Journal of Cellular Biochemistry 77:213–220 (2000)

Inhibition of Neointima Formation by a Nonpeptide $\alpha_v\beta_3$ Integrin Receptor Antagonist in a Rabbit Cuff Model

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Abstract This study was performed to determine whether a highly selective nonpeptide $\alpha_v\beta_3$ antagonist (SH306) would prove effective in inhibiting neointima formation in a rabbit cuff model. The animals were dosed with SH306, 5 mg/kg i.v., followed by 10 mg/kg s.c., 3 times daily for 3 days, or with vehicle (10% DMAC). Rabbits were sacrificed and perfused on days 1, 3, and 21; the vessels were paraffin embedded. A reduction in the intima/media (I/M) of the SH306-treated rabbits, as compared with the vehicle-treated control group, was noted (0.20 vs 0.36 [n = 4]). A significant increase in the area of the media was observed in the SH306-treated group versus the control group (0.20 vs 0.13). No difference was observed in cell proliferation between SH306 and vehicle after 1-day and 3-day dosing. Thrombi were found in 43% of the control vessels and in only 14% of the drug-treated vessels. No anticoagulant was used during the surgical procedure. No increase in inhibition of GPIIb/IIIa was observed in SH306-treated animals, as compared with the vehicle control group. We conclude that selective inhibition of $\alpha_v\beta_3$ reduced neointima formation in a rabbit model at 3 weeks. J. Cell. Biochem. 77:213–220, 2000.

Key words: restenosis; apoptosis; rabbits; neointima; integrin; $\alpha_v\beta_3$

Coronary restenosis remains an important clinical problem; clinical and quantitative angiographic follow-up studies have demonstrated that within 4–6 months of successful balloon dilation, approximately 30% of patients develop significant coronary restenosis [Popma et al., 1991]. Coronary stenting has proved effective in preventing coronary dissection, impending occlusion, and acute elastic recoil; although restenosis still occurs, the incidence is reduced [Erbel et al., 1998]. The precise mechanism remains unclear; restenosis is thought to result from a number of processes, including smooth muscle cell migration and proliferation [Bernstein et al., 1982; Forrester et al., 1991; Fingerle et al., 1989; Grotendorst et al., 1981], extracellular matrix synthesis [Yue et al., 1994; Panda et al., 1997], and mural thrombosis [Ip et al., 1990]. However, a variety of agents targeting these processes have proved largely unsuccessful in clinical studies despite positive effects in animal models of neointima hyperplasia [Jackson, 1994; Lefkovitz, 1997].

The beneficial effect of the neutralizing antibody against the β_3 integrin, c7E3, on repeat target vessel vascularization in the EPIC (Evaluation of 7E3 for the Prevention of Ischemic Complications) trial has raised the question of whether blocking the $\alpha_v\beta_3$ integrin may represent a viable antirestenotic strategy. This hypothesis was supported by studies showing that a small peptide nonselective antagonist, GpenGRGDSPCA, which blocks the $\alpha_v\beta_3$ integrin, succeeded in reducing neointimal hyperplasia in rabbit carotid arteries after balloon injury [Choi et al., 1994]. Another compound, SK&F107260, which inhibited $\alpha_v\beta_3$-mediated vascular smooth muscle cell migration also inhibited neointima formation in rat carotid artery [Yue et al., 1998]. Likewise, using a cyclic

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Received 17 August 1999; Accepted 30 November 1999

This article published online in Wiley InterScience, March 2000.

RGD peptidomimetic antagonist, selective $\alpha_v\beta_3$ integrin blockade was shown to be effective in reducing neointima formation and lumen stenosis after deep coronary arterial injury elicited by stent deployment in a pig model [Srivatsa et al., 1997].

This report describes studies of $\alpha_v\beta_3$ antagonism in a rabbit model of neointima hyperplasia [Kockx et al., 1992, 1993; Bosmans et al., 1997; Wilcox et al., 1996], using a nonpeptide $\alpha_v\beta_3$ antagonist for 3 days after surgery. Our results show that $\alpha_v\beta_3$ inhibition reduced both neointima formation and clot formation, as determined 3 weeks after cuffing.

