Regulation of Vein Graft Hyperplasia by Survivin, an Inhibitor of Apoptosis Protein

Grace J. Wang, Xin Xin Sui, Hector F. Simosa, Mukesh K. Jain, Dario C. Altieri, Michael S. Conte

Objective—Survivin (SVV) is an inhibitor of apoptosis protein (IAP) that is upregulated in cancer and has recently been implicated in vascular injury. We sought to investigate the role of SVV in vein graft hyperplasia.

Methods and Results—Adenoviral constructs expressing a dominant-negative (AdT34A) and wild-type (AdWT) SVV were used. Proliferation and apoptosis were assayed on endothelial cells (ECs) and smooth muscle cells (SMCs) from human saphenous vein. A rabbit carotid interposition vein graft model (N=31) was used, with adventitial gene transfer of SVV constructs. In vitro, overexpression of SVV was associated with protection from cytokine-induced apoptosis in ECs and SMCs; conversely, AdT34A directly induced apoptosis in these cells. SMC proliferation was increased by AdWT infection, whereas AdT34A reduced proliferation; both effects were serum-dependent. Expression of platelet-derived growth factor (PDGF) in SMCs was regulated by functional SVV expression in analogous fashion. In vivo, proliferation and apoptosis (7 days), as well as wall thickness (30 days), were modified by adenoviral-mediated SVV expression. Adventitial angiogenesis was regulated by the SVV-expressing constructs in a fashion parallel to wall thickness changes.

Conclusions—SVV is a critical regulator of multiple processes, including proliferation, apoptosis, and angiogenesis, that determine the remodeling response of vein grafts following arterialization. (Arterioscler Thromb Vasc Biol. 2005;25:2081-2087.)

Key Words: angiogenesis ■ apoptosis ■ intimal hyperplasia ■ proliferation ■ survivin

An autologous vein graft remains the preferred conduit for the reconstruction of advanced peripheral arterial disease. However, an estimated 30% to 50% of all bypass grafts develop stenosis within the first 2 years of surgery secondary to intimal hyperplasia (IH), a fibroproliferative lesion characterized by intimal thickening.1 Much research has focused on understanding the remodeling response after arterialization of the vein graft, but several important questions remain unanswered. Neointimal cells, for example, are no longer believed to originate solely from medial smooth muscle cells (SMCs), but appear to have contributions from circulating hematopoietic precursors as well as adventitial cells.2–4 The molecular signals that control the recruitment and proliferation of these cells are also incompletely understood.

The proliferative characteristics of IH suggest obvious parallels to neoplastic disease. Cancer progression is characterized by deregulated cellular proliferation, dedifferentiation, and suppressed cellular death. In addition, at a macroscopic level, after exhaustion of local nutrients and support factors, angiogenesis must be induced to support further tumor growth. Recent interest in the gene survivin (SVV), a member of the inhibitor of apoptosis protein (IAP) family, has stemmed from observations that it is expressed across a large spectrum of human malignancy and is virtually absent in terminally differentiated tissues, making it a suitable target for cancer gene therapy.5 More recently, in vitro and in vivo studies have demonstrated an important role for SVV in the regulation of endothelial cell (EC) and SMC survival, and suggest that it may be involved in the vascular injury response.6

We have recently demonstrated that SVV is transcriptionally upregulated after vascular injury and is broadly expressed across a spectrum of vascular lesions including early as well as complex atherosclerotic plaque, postangioplasty IH in arteries, and vein bypass grafts in human and animal models.7 The current study was undertaken to determine whether SVV directly modulates mitogenic and apoptotic signals in vascular cells, and if targeted manipulation of SVV expression in the arterialized vein would alter the subsequent development of IH within the graft.

Methods

Cell Culture and Adenoviral Transduction of SVV Constructs

Discarded segments of human saphenous vein (HSV) were obtained from patients undergoing cardiac and vascular surgical procedures.
under a protocol approved by the institutional review board of Brigham and Women’s Hospital. Primary EC and SMC cultures were established as previously described (please see Methods supplement at http://atvb.ahajournals.org).6 Replication-deficient adenoviruses (E1, E3-deleted, serotype 5) expressing bicistronic constructs of wild-type human SVV (AdWT) and enhanced green fluorescent protein (GFP), a dominant-negative (phosphorylation-defective Thr34 → Ala mutant; AdT34A) SVV mutant and GFP, as well as GFP alone, were kindly provided by the Core Vector Laboratory of the Harvard Gene Therapy Initiative. Details of these constructs have been provided elsewhere.9 Viral titers were determined by plaque assay on 293a cells. SMCs and ECs were infected at a multiplicity of infection (MOI) of 500 for SMCs and 250 for ECs for 12 hours at 37°C. Live cultures were examined for GFP expression using fluorescence microscopy, and successful transduction of >80% of cells was confirmed in all cases.

