A $G_{\beta\gamma}$ inhibitor reduces intimal hyperplasia in aortocoronary saphenous vein grafts

Jason A. Petrofski, MD,^{a*} Jonathan A. Hata, MD,^{a*} Matthew L. Williams, MD,^a Cyrus J. Parsa, MD,^a Richard B. Thompson, MD,^a Steven I. Hanish, MD,^a Thomas R. Gehrig, MD,^b Walter J. Koch, PhD,^c and Carmelo A. Milano, MD^a

Objective: Approximately 50% of aortocoronary saphenous vein grafts are occluded 10 years after coronary revascularization surgery. Intimal hyperplasia, a critical component in saphenous vein graft failure, is defined by vascular smooth muscle cell proliferation, which is mediated in part by $\beta\gamma$ subunits of heterotrimeric G proteins ($G_{\beta\gamma}$) and downstream effectors such as mitogen-activated protein kinases. A peptide consisting of the carboxyl-terminus of the β -adrenergic receptor kinase (β ARKct) binds $G_{\beta\gamma}$, thereby inhibiting $G_{\beta\gamma}$ signaling. Utilizing a recombinant adenovirus containing the coding sequence for the β ARKct peptide (Ad β ARKct), this study investigates whether treatment of the vein graft with Ad β ARKct reduces intimal hyperplasia in a large animal model of aortocoronary saphenous vein graft intimal hyperplasia.

Methods: Twenty-seven dogs (27-32 kg) underwent aortocoronary bypass grafting to the left anterior descending artery using autologous saphenous vein. Vein grafts were treated with saline ($n = 8$), an empty adenovirus ($n = 8$), or Ad β ARKct ($n = 8$). A subset of dogs ($n = 3$) were sacrificed on postoperative day 7 and β ARKct expression confirmed by Northern blotting.

Results: Arteriograms performed on postoperative day 90 confirmed that saphenous vein grafts were patent. At postoperative day 90, Ad β ARKct-treated grafts demonstrated reduced intimal area compared to empty virus and saline treated animals ($P < .05$). Additionally, Ad β ARKct treatment of isolated vascular smooth muscle cells in vitro inhibited mitogen-activated protein kinase activation and decreased overall vascular smooth muscle cell proliferation.

Conclusion: This study demonstrates that β ARKct expression in aortocoronary saphenous vein grafts reduces intimal hyperplasia and decreases vascular smooth muscle cell proliferation in vitro via inhibition of $G_{\beta\gamma}$ -mediated mitogen-activated protein kinase activation. Modulation of $G_{\beta\gamma}$ via β ARKct may represent a novel therapy to reduce intimal hyperplasia and saphenous vein graft failure.

From the Departments of Surgery^a and Medicine,^b Duke University Medical Center, Durham, NC, and the Center for Translational Medicine, Thomas Jefferson University, Philadelphia, Pa.^c

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Address for reprints: Carmelo A. Milano, MD, Box 3043, Department of Surgery, Duke University Medical Center, Durham, NC 27703 (E-mail: milano002@mc.duke.edu).

*J.A.P. and J.A.H. contributed equally to this manuscript and are considered coequal first authors.

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Saphenous vein grafts (SVGs) remain the most common conduit used for surgical revascularization procedures, including coronary artery bypass grafting (CABG). Unfortunately, aortocoronary grafting with SVGs is limited because of intimal hyperplasia (IH) and subsequent vein graft atherosclerosis, which leads to a 10-year failure rate of almost 50%.^{1,2} IH is a chronic structural lesion that develops after vein graft implantation and leads to luminal stenosis and occlusion. IH is defined as abnormal hypertrophy, proliferation, and migration of vascular smooth muscle cells (VSMCs) from the tunica media to the intima, with associated deposition of an extracellular connective tissue matrix.

