested for 48 h, harvested by trypsinization and centrifuged. The cells were resuspended (5×10^5 cells ml^{-1}) in Ham F10 (Gibco Ltd., Paisley, U.K.). Then 100 μl of the SMC suspension was placed in the upper chamber and 600 μl of Ham F10, containing 10% FBS and 50 ng ml^{-1} hepatocyte growth factor as chemoattractants, was placed in the lower chamber. Cytochalasin D (0, 10^{-8}, 10^{-7} or 10^{-6} M, $n=4$) was added to the lower chamber, and the chambers were incubated at 37°C in 5% CO_2 and 95% air for 6 h. Thereafter the cells attached to the upper side of the filter were removed with a cotton-wool swab; the filter was fixed in methanol and stained with haematoxylin. SMCs on the lower side were counted by light microscopy in four high-power magnification fields ($\times400$). After correction for spontaneous migration in the absence of FBS and hepatocyte growth factor, migration was expressed as a percentage of the mean number of migrated cells relative to control (0 M cytochalasin D).

Experimental model of intimal thickening

Male New Zealand white rabbits (2.5–3.5 kg) were anaesthetized with sodium pentobarbital (30 mg kg^{-1} i.v.) and both common carotid arteries were exposed surgically. Non-occlusive, biologically inert, flexible silicone collars (inlet/outlet diameter 1.8 mm, interior volume 134 mm^3, Silicone MED-4211, Nusil Technology, CA, U.S.A.) were placed around the carotid arteries and closed with silicone glue (Kockx *et al*., 1992). The interior of the collar around the left artery was connected to an osmotic minipump (Alzet, Charles River France) placed subdermally in the thoracic region. Pumps delivered cytochalasin D in different concentrations (10^{-8} M, $n=8$; 10^{-7} M, $n=8$; 10^{-6} M, $n=8$; 10^{-5} M, $n=4$; 10^{-4} M, $n=4$). Collars placed around the right carotid artery were not connected to a pump and served as internal controls. The experiment and the treatment lasted for 14 days. In a second study additional groups of animals received 10^{-4} M cytochalasin D for 3 ($n=3$), 5 ($n=3$) or 7 ($n=3$) days after collar placement. At the end of the infusion period rabbits were anaesthetized with sodium pentobarbital (30 mg kg^{-1} body weight, i.v.) and both carotid arteries were excised. Two segments were taken from the middle part of the collared section, and two segments from the region proximal to the collar. From each pair one ring was fixed in 4% formaldehyde and embedded in paraffin for determination of intimal hyperplasia and immunohistochemistry, the other ring was snap frozen in liquid nitrogen for microfilament staining. Histological evaluation of potential hepatotoxicity was performed on formaldehyde-fixed haematoxylin-eosin stained liver sections.

Immunohistochemistry and histology

Transverse paraffin sections (5 μm), cryostat sections (6 μm, vascular cell adhesion molecule-1, VCAM-1) or methanol fixed cells on culture slides were stained with monoclonal antibodies directed against rabbit macrophages (RAM-11, 1 : 1000), vimentin (1 : 10000) (Dako, Glostrup, Denmark), SMCs and myofibroblasts (α-smooth muscle actin, 1A4, 1 : 2700, Sigma, St-Louis, MO, U.S.A.), contractile SMCs (smoothelin, 1 : 300, Monosan 2094, Sanbio, Uden, The Netherlands) (van der Loop *et al*., 1996), desmin (1 : 1000, Organon Teknika-Cappel, Durham, NC, U.S.A.), Ki-67 (MIB-1, 1 : 500, Immunotech, Marseille, France), rabbit T-lymphocytes (CD43, L11/35, 1 : 300, Serotec, Oxford, U.K.) or rabbit VCAM-1 (Rb1/9, 1 : 300, gift of Dr M. Cybulski). After inactivation of endogenous peroxidase activity with 3% H_2O_2, primary antibodies against vimentin and α-SM actin were detected with a goat anti-mouse-peroxidase antibody (Jackson, West Grave, PA, U.S.A.) (Kockx *et al*., 1992; 1998), the other antibodies with the ABC technique (avidin biotinylated horseradish peroxidase macromolecular complex, Vectastain ABC kit, Vector Laboratories) as described (Matthys *et al*., 1997; Crauwels *et al*., 2000). For detection of the complex 0.1% H_2O_2 was used as substrate and 3,3′ diaminobenzidine (Sigma, St. Louis, MO, U.S.A.) as the chromogen and the sections were counterstained with haematoxylin (Merck, Darmstadt, Germany). Controls from which the primary antibody had been omitted were run for each protocol. These consistently gave negative observations. Detection of actin filaments was performed by staining cryostat sections (6 μm) or acetone fixed cells (outgrowth) with Oregon green 488-phalloidin (Molecular Probes, Leiden, the Netherlands).