Cell Proliferation Assay
SMCs were seeded onto 24-well plates at a density of 104 cells/well, then infected with adenoviral constructs (AdT34A, AdWT; and AdGFP) for 12 hours in media containing varying serum concentrations (0.5%, 1%, or 10%). Anti-PDGF neutralizing antibody10 (5 μg/mL; R&D Systems) was replenished every 2 days in selected wells. Alamar Blue (BioSource International, Inc) assays were conducted every 24 hours according to the manufacturer’s protocol. Fluorescence measurements (excitation 506 nm, emission 590 nm) were made on media aliquots; a standard curve was generated by correlating emission intensity with viable cell counts using Trypan Blue exclusion.

DNA Content Analysis of Apoptosis
SMCs and ECs were seeded onto 6-well plates at 75% confluence and infected with viral constructs as described. Apoptosis was induced in SMC by stimulation with 400 U/mL human IFN-γ (R & D Systems), 400 U/mL human tumor necrosis factor (TNF)-α (Pierce Endogen), and 100 U/mL human IL-1β (Pierce Endogen) for 48 hours. ECs were treated with 5 ng/mL TNF-α, and 5 μg/mL cycloheximide (Calbiochem,) for 48 hours. After cytokine stimulation, cells were fixed with 70% ETOH, stained with propidium iodide (0.5 mg/mL; Roche Diagnostics), and then analyzed for DNA content by flow cytometry (FACScalibur; Becton Dickinson). Data analysis was performed with Flow software (Tree Star, Inc).

Immunofluorescence for Annexin V
Please see Methods supplement at http://atvb.ahajournals.org

RNA Isolation and Reverse-Transcriptase Polymerase Chain Reaction
HSVSMCs were quiesced for 24 hours in serum-free medium, then infected with viral constructs for 12 hours. The next day, RNA was isolated with Trizol (Gibco) and 1 μg of total RNA was used in a reverse-transcriptase reaction to generate cDNA (BioRad iScript cDNA Synthesis Kit). Quantification of gene expression was performed by semi-quantitative reverse-transcriptase polymerase chain reaction, using oligonucleotide primers specific for PDGF-A, PDGF-B, and β-actin, using the Titanium TaqPCR kit (Clontech; BD Biosciences; see http://atvb.ahajournals.org for details of polymerase chain reaction primers and conditions).

PDGF Promoter Assay
PDGF-A (pACCAT12) and PDGF-B (psisCAT6A) promoter constructs and a control reporter plasmid expressing chloramphenicol acetyltransferase (pCAT3) were kindly provided by Dr Tucker Collins.11 SMCs were seeded onto 12-well plates at a density of 104 cells/well, then infected with the SVV adenoviral constructs. The next day, cells were cotransfected with one of the PDGF promoter constructs as well as a CMV–β-gal plasmid (4:1 ratio) to control for transfection efficiency. Transfections were performed with 3 μL of FuGene (Roche) and a total of 1 μg of DNA/well for 48 hours. Cells were harvested for quantitation of CAT and β-gal by enzyme-linked immunosorbent assay (Roche) and values were normalized to protein content (BCA method; Pierce).

Rabbit Vein Graft Model and In Vivo Gene Transfer
All animal experiments were performed in accordance with the requirements outlined by the Guide for the Care and Use of Laboratory Animals,12 under a protocol approved by the Harvard Medical Area Standing Committee on animals. New Zealand White (NZW) rabbits (N=31) weighing 3 to 3.5 kg were anesthetized with intramuscular ketamine (25 mg/kg) and xylazine (5 mg/kg) and maintained with intravenous dosing of a ketamine (2.5 mg/kg) and xylazine (0.5 mg/kg) mix. Animals were placed on a normal chow diet and postoperative analgesia was maintained by daily intramuscular administration of flunixamine 5 mg for at least 48 hours.

