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Short Communication

Mitochondrial Heat Shock Protein-90 Modulates Vascular Smooth Muscle Cell Survival and the Vascular Injury Response *in Vivo*

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The healing response of blood vessels from the vascular injury induced by therapeutic interventions is characterized by increased cellularity and tissue remodeling. Frequently, this leads to intimal hyperplasia and lumen narrowing, with significant clinical sequelae. Vascular smooth muscle cells are the primary cell type involved in this process, wherein they express a dedifferentiated phenotype that transiently resembles neoplastic transformation. Recent studies have highlighted the role of mitochondrial proteins, such as the molecular chaperone heat shock protein-90 (Hsp90), in promoting cancer cell survival, which leads to new candidate chemotherapeutic agents for neoplastic disease. Herein, we identify mitochondrial Hsp90 as a key modulator of the vascular injury response. Hsp90 expression is up-regulated in injured arteries and colocalizes with the apoptosis inhibitor, survivin, in vascular smooth muscle cell *in vitro* and *in vivo*. By using a proteomic approach, we demonstrate that targeted disruption of mitochondrial Hsp90 chaperone function in vascular smooth muscle cell leads to loss of cytoprotective client proteins (survivin and Akt), induces mitochondrial permeability, and leads to apoptotic cell death. Hsp90 targeting using a cell-permeable peptidomimetic agent resulted in marked attenuation of neointimal lesions in a murine arterial injury model. These findings suggest that mitochondrial Hsp90 chaperone function is an important regulator of intimal hyperplasia and may have implications for molecular strategies that promote the long-term patency of cardiovascular interventions. *(Am J Pathol 2012, 181:1151–1157; http://dx.doi.org/10.1016/j.ajpath.2012.06.023)*

Intimal hyperplasia (IH) is a vaso-occlusive pathological process that limits the efficacy of cardiovascular interventions, including angioplasty, stenting, and surgical bypass. It is characterized by increased cellularity in the vessel wall, dominated by an expansion of dedifferentiated vascular smooth muscle cells (VSMCs).[1] The mechanisms regulating IH involve multiple signaling pathways that collectively alter VSMC phenotype. The end result is a temporal and spatial disparity between proliferation and apoptosis that may lead to excessive tissue expansion and lumen compromise.

The 90-kDa heat shock protein-90 (Hsp90) is a highly abundant cellular protein that is a key molecular chaperone associated with cell cycle regulation and apoptotic pathways.[2,3] In cancer cells, direct interaction between Hsp90 and its client proteins appears central to maintaining an apoptosis-resistant cell phenotype, and has become a target for cancer therapeutics.[4–6] The role of Hsp90 in vascular injury is less well established. However, it has been recently implicated in a pro-inflammatory phenotype of VSMCs[7] and in the pathogenesis of atherosclerosis.[8] We hypothesize that neointimal VSMCs transiently use molecular pathways of proliferation and apoptosis resistance, similar to neoplasia. We, therefore, investigated whether dis-

ruption of Hsp90 chaperone function would alter VSMC survival circuits that may be essential to IH.

Shepherdin is a candidate anticancer molecule that is a direct inhibitor of Hsp90 chaperone function. It is a biotinylated, cell-permeable, retro-inverso peptidomimetic molecule incorporating residues 79 to 87 of the inhibitor of apoptosis protein survivin (SVV), an Hsp90 client. This sequence was identified as a minimal sequence of SVV that interacts with the ATP-dependent binding pocket of Hsp90, and its presence has competitively inhibited the HSP90 chaperone function of SVV.[5] SVV is an important regulator of cell cycle progression and inhibits apoptosis. It has had a role in both cancer and IH. In particular, disruption of Hsp90 chaperone function by shepherdin induces apoptosis in multiple cancer cell lines.[4,9–11] Shepherdin has been designed as a specific inhibitor of the SVV-Hsp90 interaction. In addition, there is evidence that it also inhibits Hsp90 chaperone function involving Akt. Furthermore, subcellular pools of Hsp90, particularly in the mitochondria, have demonstrated a central role in the regulation of tumor cell growth.[12,13] More importantly, the targeting of mitochondrial fractions of Hsp90 by shepherdin in prostate cancer and glioblastoma cells has demonstrated tumor cell killing *in vitro* and *in vivo*.[14] In this study, we investigate the role of mitochondrial Hsp90 in regulating VSMC survival pathways that may contribute to IH after vascular injury.