Apoptosis was detected using the terminal deoxynucleotide transferase-mediated deoxy-uridine-5′-triphosphate nick-end labelling (TUNEL) assay (ApopTag kit, Oncor) as described (Kockx *et al*., 1998). After deparaffinization and rehydration sections were incubated with 3% citric acid to remove calcium-containing vesicles causing non-specific nucleotide binding. Moreover the proteinase K step was omitted to avoid non-specific TUNEL labelling due to RNA synthesis/splicing. The labelled antibody was visualized using 3-amino-9-ethyl carbazole as chromogen. Sections were lightly counterstained with haematoxylin and mounted in glycerine-jelly. Negative controls included omission of terminal deoxynucleotide transferase or the Klenow fragment.

The cross-sectional area of intima and media was measured on elastin (Verhoeff–van Giesson) stained cross-sections using computer-assisted planimetry. For the quantification of the immunoreactive areas of α-smooth muscle actin, RAM11 and CD43 the sections were superimposed with an unbiased counting frame with a set of regularly spaced points. The points hitting immunoreactive structures were counted and expressed as percentage of the total number of points in the intima or the media (Gundersen *et al*., 1988). The number of TUNEL and Ki-67 positive nuclei in intima and media was counted and expressed as percentage of the total number of nuclei in each structure. In two sections VCAM-1 expression was scored at eight randomly selected sites along the perimeter of the lumen. The number of positive hits was divided by eight to estimate the percentage of VCAM-1 expressing endothelial cells; the average of both sections was used for further analysis.

Data analysis

Data are expressed as mean ± s.e.mean throughout, and n = number of animals or cell lines. The concentration of phenylephrine producing a half maximal response (EC_{50}) was determined by fitting the individual dose response curves to a four parameter logistic function using Graphpad Prism version 3.0 (Graphpad Software, San Diego, CA, U.S.A.) with the equation:

$$E = E_{min} + (E_{max} - E_{min})(1 + 10\hat{}[(\log EC_{50} - \log A)n_H])^{-1} \quad (1)$$

where E is the contraction (g), E_{min} the initial tension, E_{max} the maximal contraction, A the phenylephrine concentration and n_H the Hill slope. The phenylephrine-induced contractions were expressed as percentage of the contraction induced by 50 mM KCl. The *in vitro* data (E_{max}, $-\log EC_{50}$, cellular outgrowth and migration assay) were evaluated by means of a one-way analysis of variance (ANOVA) and Dunnett's multiple comparison *post-hoc* test. In the *in vivo* experiments the treated artery was compared to the contralateral, collared artery using Student's *t*-test, unless variances of the groups were unequal. In the latter situation the non-parametric Wilcoxon's test was used. Statistical significance was assumed at $P<0.05$.

Drugs

Phenylephrine was from Sigma (St. Louis, MO, U.S.A.), cytochalasin D from Alexis (San Diego, CA, U.S.A.). The Krebs-Ringer physiological salt solution contained (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $KHPO_4$ 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, CaEDTA 0.025 and glucose 11.1. The 50 mM KCl solution was prepared by equimolar replacement of sodium by potassium in the physiological salt solution.

Results

Contractile responses of the isolated rabbit carotid artery

The E_{max} of phenylephrine decreased by about 16% and 80% after 30 min incubation with 10^{-7} M and 10^{-6} M cytochalasin D respectively (Figure 1A). The sensitivity to phenylephrine ($-\log EC_{50} = pD_2$) was not influenced by cytochalasin D at any concentration (Table 1). The contractile force showed a partial recovery after repeated washing. In the presence of 10^{-6} M cytochalasin the contraction induced by 50 mM KCl was $32 \pm 1\%$ of the preceding control response (4.1 ± 0.05 g, $n=4$). After repeated washing the force increased to $64 \pm 2\%$, $78 \pm 3\%$, $81 \pm 3\%$ and $82 \pm 4\%$, respectively at 1, 2, 3 and 4 h ($n=4$).

Generally, the same results were obtained after 3 days of exposure ('chronic') to cytochalasin D (Figure 1B). Force development was smaller in control segments, but a similar 70% decrease was also seen for 50 mM KCl: 4.0 ± 0.9 g ($n=6$) *vs.* 5.6 ± 1.3 g ($n=7$) in freshly isolated segments. Though the effect of 10^{-8} M cytochalasin D seemed slightly more pronounced, the E_{max} was not influenced (-6.6%, $p>0.05$) but force development decreased after 3 days exposure to 10^{-7} M (-20%) or 10^{-6} M cytochalasin D (-52%). In contrast to the acute experiment, the sensitivity to phenylephrine declined after 3 days incubation with 10^{-6} M cytochalasin D, but not at the lower concentrations (Table 1). Cytochalasin D did not change the expression pattern of α-smooth muscle actin, vimentin, desmin and Oregon green 488-phalloidin in the vascular segments in either acute or 'chronic' experiment (data not shown).

Cellular outgrowth and migration assay

Cellular outgrowth from carotid artery explants could be detected microscopically after approximately 4 days. At 10 days the cells had almost reached confluence. Therefore, all experiments were terminated after 10 days of organ culture. Incubation with 10^{-7} or 10^{-6} M cytochalasin D decreased the cellular outgrowth by respectively $69 \pm 4\%$ or $99.7 \pm 0.13\%$ (Figure 2). Outgrowth was not influenced by 10^{-8} M cytochalasin D ($-6.2 \pm 6.5\%$). Cells treated with cytochalasin D were viable as shown by the exclusion of 0.4% Trypan blue. Furthermore there were neither changes in the frequencies of Ki-67 or TUNEL labelling, nor in the staining patterns of α-smooth muscle actin, vimentin, desmin or Oregon green 488-phalloidin at any concentration of cytochalasin D (data not shown). The latter data indicate that cells that do migrate possess intact actin microfilaments.

