Antisense basic fibroblast growth factor gene transfer reduces early intimal thickening in a rabbit femoral artery balloon injury model

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Purpose: **The purpose of this study was to determine whether endogenous basic fibroblast growth factor (bFGF) production contributes to the intimal hyperplastic response to injury in a model of rabbit femoral artery balloon injury. Inhibition of de novo production of bFGF protein was targeted by intramural adenoviral gene transfer of antisense bFGF (Ad.ASbFGF) RNA. The adenovirus was delivered locally intraluminally at the time of arterial injury.**

Methods: **New Zealand White rabbits underwent balloon injury of the superficial femoral artery, followed by intraluminal delivery of an adenoviral vector encoding a rat antisense bFGF *(ASbFGF)* transcript at a concentration of 1×10^{10} plaque-forming units per milliliter. Control animals were treated in a similar fashion, using either an adenovirus encoding the *lac Z* reporter gene (Ad.lacZ) or phosphate-buffered saline solution (PBS; vehicle) alone. Two weeks after balloon injury, rabbits were killed and perfusion fixed. Femoral arteries were harvested for histomorphometric analysis. Intimal and medial wall thickness was measured at eight points around the vessel perimeter, and mean intimal/medial (I/M) thickness ratios were compared by analysis of variance and Student's *t* test. In addition, medial cell proliferation in Ad.ASbFGF and Ad.lacZ treated arteries was evaluated 4 days and 2 weeks after balloon injury by 5-Bromo-2'-deoxyuridine labeling.**

Results: **At 14 days (n = 25) after balloon injury, histomorphometric analysis revealed a significant inhibition of intimal thickening in Ad.ASbFGF-treated arteries as compared with Ad.lacZ-treated and PBS-treated controls (I/M thickness ratios ± SD, 0.43 ± 0.22 for Ad.ASbFGF vs 1.03 ± 0.28 for Ad.lacZ and 0.86 ± 0.19 for PBS; $p < 0.0001$ and $p = 0.004$, respectively). There was no significant difference in the I/M thickness ratios of Ad.lacZ-treated and PBS-treated vessels ($p = 0.27$). Although there was no significant difference in the proliferation index of Ad.ASbFGF-treated and Ad.lacZ-treated vessels 4 days after injury, an increase in apoptosis was noted in the Ad.ASbFGF-treated vessels 4 days after balloon injury.**

Conclusions: **The use of *ASbFGF* RNA gene transfer, designed to inhibit de novo bFGF synthesis after balloon injury, results in a significant inhibition of neointimal formation. This suggests that continued bFGF synthesis contributes to the arterial response to injury in rabbits. *ASbFGF* gene transfer may be an effective strategy in limiting the intimal hyperplastic response after vascular reconstructive procedures. (J Vasc Surg 1998;27:126-34.)**

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Despite numerous advances in the treatment of the lesions of atherosclerosis by both bypass grafting and angioplasty, restenosis continues to threaten clinical results. Recent reports describe the restenosis rate 6 months after coronary angioplasty to be as high as 43% to 57%.[1,2] A variety of strategies have been developed to target the hyperplastic response to injury in an effort to minimize the resulting restenotic lesion. The process of intimal hyperplasia is generally accepted to involve the adherence and activation of platelets at the site of vascular injury with the subsequent release of growth factors from both platelets and injured smooth muscle cells (SMCs), resulting in SMC migration to the neointima associated with cellular proliferation and extracellular matrix deposition.

In recent years, the targeting of growth factors involved in the injury response has been the subject of intense investigation. Lindner and Reidy[3] have demonstrated that the systemic administration of a neutralizing antibody against basic fibroblast growth factor (bFGF) at the time of carotid balloon injury in rats results in a significant reduction in the SMC proliferation index 2 days after balloon injury without a significant reduction in intimal thickening 8 days after injury. Their study further identifies bFGF as an important mitogen that controls the growth of vascular SMCs, but the study also demonstrates that neutralization of bFGF released from cells at the time of injury alone is insufficient in reducing intimal thickening after balloon injury. Along with other studies, their data provide strong evidence that endogenous bFGF released by injured and dying cells is a major factor in the intimal hyperplastic response.[4,5]