Materials and Methods

Cell Isolation and Culture

Primary human VSMC (HVSMC) cultures were established from discarded saphenous vein segments after cardiac or vascular surgical procedures using an explant protocol, as previously described.[10] Primary rabbit aortic VSMC cultures were established by explant culture of thoracic aorta from New Zealand white rabbits. Cells were used in passages 2 to 6.

Peptidomimetics

All peptides were synthesized by the W. M. Keck Biotechnology Research Center at Yale University School of Medicine.[5] The SVV sequence K79-L87 (KHSSGCAFL) was attached to a cell-permeable *Antennapedia* homeodomain (italicized sequence following) in a retro-inverso sequence, resulting in the final sequence: free/biotin-*RQIKIWFQNRRMKWKK*LFACGSSHK-COOH. Similarly, a scrambled peptide (SKLACFSHG) was constructed to serve as a control with the sequence free/biotin-*RQIKI-WFQNRRMKWKK*GHSFCALKS-COOH. Reconstituted peptides were diluted under sterile conditions for *in vitro* and *in vivo* experiments.

Cell Viability Assay

A total of 40,000 VSMCs per treatment group were treated with or without peptide for 8 hours. An MTT viability assay was performed per manufacturer's instructions (TOX1 assay; Sigma-Aldrich, St. Louis, MO). For experiments using

cyclosporin A (CsA), cells were incubated for 24 hours in 1 μmol/L CsA before treatments, as noted.

DNA Content Analysis

Peptide-treated HVSMCs were fixed, stained with propidium iodide (0.5 mg/mL; Roche Diagnostics, Indianapolis, IN), and analyzed for DNA content by flow cytometry (FACScalibur; Becton Dickinson, Franklin Lakes, NJ). Data analysis was performed with FlowJo (Tree Star, Ashland, OR).

Annexin V Assay

VSMCs were seeded into a four-chamber cell culture slide (Nalge Nunc International, Rochester, NY) at 75% confluence; treated with peptide for 8 hours; stained for annexin V, per manufacturer's instructions (BioVision, Mountain View, CA); and imaged via fluorescence microscopy.

Co-Immunoprecipitation

Total protein lysate, 300 μg, was collected from 3 million HVSMCs and incubated with 5 mg/mL anti-SVV rabbit polyclonal antibody (Novus Biologicals, Littleton, CO) at 4°C for 12 hours. Bound protein was precipitated with protein A slurry at 4°C. After washing and boiling the bound fraction, all fractions were assayed using Western blot analysis, as described later.

In Vitro *Shepherdin Assay and Western Blot Analysis*

VSMCs were plated at 75% confluence; after 24 hours, they were treated with either shepherdin or scrambled peptide for 8 hours, followed by harvesting and lysis in the presence of protease inhibitor (Sigma-Aldrich). The lysate supernatant was extracted by centrifugation, and protein concentrations were checked by modified Lowry assay (Detergent Compatible Assay; Bio-Rad, Hercules, CA), per manufacturer's instructions. Total protein, 30 μg per plate, was separated by SDS-PAGE gel and transferred to a polyvinylidene difluoride membrane using a semidry technique (Bio-Rad). The membrane was incubated overnight at 4°C with primary antibodies [anti-SVV, 1:1000 (Novus Biologicals); anti-Akt, 1:1000 (Santa Cruz Biotechnology, Santa Cruz, CA); anti-Hsp90, 1:1000 (Becton Dickinson); and anti-β-actin, 1:2500 (Sigma-Aldrich)], followed by corresponding secondary antibody. Bands were detected using Super-Signal Pico chemiluminescence reagent (Pierce, Rockford, IL), according to the manufacturer's instructions.