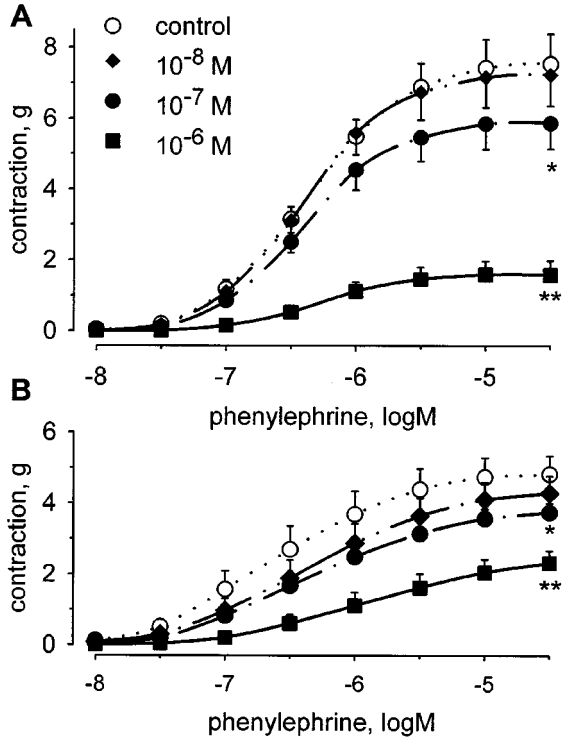

Figure 1 Phenylephrine contractions in segments of the rabbit carotid artery after *in vitro* exposure to cytochalasin for 30 min (A, acute) or 3 days (B, chronic). Data represent mean ± s.e.mean, $n=6-7$. $^*P<0.05$, $^{**}P<0.01$, different from control (0 M), Dunnett's test.

Table 1 Effect of cytochalasin D on the sensitivity of the rabbit carotid artery to phenlyephrine after *in vitro* exposure to cytochalasin for 30 min (acute) or 3 days (chronic)

Cytochalasin D (M)	*Acute* $-log\ (EC_{50})$	n	*Chronic* $-log\ (EC_{50})$	n
0	6.44 ± 0.10	7	6.56 ± 0.17	6
10^{-8}	6.46 ± 0.08	6	6.35 ± 0.16	6
10^{-7}	6.43 ± 0.04	7	6.36 ± 0.18	6
10^{-6}	6.33 ± 0.10	7	$5.89 \pm 0.20^*$	6

Data represent mean ± s.e.mean. *Significantly different from 0 M cytochalasin D: $P<0.05$, ANOVA, Dunnett's test.

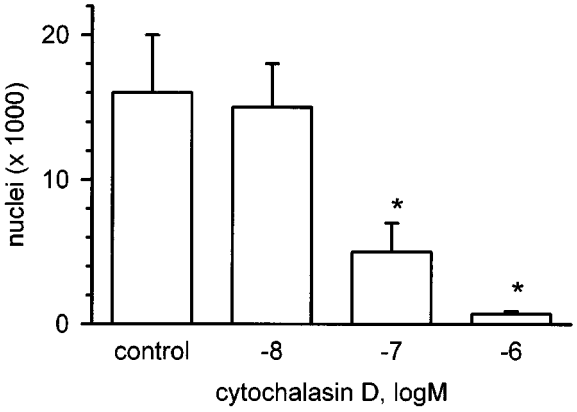

Figure 2 Cytochalasin D inhibits cellular outgrowth from rabbit carotid artery segments in culture. $^*P<0.05$, different from control (0 M), Dunnett's test.

In the microchemotaxis chambers 10^{-6} M cytochalasin D prevented SMC migration completely ($P<0.01$), whereas 10^{-7} M caused inhibition and 10^{-8} M was without effect (Table 2).

In vivo *effects of cytochalasin D*

To investigate whether cytochalasin D administered by perivascular infusion had reached the inner media on day 14, the organization of actin filaments was examined upon staining with Oregon green 488-phalloidin. Disruptions of the actin filaments of the medial SMCs were evident in segments treated with 10^{-5} M or 10^{-4} M cytochalasin D (Figure 3B), but not at lower concentrations. In segments treated with 10^{-5} M and 10^{-4} M cytochalasin D α-smooth muscle actin and smoothelin positive cells could still be detected in the media (Figure 3D,F), but the staining pattern of both SMC markers was different from the contralateral controls (Figure 3C,E).

In the central segments the collar evoked intimal thickening on day 14 (Figure 3, Table 3). This thickening constisted of α-SMC actin and smoothelin-expressing cells under a continuous layer of endothelial cells in control segments (Figure 3C,E). In contrast, the myofibroblasts in the perivascular granulation tissue stained for α-SMC actin, but not for smoothelin. The expression of α-SMC actin and smoothelin (Figure 3D,F) was strongly suppressed in the intima of segments treated with 10^{-5} or 10^{-4} M cytochalasin D. The α-SMC actin reduction was 69 and 84% respectively in comparison to the contralateral control (Figure 4). Lower concentrations (10^{-8} to 10^{-6} M) were without effect on the expression of α-SMC actin (Figure 4) or smoothelin (results not shown) in the intima.

