

# Different Vascular Smooth Muscle Cell Apoptosis in the Human Internal Mammary Artery and the Saphenous Vein

## Implications for Bypass Graft Disease

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### Key Words

Apoptosis · Atherosclerosis · Cell cycle · Proliferation · Signal transduction

### Abstract

**Background:** The remarkable patency of internal mammary artery (MA) grafts compared to saphenous vein (SV) grafts has been related to different biological properties of the two blood vessels. We examined whether proliferation and apoptosis of vascular smooth muscle cells (VSMC) from human coronary artery bypass vessels differ according to patency rates. **Methods and Results:** Proliferation rates to serum or platelet-derived growth factor (PDGF)-BB were lower in VSMC from MA than SV. Surface expression of PDGF  $\beta$ -receptor was slightly lower, while that of  $\alpha$ -receptor was slightly higher in MA than SV. Cell cycle distribution, expression of cyclin E, cdk2, p21, p27, p57, and cdk2 kinase activity were

identical in PDGF-BB-stimulated cells from MA and SV. However, apoptosis rates were higher in MA than SV determined by lactate dehydrogenase release, DNA fragmentation, and Hoechst 33258 staining. Moreover, caspase inhibitors (Z-VAD-fmk, Boc-D-fmk) abrogated the different proliferation rates of VSMC from MA versus SV. Western blotting and GSK3- $\beta$  kinase assay revealed lower Akt activity in VSMC from MA versus SV, while total Akt expression was identical. Adenoviral transduction of a constitutively active Akt mutant abrogated the different proliferation rates of VSMC from MA versus SV. **Conclusions:** Higher apoptosis rates due to lower Akt activity rather than different cell cycle regulation account for the lower proliferation of VSMC from MA as compared to SV. VSMC apoptosis may protect MA from bypass graft disease.

### Introduction

Coronary artery disease is often treated by bypass grafting. Internal mammary artery (MA) and saphenous vein (SV) are routinely used bypass grafts; patency rates

The study was supported by the Swiss National Science Foundation (grant No. 3200B0-102232/1 to F.C.T. and No. 32-51069.97/2 to T.F.L.), the Swiss Heart Foundation, the Novartis Foundation and the Schweizerische Rentenanstalt.

of MA grafts, however, are higher than those of SV [1]. Vascular smooth muscle cells (VSMC) accumulate in the subendothelium of grafted vessels and lead to neointima formation; therefore, VSMC proliferation plays a major role in long-term patency [2]. Interestingly, VSMC are a heterogeneous population; indeed, VSMC from different vascular beds exhibit different intrinsic properties [3]. Hence, VSMC from MA and SV may differ in their intrinsic properties as well, thereby determining patency rates of grafted vessels.

Growth factors such as platelet-derived growth factor (PDGF) are released from aggregating platelets leading to stimulation of VSMC proliferation. As platelet adhesion to bypass grafts is initiated within minutes after grafting, VSMC of grafted vessels are exposed to PDGF from a very early time point on; not surprisingly, such growth factors are important regulators of neointima formation [4]. However, the effect of growth factors on different bypass grafts may be modulated by intrinsic properties of the grafts, as VSMC from MA and SV may differ in these properties resulting in a differential response to growth factors. Moreover, not only proliferation, but also apoptosis of VSMC plays an important role in the pathogenesis of vascular diseases such as atherosclerosis and bypass graft disease [5, 6]. Indeed, VSMC apoptosis may reduce neointima formation [7], and the balance of VSMC proliferation versus apoptosis may be a more important determinant of long-term patency of grafted vessels than either parameter alone.

Therefore, the present study was designed to analyze proliferation and apoptosis of VSMC from MA as compared to SV. We observed that proliferation rates of VSMC from MA and SV are consistent with the different patency rates. Neither PDGF receptor expression nor cell cycle regulation could account for this difference; instead, different apoptosis rates due to different Akt activation were identified.

## Methods

### *Cell Culture and Proliferation*

VSMC and endothelial cells (EC) of MA, SV, and aorta were isolated from patients undergoing coronary artery bypass grafting, which was approved by the Ethics Committee of Human Research at the University Hospital. Cells were obtained from 32 patients and 34 vessels (MA: 13 vessels from 13 patients; SV: 16 vessels from 14 patients; aorta: 5 vessels from 5 patients). VSMC were isolated by explant generation, EC by the enzymatic method. The identity of each VSMC and EC isolate was confirmed by immunofluorescent staining for smooth muscle  $\alpha$ -actin (No. 1148818, Roche Diagnostics, Mannheim, Germany) and uptake