MATERIALS AND METHODS

Cell Adhesion Assay

For determination of endothelial cell IC_{50} values, plates were coated overnight with fibrinogen and blocked with 5% bovine serum albumin (BSA) for 1 h. Confluent cells (rabbit endothelial or human umbilical vein endothelial cells [HUVECs]), passages 2–6, were grown in EGM media (Clonetics, San Diego, CA) and detached with trypsin EDTA. Cells (1×10^5 cells/well) were loaded with Calcein AM; free Calcein AM was then washed away. Cells were preincubated with inhibitor for 15 min. Cells and the inhibitor were added to the plate for 1 h. The nonadherent cells were gently washed away and the adherent cells measured fluorometrically [Srivatsa et al., 1997].

Platelet Aggregation Assay

Human and rabbit blood, drawn in citrate to determine platelet aggregation IC_{50} values, was centrifuged for 8 min at 1,000 rpm. Platelet-rich plasma was removed. A total of 20 μl of ADP (10 μM for human and 100 μM for rabbit) was added to 200 μl of platelet-rich plasma; aggregation was determined using a PAP-4 aggregometer [Srivatsa et al., 1997]. For the determination of ex vivo platelet aggregation from the rabbits dosed with SH306, blood was drawn at 80 min after subcutaneous injection and 6 h after the last injection.

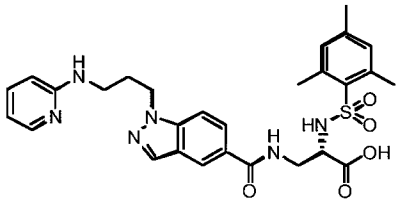
$\alpha_v\beta_3$ ELISA

To measure plasma levels of SH306 after dosing, a competitive enzyme-linked immunosorbent assay (ELISA) with $\alpha_v\beta_3$ was used. The 96-well plates were coated with the $\alpha_v\beta_3$ protein, which was purified from human placenta and then blocked with BSA. SH306 and biotinylated vitronectin were added to $\alpha_v\beta_3$-coated wells for 20 min at room temperature. Goat anti-biotin alkaline phosphatase conjugate was added to the plate and incubated for 1 h at room temperature with gentle shaking. The wells were washed three times, the phosphatase substrate was added, and plates were read at 405 nm.

Experimental Protocol

All procedures performed in this study were approved by the DuPont Pharmaceuticals Animal Care and Use Committee and conform to the Guide for the Care and Use of Laboratory Animals [NIH, 1996]. Two groups of male New Zealand White rabbits (2.9–3.2 kg), a vehicle-treated group (n = 7) that received 10% N,N-dimethylacetamide (DMAC) and an SH306-treated group (n = 7) were included in the study. The rabbits were given an intravenous dose of SH306 (5 mg/kg i.v.) or vehicle 30 min before surgery). Surgery was performed under aseptic conditions. The left femoral artery was surgically exposed and dissected from the surrounding tissue under isoflurane anesthesia in addition to ketamine (40 mg/kg i.m.) and xylazine (5 mg/kg i.m.). A piece of flexible silicone tubing (1-cm length, 1.6-mm internal diameter, 0.8-mm wall) was cut longitudinally, cold sterilized for 12 h and then placed around the left femoral artery by opening the tubing using curved forceps. Care was taken to avoid crushing or damaging the vessel and to place the cuff so that the artery was not occluded. The rabbits were given a central-acting analgesic, Nubain (2 mg/kg s.c.). No anticoagulation was used during the study.

The animals were returned to their cages and were administered either SH306 10 mg/kg s.c. or 10% DMAC three times daily for 3 days. A volume of 5 ml SH306 or vehicle was injected into each rabbit. Blood was drawn on day 3 (through the central ear artery) 80 min after the first subcutaneous injection and 6 h after the last injection for measurement of plasma levels of the compound. Three weeks after cuffing, the rabbits were injected subcutaneously with 45 mg/kg bromodeoxyuridine (BRDU) 18 h before perfusion, followed by a second dose (30 mg/kg i.v.) 2 h before perfusion. Three weeks later, the 1-cm vessel segment directly



Integrin	IC50 (μM)	
	Human	Rabbit
$\alpha_v\beta_3$ (endothelial cell adhesion)	0.02	0.5
$\alpha_{IIb}\beta_3$ (platelet aggregation)	71.0	730.0

Fig. 1. Structure and integrin specificity profile of SH306, a small molecule $\alpha_v\beta_3$ antagonist in humans and in rabbits. Integrin $\alpha_v\beta_3$ specificity was determined using a cell adhesion assay, while $\alpha_{IIb}\beta_3$ specificity was determined using platelet aggregation.