Current approaches to limit SVG failure include technical considerations, long-term aspirin therapy, and lipid-lowering medications.^{3,4} Despite these interventions,

Abbreviations and Acronyms

Ad	= adenovirus
AdGFP	= adenoviruses containing the coding sequence for the green fluorescent protein
ANOVA	= analysis of variance
β ARK1	= β -adrenergic receptor kinase 1
CABG	= coronary artery bypass grafting
DNA	= deoxyribonucleic acid
EGF	= epidermal growth factor
ERK	= extracellular signal-regulated receptor kinase
EV	= empty virus
FBS	= fetal bovine serum
IH	= intimal hyperplasia
LPA	= lysophosphatidic acid
MAP	= mitogen-activated protein
POD	= postoperative day
SVC	= saphenous vein graft
VMSC	= vascular smooth muscle cell

however, SVG failure after CABG remains an important clinical problem, leading to recurrent angina and a 10% to 15% incidence of need for reoperative CABG.⁵

Although they have not been completely characterized, molecular signaling mechanisms important in VSMC proliferation have been studied in experimental models of vessel injury. G proteins have been identified as important mediators of this process, because many growth factors that induce VSMC mitogenesis act through G protein-coupled receptors.⁶ With ligand binding, G protein-coupled receptors interact with heterotrimeric G proteins, triggering the dissociation of the G protein into individual α and $\beta\gamma$ subunits. Specifically, G protein $\beta\gamma$ subunits ($G_{\beta\gamma}$) have been shown to trigger intracellular signaling events leading to activation of p21^{ras} (ras) and subsequent phosphorylation of the p42 and p44 mitogen-activated protein (MAP) kinases in VSMCs.⁷ Stimulation of the ras-MAP kinase pathway is important for the induction of VSMC proliferation. Specifically, extracellular signal-regulated receptor kinase (ERK) is a member of the MAP kinase family that has been previously implicated as a key mediator of VSMC growth, proliferation, and survival.⁸

$G_{\beta\gamma}$ subunits have also been shown to bind to the carboxyl terminus of the cytosolic enzyme β -adrenergic receptor kinase 1 (β ARK1), enabling translocation of β ARK1 to the cell membrane, where the enzyme is responsible for phosphorylation of activated receptors. This membrane-targeting event is mediated through binding of the carboxyl terminus of β ARK1 with $G_{\beta\gamma}$.⁹ Previous studies have shown that peptides consisting of the carboxyl-terminal $G_{\beta\gamma}$ -binding domain act as inhibitors of both in vitro and in vivo $G_{\beta\gamma}$ -dependent processes.⁷ A peptide known as

β ARKct, which consists of the last 194 amino acid residues of β ARK1, acts as a competitive inhibitor of $G_{\beta\gamma}$ -mediated processes. In vivo delivery of β ARKct has been accomplished with a replication-deficient recombinant adenoviral vector containing the coding sequence for β ARKct (Ad β ARKct). Previous investigations by our laboratory have demonstrated that Ad β ARKct inhibits VSMC proliferation in vitro and in a small-animal model of arterial injury.⁶

The effectiveness of the Ad β ARKct in inhibiting aortocoronary SVG IH has not yet been examined in a clinically relevant large-animal model. This study was designed to provide preclinical data that could support the clinical application of Ad β ARKct as a treatment for SVG IH. Previously, we characterized a canine model of aortocoronary SVG IH and demonstrated efficient transgene expression in the SVG vessel wall after an ex vivo treatment with adenoviral vectors.¹⁰ This study tests the hypothesis that β ARKct could reduce aortocoronary SVG IH in this large-animal model of aortocoronary SVG IH. In addition, the effects of β ARKct expression in cultured canine saphenous vein smooth muscle cells were investigated.

Methods**Experimental Design**

Animals used in this study were mongrel dogs ($n = 27$, 27–32 kg). Each animal underwent CABG with a reversed SVG from the aorta to the left anterior descending coronary artery as previously described.¹⁰ Before grafting, veins were treated with 1×10^{12} total virus particles of either Ad β ARKct ($n = 8$) or empty virus (EV, $n = 8$). A third group of animals did not have their vein grafts treated with any adenoviral vector and served as a negative control group (control, $n = 8$). Graft blood flow was confirmed by ultrasonic probe (Transonic Systems, Inc, Ithaca, NY) immediately after completion of the distal anastomosis. SVG patency was confirmed by an arteriogram performed on postoperative day (POD) 90. On POD 90, dogs were sacrificed and their SVGs explanted, sectioned, and stained with hematoxylin and eosin. SVG intimal and medial areas were calculated with Image Tool v.3.0 (The University of Texas Health Sciences Center, San Antonio, Tex). A separate subset of animals treated with Ad β ARKct ($n = 3$) were sacrificed on POD 7 to assess graft and systemic transgene expression.