Subcellular Protein Fractionation

HVSMC lysates were fractionated into cytosolic and mitochondrial fractions for Western blot analysis using the Qproteome Mitochondria Isolation Kit (Qiagen, Valencia, CA). After separation by SDS-PAGE gel and membrane transfer, the membranes were probed to quantify protein expression of Hsp90 (Cell Signaling Technology, Danvers, MA) and SVV (Santa Cruz Biotechnology). Glyceraldehyde-3-phosphate dehydrogenase (Santa Cruz Biotechnology) and cytochrome C (Clontech, Mountain

View, CA) were used for cytosolic and mitochondrial protein loading controls, respectively.

JC-1 Assay

The JC-1 potentiometric dye (Invitrogen, Carlsbad, CA) was used to measure mitochondrial membrane potential. HVSMCs were cultured in Dulbecco's modified Eagle's media with 0.5% fetal bovine serum, 5 μmol/L 17-allyl-amino-17-demethoxygeldamycin (17-AAG), 50 nmol/L shepherdin, 50 nmol/L scrambled peptide, or 50 μmol/L peroxynitrite for 24 hours. Cells were treated with JC-1 dye and analyzed using flow cytometry, per manufacturer's instructions. Mitochondrial membrane potential was estimated by calculating a ratio of red/green fluorescence.

Balloon Injury and Local Peptidomimetic Delivery in Rabbits

All animal experiments were performed in accordance with the standards of the Harvard Medical Area Standing Committee on Animals. New Zealand white rabbits (Charles River Laboratories, Wilmington, MA), weighing 3.0 to 3.5 kg, underwent bilateral external iliac artery injury with a number 2 Fogarty balloon.[10] A 40% Pluronic gel F127 matrix (BASF, Florham Park, NJ), with or without 50 to 250 μmol/L shepherdin, was applied to the external iliac artery segment. The external iliac arteries were harvested at days 3 and 5 postoperatively for evaluating the early vessel changes (via cryosectioning) or at day 28 for evaluating IH (via 80 to 100 mmHg, 10% normal-buffered formalin perfusion fixation, paraffin embedding, and sectioning).

Wire Injury and Systemic Peptidomimetic Delivery in Mice

Male C57BL/6 mice, aged 8 to 10 weeks and weighing 20 to 30 g (Charles River Laboratories), underwent common femoral artery injury via exposure of the superficial femoral artery using six passes of a 0.014-inch diameter angioplasty guide wire (CROSS-IT 100 XT; Abbott Vascular, Santa Clara, CA). The arteriotomy site in the superficial femoral artery was subsequently ligated. For the first postoperative set, day 14 after injury, mice were administered 50 mg/kg shepherdin or vehicle (saline) via i.p. injection. At day 21 after injury, the mice were euthanized and perfusion fixed with 10% normal-buffered formalin. The injured common femoral artery was harvested and processed into paraffin for sectioning.