TABLE I. Plasma Levels of SH306 After Subcutaneous Injection*

Day 3	μM
Peak	1.0 ± 0.05
Trough	0.180 ± 0.030

*Blood was drawn from rabbits dosed with SH306 80 min after subcutaneous injection and 6 h after subcutaneous injection.

inside the cuff was excised after perfusion with 4% paraformaldehyde, transected into three equal segments, and embedded in paraffin. Ten sections containing the trisected vessel were cut.

Immunohistochemistry

Bromodeoxyuridine incorporation was determined by the following method. Tissue sections were deparaffinized, incubated in methanol/H_2O_2, incubated in proteinase K, and incubated with the primary antibody to BRDU (Dako, A/S, Denmark) which was detected using the Vectastain ABC (Vector Laboratories) [Bosmans et al., 1997].

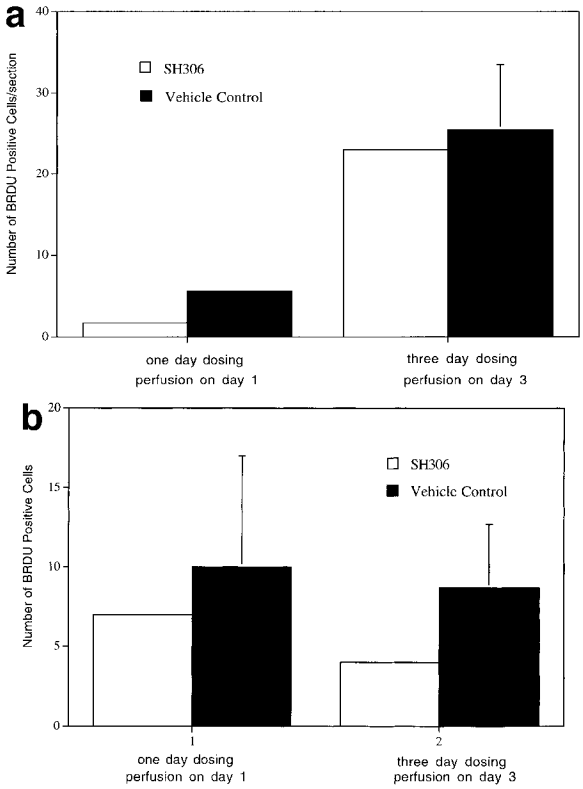


Fig. 2. Cell proliferation was determined using bromodeoxyuridine (BRDU) incorporation on vessels perfused (**a**) 1 or 3 days after injury in the media, and (**b**) at 1- or 3-day dosing in adventitia with SH306 10 mg/kg s.c. 3 times daily. n = 4/group. Data represent mean ±SEM.

Quantification of Intima and Neointima

For determination of intima and media areas, slides were stained with Verhoeff's elastica stain. The cross-sectional area of intima and media were determined in a blinded fashion using a Colourmorph 5.2 (Perceptive Instruments) System. In certain sections in which the internal elastic lamina was disrupted, the junction between the neointima and media was used to delineate the media from the neointima. The morphology of the cells in this area was different enough to make this differentiation. Three sections from each slide containing six sections were averaged. A minimum of 10 slides were read per treatment group. Areas were determined from three different segments within the cuffed region and averaged.

Data Analysis

All data are presented as means ±SEM. Statistical significance was determined by a one-