One animal from the control group died of a fatal arrhythmia immediately after the operation. One animal from the EV group died of hemorrhage on POD 2. Accordingly, 22 dogs completed the full 90-day protocol. Animal care complied with Duke University Medical Center animal care and use guidelines, the “Principles of Laboratory Animal Care” of the National Society for Medical Research, and the “Guide for the Care and Use of Laboratory Animals” (<http://www.nap.edu/catalog/5140.html>).

Adenovirus Construction

The structure of a recombinant, replication-deficient adenovirus directing the expression of β ARKct (Ad β ARKct) has been described previously.¹¹ The adenoviral backbone for the vectors is a

second-generation, replication-deficient serotype-2 adenovirus with deletions of E1 and E4.¹¹ These gene deletions render the adenovirus unable to replicate. The complementary DNA for β ARKct was cloned into this vector, generating the vector Ad β ARKct. Control viruses were also constructed, including an empty adenovirus containing no complementary DNA insert (EV) and adenoviruses containing the coding sequence for the green fluorescent protein (AdGFP).

Operative Protocol

Saphenous vein harvest, SVG gene delivery, and CABG were performed as previously described.¹⁰ In brief, each animal was sedated and intubated, and approximately 10 cm of SVG was harvested from the left hind leg. An aliquot of 1×10^{12} total virus particles was thawed and suspended in 2 mL phosphate-buffered saline solution. This suspension was delivered intraluminally with a measured distention pressure of approximately 10 mm Hg for a 20-minute incubation period. After the treatment, the SVG was submerged in 3% albumin. For the control group, each SVG was gently flushed and submerged in 3% albumin. A partial lower sternotomy was performed, a chest retractor was placed, the pericardium was opened, intravenous heparin was administered (50 U/kg), and a Satinsky clamp was placed to partially occlude the ascending aorta. An aortotomy was created, and an end-to-side running anastomosis was performed with 6-0 Prolene (Ethicon, Inc, Somerville, NJ). A myocardial stabilizer was positioned over the distal left anterior descending coronary artery, a coronary arteriotomy was created, and an end-to-side running anastomosis was performed with 7-0 Prolene. The proximal left anterior descending coronary artery was ligated, rendering the anterior left ventricle SVG dependent. An ultrasonic vascular probe (Transonic Systems) confirmed flow through the SVG. All grafts had blood flow greater than 30 mL/min. The sternum was reapproximated, and the chest was closed in layers. Animals were maintained with buffered aspirin (325 mg/d) throughout the study.

Tissue Preparation and Analysis

Animals were sacrificed with a lethal dose of intra-arterial Euthosol (pentobarbital 390 mg/mL and phenytoin 50 mg/mL) on either POD 7 or POD 90. SVG, liver, and lung specimens were collected. The SVG was placed into 10% formalin for a minimum of 24 hours. Segments were embedded in paraffin and cut in cross-section for histologic staining and measurements. Cross-sections (5 μ m) were taken every 0.5 cm and prepared with a modified hematoxylin and eosin stain. For animals at both POD 7 and POD 90, Northern analysis was also performed on lung, liver, non-grafted saphenous vein, and SVG samples from Ad β ARKct-treated animals to assay for expression of β ARKct as previously described.¹²

Angiographic Confirmation of SVG Patency

On PODs 30 and 90, the dogs underwent coronary arteriography through the femoral artery. A 6F coronary catheter was placed into the left coronary artery under fluoroscopic guidance, and radiopaque dye was used to confirm patency of the SVG.

Measurement of SVG Wall Dimensions

All SVG vessel sections were digitally photographed at $5\times$ magnification. Photomicrographs demonstrating maximal IH from each third of the SVG were analyzed. For each SVG section analyzed, intimal area, medial area, maximal wall thickness, and minimal wall thickness were determined by a blinded operator with Image Tool v.3.0. For each SVG, mean values were calculated as previously described.¹² The ratio between the intimal and medial areas was calculated.

Cell Harvest and Culture

VSMCs were isolated from canine saphenous veins harvested under sterile conditions. In brief, the adventitia was stripped away and the intima removed by blunt dissection. The media was cut into 1-cm² sections and placed in culture dishes containing a small amount of growth medium as previously described.¹³ After 10 days, veins were removed, and monolayers of smooth muscle cells were trypsinized and passaged. In this study, only cells between passages 3 and 5 were used.