We used the anastomotic cuff technique previously described for the creation of a carotid artery interposition vein graft using jugular vein.15 After completion of both anastomoses, adenoviral vectors were applied to the outside of the vein graft using a 200 μL mixture of 30% pluronic gel F127 (polyoxyethylene–polyoxypropylene block copolymer; BASF) and the viral construct (5×107 plaque-forming units/mL). Once the gel was solidified, the incision was closed.

Harvest and Processing of Tissue Samples
After induction of anesthesia and after confirming patency of the vessels studied, a heparin (100 units intravenous) bolus was given, animals were euthanized with an overdose of anesthetic. Vein grafts harvested at the 1-week time point were immediately snap-frozen in OCT compound (Sakura Finetek, Inc) with liquid nitrogen (LN2) and 2-methylbutane (Sigma) and stored at −80°C for later sectioning. Vein grafts harvested at 4 weeks were perfusion-fixed (80 to 100 mm Hg) with 10% neutral-buffered formalin (NBF) (Sigma) and subsequently paraffin-embedded for sectioning.

Quantitative Morphometry-Elastin Staining
Six-μm-thick sections (15 per graft) from the middle segment of paraffin-embedded vein grafts (N=16) were stained with Verhoff’s Van Gieson elastin stain. Images were collected using a microscope (Olympus BX41) and online digital camera (Kodak). Outlines of the vessel lumen, internal elastic lamina, and external graft wall (as judged by the boundary between circumferentially oriented medial SMC and adventitial microvessels) were measured using image analysis software (Scion Software) and intimal, medial, and total wall thickness were calculated. Mean values from the 15 measured sections were assigned to each graft.

Immunohistochemistry and Quantitation of Proliferation, Apoptosis, and Microvessels
Immunohistochemistry was performed on 6-μm-thick sections from frozen 1-week-old vein grafts (N=15) and formalin-fixed, paraffin-embedded 4-week-old grafts (N=16). For details, please see http://atvb.ahajournals.org.

Double Immunofluorescent Staining for Colocalization of SVV with CD31 and PDGF
Please see http://atvb.ahajournals.org for detailed protocol.

Statistical Methods
Comparisons between treatment groups were made using 1-way ANOVA, with unpaired t tests (and Bonferroni’s correction) as appropriate.

Results
SVV Regulates Proliferation of Human Saphenous Vein Smooth Muscle Cells
SVV expression is known to be upregulated at the G2/M phase of the cell cycle and is stimulated by mitogens, but a
SVV Regulates Apoptosis of Primary Human Saphenous Vein SMCs and ECs

Previous studies have demonstrated that SVV regulates apoptosis in nonhuman SMC cell lines. To directly investigate this role in primary cultures from human saphenous vein, cell-cycle analysis was performed. Infection with AdGFP did not induce apoptosis in either HSEC or HSVSMC cultures (not shown). Cytokine stimulation of apoptosis in ECs and in SMCs resulted in the reproducible induction of apoptosis as reported elsewhere (Figure IIA and IIB, available online at http://atvb.ahajournals.org). As expected, infection with the dominant-negative AdT34A construct directly induced apoptosis in both ECs and SMCs, and the combination of T34A-mutant expression and cytokine exposure produced an additive pro-apoptotic effect. In contrast, ECs and SMCs that were pretreated with AdWT were protected from cytokine-mediated apoptosis.

Annexin V staining was performed to confirm the DNA content analysis and to directly assess the colocalization of transgene expression (assessed by GFP fluorescence) and apoptosis in individual cells (Figure IIC). These studies confirmed a high degree of colocalization of T34A expression and Annexin V, with minimal or no Annexin V staining seen in cells expressing the WT SVV construct, with or without cytokine stimulation.

Local Periadventitial Delivery of SVV Constructs Modulates Proliferation and Apoptosis in the Early Phase of Vein Graft Healing

Rabbit vein grafts treated with the adenoviral SVV constructs were examined 1 week after implantation to evaluate transgene expression and cellular kinetics. Immunohistochemistry for GFP demonstrated transgene expression in all adenovirus treated grafts, with the majority of expression localized to the adventitia and scattered areas of the neointima and media treated grafts, with the majority of expression localized to the adventitia and scattered areas of the neointima and media.