Cytochalasin D infusion (10^{-8} to 10^{-6} M) did not influence the cross sectional areas of intima or media (Table 3), but both media and intima increased after exposure to the higher concentrations (10^{-5} M, 10^{-4} M). The intima–media ratio did not change at any cytochalasin D concentration. However, an increase was noted in comparison to the contralateral control when 10^{-5} M and 10^{-4} M cytochalasin D were combined ($P<0.05$, $n=8$, Student's t-test). The intima had however, a very different cellular composition and both T-lymphocytes (CD43) and macrophages (RAM11) were very abundant. These cell types were absent from the contralateral controls (Figure 3G–J). Quantification of the immunoreactive areas confirmed that the intima of control arteries was composed of α-SMC actin expressing cells, whereas the intima of segments treated with 10^{-5} or 10^{-4} M cytochalasin D consisted mainly of T-lymphocytes (CD43) and macrophages (RAM11, Figure 5). Interestingly, the influx of lymphocytes and macrophages was largely confined to the intima. Relatively few CD43 or RAM11 immunoreactive cells were found in the media, and these were confined to the inner layers (Figure 3H,J). The leukocyte influx in the intima was seen as early as 3 days after infusion of 10^{-4} M cytochalasin D and still progressed on day 5 (Figure 3I,J) and day 7 after collar implantation. In contrast, the intima in the contralateral control (collar only) developed from day 3 onward and consisted purely of α-smooth muscle actin positive cells without signs of inflammatory cells.

Table 2 Influence of cytochalasin D on SMC migration in a microchemotaxis chamber

Cytochalasin D (M)	*Cells (1 field)*	*%*
0	25±6	100
10^{-8}	25±7	101±10
10^{-7}	20±6	77±8*
10^{-6}	0	0*

Mean±s.e.mean, $n=4$. *Different from 0 M, $P<0.05$.

The leukocyte infiltration was associated with increased expression of VCAM-1 by the endothelial cells. The percentage of VCAM-1 expressing endothelial cells was low (4±2%, $n=24$) on day 14 after collar placement and not influenced by low cytochalasin D concentrations (6±4, $n=24$, $10^{-8}-10^{-6}$ M combined). However VCAM-1 expression was raised in segments exposed to 10^{-5} M (84±10%, $n=4$) or 10^{-4} M (71±6%, $n=4$) cytochalasin D when compared to the contralateral controls (0±0%, $n=4$ and 20±14%, $n=4$ respectively, $P<0.05$, Wilcoxon test).

Perivascular cytochalasin D infusion did not affect the number of nuclei in cross sections of the media as compared to the control (Table 4). Due to the inflammatory infiltrate the nuclear count increased in the intima. The effect was statistically significant at 10^{-4} M cytochalasin D. Expression of the cell replication marker Ki-67 was low in both intima and media of control arteries, confirming earlier observations on day 14 (Kockx *et al*., 1992). In segments treated with 10^{-5} or 10^{-4} M cytochalasin D the frequency of Ki-67 expressing nuclei was augmented in the media and particularly in the intima. Stringent TUNEL labelling of the cross sections showed that apoptosis was rare in media and intima of control segments and that it was not influenced by cytochalasin D (Table 4).

Evaluation of segments taken proximally from the collared sites showed no effect of cytochalasin D infusion on any parameter (data not shown). This indicated that its effects were confined exclusively to the segment enclosed by the collar, irrespective of the concentration of cytochalasin D that was used. Finally, microscopic inspection of the liver by an experienced pathologist, who was unaware of the treatments, did not reveal histological or cytological abnormalities in any of the groups on day 14.

Discussion

The major findings in this study are that cytochalasin D: (i) impaired phenylephrine induced contractions, (ii) inhibited *in vitro* SMC migration, (iii) suppressed collar-induced SMC migration *in vivo*, (iv) without reducing intimal thickening. The study of the rabbit vessels confirmed previous results in which contractions of isolated rat and bovine arteries were attenuated by cytochalasins without influencing the sensitivity to the agonist (Adler *et al*., 1983; Tseng *et al*., 1997). The force measurements were mainly used as a quick bioassay to determine biologically effective concentrations of cytochalasin D. The concentration-effect relationship was rather steep. The arteries gradually regained the capacity to constrict after repeated washing. This indicated that the effect of cytochalasin D was partly reversible. Upon prolonged exposure (3 days) the efficacy of cytochalasin D was not raised.