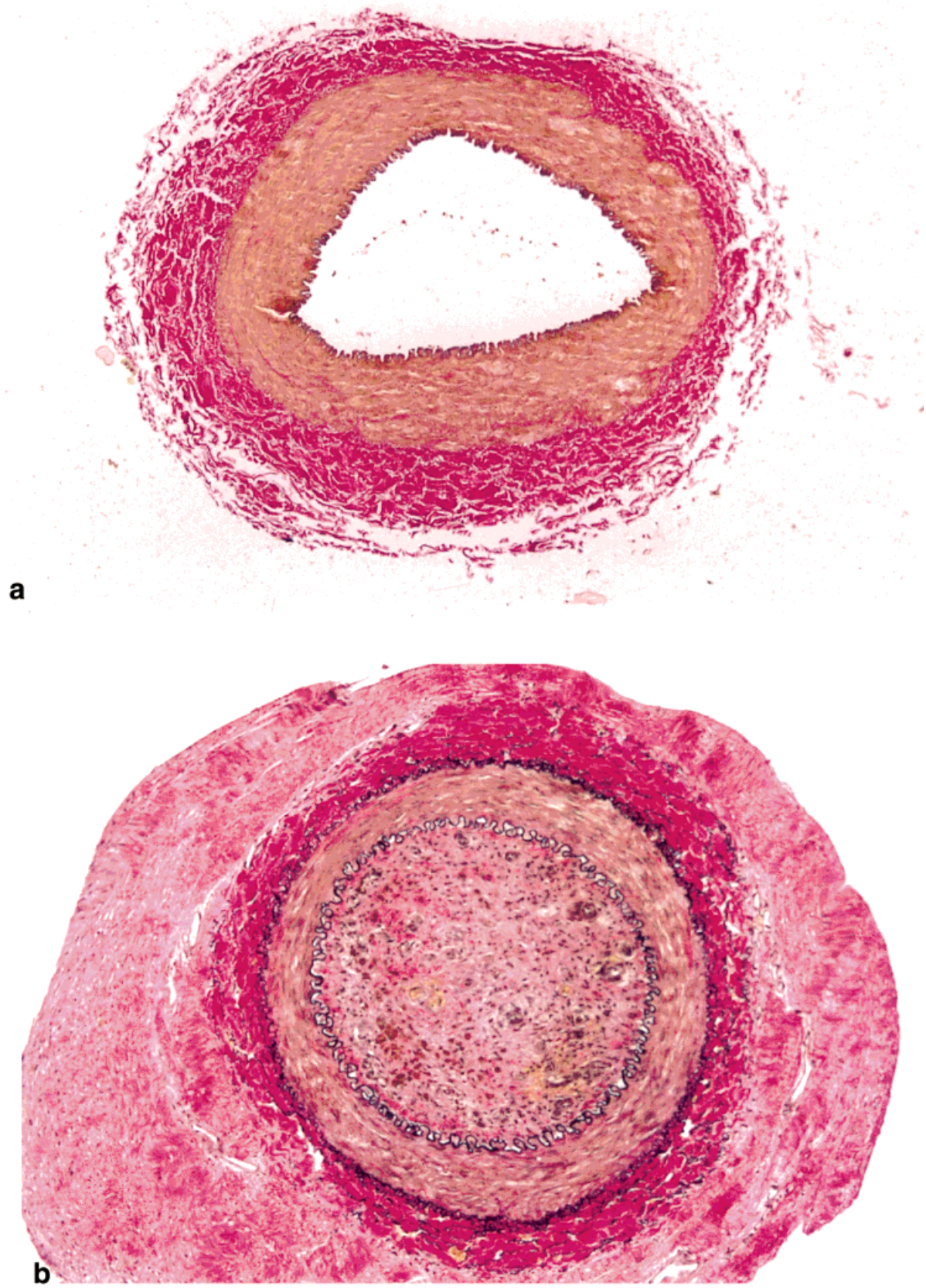


Fig. 3. Photomicrographs of a femoral artery section. **a:** Normal vessel. **b:** Cuffed vehicle control, perfused 3 weeks after injury. **c:** Cuffed rabbit treated with SH306 10 mg/kg s.c. 3 times daily for 3 days after a bolus of SH306 5 mg/kg i.v. before cuffing surgery. Verhoeff's elastica stain, ×10.