of acLDL (No. L3484, Molecular Probe, Leiden, The Netherlands), respectively. VSMC and EC were cultured as described. VSMC were used for experiments up to passage 12 and EC up to passage 5. To induce G0/G1 arrest, VSMC were kept in serum-free medium containing 0.1% BSA fraction V (Sigma, St. Louis, Mo., USA) for 48 h prior to stimulation with 10% FCS or 10 ng/ml human PDGF-BB (No. 220-BB, R&D Systems, Minneapolis, Minn., USA). Cell number was determined after 0, 2, 4, and 6 days using an improved Neubauer hemacytometer. G1/S-progression was evaluated by  $^3\text{H}$ -thymidine incorporation; 20 h after mitogenic stimulation, VSMC were pulsed with  $^3\text{H}$ -thymidine for 4 h, and incorporated  $^3\text{H}$ -thymidine was detected by a  $\beta$ -counter (Beta-matic V; Kontron Instruments, Milan, Italy) [8]. This time window for  $^3\text{H}$ -thymidine incorporation was chosen because preliminary experiments revealed a progressive decline in the cellular level of the cyclin-dependent kinase inhibitor p27 over 30 h after mitogenic stimulation (data not shown); p27 expression is indeed known to be high in arrested cells and to decrease during G1-progression and S-phase entry.

### *Western Blot Analysis and Kinase Assays*

Western blot analysis of cell cycle proteins was performed on whole-cell lysates as described [9]. Membranes were incubated with antibodies against cyclin E (No. sc-198), cdk2 (No. sc-163), p27<sup>Kip1</sup> (No. sc-528; all from Santa Cruz Biotechnology, Santa Cruz, Calif., USA), p21<sup>Cip1</sup> (No. 65961A), p57<sup>Kip2</sup> (No. 556346; both from Pharmingen, San Diego, Calif., USA), Akt (No. 9272), p-Akt (No. 9271; both from Cell Signaling Technology, Beverly, Mass., USA) and acetylated tubulin (No. T-6793, Sigma). Immunoprecipitations for cdk2 kinase assays were performed as described [9]. Labeled proteins were resolved on 12% SDS-PAGE and subjected to autoradiography. Akt kinase activity was determined by GSK3- $\beta$  phosphorylation according to the manufacturer's recommendations (No. 9840, Cell Signaling Technology).

### *Flow Cytometry and Cell Death Assays*

For analysis of PDGF receptor expression, VSMC were detached with 2 mM EDTA (pH 8.0) in PBS, collected, and washed with PBS containing 20% FCS. Cells were stained with anti-PDGF receptor  $\alpha$  (No. 556001, Pharmingen) or  $\beta$  antibody (No. MAB1263, R&D Systems) for 1 h on ice, and then incubated with rabbit anti-mouse IgG phycoerythrin-conjugated antibody (No. P9287, Sigma) for 1 h on ice. Cells were washed, fixed with 1% paraformaldehyde, and analyzed on a FACSCalibur cytometer using CellQuest software (Becton Dickinson, Franklin Lakes, N.J., USA).

For analysis of cell cycle distribution as well as quantification of fragmented DNA, cells were harvested, washed twice in PBS, fixed in 2% paraformaldehyde for 60 min, and permeabilized in 0.2% Tween-20. The cells were then treated with 1 U RNase in 1 ml PBS for 30 min at 37°C, resuspended in 0.03 mg/ml propidium iodide, and analyzed by flow cytometry [10]. Cell death was also examined using a colorimetric assay for lactate dehydrogenase activity (Roche Diagnostics) according to the manufacturer's instructions. Apoptosis was determined by staining trypsinized VSMC with 10  $\mu\text{g}/\text{ml}$  Hoechst 33258 (No. B1155, Sigma) for 5 min, which were then washed twice with PBS and 10% FCS, resuspended in 25  $\mu\text{l}$  PBS and transferred to a microscope slide. 300 cells were analyzed by fluorescent microscopy, and the number of Hoechst-positive cells showing condensed chromatin was determined [11].

**Table 1.** Growth of VSMC and EC from human coronary artery bypass vessels in response to different stimuli

Vessel	MA	SV	p value
Explant outgrowth of VSMC, %	55.6	100	
VSMC, n × 1,000			
PDGF (day 0)	13.2 ± 5.1	15.6 ± 5.4	NS
PDGF (day 2)	17.0 ± 6.6	29.1 ± 12.7	<0.01
PDGF (day 4)	21.7 ± 11.9	45.6 ± 17.8	<0.005
PDGF (day 6)	28.4 ± 22.7	69.8 ± 43.4	<0.001
EC, n × 1,000			
VEGF (day 4)	6.4 ± 0.6	6.6 ± 1.8	NS
FGF (day 4)	3.7 ± 0.2	4.5 ± 1.0	NS

NS = Nonsignificant; VEGF = vascular endothelial growth factor; FGF = fibroblast growth factor.

#### Caspase Inhibition

Cells were growth arrested and then stimulated with 10 ng/ml PDGF-BB plus 50 μM of the specific caspase inhibitor Z-VAD-fmk (Bachem, Heidelberg, Germany) or 30 μM of another specific caspase inhibitor Boc-D-fmk (No. 218745, Calbiochem, Darmstadt, Germany). Media were changed daily, and cell number was determined after 4 days of mitogenic stimulation.