SVV Regulates PDGF Expression

Given the noted effects of SVV constructs on early proliferation in the vein graft, we investigated the expression of PDGF as a potential downstream mediator. One week (non-infected control) vein grafts immunostained for PDGF-A and SVV revealed significant overlap within the intima and media.

Figure 1. Local, periadventitial delivery of survivin constructs modulates proliferation and apoptosis in the early phase of vein graft healing. A, Vein grafts explanted 1 week after implantation and adventitial delivery of indicated adenoviral constructs, immunostained for GFP (upper left) and for the proliferation marker Ki-67 (other panels; L=lumen; 100×). Positive staining for GFP transgene is evident throughout the adventitia and in scattered areas of the intima and media. B, Bar graph summarizes quantitative assessment of the proliferative index (Ki-67+/total number of nuclei per graft). *P<0.05 vs control groups. C, Bar graph summarizes quantitative assessment of the apoptotic index, apoptotic nuclei/total number of nuclei per graft). *P<0.005 vs control groups.

(Figure 1A, upper left panel). Control vein grafts that were untreated or exposed to pluronic gel alone (without vector) did not demonstrate any staining for GFP (not shown).

Ki-67 staining (Figure 1A and 1B) of 1-week-old rabbit vein grafts demonstrated that treatment with the AdT34A construct decreased cellular proliferation, whereas the AdWT construct dramatically enhanced proliferation in the early phase of graft remodeling (proliferative index 24% control, 9.8% T34, 66% WT; P<0.005 ANOVA). Interestingly, the number and distribution of proliferating cells within the AdWT-treated graft appeared to exceed the distribution of transgene expression, suggesting the possibility of a paracrine effect. There was no difference in Ki-67 index between AdGFP- treated grafts and grafts treated with 20% pluronic gel without virus.

Apoptotic index in untreated controls, gel alone, or Ad GFP treated grafts at one week was similar (5% to 8%; Figure 1C). We observed an increased apoptotic rate in AdT34A-treated grafts, and a reciprocal decrease in AdWT-treated grafts (2.75-fold versus 0.66-fold; P<0.005). In a similar fashion to that observed for the Ki-67 stained grafts, the AdT34A-treated grafts demonstrated apoptotic cells throughout the intima, media, and adventitia.
sis and/or myofibroblast recruitment, and supporting the suggestion of a possible role for SVV in adventitial angiogene-
sis. Previous studies had demonstrated strong endogenous ex-
pression of SVV in the adventitia of healing vein grafts, leading to a reduction in intimal thickness, whereas the enhanced mediator, strongly suggestive of colocalization (Figure 2A through 2C). Comparison among the adenovirus treatment groups (Figure 2D through 2F) revealed diminished expression of PDGF-A in the AdT34A-treated grafts in contrast to a marked increase in PDGF-A expression in the AdWT grafts.

In vitro, expression of PDGF-A and PDGF-B transcripts in SMCs was regulated by functional SVV expression. Infection with the AdT34A construct reduced the serum-dependent increase in PDGF transcription, whereas overexpression of SVV with AdWT markedly unregulated PDGF even in serum-free conditions (Figure 3A). Supporting a direct link between SVV and PDGF expression, overexpression of SVV resulted in a 2.8- and 2.4-fold increase in PDGF-A and PDGF-B promoter activation (Figure 3B; \( P < 0.005 \)). Conversely, SMCs infected with the AdT34A construct demonstrated a 60% and 70% reduction in PDGF-A and PDGF-B promoter activity, respectively (\( P < 0.05 \)). These findings imply that SVV regulation of vein graft remodeling may be mediated, at least in part, by PDGF.

Local, Periadventitial Delivery of SVF Constructs Modulates Vein Graft Hyperplasia at 1 Month
Morphometric analysis of elastin-stained sections was performed to quantitate structural changes in rabbit vein grafts at 30 days after implantation (Figure 4A). Treatment with the AdT34A construct resulted in a significant reduction (41%) in vein graft wall thickness, whereas treatment with the AdWT construct reciprocally augmented (137%) the hyperplastic response. Separation of the effect into intimal and medial compartments demonstrated that the inhibition observed in the AdT34A treatment group was largely attributable to a reduction in intimal thickness, whereas the enhanced response observed in the AdWT-treated grafts was mostly caused by medial growth (Figure 4B).