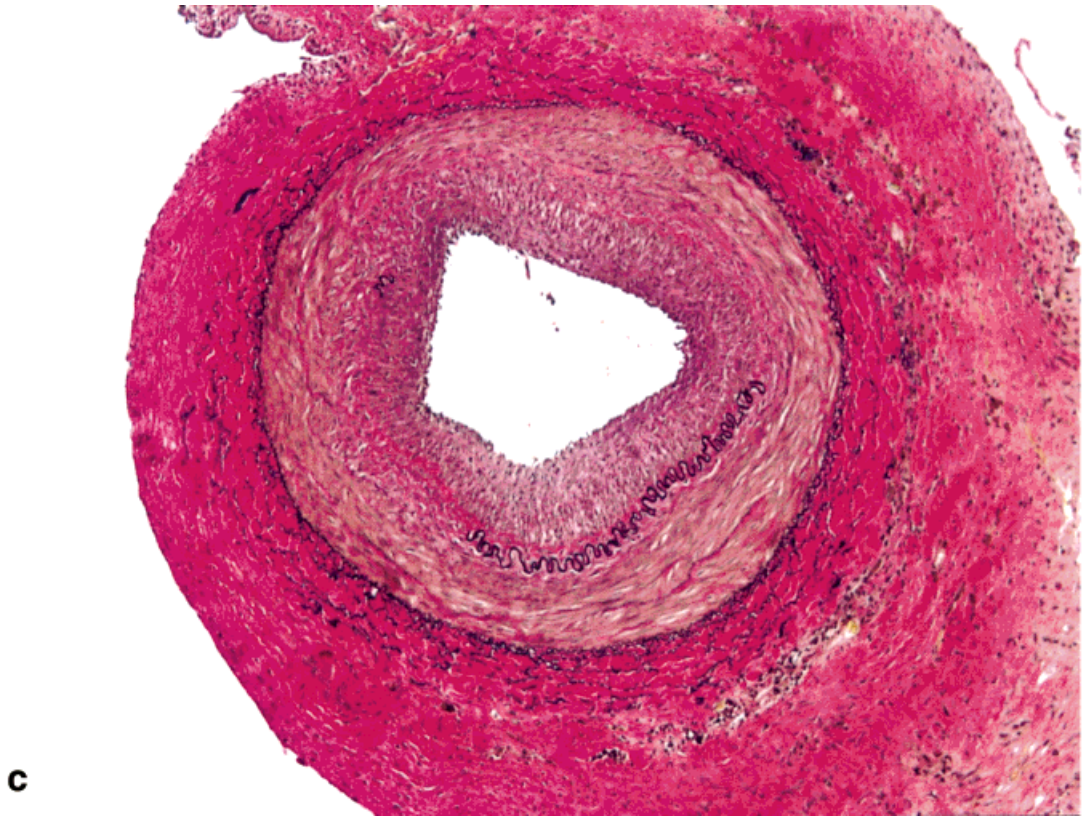


Figure 3. (Continued.)

way analysis of variance (ANOVA), followed by a Tukey multiple comparison test.

RESULTS

The structure and specificity profile of SH306 are shown in Figure 1. SH306 is a highly selective $\alpha_v\beta_3$ antagonist in both human and rabbit endothelial cell adhesion assays, with an IC_{50} value of 0.02 μM for human $\alpha_v\beta_3$ and an IC_{50} value of 0.5 μM for rabbit $\alpha_v\beta_3$. In both species, SH306 exhibited greater than 1,000-fold selectivity for $\alpha_v\beta_3$, compared with GPIIb/IIIa, as determined by comparing IC_{50} values in the fibrinogen adhesion assay, using rabbit endothelial cells or HUVECs, and in platelet aggregation assays, using human or rabbit platelet-rich plasma (Fig. 1).

Plasma levels obtained after dosing with SH306 at 5 mg/kg i.v., followed by 10 mg/kg s.c. three times daily, are shown in Table I. Compound levels peaked at approximately 1.0 μM, as determined by ELISA. This value is twice the IC_{50} value for $\alpha_v\beta_3$ in the rabbit endothelial cell adhesion assay (IC_{50} = 0.5 μM). Trough levels obtained between subcutaneous doses were approximately 0.180 μM. No effects on inhibition of platelet aggregation were seen for SH306 as compared with the vehicle control.

Despite an observed trend toward decreased BRDU incorporation, no significant change was noted in cell proliferation, as determined by BRDU incorporation in the various treatment groups during the first 3 days after surgery, in either the media or adventitia (Fig. 2a,b).

Figure 3 presents three photomicrographs from the different treatment groups. Figure 3a shows a normal vessel stained with Verhoeff's elastica stain, and Figure 3b shows a representative vessel in rabbits treated with 10% DMAC from the vehicle control group, harvested 3 weeks after the vascular injury. The lumens of three of seven vessels in the control group were completely occluded by neointima

TABLE II. Neointima Area and Media Area of the Rabbit Femoral After Cuff Placement

Rabbit no.	Intima (mm^2)	Media (mm^2)	I/M
Vehicle control-clotted			
R45	0.03	0.07	0.4
R47	0.03	0.06	0.5
R51	0.1	0.1	1.0
Mean	0.05	0.08	0.64
SEM	0.02	0.01	0.17
Vehicle control			
R53	0.04	0.12	0.3
R55	0.06	0.13	0.5
R57	0.06	0.12	0.5
R59	0.02	0.13	0.2
Mean	0.05	0.13	0.36
SEM	0.01	0.00	0.08
SH306			
R29	0.10	0.24	0.44
R31	0.04	0.22	0.19
R33	0.04	0.20	0.20
R35	0.01	0.20	0.04
R37	0.03	0.23	0.15
R39	0.02	0.16	0.11
Mean	0.04	0.20	0.20
SEM	0.01	0.01	0.00

after 3 weeks, as determined by sections cut throughout the cuffed region. The remaining four vessels had varying degrees of neointima formation. Those vessels that were completely occluded had visible evidence of clot remodeling, including angiogenesis and smooth muscle cell infiltration. Figure 3c shows a representative vessel harvested 3 weeks after cuff placement after subcutaneous treatment with SH306 for 3 days, in which neointima formation was inhibited. Only one of seven vessels was completely occluded in the treatment group, as compared with three vessels in the control group (as determined by analysis of 60 continuous sections).