Immunofluorescent Staining and TUNEL Assay

All of the immunofluorescent staining was completed on frozen sections (6 μm thick) fixed by ice cold acetone for 10 minutes. Goat anti-mouse IgG, conjugated with Alexa Fluor 488 or 568 (1:150, respectively; Invitrogen), was selected as secondary antibody. Mouse IgG$_1$ (number MAB002; R&D Systems, Minneapolis, MN) or IgG2a, κ (number NB600-986; Novus Biologicals) was used as isotype negative control. Hsp90 (AC88 anti-Hsp90, 1:100, number ab13492; Abcam, Cambridge, MA) *in vivo*–localized expression or colocalization with SVV (anti-SVV, 1:500, number NB500-205; Novus Biologicals) was examined on day 5 injured rabbit iliac arteries. Anti-Ki-67 (1:100, number NCL-Ki-67-MM1; Novocastra Laboratories, Newcastle Upon
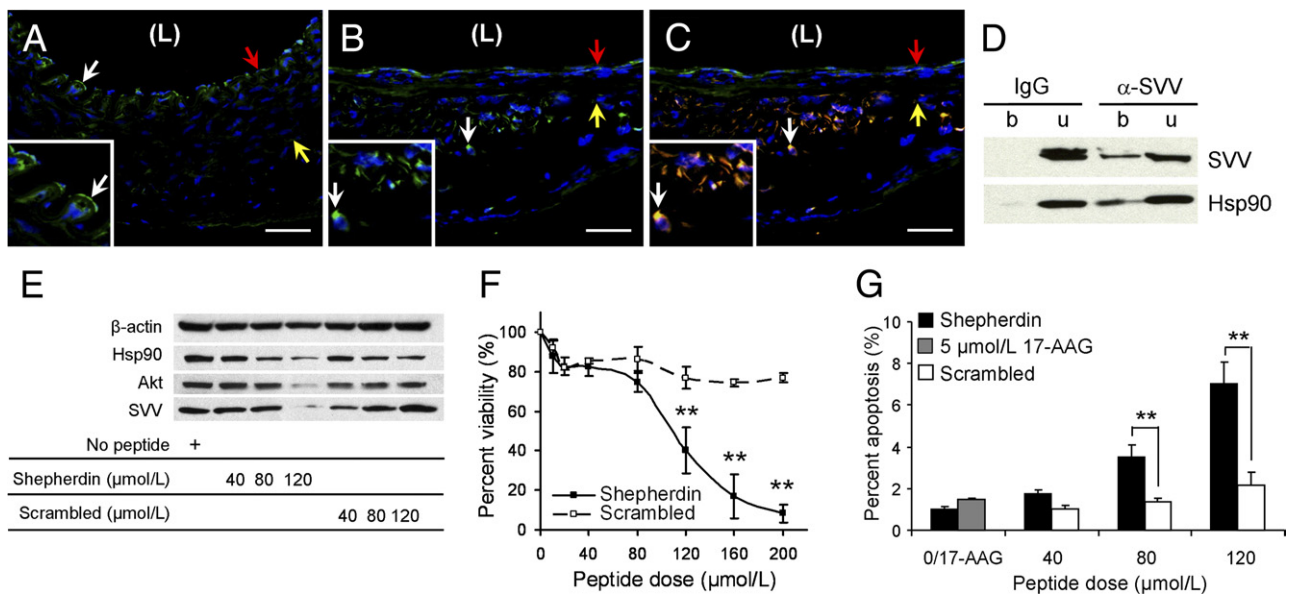

Figure 1. Hsp90 and SVV associate in VSMCs, and the targeted disruption of this interaction with shepherdin rapidly induces apoptotic cell death. **A:** Uninjured rabbit iliac artery demonstrates predominant Hsp90 staining in the arterial intima. **B:** At day 3 after balloon injury, rabbit iliac arteries demonstrate increased Hsp90 expression in the media and inner adventitial layers. **C:** Hsp90 expression after balloon injury localizes extensively with SVV. **A–C:** Bright green, Hsp90; red, SVV; yellow, colocalized red and green fluorescence; blue, nuclei; **red arrows**, internal elastic lamina; **yellow arrows**, external elastic lamina; **white arrows**, samples of positive cells; **white arrows** in the **insets** point to the same cell as the **white arrows** in A–C at a higher resolution. (L), vessel lumen. Scale bar = 50 μm. **D:** Hsp90 and SVV interaction in VSMCs is shown by co-immunoprecipitation. b, bound fraction; u, unbound fraction. Data are representative of three experiments. **E:** Treatment of VSMCs with shepherdin for 8 hours leads to degradation of known Hsp90 client proteins SVV and Akt by Western blot analysis. **F:** Loss of VSMC viability with 8-hour shepherdin exposure by MTT assay, compared with a scrambled peptide ($n = 6$). **P < 0.01. **G:** Dose-dependent induction of apoptosis in VSMCs, as evaluated by DNA content flow cytometry after 6-hour shepherdin treatment. **P < 0.01.

Tyne, UK) was used for cell proliferation detection. The penetration and viability of biotinylated shepherdin were assessed via Alexa Fluor 594–conjugated streptavidin (1: 400; Invitrogen). The apoptosis TUNEL assay was used on 4% paraformaldehyde fixed frozen sections, using *in situ* cell death detection kit fluorescein (number 11684795910; Roche Diagnostics), per manufacturer's instructions. DAPI (1 μg/mL, number D1306; Invitrogen) nuclear counterstaining was used on all of the immunofluorescent staining and TUNEL assay.