#### Adenoviral Transduction

Cells were transduced with an adenoviral vector expressing a constitutively active Akt mutant (Ad-HA-m/p-PKB) or a control vector harboring the green fluorescent protein (GFP) at a multiplicity of infection of 800 pfu/cell. For some experiments, a multiplicity of infection of 100 pfu/cell was used. The vectors were kindly provided by Brian A. Hemmings, PhD (Friedrich Miescher Institute Basel, Switzerland) and Zhihong Yang, MD (University of Fribourg, Switzerland) [12, 13]. Medium was then removed and cells were stimulated with proliferation medium. Increase in cell number was determined after 4 days of mitogenic stimulation.

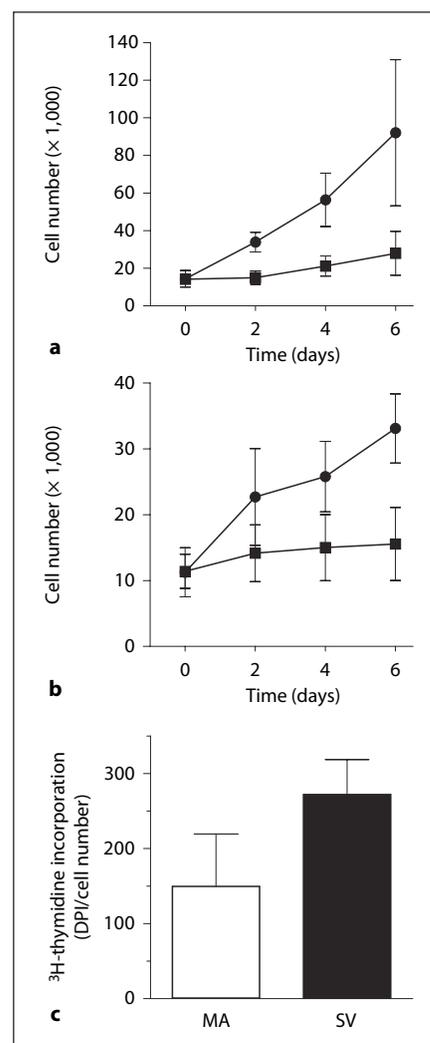
#### Statistics

Values represent means ± SD of 4–5 independent experiments. For each experiment, VSMC from a different patient were used. Statistical evaluation was performed by unpaired Student's t test or ANOVA as appropriate. A p value <0.05 was considered to indicate a significant difference.

## Results

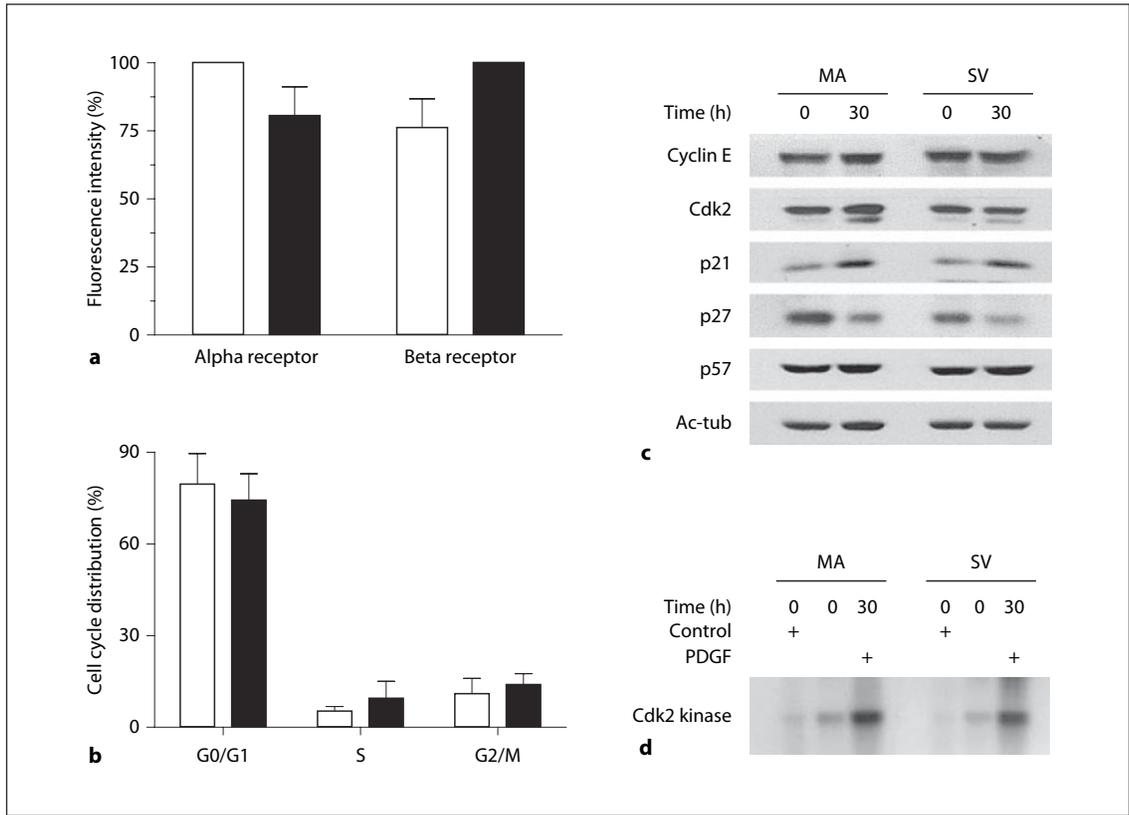
#### Proliferation

Isolation of VSMC was successful in 56% of explants from MA and in 100% from SV, respectively (table 1). This difference was related to both a lower number of explants with outgrowth and a slower growth in MA as compared to SV. Therefore, the proliferation pattern of