Adenoviral Treatment

VSMCs were grown in 12-well plates in Dulbecco's modified Eagle's medium and F12 Ham's medium containing 10% fetal bovine serum (FBS). When the cells were nearly confluent, the medium was changed to 2% FBS, and viral vectors were added at a multiplicity of infection of 100. After 24 hours, the medium was changed to be serum free, followed by different treatments or stimuli as indicated. As a control in all experiments, identical groups of cells were left uninfected but incubated 24 hours in 2% FBS.

Immunoblotting

After overnight infection with adenoviruses, VSMCs were serum starved for 5 hours and then stimulated 5 minutes with lysophosphatidic acid (LPA, 10 μ mol/L), epidermal growth factor (EGF, 10 μ mol/L), or FBS (5%). Cells were lysed in Triton lysis buffer (The Dow Chemical Company, Midland, Mich),¹⁴ and samples were separated by sodium dodecylsulfate 8% to 16% polyacrylamide gel electrophoresis and transferred to nitrocellulose. Membranes were Western blotted with the following antibodies: anti-GRK2 polyclonal (SC-562, Santa Cruz Biotechnology, Inc, Santa Cruz, Calif), anti-phospho-p44/42 ERK (Thr202/Tyr204; Cell Signaling Technology, Inc, Beverly, Mass), anti-p44/42 ERK (Cell Signaling Technology), and rat monoclonal anti- α -tubulin (clone YL1/2; Abcam Inc, Cambridge, Mass).

Thymidine Incorporation and Cell Counts

To evaluate the effect of β ARKct expression on serum-mediated DNA synthesis in VSMCs, tritiated thymidine incorporation was assayed as previously described.¹⁴ In brief, VSMCs were plated in triplicate in 12-well plates at a concentration of 20,000 cells/well. The following day, the cells were either left uninfected or were infected with Ad β ARKct or AdGFP. The next day, cells remained quiescent in serum-free medium for another 24 hours. The medium was replaced with fresh serum-free medium with or without agonists, and the cells were incubated 18 hours. The cells were pulse labeled with tritiated thymidine (2 μ Ci/mL; Amersham Pharmacia

Figure 1. Northern blot analysis of β ARKct expression. RNA was extracted from tissues harvested from animals killed on POD 7 whose SVGs had been treated with Ad β ARKct (1×10^{12} total virus particles). Northern blot analysis was performed with radiolabeled β ARKct probe. Lane 1, lung; lane 2, liver; lane 3, nongrafted saphenous vein (NGSV); lane 4, Ad β ARKct-treated SVG.

Biotech, Inc, Piscataway, NJ) for 3 hours, and thymidine incorporation was then assessed by liquid scintillation counting.

For cell counts, VSMCs were plated in triplicate on 12-well plates and either left uninfected or infected with Ad β ARKct or AdGFP. The medium was changed to be serum free, and the cells were incubated for 48 hours with or without agonists. The cells were trypsinized and counted on a hemocytometer (Fisher Scientific Worldwide, Hampton, NH).

Statistical Analysis

All data are presented as mean $\pm$ SEM. Statistical significance was determined by the Student *t* test or 1-way analysis of variance (ANOVA) where appropriate. After ANOVA, pairwise comparisons were then made with the Bonferroni or Student-Newman-Keuls post hoc tests.

Results

Tissue Analysis

Northern analysis was performed on total RNA isolated from lung, liver, nongrafted saphenous vein, and SVG from Ad β ARKct-treated dogs killed on POD 7 ($n = 3$). The β ARKct messenger RNA was detected by Northern analysis in all 3 SVG samples. Lung, liver, and nongrafted saphenous vein RNA samples from the Ad β ARKct-treated group did not demonstrate any transgenic expression (Figure 1). Northern analysis was also performed on tissue samples from dogs treated with Ad β ARKct who were killed on POD 90 ($n = 8$). As expected, because of the transient nature of adenoviral vectors, no appreciable β ARKct transgene expression was observed (data not shown).

Assessment of SVG Patency

All SVGs in this study ($n = 27$) were patent after completion of the distal anastomosis, as documented with an ultrasonic vascular flow probe. Flow values for each animal were at least 30 mL/min. Angiograms obtained on POD 90 demonstrated that the SVGs of all surviving animals were patent ($n = 22$). Extensive filling defects consistent with

graft wall thickening were noted throughout the control and EV-treated SVGs relative to the Ad β ARKct treated SVGs. (Figure 2, A and B).