Image Acquisition and Measurements

Photography was completed via a Nikon Eclipse 80i fluorescent microscope (Nikon Instruments, Melville, NY), with SPOT RTke Camera and SPOT Windows version 4.1.2 software (Diagnostic Instruments, Sterling Heigths, MI). Four vessel zones (original magnification, ×200) were selected randomly on four coordinate axes of every stained rabbit arterial cross section. The proportion of Ki-67– or TUNEL-positive cells was calculated by absolute positive cell number/DAPI-positive nuclei.

Elastin Staining

Modified Verhoeff–Van Gieson elastic staining was performed on day 28 rabbit iliac arterial and day 21 mouse femoral arterial cross sections. Lumen circumference, internal elastic lamina, and external elastic lamina were delineated by hand, and planimetry was completed by AxioVision Rel 4.4 software (Carl Zeiss, Oberkocken, Germany).

Statistical Analysis

Data are expressed as mean ± SEM, where appropriate. Statistical tests were completed with Stata/IC version 10.0 (StataCorp LP, College Station, TX). Differences among more than two groups were analyzed by one-way analysis of variance. A comparison for two homogeneous groups was performed via a two-tailed paired Student's *t*-test. $P < 0.05$ was considered statistically significant.

Results

Hsp90 Is a Chaperone of SVV in VSMCs and Disruption by Shepherdin Results in Apoptotic Cell Death

In the healthy arterial wall, Hsp90 was prominently expressed in the intima (Figure 1A), where it has been associated with the regulation of endothelial nitric oxide
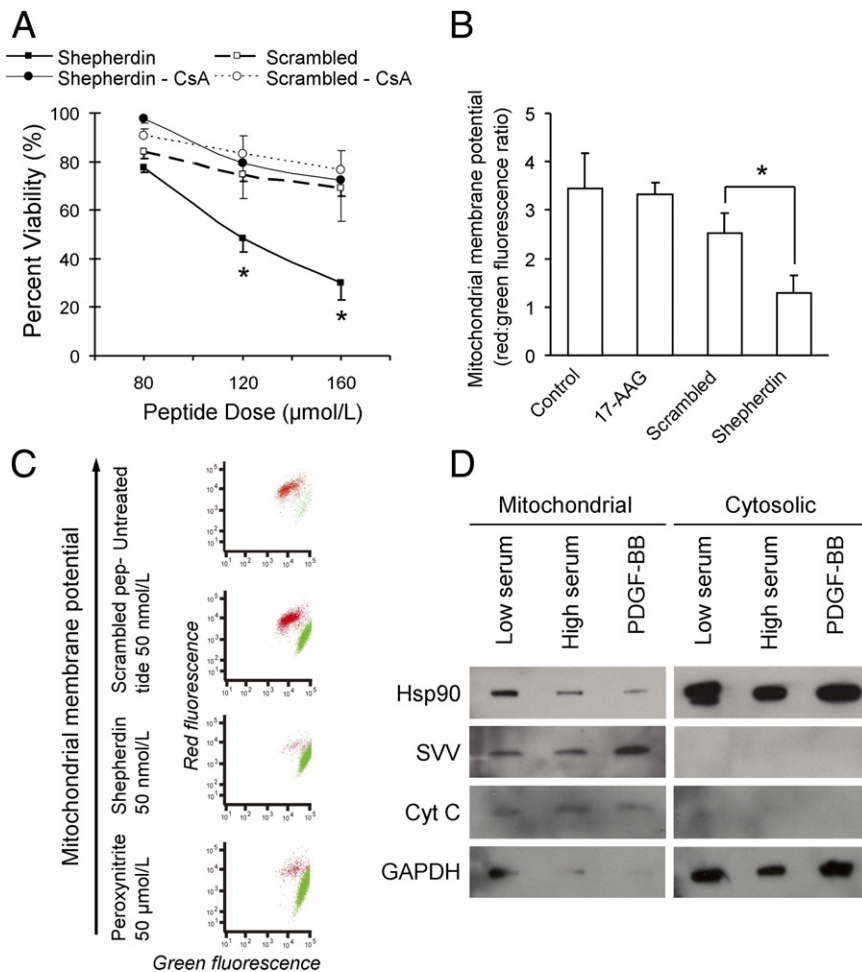