**Fig. 1.** Increase in the VSMC number from MA (■) and SV (●). Cells were serum starved for 48 h and then stimulated with a mitogen. **a** Stimulation with 10% FCS. **b** Stimulation with 10 ng/ml PDGF-BB. **a, b** Cell numbers were lower in MA than SV ( $p < 0.05$ ). **c** Stimulation with 10 ng/ml PDGF-BB; G0/G1 phase arrest measured by <sup>3</sup>H-thymidine incorporation was lower in MA (□) than SV (■);  $p < 0.05$ .

VSMC isolates from MA and SV was examined. Cell number was determined every other day for up to 6 days. Proliferation of MA VSMC to 10% FCS was lower than that of SV (cell number on day 6: MA: 28,365 ± 22,700 and SV: 69,750 ± 43,400;  $p < 0.01$ ;  $n = 13-15$ ; table 1). A similar pattern was observed when VSMC from both vessels were derived from the same patient (cell number on day 6: MA: 27,938 ± 11,663, SV: 92,031 ± 38,853;  $p < 0.05$ ;  $n = 4$ ; fig. 1a). Thus, proliferation of VSMC from MA was lower than that of SV in response to FCS.



**Fig. 2.** Cell cycle regulation of VSMC from MA and SV. Cells were serum starved for 48 h and then stimulated with PDGF-BB for 30 h. **a** PDGF receptor expression analyzed by FACS.  $\alpha$  expression was slightly higher in MA (□) than SV (■;  $p < 0.01$ ).  $\beta$  expression was slightly lower in MA (□) than SV (■;  $p < 0.005$ ). **b** Cell cycle distribution was determined by propidium iodide incorporation and analyzed by FACS. A similar distribution was observed between MA (□) and SV (■;  $p = \text{nonsignificant}$ ). **c** Cell-cycle-regulatory protein expression was determined by Western blotting and observed to be similar between MA and SV. **d** Cdk2 kinase activity was determined by H1 kinase assay and observed to be similar between MA and SV.

To study the effect of a specific mitogen, VSMC were stimulated with 10 ng/ml PDGF-BB. MA VSMC exhibited only minimal proliferation, while the number of SV VSMC increased faster (cell number on day 6: MA:  $15,446 \pm 5,528$  and SV:  $33,094 \pm 5,244$ ;  $p < 0.01$ ;  $n = 4$ ; fig. 1b). PDGF-BB-induced exit from G0/G1 phase was determined by  $^3\text{H}$ -thymidine incorporation. Consistent with proliferation rates,  $^3\text{H}$ -thymidine incorporation was lower in MA VSMC as compared to SV (MA:  $149.6 \pm 69.8$  and SV:  $271.9 \pm 46.6$ ;  $p < 0.05$ ;  $n = 4$ ; fig. 1c). Thus, the increase in the number of MA VSMC was lower than that of SV after stimulation with PDGF-BB. VSMC from MA were also compared to aorta. Again, MA VSMC exhibited only minimal proliferation in response to 10 ng/ml PDGF-BB over 6 days, while the number of aorta VSMC increased faster (cell number on

day 6: MA:  $6,250 \pm 5,796$  and aorta:  $21,188 \pm 13,097$ ;  $p < 0.05$ ;  $n = 5$ ). Thus, the lower increase in cell number was specific for MA rather than being a general property of arterial vessels.

#### Receptor Expression

The different proliferation rates of VSMC from MA versus SV in response to PDGF-BB may be related to different expression of PDGF receptors. Therefore, surface expression of PDGF receptor  $\alpha$  and  $\beta$  was determined by FACS analysis. Expression of receptor  $\alpha$  was slightly higher in VSMC from MA than SV (MA: 100% and SV:  $80.6 \pm 10.5\%$ ;  $p < 0.01$ ;  $n = 4$ ; fig. 2a). PDGF receptor  $\beta$  expression was slightly lower in VSMC from MA than SV (MA:  $76.2 \pm 10.5\%$  and SV: 100%;  $p < 0.005$ ;  $n = 4$ ; fig. 2a). Thus, the pattern of PDGF receptor expression