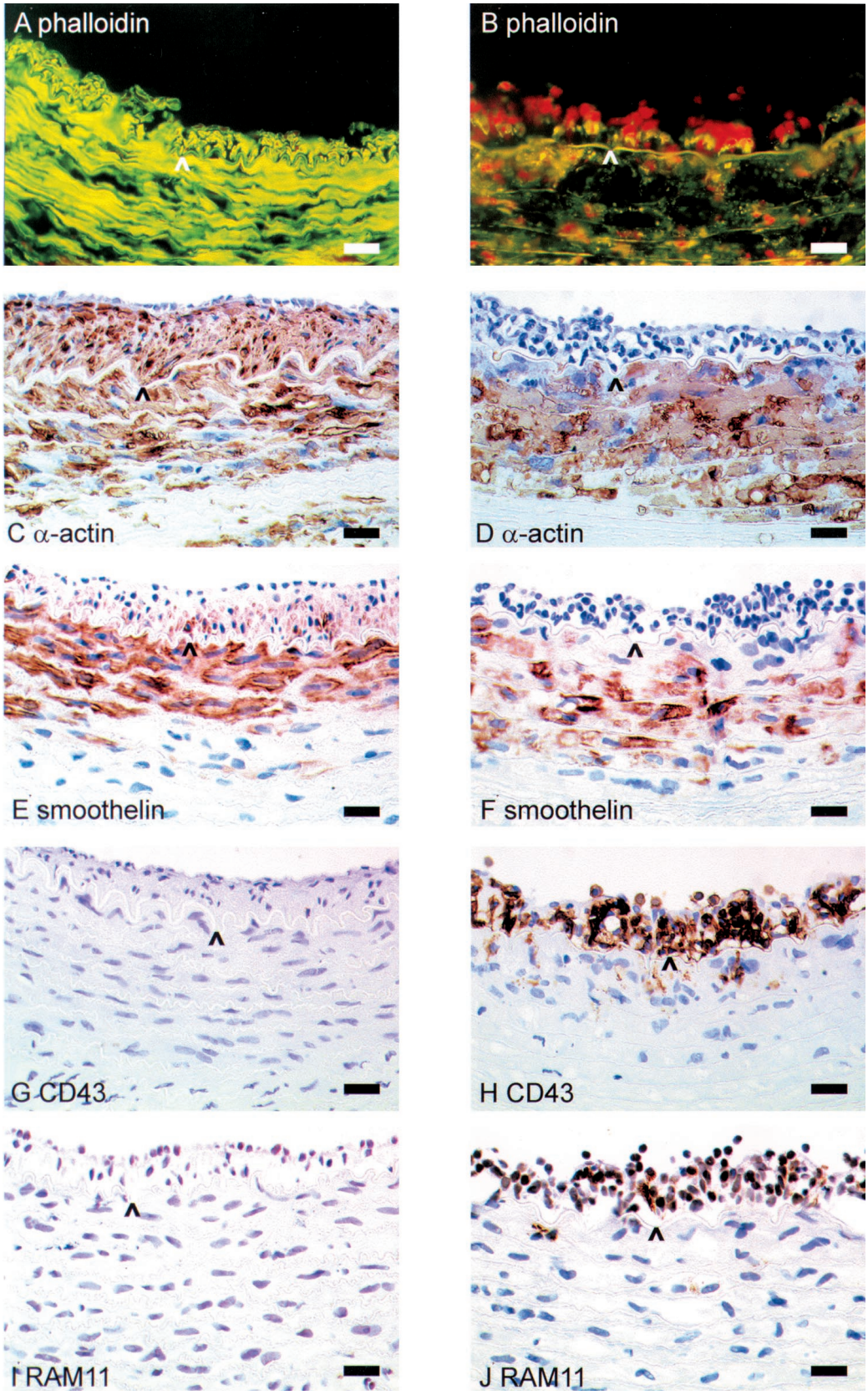

Figure 3 Photomicrographs of cross-sections of rabbit carotid arteries infused for 14 days (B, D, F, H) or 5 days (J) with 10^{-4} M cytochalasin D (right panels) and their contra-lateral controls (A, C, E, G, I, left panels). Phalloidin: Oregon green 488-phalloidin; α-actin: α-smooth muscle actin; RAM11: macrophage marker; CD43 T-lymphocyte marker. ∧: internal elastic lamina. Bar = 10 μm.

Table 3 Effect of perivascular cytochalasin D infusion (treated) on cross-sectional areas of intima and media of rabbit carotid arteries surrounded by a collar for 14 days

		Intima		*Media*		*Intima Media*$^{-1}$	
Cytochalasin D (M)	n	*Control* (mm^2)	*Treated* (mm^2)	*Control* (mm^2)	*Treated* (mm^2)	*Control* (ratio)	*Treated* (ratio)
10^{-8}	8	0.08±0.02	0.09±0.03	0.60±0.03	0.58±0.04	0.13±0.03	0.17±0.07
10^{-7}	8	0.09±0.03	0.11±0.03	0.63±0.02	0.60±0.03	0.14±0.04	0.18±0.04
10^{-6}	8	0.10±0.02	0.15±0.04	0.54±0.02	0.60±0.03	0.19±0.03	0.25±0.06
10^{-5}	4	0.12±0.04	0.34±0.14	0.65±0.02	0.79±0.06	0.20±0.06	0.40±0.14
10^{-4}	4	0.10±0.02	0.29±0.08*	0.52±0.04	0.70±0.05*	0.20±0.03	0.40±0.08

Data represent mean±s.e.mean. Control: contralateral artery not connected to an osmotic minipump. *Different from control: $P<0.05$, Student's *t*-test.