Figure 2. A mitochondrial Hsp90-SVV complex modulates organelle injury and is cytoprotective in VSMCs. **A:** Mitochondrial stabilization of VSMCs with CsA preserves cell viability in the presence of shepherdin. *$P < 0.05$. Rapid mitochondrial depolarization after exposure to 50 nmol/L shepherdin (**B**, $n = 6$, analysis of variance $P < 0.05$, and *$P < 0.05$) demonstrated by red to green fluorescence shift in JC-1 assay (**C**). Representative JC-1 assay showing red to green dye shift consistent with mitochondrial permeability. **D:** Representative Western blot analysis demonstrating mitochondrial pool of SVV that is increased after 24-hour exposure to platelet-derived growth factor–BB (100 ng/mL). GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

synthase.[15] After arterial balloon injury in the rabbit, Hsp90 expression increased in all layers of the arterial wall, including within the VSMC-rich medial layer of the artery (Figure 1B). The highly abundant expression of Hsp90 demonstrated extensive colocalization with SVV (Figure 1C). Immunoprecipitation of protein lysates from primary cultured HVSMCs with an anti-SVV antibody demonstrated Hsp90 in the precipitate, confirming direct interaction between Hsp90 and SVV in these cells (Figure 1D).

Shepherdin rapidly accumulated intracellularly in a broad spectrum of cell types,[16] including HVSMCs (see Supplemental Figure S1 at *http://ajp.amjpathol.org*). Western blot analysis demonstrated that shepherdin treatment of HVSMCs led to a rapid, dose-dependent degradation of the Hsp90 client proteins SVV and Akt, whereas treatment with a scrambled control peptide did not decrease these proteins (Figure 1E). Exposure of HVSMCs to a 120 μmol/L dose of shepherdin for 8 hours resulted in a 60% $\pm$ 11% loss of viability compared with scrambled (control) peptide, which demonstrated 23% $\pm$ 5% loss of viability ($P < 0.01$) by MTT assay. This sequence-specific cytotoxicity was dose dependent (Figure 1F). Similar dose-dependent cell death was seen in primary cultured rabbit aortic VSMCs (eg, 34% versus 2% loss of viability at 80 μmol/L shepherdin versus scrambled peptide; data not shown). To capture the cell death mechanisms at an early time point, HVSMCs were exposed to peptides (40, 80, or 120 μmol/L) for 6 hours.

DNA content analysis showed a significant increase in the apoptotic fraction at both 80 and 120 μmol/L doses of shepherdin ($P < 0.01$, Figure 1G). Annexin V staining after 8-hour exposure to shepherdin provided qualitative confirmation of apoptosis induction in VSMCs (see Supplemental Figure S2 at *http://ajp.amjpathol.org*). In contrast, treatment of VSMCs with the Hsp90 inhibitor 17-AAG at doses shown in cancer cells to induce apoptosis (5 μmol/L)[17] did not initiate VSMC cell death (Figure 1G). Any appreciable loss of VSMC viability (22%) was observed only at high concentrations of 17-AAG (120 μmol/L) (data not shown), similar to the degree seen with scrambled peptide.

Mitochondrial Hsp90 and SVV Modulated Organelle Injury and Are Cytoprotective in VSMCs