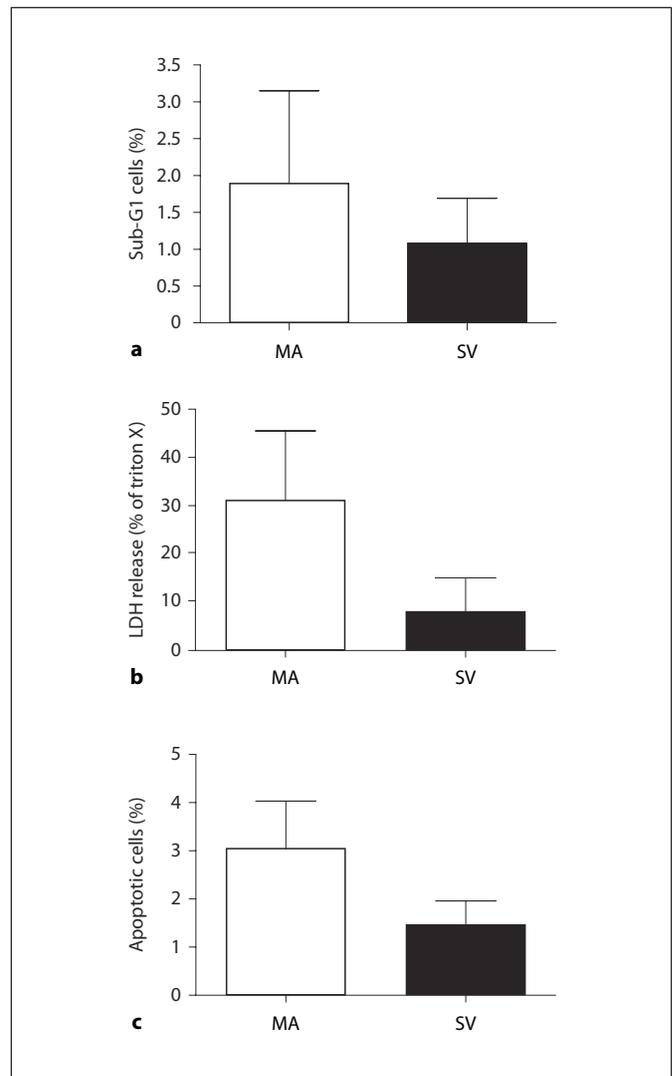
did not account for the different proliferation rates of VSMC from MA and SV.

### Cell Cycle Progression

The different proliferation rates of VSMC from MA versus SV may be due to differences in signal transduction or cell cycle regulation. In either case, differences in cell cycle progression would be expected. Therefore, cell cycle distribution and G1 progression was compared in VSMC from MA and SV. Preliminary experiments examining cell cycle protein expression at 0, 6, 12, 18, 24, and 30 h of mitogenic stimulation revealed that differences in the expression of these proteins progressively develop in VSMC over the 30-hour observation period (data not shown); hence, the analysis was performed at 0 and 30 h. Propidium iodide incorporation demonstrated similar cell cycle distribution in VSMC from both vessels (% of cells in G0/G1 phase: MA:  $79.5 \pm 10.0$ , SV:  $74.25 \pm 8.7$ ; S phase: MA:  $5.25 \pm 1.5$ , SV:  $9.5 \pm 5.5$ ; G2/M phase: MA:  $11 \pm 5.0$  and SV:  $14 \pm 3.5$ ;  $n = 4$ ;  $p =$  nonsignificant; fig. 2b). Next, Western blot analysis for expression of cell cycle proteins regulating G1 progression was performed. Consistent with cell cycle distribution, VSMC from MA and SV exhibited an identical pattern of cell cycle protein expression (fig. 2c). Indeed, in VSMC from both vessels, cyclin E expression remained unaltered by PDGF stimulation, the lower part of the cdk2 double band representing threonine-phosphorylated and hence activated cdk2 was induced, the cyclin-dependent kinase inhibitor (CKI) p21 was slightly upregulated, p27 was downregulated, and p57 remained unchanged. Consistent with these observations, cdk2 kinase activity was identical in VSMC from MA and SV (fig. 2d). Thus, both cell cycle distribution and G1 progression were identical in VSMC from MA and SV, and therefore did not account for the different proliferation rates.

### Cell Death

As neither PDGF receptor expression nor cell cycle progression could account for the different proliferation rates of VSMC from MA and SV, cell viability after 30 h of PDGF-BB stimulation was examined. FACS analysis of fragmented DNA revealed a higher number of dead cells in MA as compared to SV (% dead cells: MA:  $1.89 \pm 1.26$  and SV:  $1.08 \pm 0.61$ ;  $p = 0.20$ ;  $n = 5$ ; fig. 3a). Quantification of cell death by lactate dehydrogenase activity confirmed this observation; indeed, cell death was higher in VSMC from MA as compared to SV (% of maximal release: MA:  $31.12 \pm 14.38$  and SV:  $8.04 \pm 7.03$ ;  $p < 0.02$ ;  $n = 5$ ; fig. 3b). To differentiate apoptosis from necrosis,



**Fig. 3.** Different cell death rates of VSMC from MA (□) and SV (■). Cells were serum starved for 48 h and then stimulated with PDGF-BB for 30 h. **a** Fragmented DNA was analyzed by FACS and was higher in MA than SV ( $p = 0.20$ ). **b** Lactate dehydrogenase (LDH) release was determined by ELISA and was higher in MA than SV ( $p < 0.02$ ). **c** Hoechst-positive cells were identified by fluorescence microscopy;  $p < 0.05$ , MA vs. SV.