Table II presents data for the intima, media, and intima/media areas of the two treatment groups. Because three vessels clotted in the vehicle control group, the vessels that clotted are considered separately from the vehicle control vessels that did not clot. One vessel that clotted is not considered in the SH306-treated group, hence n = 6 for this group. No significant differences were noted between the intimas of the treatment groups, although a strong trend was noted. However, the media of the clotted vehicle control group was significantly less than that of the other groups. This is most likely attributable to medial necrosis after complete occlusion in this group. An increase in the area of the media is seen in the SH306 group, which may be related to the inhibition of migration with continued cell proliferation. The intima/media (I/M) ratio for the SH306 group was significantly different from that of the other groups.

DISCUSSION

Neointima formation in the cuff-injury rabbit model, as assessed by the I/M ratio, was significantly decreased after treatment with SH306, a highly selective antagonist of the $\alpha_v\beta_3$ integrin. Intimal thickening occurs in this model after a nonocclusive silicone cuff is positioned around the femoral artery, resulting in vascular injury and hindering transmural flow by the cuff [DeMeyer et al., 1997]. The time course of neointima formation in this model follows three phases: (1) early infiltration of the vessel wall by polymorphonuclear neutrophil leukocytes (PMNs) at 2–24 h; (2) by smooth muscle cell replication in the media; and (3) a gradual subendothelial accumulation of smooth muscle cells, with deposition of collagen fibers and fibronectin within the neointima [Kockx et al., 1992]. During the 3- to 5-day time interval, when smooth muscle cells are proliferating and beginning to migrate, a dramatic increase in medial expression of $\alpha_v\beta_3$, $\alpha_v\beta_5$, and osteopontin was noted. At later time points (2 weeks), integrin and osteopontin expression return to normal [Corjay et al., 1999].

Mechanisms by which an $\alpha_v\beta_3$ antagonist may exert inhibition of neointima formation include inhibition of the migration of smooth muscle cells [Yue et al., 1994]. High levels of the adhesive phosphoprotein, osteopontin, have been identified during neointima formation. Osteopontin is expressed by numerous vascular cells, including endothelial cells, vascular smooth muscle cells, and macrophages in both atherosclerotic and restenotic neointima [Giachelli et al., 1993]. Spatiotemporal induction of osteopontin expression is observed in porcine and rodent models and in this rabbit model of balloon or cuff injury [Corjay et al., 1999; O'Brien et al., 1994]. Osteopontin promotes the migration of macrophages and smooth muscle cells through binding to $\alpha_v\beta_3$

receptors [Yue et al., 1994]. Therefore, antagonizing $\alpha_v\beta_3$ receptors may reduce neointima formation by decreasing the migration of smooth muscle cells. This result suggests a potential mechanism contributing to the decreased neointima formation after compound treatment.

A greater degree of neointima formation was seen in the vessels that were occluded by clots, as compared with the patent vessels. This difference may be related to the clot acting as a surface onto which the smooth muscles migrate during the process of neointima formation. Nonetheless, whether the vessels occluded or remained patent, rabbits treated with SH306 had lower I/M ratios. As the model was performed in the absence of any anticoagulant, any manipulation of the vessel placed it at risk of clotting. It was interesting to note that SH306 protected a large percentage of the vessels from clotting (14% clotted in drug-treated vs 43% in the control group). The antithrombotic effect may be related to inhibition of platelet adhesion by the $\alpha_v\beta_3$ antagonist. Platelet adhesion to endothelium is mediated through fibrinogen bridging $\alpha_v\beta_3$ located on activated endothelial cells to GPIIb/IIIa on platelets [Gawaz et al., 1997]. Because adhesion is an initial step in the process of platelet aggregation, an $\alpha_v\beta_3$ antagonist may inhibit thrombosis by inhibiting this adhesive interaction.

In conclusion, a trend in reducing neointima formation was observed with an $\alpha_v\beta_3$ antagonist in a rabbit cuff model, even though the rabbits were dosed for only 3 days. These results add to the growing body of evidence suggesting that $\alpha_v\beta_3$ antagonists can inhibit neointima formation.

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