The differential potency between two Hsp90 inhibitors in cancer, shepherdin and 17-AAG, was postulated to relate to subcellular pools of Hsp90 and SVV with distinct functions.[5] Unlike 17-AAG, the cell-permeability (antennapedia) domain of shepherdin allowed rapid penetration of the mitochondrial envelope. Mitochondrial-specific Hsp90 chaperone function was critical for survival of malignant cells.[12] To investigate the possibility of a similar relationship in VSMC survival, we blocked the mitochon-
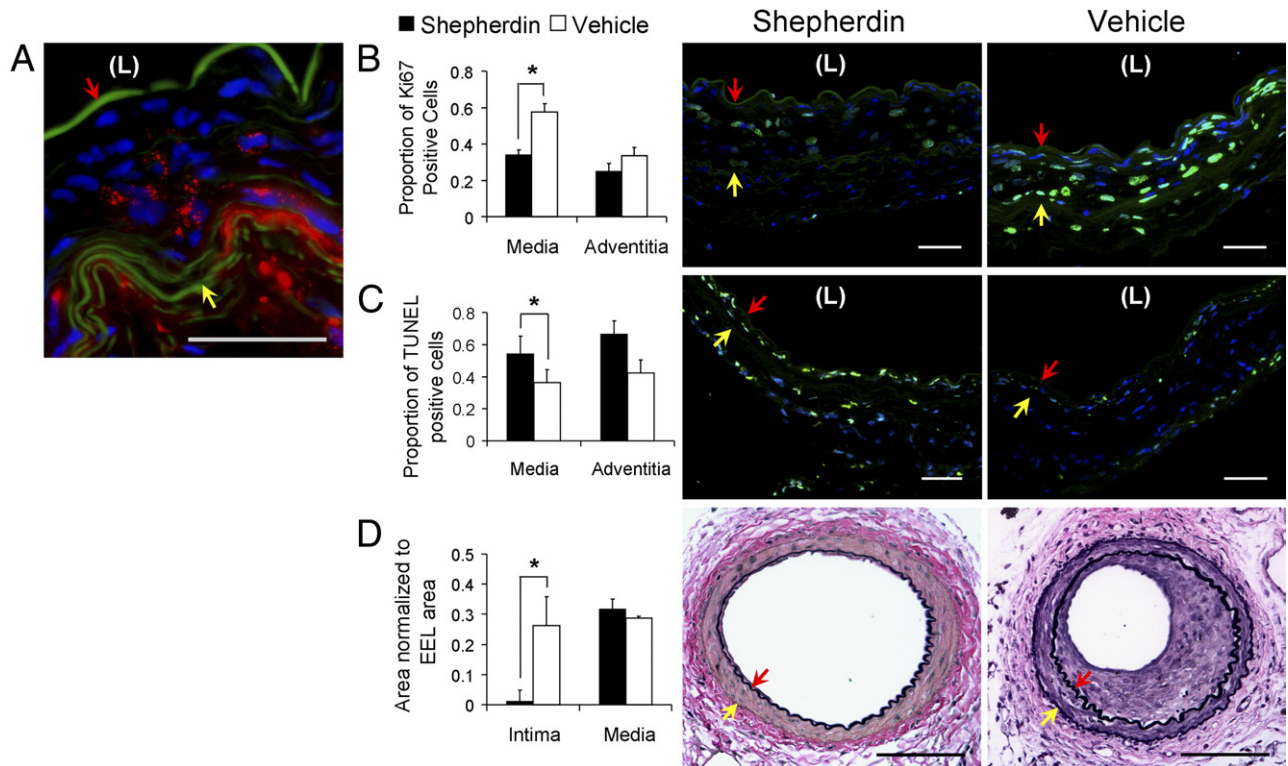

Figure 3. Targeted disruption of Hsp90-SVV with shepherdin modulates the vascular injury response *in vivo*. **A–C:** After iliac artery balloon injury in rabbits, shepherdin peptide is applied to the adventitial surface of the injured segment in a Pluronic gel vehicle. (L), vessel lumen. The following results are for day 5 after injury. **A:** Streptavidin staining for the biotinylated shepherdin peptide demonstrates diffusion into the media of the vessel wall. Red, shepherdin; blue, nuclei. **B:** Ki-67 staining of shepherdin-treated vessels demonstrates attenuation of the postinjury proliferative response ($n = 9$). *$P < 0.05$. Bright green, Ki-67; blue, nuclei. **C:** TUNEL assay of shepherdin-treated vessels demonstrates increased apoptosis ($n = 9$). *$P = 0.06$. Bright green, TUNEL; blue, nuclei. **D:** Systemic, i.p. delivery of shepherdin (50 mg/kg) after iliac artery wire injury in mice ($n = 6$) results in marked attenuation of IH at day 21. *$P < 0.05$. **Red arrows**, internal elastic lumen; **yellow arrows**, external elastic lamina (EEL). Scale bars: 50 μm (**A–C**); 500 μm (**D**).