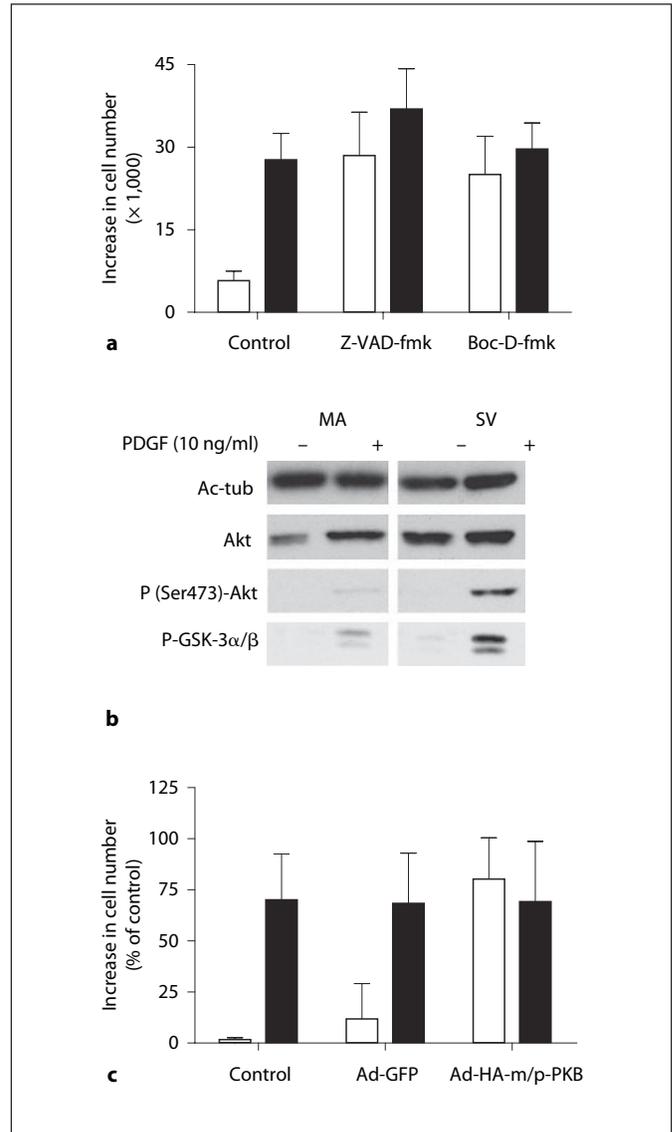
VSMC were stained with Hoechst 33258, and the percentage of Hoechst-positive cells exhibiting condensed chromatin was determined. Consistent with the other death assays, apoptosis rates were higher in VSMC from MA than SV (MA:  $3.04 \pm 0.99\%$  and SV:  $1.46 \pm 0.50$ ;  $p < 0.05$ ;  $n = 4$ ; fig. 3c). This pattern of cell death was consistent with the different increase in VSMC number from MA and SV.

### Caspase Inhibition

As activation of caspases is a key element in apoptosis, VSMC were treated with specific caspase inhibitors. VSMC were stimulated with PDGF-BB over 4 days (increase in cell number: MA:  $5,781 \pm 4,280$  and SV:  $27,734 \pm 11,777$ ;  $p < 0.01$ ;  $n = 6$ ). Addition of the caspase inhibitor Z-VAD-fmk abolished the difference in proliferation of VSMC from MA versus SV, confirming that the different cell death rates are determined by apoptosis (increase in cell number in the presence of Z-VAD-fmk: MA:  $28,520 \pm 19,270$  and SV:  $36,953 \pm 17,888$ ;  $p =$  nonsignificant; MA vs. MA+VAD:  $p < 0.05$ ; SV vs. SV+VAD:  $p =$  nonsignificant;  $n = 6$ ; fig. 4a). Another specific caspase inhibitor, Boc-D-fmk, had a similar effect (increase in cell number in the presence of Boc-D-fmk: MA:  $25,078 \pm 16,980$  and SV:  $29,688 \pm 11,570$ ;  $p =$  nonsignificant; MA vs. MA+Boc:  $p < 0.05$ ; SV vs. SV+Boc:  $p =$  nonsignificant;  $n = 6$ ; fig. 4a). Thus, these data indicate that the different proliferation rates of VSMC from MA and SV are due to different caspase-dependent apoptosis rates.