Histologic Analysis

Histologic analysis is summarized in Table 1. SVG mean intimal area was decreased by approximately 50% in the Ad β ARKct-treated groups relative to the control and EV groups (1.32 ± 0.44 mm² vs 2.83 ± 0.53 mm² vs 2.41 ± 0.67 mm², $P < .05$). Similar reductions in intima/media ratio (0.59 ± 0.16 vs 1.22 ± 0.43 vs 1.47 ± 0.41 , $P < .05$) and maximal wall thickness (1.14 ± 0.31 mm vs 1.94 ± 0.90 mm vs 1.87 ± 0.45 mm, $P < .05$) were noted in the Ad β ARKct-treated group relative to the control and EV groups, respectively. No differences in medial thickness were noted among the three groups ($P > .1$). Representative photomicrographs of SVGs from control and Ad β ARKct-treated animals are shown in Figure 2, C and D.

In Vitro Adenovirally Mediated Gene Transfer

To investigate the mechanism of action of Ad β ARKct in SVGs, VSMCs derived from canine and human saphenous veins were cultured and infected with Ad β ARKct. Control preparations consisted of AdGFP-infected and uninfected cells. Treatment with adenoviral vectors resulted in nearly 100% infection efficiency, as assessed by green fluorescent protein immunofluorescence (data not shown), which is consistent with previously published data in cultured VSMCs and myocytes.¹⁵ After Ad β ARKct infection, protein immunoblotting of whole-cell lysates revealed robust expression of β ARKct (Figure 3, A).

In Vitro Effects of β ARKct Inhibition of MAP Kinase Activation and Cellular Proliferation

To investigate the role of β ARKct in mitogenic signaling, p42/p44 MAP kinase activation was measured in response to a number of stimuli: LPA, EGF, and serum (FBS). LPA is a mitogen expressed in serum that has been shown to activate the ras-MAP kinase pathway exclusively through $G_{\beta\gamma}$.¹⁶ EGF is a tyrosine kinase receptor agonist that stimulates MAP kinase independent of $G_{\beta\gamma}$.¹⁷ Figure 3, B, shows MAP kinase activation, as demonstrated by ERK phosphorylation, in VSMCs infected with either AdGFP or Ad β ARKct. Treatment with Ad β ARKct significantly inhibited MAP kinase activity (as evidenced by decreased phosphorylated ERK) in response to LPA and serum but not EGF. These data correlate with similar findings by Iaccarino and colleagues¹² in rat aortic VSMCs, suggesting that MAP kinase activity in response to serum is mediated primarily by $G_{\beta\gamma}$.

In vitro proliferative assays were also conducted. First, tritiated thymidine incorporation in VSMCs was measured after infection with either AdGFP or Ad β ARKct. As shown in Figure 3, C, thymidine incorporation after exposure to

Figure 2. POD 90 angiograms from representative animals. A, Control (untreated) SVG reveals multiple filling defects on angiography, suggestive of advanced disease and IH. B, AdβARKct-treated SVG reveals SVG devoid of significant filling defects on angiography. POD 90 hematoxylin and eosin-stained slides from representative animals. Arrows indicate intimal borders. C, Control (untreated) SVG specimen. D, AdβARKct-treated SVG specimen.

LPA or 5% serum was significantly attenuated by treatment with AdβARKct. To confirm these findings, cell counts were performed on uninfected and AdGFP- or AdβARKct-infected VSMCs treated with or without LPA, EGF, or FBS. As in the thymidine incorporation experiments, LPA and FBS induced robust proliferation of uninfected or AdGFP-infected VSMCs, whereas βARKct expression significantly inhibited cell proliferation (Figure 3, D). Conversely, EGF-stimulated cell proliferation was not affected by the presence of βARKct (Figure 3, D).