drial pore complex in VSMCs with the cyclophilin D inhibitor, CsA. Pretreatment of VSMCs with CsA abrogated the shepherdin-induced loss of viability by MTT assay (Figure 2A). Direct measurement of mitochondrial membrane potential in cultured VSMCs confirmed that a rapid depolarization occurred with exposure to shepherdin, similar to that observed after short-term exposure to peroxynitrite, and not seen with 17-AAG or scrambled peptide (Figure 2, B and C). Subcellular protein fractionations demonstrated that most total cellular SVV resided within the mitochondrial compartment of cultured VSMCs. Notably, this mitochondrial SVV pool was up-regulated by the prototypic vascular growth factor, platelet-derived growth factor–BB (Figure 2D), which promoted VSMC activation and was established as a mediator of IH.[18,19] Our data suggested that mitochondrial Hsp90 and SVV functioned as important regulators of mitochondrial homeostasis and cytoprotection in VSMCs.

Modulation of Injury Response, VSMC Survival, and IH in Rabbits and Mice

We examined the *in vivo* relevance of Hsp90 chaperone function in two established animal models of vascular injury. In the first model, balloon injury of rabbit iliac artery was performed, followed directly by local, peri-adventitial application of shepherdin (50 to 250 μmol/L) in 40% Pluronic gel to the vessel. Fluorescence microscopy at day 5 after the procedure demonstrated circumferential delivery with penetration of peptide into the vessel wall to the tunica media (Figure 3A). Dose-ranging experiments demonstrated an increase of vessel wall staining intensity at 100 and 250 μmol/L, with a similar extent of penetration (data not shown). Five days after injury, shepherdin-treated arteries demonstrated a 40% reduction in cell proliferation by Ki-67 staining compared with control arteries exposed to Pluronic gel alone ($n = 9$, $P < 0.05$, Figure 3B). The TUNEL assay demonstrated a concomitant 33% increase in apoptosis in the media and adventitial layers under the same conditions ($P = 0.06$, Figure 3C). The development of IH in this rabbit model was evaluated at day 28 after angioplasty ($n = 7$), and demonstrated a nonsignificant reduction in intimal thickness (60.52 ± 10.42 μm for shepherdin versus 67.65 ± 8.58 μm for control; $P = 0.61$) and intimal/medial thickness ratio (0.98 ± 0.09 for shepherdin versus 1.18 ± 0.17 for control; $P = 0.33$). Because we observed a robust effect on the early cellular response, we hypothesized that a single, local application of peptide to the adventitia might be inadequate to fully exploit mitochondrial Hsp90 inhibition in this model. This was supported by the incomplete intracellular delivery of shepherdin to the vessel media, as shown in Figure 3A. Therefore, we next took a multiple-dose systemic delivery approach previously established in a malignancy model[13] and applied it to a murine model of arterial injury. After unilateral common femoral artery wire injury, male C57BL/6 mice were treated with daily i.p. injections of either 50 mg/kg shepherdin or saline ($n = 6$ per group) for 14 days. Analysis of arterial segments harvested 21 days after injury demon-

strated a marked (95%) attenuation of IH in the shepherdin-treated mice ($P < 0.05$, Figure 3D).

Discussion

Previous work has suggested that VSMCs temporally express an apoptosis-resistant phenotype that may directly contribute to the pathogenesis of vasoproliferative disorders, such as IH and atherosclerosis.[20] Furthermore, the persistence of this phenotype in isolated VSMC culture[21] readily allows study of their cellular mechanisms. In neoplasia, a mitochondrial pool of Hsp90 is critical to the cellular stress response, and is required to maintain mitochondrial integrity.[22] After acute vascular injury, VSMCs in the vessel wall are subject to a host of inflammatory, proliferative, and anti-apoptotic signals that are integrated in the early phase of healing. Herein, we demonstrate the importance of a mitochondrial cytoprotective protein complex including Hsp90 in the regulation of this short-term response. Growth factors and inflammatory cytokines generated locally may directly modulate the activity of this mitochondrial protein network. Because IH represents the net effect of alterations in cell survival, proliferation, and matrix metabolism, this pathway may be an important regulator of lesion development. We demonstrate that this organelle-specific network may be selectively exploited by mitochondrial targeting of Hsp90 chaperone activity in VSMCs using a proteomic approach. Therefore, modulation of mitochondrial membrane integrity in VSMCs may represent an important new avenue of investigation for anti-restenosis therapies.

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