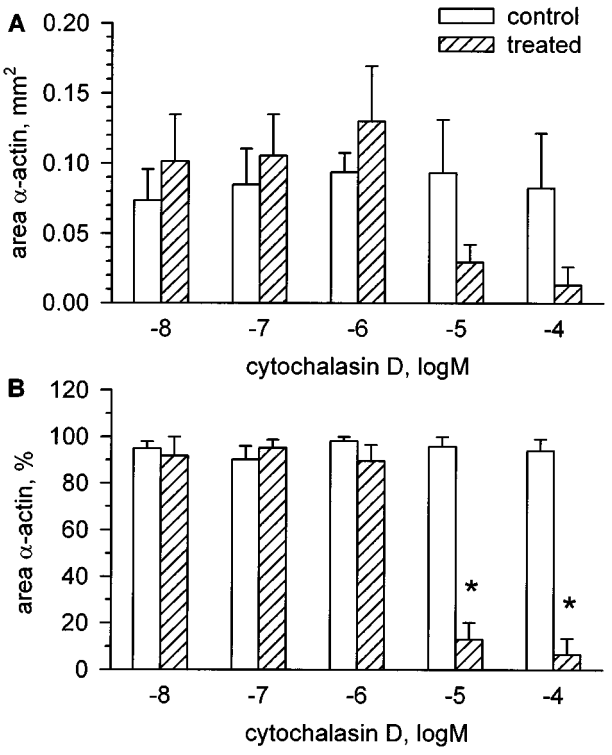

Figure 4 Effect of local cytochalasin D infusion for 14 days on absolute (A) and relative (B) α-smooth muscle actin immunoreactive areas in the intima. Data represent mean±s.e.mean, $n=8$ (10^{-8}–10^{-6} M) or $n=4$ (10^{-5}–10^{-4} M). Control: contralateral artery with a collar not connected to an osmotic minipump. * Different from control: $P<0.05$, Student's *t*-test.

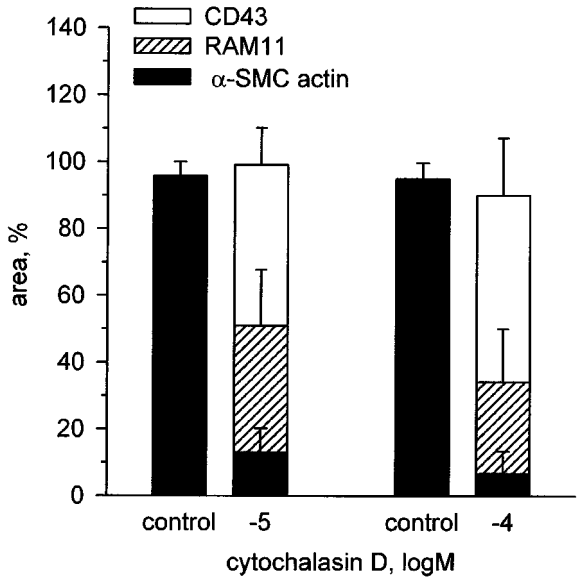

Figure 5 Effect of local cytochalasin D infusion for 14 days on α-smooth muscle actin, RAM11 and CD43 immunoreactive areas in the intima of rabbit carotid arteries. Data represent mean±s.e.mean, $n=4$. Control: contralateral artery with a collar not connected to an osmotic minipump.

The same concentrations of cytochalasin D which impaired vessel contractility also decreased (10^{-7} M) and even abolished (10^{-6} M) cellular outgrowth from explants of the rabbit carotid artery stimulated with 10% FBS. Evidence for the importance of actin in cell migration originally came from studies in living cells where fluorescently labelled actin molecules were microinjected into migrating fibroblasts (Wang, 1984). The labelled actin was found to be incorporated into the filaments at the leading edge of the cell. We were, however, not certain that the decreased outgrowth of cells seen after cytochalasin D incubation was entirely due to impaired migration. Dihydrocytochalasin B, a related drug that interferes with actin assembly, retains the machinery for cell cleavage for up to 1 h into G_1 and cleavage cannot proceed. This is illustrated by the retention of a 'post mitotic' spindle and a telophase disc, a structure templated by the mitotic spindle in anaphase that may determine the position and timing of the cleavage furrow (Martineau *et al*., 1995). In other words cytochalasins may cause mammalian cells to fail in cell proliferation (Martineau *et al*., 1995; Aubin *et al*., 1981) and this could explain the decreased cellular outgrowth from the vessel segments. However, no differences in the expression of the proliferation marker Ki-67 were seen after cytochalasin D incubation as compared to controls in this *in vitro* experiment, and increased Ki-67 labelling rather than a decrease was noted in the *in vivo* study.