Discussion

We previously characterized a canine model of aortocoronary SVG IH. By POD 90, histologic analysis revealed that mean intimal area was approximately 30 times greater in aortocoronary SVGs than in nongrafted saphenous vein control preparations.¹⁰ Additionally, average medial thickening, intima/media ratio, maximal wall thickness, and minimal wall thickness were similarly increased in the SVGs at POD 90. Although the intimal thickening in this model is more robust than the average change seen with human

TABLE 1. Histologic analysis of POD 90 aortocoronary SVGs

	AdβARKct (n = 8)	Control (n = 7)	EV (n = 7)	P Value
Intimal area (mm ²)	1.32 ± 0.44*†	2.83 ± 0.53*	2.41 ± 0.67†	<.05
Medial area (mm ²)	2.46 ± 0.61	2.21 ± 0.70	1.82 ± 0.91	NS
Intima/media ratio	0.59 ± 0.16*†	1.22 ± 0.43*	1.47 ± 0.41†	<.05
Maximum wall thickness (mm)	1.14 ± 0.31*†	1.94 ± 0.90*	1.32 ± 0.44†	<.05
Minimum wall thickness (mm)	0.59 ± 0.16	0.71 ± 0.19	1.32 ± 0.44	NS

Data represented as mean ± SEM.

NS, Not significant. Statistical significance determined by analysis of variance, with the Newman-Keuls multiple comparison test to identify differences between subgroups. *Statistically significant difference between AdβARKct-treated and uninfected (control) SVGs. †Statistically significant difference between AdβARKct- and EV-treated grafts.

ACD

Figure 3. Expression of β ARKct inhibits ERK phosphorylation and VSMC proliferation in isolated canine saphenous vein smooth muscle cells. VSMCs were treated with AdGFP or Ad β ARKct and then exposed to agonists LPA (10 μ mol/L), EGF (10 μ mol/L), or FBS (5%) for 5 minutes. GFP, Green fluorescent protein. **A**, Lysates were Western blotted sequentially with antibodies specific for β ARKct, phosphorylated ERK (pERK-1/2), and ERK (ERK-1/2). Equal protein loading was confirmed with antibody against α -tubulin. Similar results were observed in four different experiments. **B**, Histogram displaying inhibition of LPA- and FBS-induced ERK phosphorylation in canine VSMCs expressing β ARKct. Data are expressed as percentage increase (mean $\pm$ SEM) in ERK phosphorylation relative to unstimulated AdGFP-infected control cells (n = 4 experiments). Asterisk indicates $P < .05$ for inhibitory effect of β ARKct versus AdGFP-treated cells when stimulated by LPA or FBS (Student t test). **C**, VSMCs were either untreated, treated with Ad β ARKct, or treated with AdGFP. VSMCs were then left quiescent in serum-free medium for 24 hours; treated with LPA (10 μ mol/L), EGF (10 μ mol/L), and FBS (5%) for 18 hours; and pulse labeled with tritiated thymidine. Thymidine incorporation was assessed by scintillation counting of precipitated DNA. Data are reported as mean $\pm$ SEM for n = 3 experiments, each performed in triplicate. Asterisk indicates $P < .001$ for decreased proliferation versus uninfected and AdGFP-treated groups (analysis of variance, Bonferroni post hoc test). **D**, VSMCs were treated as in **A**, except cells were grown 48 hours in serum-free media or in presence of agonists, then trypsinized and counted. Data reported as mean $\pm$ SEM for n = 3 experiments, each performed in triplicate. Asterisk indicates $P < .001$ for decreased proliferation versus uninfected and AdGFP-treated groups.

aortocoronary SVGs, the changes in the model likely parallel those seen in some patients with early failure of vein grafts.

In this study, an ex vivo transgene delivery technique was used. This technique involved adenoviral vectors delivered intraluminally at low-distention pressures. Significant transgene expression is achieved in the SVG with this delivery strategy.¹⁰ Furthermore, in this investigation trans-

gene expression was not observed in liver and lung specimens from Ad β ARKct-treated animals. This observation suggests focal, efficient transgene delivery to the SVG. This result is not unexpected, because adenovirus survival at room temperature is brief, perhaps as short as 15 minutes. The transgene delivery method used a 20-minute intraluminal, ex vivo incubation time followed by a heparinized saline solution flush. Completion of the aortic and coronary

anastomoses required approximately another 20 minutes before the grafts were perfused. This period at room temperature (approximately 40 minutes) minimized surviving adenoviral vector and prevented systemic delivery, with potentially deleterious extragraft expression.

This ex vivo transgene delivery technique is probably clinically applicable. In this study, subphysiologic distention pressures were used, the incubation period was limited to 20 minutes, and the delivery method itself did not appear to induce any negative effects. Notably, the EV control grafts (which underwent the delivery process) and the grafts that received no treatment (no distention) displayed similar histologic characteristics.