**SVV Regulates Adventitial Angiogenesis in the Healing Vein Graft**

Previous studies had demonstrated strong endogenous expression of SVV in the adventitia of healing vein grafts, suggesting a possible role for SVV in adventitial angiogenesis and/or myofibroblast recruitment, and supporting the experimental approach of periadventitial gene delivery. Histological examination revealed a significant reduction in adventitial microvessels in AdT34A-treated grafts, as compared with AdGFP-treated grafts at 7 and 30 days (38% and 17% reduction versus GFP at 7 and 30 days; \( P < 0.05 \); Figure 5A). In contrast, AdWT-treated grafts demonstrated a dramatically enhanced angiogenic response at both time points examined (82% and 42% increase over GFP at 7 and 30 days; \( P < 0.05 \)). There were no differences in microvessel number between AdGFP-treated grafts, pluronic gel-treated grafts, or grafts without gel. Regression analysis revealed a positive correlation between microvessel number and total wall thickness (\( r^2 = 0.5259; P < 0.005 \)) at 30 days, suggesting a direct association between adventitial angiogenesis and the progression of vein graft hyperplasia in this model. Double immunofluorescent staining demonstrated considerable overlap in expression of CD31 and SVV in the vein graft adventitia, notably within the walls of microvessels (Figure 5B).

**Discussion**

These data provide compelling evidence for an important role for SVV in the regulation of vein graft remodeling and suggest several relevant mechanisms. We have demonstrated that SVV regulates proliferation as well as apoptosis in vascular SMCs using both in vitro and in vivo models in which SVV was overexpressed (AdWT) or functionally inhibited (AdT34A) in target cells. Targeting SVV expression...
Despite its important role in mitosis, however, there is disorganization of mitotic spindles resulting in failure of chromosomes. SVV knockout embryos exhibit complete binding to microtubules at G2/M in the cell cycle, SVV acts of vein grafts after implantation.

expression is strongly upregulated in the adventitia and media in recent studies. Our data, both in vitro and in vivo, suggest the relationship between SVV and p53 has been suggested in some recent studies. Our data, both in vitro and in vivo, suggest that SVV expression may directly regulate a novel mitogenic pathway in SMCs. The proliferative response observed after exposure to the SVV constructs in vivo appeared to exceed both the localization and magnitude of transgene expression, suggesting the possibility of paracrine signaling. To this end, we examined the expression of PDGF, a prototypic SMC growth factor that is known to be associated with vascular injury and vein graft hyperplasia. The data presented provide strong evidence that SVV regulates PDGF expression, both in vitro (by reverse-transcription polymerase chain reaction and promoter activity assay) and in vivo (immuno-staining). Previous work has shown that PDGF induces SVV expression in SMCs. This leads us to hypothesize a potential positive feedback loop in which SVV and PDGF may amplify proliferative signals in a paracrine fashion. The mitogenic effect of the AdWT construct on SMCs was markedly reduced, but not completely abolished, in the presence of the pan anti-PDGF antibody, suggesting that other factors in serum are required for full expression of the SVV-induced proliferative phenotype. Further studies are required to elucidate the specific pathways by which SVV may be linked to other cytokine or growth factor-mediated cellular events.

Previous studies have established the anti-apoptotic function of SVV, although the mechanism remains incompletely defined. SVV is known to bind to pro-caspase 9 and prevent its activation by cytochrome c, thus inhibiting the intrinsic pathway of apoptosis. Additionally, SVV inhibits effector caspases 3, 6, and 7, which mediate the extrinsic, or TNF-α/ Fas receptor pathway of apoptosis. Recent studies have identified a novel mitochondrial pool of SVV that mediates cytoprotection. Because much of the work on SVV has used cancer cell lines, questions regarding its role in normal cells
have persisted.24 Here we confirm that SVV protects against cytokine-induced apoptosis in primary cultures of ECs and SMCs, as well as in the early phase of vein graft healing in vivo. Our in vitro and in vivo data further confirm a direct pro-apoptotic effect of the dominant-negative AdT34A construct, consistent with a critical role for endogenous SVV in cell survival. In a fashion analogous to proliferation, apoptosis within the T34A-treated vein grafts appeared to exceed the distribution of adenosine transduction, suggesting that inhibition of SVV in a critical population of cells may modulate signaling pathways that control neighboring cell survival (eg, PDGF). These data imply that SVV expression plays an important role in controlling EC and SMC apoptosis with direct relevance to vascular injury.