In addition, increased apoptosis could explain the decreased cell numbers found after stimulating cellular outgrowth in the presence of cytochalasin D. An active involvement of the cytoskeleton in the apoptotic process has been suggested by several studies. A special role has been proposed for the monomeric form, G-actin. G-actin binds to and almost completely inhibits the nucleolytic activity of DNase I (Kreuder *et al*., 1984), the endonuclease possibly involved in DNA degradation during apoptosis (Peitsch *et*

Table 4 Effect of perivascular cytochalasin D infusion on nuclear count, K_i-67 and TUNEL reactive nuclei in media and intima

	Cytochalasin D (M)	*Media*		*Intima*	
		Control	*Treated*	*Control*	*Treated*
Nuclei $\times 10^3$	10^{-5}	0.96±0.03	1.13±0.10	0.75±0.19	1.43±0.31
	10^{-4}	0.77±0.07	1.72±0.08	0.78±0.14	1.54±0.34*
Ki-67, %	10^{-5}	0.9±0.2	2.9±1.2	0.3±0.2	11.1±2.7*
	10^{-4}	0.7±0.3	5.8±1.5*	0.8±0.5	11.2±1.1*
TUNEL, %	10^{-5}	0.10±0.04	0.20±0.10	0.02±0.02	0.15±0.05
	10^{-4}	0±0	0.04±0.04	0±0	0.03±0.03

Data represent mean±s.e.mean, $n=4$. *Different from contralateral control, $P<0.05$, Wilcoxon test.

al., 1993; Polzar *et al*., 1994). G-actin has also been shown to be a substrate for the proapoptotic cysteine protease interleukin-β-converting enzyme (Kayalar *et al*., 1996) and other caspases (Mashima *et al*., 1995). Caspase-cleaved G-actin can no longer inhibit DNase I activity and apoptosis becomes eminent (Kayalar *et al*., 1996). However, no signs of increased apoptosis (TUNEL-reactivity) were found in either *in vitro* or *in vivo* experiments.

A drawback of the outgrowth assay as a model for cell migration is the heterologous nature of the cell populations. Although the cells migrating from the explants stained for α-smooth muscle actin, they could be myofibroblasts (Shi *et al*., 1996) as well as SMCs. Therefore, we performed a migration assay with a cell culture consisting solely of rabbit aortic SMCs. These experiments gave identical results: no migration at 10^{-6} M, decreased migration at 10^{-7} M whereas 10^{-8} M cytochalasin D was without effect. Since the time window (6 h) of this assay was not sufficient for the completion of cell replication or apoptosis, this provides more direct proof that the effect of cytochalasin D on SMCs is solely on migration. To our knowledge this is the first study describing that SMC migration *in vitro* is impaired after cytochalasin D incubation.

To determine the *in vivo* effect of cytochalasin D on SMC migration, collar-induced intimal thickening was studied in the rabbit carotid artery. As early as 2 days after collar placement SMCs start migrating from the media to the intima (Kockx *et al*., 1992). This model has the advantage that local application of substances at the site of intima formation can be achieved by connecting the interior of the collar to an osmotic minipump (Matthys *et al*., 1997; Crauwels *et al*., 2000), thereby reducing systemic influences, such as potential hepatotoxic effects of cytochalasins. Perivascular infusion of cytochalasin D up to 10^{-6} M did not affect SMC migration or intima formation, but there were also no indications of actin filament disruption upon staining with Oregon green 488-phalloidin. Therefore, we concluded that the concentration of cytochalasin D in the arterial wall was insufficient. This can be explained by dilution of the drug in the perivascular space inside the collar and by the marked rise of transmural flow in response to collar placement (Coleman *et al*., 1993; Bosmans *et al*., 1997). In addition to causing further dilution, the latter factor will also oppose its diffusional transfer across the arterial wall. In view of this the concentration of cytochalasin D was raised (10^{-5} M and 10^{-4} M). Cytochalasin D then clearly reached the inner media, as indicated by the disruption of the filamentous-actin outline (phalloidin staining) and the changed staining patterns for α-smooth muscle actin and smoothelin. The latter findings could suggest that the epitopes recognized by the antibodies were still present but that their distribution was different due to the severing action of cytochalasin D on actin filaments. At those concentrations cytochalasin D effectively inhibited SMC migration, since cells staining for α-smooth muscle actin or smoothelin, a cytoskeletal protein specific for contractile SMCs (van der Loop *et al*., 1996), were virtually absent from the intima. As cell proliferation (Ki-67) was not inhibited by cytochalasin D and apoptosis (TUNEL) was not influenced, these data collectively indicated that cytochalasin D prevented *in vivo* SMC migration towards the intima. The concentration-effect relationship appeared to be steep, an unexplained finding that was also seen our *in vitro* studies.

In spite of the reduced SMC migration, the cross-sectional area and cell number of the intima increased rather than decreased. The explanation was that the high cytochalasin D concentrations elicited an inflammatory response. Indeed, the intima of those segments consisted predominantly of T-lymphocytes and macrophages. Leukocytes are rarely seen after collar placement, in contrast to rabbit models employing more extensive injury, such as balloon denudation or angioplasty (Kling *et al*., 1995; Golino *et al*., 1997; Welt *et al*., 2000; Miller *et al*., 2001; Bosmans *et al*., 1997). Immediately after collar placement neutrophils invade the media (Kockx *et al*., 1992), but this brief, transient influx has little impact on intimal hyperplasia. Indeed, prevention of the neutrophil extravasation by the administration of a monoclonal antibody against the adhesion molecule CD11/CD18 did not reduce collar-induced intimal thickening (van put *et al*., 1998) in contrast to models with more pronounced vascular injury (Kling *et al*., 1995; Golino *et al*., 1997; Welt *et al*., 2000). However, perivascular application of oxidized low density lipoproteins (Matthys *et al*., 1997) or advanced glycation end products (Crauwels *et al*., 2000) elicits large numbers of T-cells and macrophages to the collared segment. In accordance with other models (Kling *et al*., 1995; Golino *et al*., 1997; Welt *et al*., 2000; Miller *et al*., 2001), this marked leukocyte infiltrate caused adverse effects by promoting intimal hyperplasia and collagen deposition (Crauwels *et al*., 2000; Matthys *et al*., 1997).