### Akt Activity

Akt plays a key role in the cellular balance between proliferation and apoptosis. Therefore, levels of total and activated Akt were determined by Western blotting, which revealed similar expression, but lower activation in VSMC from MA than SV ( $n = 4$ ; fig. 4b). These data were confirmed by an Akt activity assay determining the levels of phosphorylated GSK-3 $\beta$ ; MA VSMC exhibited a lower GSK-3 $\beta$  activation as compared to SV ( $n = 3$ ; fig. 4b). Similar to the effect of caspase inhibitors, adenoviral transduction of the constitutively active Akt mutant Ad-HA-m/p-PKB abrogated the growth differences in VSMC from MA and SV [13]. The VSMC number was lower in MA than SV after PDGF stimulation (increase in cell number in %: MA:  $0 \pm 13.19$  and SV:  $70.16 \pm 22.37$ ;  $p < 0.02$ ). Transduction with the Akt mutant Ad-HA-m/p-PKB revealed a similar VSMC number in MA and SV (increase in cell number in %: MA+AdPKB:  $80.27 \pm 21.2$  and SV+AdPKB:  $69.17 \pm 29.52$ ;  $p =$  nonsignificant; MA vs. MA+AdPKB:  $p = 0.01$ ; SV vs. SV+AdPKB:  $p =$  nonsignificant). No effect of the control vector was observed (increase in cell number in %: MA+AdGFP:  $11.87 \pm 17.20$  and SV+AdGFP:  $68.42 \pm 24.57$ ;  $p < 0.01$ ; MA vs. MA+AdGFP and SV vs. SV+AdGFP:  $p =$  nonsignificant;  $n = 4$ ; fig. 4c). To confirm the specificity of these data, the effect of transduction with Ad-HA-m/p-PKB on MA VSMC was also examined at a lower multiplicity of infection (100 pfu/cell). After transduction, the cell number



**Fig. 4.** Caspase inhibitors abolish growth differences in VSMC from human MA and SV. **a** VSMC from MA ( $\square$ ) and SV ( $\blacksquare$ ) were serum starved for 48 h and then stimulated with PDGF-BB plus either the caspase inhibitor Z-VAD-fmk or Boc-D-fmk. Cell number was determined on day 4 after stimulation. Caspase inhibition enhanced cell number in MA ( $p < 0.05$ ) but not SV ( $p =$  nonsignificant). **b** Akt activity of VSMC from MA and SV. Cells were lysed, levels of phosphorylated Akt were determined by Western blotting, and Akt activity was also measured by GSK3- $\beta$  phosphorylation. **c** VSMC from MA ( $\square$ ) and SV ( $\blacksquare$ ) were transduced with 800 pfu of an adenoviral vector expressing a constitutively active Akt mutant (Ad-HA-m/p-PKB) or control vector (Ad-GFP) and stimulated with PDGF-BB. Cell number was determined 4 days after stimulation. Constitutively active Akt enhanced cell number in MA ( $p = 0.01$ ) but not SV ( $p =$  nonsignificant).

was higher as compared to control conditions (Ad-PKB:  $16,625 \pm 2,604$ , Ad-GFP:  $11,844 \pm 2,040$  and control:  $10,312 \pm 2,632$ ;  $p < 0.05$  for Ad-PKB vs. Ad-GFP,  $p < 0.05$  for Ad-PKB vs. control,  $p =$  nonsignificant for Ad-GFP vs. control). These data demonstrate that different Akt activities determine the different apoptosis rates of VSMC from MA and SV.

## Discussion

This study demonstrates that VSMC from MA and SV exhibit different intrinsic properties, indicating that there is an intrinsic heterogeneity of these vessels. Indeed, VSMC number increases slower in MA than SV, which is neither related to PDGF receptor expression nor PDGF-BB-induced cell cycle progression, but is rather determined by higher apoptosis rates due to lower Akt activation. This observation is consistent with the different propensity of MA and SV bypass grafts to accelerated atherosclerosis.

The increase in MA VSMC number was lower in response to both FCS and PDGF-BB. This observation is consistent with patency rates of the respective bypass grafts; indeed, patients with an MA graft have a lower long-term risk of death as compared to those with only SV grafts [1]. Hence, this observation suggests that VSMC are important for the pathogenesis of bypass graft disease and influence long-term patency rates. The lower increase in VSMC number from MA as compared to aorta indicates that VSMC from different arteries exhibit different properties; moreover, these data support the interpretation that the lower proliferation of MA VSMC may protect this vessel from atherosclerosis. The difference between MA and SV as well as MA and aorta must be related to intrinsic cellular properties, as it was observed in isolated VSMC. Interestingly, proliferation of EC from MA versus SV was identical in response to different mitogens, suggesting that this aspect of endothelial function may play a less important role than the well-documented differences in the release of vasoactive mediators such as nitric oxide; however, more insight into the regulation of endothelial proliferation in MA versus SV has to be gained before definitive conclusions regarding its role in the pathogenesis of bypass graft disease can be drawn.