Adventitial delivery of the adenosine SVV constructs produced significant, and reciprocal, changes in vein graft wall thickness at 1 month. Recent evidence has suggested that cells derived from the adventitia (eg, myofibroblasts),3,25,26 as well as circulating progenitor cells,4 may play a significant role in the development of intimal hyperplasia. Though we are unable to provide direct evidence of SVV expression in adventitial myofibroblasts, we hypothesize that a critical population of adventitial-derived cells express SVV as they are activated in the injury response. SVV has been found to be important in regulating the proliferation of circulating CD34+ hematopoietic stem cells27 and granulocyte macrophage colony-forming unit (CFU-GM) cells.28 Of interest, treatment with AdT34A reduced wall thickness primarily by attenuating neointima formation, whereas the AdWT construct produced its most marked effect in enhancing medial thickness. A potential explanation for this differential response may be that endogenous SVV expression is critical to the early proliferative burst that accompanies the formation of a nascent neointima; subsequent remodeling of the graft to reduce wall tension may then occur by medial thickening. Similar findings were reported by Ehsan et al using an alternative approach of transient cell-cycle blockade in the acute phase of vein graft healing.29

The present studies also highlight an important role for SVV in the angiogenic response to vein grafting. Adventitial microvessel formation was potently inhibited by AdT34A, and enhanced by AdWT infection. SVV expression in the adventitia strongly colocalized with CD31(+) microvessels. Microvessel count was in turn correlated with wall thickness, suggesting that the development of graft hyperplasia requires an enhanced blood supply. This concept is supported by other recent studies linking intimal hyperplasia and adventitial angiogenesis in arterial injury models30 as well as human vein graft lesions.31

In conclusion, the present studies demonstrate that SVV is a critical regulator of apoptosis, proliferation, and angiogenesis within the healing vein graft, paralleling its roles in neoplasia.32 Further studies are required to clarify the signaling pathways involved in these distinct yet complementary effects. Given this broad profile of activity, SVV appears to be a relevant molecular target for modulating the vascular injury response.

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On-line Methods supplement

**Cell culture**

EC were grown on a 1% gelatin substrate using complete EC medium (Medium-199, BioWhittaker) with 20% fetal bovine serum (BioWhittaker), endothelial cell growth supplement (50 µg/ml ; Collaborative Biomedical), L-glutamine (2 mmol ;Gibco), porcine intestinal-derived heparin (17.5 units/ml; Sigma) and antibiotic/antimycotic (PSF; Sigma). SMCs were grown in uncoated tissue culture dishes and maintained with medium composed of Dulbecco’s Modified Eagle’s Medium (BioWhittaker), 10% FBS, and PSF.

**Immunofluorescence for Annexin V**

SMCs were seeded into an 8-chamber cell culture slide (Nalge Nunc International) at 75% confluence. Select wells were infected with adenoviral constructs at an MOI of 500 for 12 hours. Select wells were then treated with the cytokine cocktail described (human IFN-γ 400 u/ml, human TNF-α 400 u/ml, human IL-1β 100 u/ml) for 48 hours. The cells were then stained with a rhodamine-conjugated antibody to Annexin V (BioVision), fixed with 2% formalin and examined using fluorescent microscopy (Zeiss Axioplan 2).

**RT-PCR Methods**

PDGF-A primers employed were: forward primer: 5’AAT TTC GCC GCC ACA GGA GA-3’, reverse primer: 5’- ACG GGG GCC AGA TCA GGA AG-3’. PDGF-B primers employed were: forward primer: 5’ GAT CCG CTC TTT TGA TGA TC-3’, reverse primer: 5’-GTC TCA CAC TTG CAT GCC AG-3’. Beta-actin primers employed were: forward primer: 5’- GAG ACC TTC AAC ACC CCA GCC ATG-3’, reverse primer: 5’- AGC CAG GTC CAG ACG CAG GAT-3’. PCR conditions included an initial 5’ denaturation step at 95 degrees, a 40-cycle amplification program: 95 degrees, 1’, 60 degrees, 2’, and 72 degrees, 3’. Reaction products were subjected to agarose (1.2%) gel electrophoresis after each experiment, to confirm the presence of a single band of expected size for PDGF-A (449 bp), PDGF-B (435 bp,) and beta-actin (171 bp).