There was, however, a major difference with those previous experiments, in which the outer media and adventitia contained a dense infiltrate of T-cells and macrophages (Matthys *et al*., 1997; Crauwels *et al*., 2000). In striking contrast, the deeper layers of the media and the adventitia remained free of leukocytes during cytochalasin D treatment (Figure 3b, J). This suggests that the drug caused paralysis of the leukocytes that were infiltrating the vessel wall from the

lumen. An indication for augmented cellular influx from the blood was the increased endothelial VCAM-1 expression after infusion of 10^{-5} M and 10^{-4} M cytochalasin D. On day 14 VCAM-1 expression was infrequent in control segments or upon infusion of lower concentrations of cytochalasin D. From our experiments it is not clear whether cytochalasin D raised endothelial VCAM-1 expression directly, or whether it was the consequence of cytokines released from SMCs in the media. The abundance of T-cells and macrophages could also account for the increased labelling with the proliferation marker Ki-67. Furthermore, oedema formation may form another explanation for the increased intimal and medial areas in addition to the increased cellularity.

The leukocytes could indirectly influence the apparent SMC accumulation in the intima, e.g. by suppressing α-actin expression. However, the quantification of the immunoreactive areas in the intima did not show evidence for a loss of α-actin expression in the SMCs, since T-cells and macrophages accounted for the α-actin negative cells. In addition, it has been reported that interferon-γ secreted by T-lymphocytes may inhibit SMC migration and proliferation (Tellides *et al*., 2000; Hansson, 1993). Yet, in previous studies the accumulation of α-smooth muscle actin positive cells in the intima was not reduced at all when large numbers of T-cells and macrophages infiltrated intima and media (Kling *et al*., 1995; Matthys *et al*., 1997; Crauwels *et al*., 2000). Those data indicate that the mere presence of intramural leukocytes is not sufficient to reduce the number of α-smooth muscle actin expressing cells. Though this does not exclude possible effects of leukocyte-derived cytokines, both *in vitro* assays clearly documented a direct, leukocyte-independent suppression of SMC migration by cytochalasin D.

In a randomized clinical trial different concentrations of cytochalasin B and placebo were administered to the site of balloon angioplasty using a microporous local delivery infusion balloon (Lehmann *et al*., 2000). The minimal stenosis diameter after the procedure was slightly better in the active drug group, but the difference was not seen at 4 to 6 weeks. The inhibition of the transient vasoconstriction immediately after balloon angioplasty reported by Lehman *et al*. (2000) is in line with the reduced constrictor capacity of cytochalasin D-treated arteries seen in the present study. This small trial ($n=43$) was not powered to detect differences in clinical outcomes. Yet, the 47% reduction in the combined primary end point (death, non fatal infarction or repeat vascularization) and the 19% reduction in clinical restenosis observed with cytochalasin B showed favourable tendencies.

The potential anti-restenotic activity of other drugs that interfere with SMC migration and replication are currently explored. The microtubule network constitutes another important component of the cytoskeleton. The antineoplastic compound paclitaxel (Taxol) causes an increased assembly of extraordinarily stable microtubules, resulting in reduced proliferation, migration and signal transduction in SMCs *in vitro* (Axel *et al*., 1997). Local delivery of paclitaxel to the rabbit carotid artery by use of microporous balloons resulted in reduced neointima formation and enlargement in vessel size after balloon angioplasty (Axel *et al*., 1997; Herdeg *et al*., 2000). Sirolimus (rapamycin), a macrolide antibiotic known to prevent allograft rejection, also inhibits SMC migration, in addition to being a potent immunosuppressant and anti-proliferative agent (Poon *et al*., 1996). Systemic administration of sirolimus has been shown to produce significant reductions in neointimal area and thickness of pig coronary and carotid arteries subjected to balloon angioplasty when compared with vehicle-treated animals (Burke *et al*., 1999; Gallo *et al*., 1999). Furthermore, sirolimus-eluting stents caused only minimal intimal hyperplasia and in-stent restenosis was not observed in a small clinical trial (Sousa *et al*., 2001), but it is unclear to which extent SMC paralysis contributed to these beneficial tendencies.

In conclusion, perivascular application of cytochalasin D inhibited collar-induced SMC migration, but failed to suppress the size of the intima due to the concurrent development of an inflammatory response. Further studies of cytochalasin D in models of balloon angioplasty, using intra-luminal delivery by means of porous balloons or drug-eluting stents, are required to test whether smooth muscle paralysis is a potential approach against restenosis.

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