PDGF receptor  $\alpha$  expression was slightly higher, while receptor  $\beta$  expression was slightly lower in VSMC from MA and SV. Thus, PDGF receptor expression cannot account for the difference in response to PDGF-BB; hence,

the reason for the different proliferation rates must be located below the membrane level. As mitogenic signals are integrated in G1 phase, differences in signal transduction or cell cycle regulation would be reflected in a different G1 progression. The latter is mediated by protein complexes consisting of a cyclin and a cyclin-dependent kinase (cdk) [14]. Indeed, cyclin E and cdk2 promote late G1 progression and S-phase entry. Endogenous protein inhibitors of cyclin-cdk complexes (CKI) can modulate cdk2 activity; the members of the p21 family of CKI inhibit cell cycle progression in such a potent manner that they have been used for reducing neointima formation in restenosis models [15]. Therefore, regulation of G1 progression in VSMC from MA versus SV was examined by assessing expression and activity of cyclin E, cdk2, p21, p27, and p57. No difference in cell cycle distribution nor expression and activity of cell-cycle-regulatory proteins was observed, indicating that cell cycle progression is similar in VSMC from both vessels. In a previous report, a lower degree of p27 downregulation by PDGF-BB was observed in MA as compared to SV [16]. We were not able to confirm this observation; in all experiments, there was a tendency towards a higher p27 downregulation in MA as compared to SV. A transient downregulation of p27 in SV within the 30-hour window can be excluded, as preliminary experiments clearly demonstrated a continuously increasing downregulation over 30 h. The difference to our observation is most probably related to the fact that the protein lysates of MA and SV were made from different patients in the previous paper, while they were from the same patient in our experiments.

As demonstrated by different methods, cell death due to apoptosis rather than cell cycle progression determines the lower increase in VSMC numbers to both FCS and PDGF-BB in MA versus SV, which suggests that VSMC apoptosis is involved in the higher patency rates of MA than SV. A role for VSMC apoptosis in vascular diseases was recognized when the reaction to balloon dilatation was examined; the VSMC number in injured arteries does not necessarily increase in spite of high levels of proliferative activity [6]. Consistent with this observation, a higher rate of apoptosis may counteract the development of neointima formation in the MA as well. In line with this interpretation, the neointima of bypass grafts develops rapidly, and this response shows many properties similar to the response to balloon dilatation. In addition to neointima formation, the higher propensity towards apoptosis of VSMC from MA may reduce atherogenesis in this vessel, as the accelerated atherosclerosis of bypass

grafts occurs on top of the neointima formed in the grafted vessels. Hence, VSMC apoptosis may indeed protect the MA from bypass graft disease.

VSMC apoptosis is also observed in atherosclerotic lesions from human coronary and peripheral arteries [5–7, 17]. The functional importance of VSMC apoptosis in atherosclerosis is, however, under some debate. Indeed, the vulnerability of atherosclerotic plaques may increase with VSMC apoptosis, as the fibrous cap of lipid-rich plaques may be destabilized through proinflammatory interactions of apoptotic VSMC with macrophages, leading to remodeling of the extracellular matrix [5]. Therefore, VSMC apoptosis may promote plaque rupture, and apoptosis in the vessel wall is considered to be a two-edged sword depending on the stage of atherosclerosis [17, 18]. VSMC apoptosis primarily reduces neointima formation in the MA, as the latter only rarely becomes atherosclerotic; hence, apoptosis seems to play a beneficial role in this vessel. Indeed, later stages of plaque formation are an exception in the MA, so that a potential role of apoptosis in the rupture of plaques may not be relevant for this vessel.

Akt is an important signaling molecule, which plays a key role in cell survival. It is known to be activated in VSMC by various growth factors such as PDGF-BB [19, 20]; indeed, Akt mediates anti-apoptotic effects after stimulation with PDGF-BB [12]. Consistent with these observations, Akt activity was lower in VSMC from MA than SV, while expression was identical. Moreover, transduction of a constitutively active Akt mutant abrogated the growth differences between VSMC from MA and SV, indicating that the PDGF-induced increase in cell num-

ber critically depends of Akt activity in MA versus SV, and suggesting that modulation of Akt activity may have a therapeutic potential. Apart from Akt activity, other differences in the properties of arterial versus venous VSMC may contribute to the lower incidence of bypass graft disease in MA versus SV as well. Indeed, venous VSMC are less differentiated and synthesize lower levels of the inhibitory proteoglycan, decorin, as compared to arterial cells [21].

The major limitation of this study consists in its *in vitro* nature. Cultured VSMC may not be representative of the VSMC population *in vivo*, as the isolated cells may be a selection of those growing best on culture dishes or in response to a defined mitogen. In addition, the extracellular matrix of the intact vessel may differ from that synthesized by VSMC in culture and thereby modulate cellular properties. Moreover, VSMC *in vivo* are subjected to pulsatile stretch and exposed to cardiovascular risk factors. Therefore, this study suggests an important difference in Akt activity of VSMC from MA versus SV, which should be confirmed in an *in vivo* model.

In summary, this study demonstrates that the balance between proliferation and apoptosis determines the response of VSMC from MA versus SV to mitogenic stimulation. The higher apoptosis rates in VSMC from MA indicate that apoptosis of these cells may play a beneficial role in bypass graft disease by primarily reducing neointima formation. Thereby, the lower Akt activity in VSMC from MA seems to play a major role, suggesting that modulation of Akt activity may have a therapeutic potential for bypass graft disease.

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