**Immunohistochemistry and quantitation of proliferation, apoptosis, and microvessels**

Cryosections were first thawed at RT and fixed in acetone for 10 minutes at 4°C. Paraffin-embedded grafts were deparaffinized in xylene, brought to water point and boiled in 0.01 M citrate buffer (pH6.0) inside a pressure cooker for antigen retrieval. The sections were quenched with 1.5% H₂O₂ solution for 10 minutes, blocked in 10% horse serum and incubated with antibodies to survivin (60.1, NOVUS, 1:500), Ki -67 (MIB-1, Cell Marque Corp., 1:100), CD31 (JC70A, DAKO,1:30), or GFP (JL-8, Becton Dickinson, 1:50) for 1 hour at RT. Bound primary antibodies were detected using avidin-biotin-peroxidase (NovaRed Peroxidase Substrate Kit, Vector Laboratories). Sections were counterstained with Gill’s hematoxylin. followed by dehydration in graded ethanol.
and xylene. Staining for apoptosis was performed per manufacturer’s protocol (VasoTACS, Trevigen).

To obtain proliferative or apoptotic indices, positively stained cells within the media/neointima of 3 evenly spaced sections per vessel were counted manually and divided by the total number of nuclei. Adventitial microvessel density was determined by manual count of CD31 stained endothelial cell clusters as described by Weidner et al.\(^\text{15}\).

**Double immunofluorescent staining for co-localization of SVV with CD31 and PDGF**

Six µm-thick cryosections of vein grafts (N=15) were thawed at RT, fixed in acetone (Sigma) for 10 minutes at 4°C, blocked in 10% horse serum and incubated with the antibody to CD31 (JC70A, DAKO1:30) or PDGF-A (sc-9974, Santa Cruz Biotechnology, 1:100) for 1 hour. The first bound primary antibody was detected using Fluorescein anti-mouse IgG (Vector Labs 1:30). The Mouse IgG Blocking Reagent was then applied for 1 hour (Vector Labs) followed by application of the second primary antibody to survivin (60.11, NOVUS, 1:500 or 0.004 ug/l). The second primary was detected using Texas Red anti-mouse IgG. Double-stained sections were examined on a fluorescent microscope (Zeiss Axioplan 2).

**On-line Data supplement**

**Figure I. Survivin regulates proliferation of HSV SMC**

Proliferation assays were performed in primary cultured HSVSMC maintained in 0.5% (A) and 10% (B) serum. Assays were performed in triplicate and results shown are mean of three individual experiments. (*p<0.05 for AdWT and AdT34 vs AdGFP). The increase in proliferation in AdWT infected cells was blocked by anti-PDGF antibody (2.5-fold vs 1.2-fold increase p<0.005).

**Figure II. Survivin regulates apoptosis of HSV EC and SMC**

A. Summary of DNA content analyses in SMC and EC. Control: no apoptotic stimulus or virus; S indicates cytokine stimulation; V indicates infection with indicated viral construct. Data shown are mean values from three separate experiments. *p<0.003 vs no virus and GFP; and #p<0.01 vs (+S-V) group.

B. Representative histograms of SMC DNA content analysis by flow cytometry. AdT34A infection resulted in a direct induction of apoptosis, and was additive to cytokine stimulation. Conversely, infection with AdWT had a protective effect. Data shown for SMC is representative of three independent experiments.

C. Smooth muscle cells infected with AdGFP, AdWT or AdT34A constructs, with or without cytokine stimulation (+/- S) to induce apoptosis were stained with a rhodamine-conjugated antibody to Annexin V. Green fluorescence (indicating expression of the GFP-containing viral constructs) and red fluorescence (Annexin V) are overlaid to examine transgene expression and apoptosis in individual cells. SMCs expressing the WT SVV construct were notably less susceptible to apoptosis than cells expressing the dominant-negative T34A mutant, with and without cytokine induction. (100x)
C  \(\alpha\)-GFP (green)/\(\alpha\)-Annexin V (red)

- AdGFP
- +S-V
- -S+AdWT
- -S+AdT34
- +S+AdWT
- +S+AdT34