SVGs treated with Ad β ARKct demonstrated an approximately 50% reduction in intimal area relative to grafts treated with no virus or EV ($1.32 \pm 0.44 \text{ mm}^2$ vs $2.83 \pm 0.53 \text{ mm}^2$ vs $2.41 \pm 0.67 \text{ mm}^2$, $P < .05$). Differences in intima/media ratio (0.59 ± 0.16 vs 1.22 ± 0.43 vs 1.47 ± 0.41 , $P < .05$) and maximal wall thickness ($1.14 \pm 0.31 \text{ mm}$ vs $1.94 \pm 0.90 \text{ mm}$ vs $1.87 \pm 0.45 \text{ mm}$, $P < .05$) were also significant. These findings are interesting because they demonstrate that the histologic effects of Ad β ARKct treatment extend well beyond the period of transgene expression. Indeed, transgene expression with adenoviruses is typically short-lived, usually lasting between 1 and 3 weeks. Significant histologic changes were seen on POD 90 in this study. This suggests that inhibition of early events may be enough to produce long-term histologic changes. One hypothesis explaining this observation is that inhibition of VSMC proliferation is required only until the SVG endothelium—which may sustain damage during the harvest and implantation procedures—can reestablish itself. Once the endothelium has been reestablished as a complete monolayer, VSMCs may be less likely to undergo pathologic proliferation. Conceivably, then, retreatment with the transgene may not be necessary to achieve continued, beneficial effects. Importantly, this hypothesis is being investigated by studying a 1-year time point.

Because of the efficacy of the β ARKct transgene in attenuating the development of IH in vivo, its effect on the molecular and cellular responses of saphenous vein VSMCs in vitro was also investigated. It is known that the inhibition of $G_{\beta\gamma}$ signaling can inhibit MAP kinase activation^{6-7,12} and thereby effect changes in cell growth and proliferation pathways. In this study, the effect of β ARKct on the responses of VSMCs was evaluated in the presence of several well-known stimulants of cell proliferation: LPA, EGF, and FBS. LPA has been shown to activate the ras-MAP kinase pathway exclusively through $G_{\beta\gamma}$,¹⁶ whereas EGF is a tyrosine kinase receptor agonist that stimulates MAP kinase independent of $G_{\beta\gamma}$.¹⁷ Serum contains a host of other growth factors and hormones also known to activate $G_{\beta\gamma}$. The ability of β ARKct to attenuate VSMC proliferation in re-

sponse to both LPA and FBS at a cellular level suggests that $G_{\beta\gamma}$ and MAP kinases play significant roles in the pathogenesis of IH and provides a potential mechanism for the observed in vivo effects.

Undoubtedly, multiple biochemical pathways are involved in the pathologic process of IH. Theoretically, different transgenes targeting different pathways could be combined to act synergistically and thus more effectively reduce IH. Other potentially important transgenes, such as nitric oxide synthase, could augment nitric oxide within the graft and help preserve endothelial function during the grafting process.¹⁸ Tissue inhibitors of metalloproteinase might also reduce IH by preventing migration of VSMCs.¹⁹ Inhibition of phosphatidylinositol 3 kinase signaling by the tumor suppressor protein PTEN (Phosphate and Tensin homolog detected on chromosome Ten) has been shown to inhibit VSMC proliferation and the development of IH.¹⁴ It remains to be determined whether combining these transgenes with β ARKct might produce a more complete inhibition of SVG IH.

Finally, it is important to recognize that even though many investigators believe that there is an important connection between early IH and subsequent graft atherosclerosis and thrombosis, this connection has not been conclusively proved.²⁰ In other words, it is possible that IH may be a pathologic entity independent of these two processes. At POD 90, the major end point of this manuscript, the incidence of thrombosis is very low and this study therefore does not help to answer this important question. Accordingly, ongoing studies examining canine aortocoronary SVGs 1 year after grafting are being conducted. Such studies may relate early IH to subsequent SVG failure.

Conclusion

VSMC proliferation and associated IH are early factors leading to aortocoronary SVG failure. In this study, adenovirus-mediated expression of β ARKct inhibited aortocoronary SVG IH in a large-animal model. The mechanism for these changes is not clear but may be through the antiproliferative effect of β ARKct and inhibition of MAP kinase signaling, as shown in cultured canine VSMCs. These results suggest that modulation of the MAP kinase pathway through β ARKct expression may represent a novel therapy to prevent IH in vascular conduits, including aortocoronary SVGs.

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