The role of de novo bFGF production by cells in the arterial wall after initial injury is less clear. Although Lindner and Reidy[3] did not demonstrate a reduction in intimal thickening 8 days after injury in rats that received neutralizing antibody against bFGF at the time of carotid balloon injury, Nguyen and colleagues[6] were able to demonstrate suppression of the neointimal lesion 14 days after aortic injury in rabbits that received anti-bFGF antibody daily from 30 minutes before injury to 5 days after injury. It had been previously thought because of the absence of a classic secretory signal peptide that bFGF is not a secreted cell product and is therefore only released by cells that undergo injury or death.[7] However, there is evidence that bFGF can exit viable cells by a mechanism independent of the classic endoplasmic reticulum–Golgi complex secretory pathway.[8-10]

We have previously shown, using an adenoviral vector encoding a full-length antisense bFGF (Ad.ASbFGF) sequence, that there is a dose-dependent reduction in intimal thickening in rat carotid arteries treated locally with Ad.ASbFGF at the time of balloon injury.[11] These results suggest that de novo production of bFGF in surviving SMCs plays an important role in the rat carotid arterial response to injury.

We believed it was important to study the role of de novo bFGF synthesis in the arterial response to injury in a second animal model, particularly the rabbit femoral artery model, for several reasons. The rat carotid balloon injury model is well described and has been a major tool in the study of the arterial response to injury.[12] A limitation of this model lies in the observation of significant variation between species in the response to a particular intervention. It has been reported that profound effects on intimal thickening in rats as a result of a drug or other intervention have not always been reproduced in larger animals.[13,14] An ability to reproduce a biologic effect in a second, larger, animal model may suggest an increased likelihood of a similar effect in the clinical setting. Because there are differences in the biologic properties of different vessels, concern was raised as to whether the response of the carotid artery would predict the response of another vessel such as the femoral artery, which currently is a more common site for procedures involving balloon injury. In addition, as it has been suggested that the desired effect of adenoviral based gene therapy may be limited, particularly in rabbit femoral arteries, by the associated inflammatory response,[15] studies of potential gene therapeutics in similar models are warranted. The current study was designed to evaluate the role of de novo bFGF production in the arterial response to injury in a rabbit femoral balloon injury model. In addition to studying the altered response to injury in terms of intimal thickening, we studied the effects of *ASbFGF* on cellular proliferation and the role of continued bFGF production on cellular viability and apoptosis.

METHODS

Rabbit femoral artery injury model. All animal procedures were approved by the Animal Care and Use Committee of the University of Pennsylvania, and complied with the *Guide for the Care and Use of Laboratory Animals* from the Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council (Washington: National Academy Press,

1996). Adult male New Zealand White rabbits (Hazelton Research Products, Inc., Denver, Pa.) weighing approximately 3 to 4 kg were used in all experiments. The animals were housed in a virus-free and Pasteurella-free facility. Anesthesia was induced by intramuscular injection of ketamine (50 mg/kg; Phoenix Scientific Inc., St. Joseph, Mo.), xylazine (5 mg/kg; Phoenix Scientific Inc.), and acepromazine maleate (1 mg/kg; Fermenta Animal Health Co., Kansas City, Mo.). Additional doses equivalent to one half the initial dose of ketamine and xylazine were given when required.

Both superficial femoral arteries were exposed medially and isolated from the inguinal ligament to the knee. Microvascular clamps were placed on the superficial femoral artery proximally and on the popliteal and genu suprema arteries distally. The great saphenous artery was tied off distally with braided polyester suture. All small side branches were ligated, as well. An arteriotomy was made in the great saphenous artery proximal to the tie, and a 2F Fogarty arterial embolectomy catheter (Baxter Healthcare Corp., Irvine, Calif.) was passed up the superficial femoral artery. The balloon was inflated and withdrawn three times to create a controlled injury of the vessel wall. After removal of the catheter, the isolated superficial femoral artery was irrigated with sterile phosphate-buffered saline solution (PBS; Bio-Whittaker, Walkersville, Md.) via a 24-gauge Angiocath (Becton-Dickinson Vascular Access, Sandy, Utah). After irrigation, approximately 200 μl of either Ad.ASbFGF at 1×10^{10} plaque-forming units (pfu)/ml concentration, control virus encoding the β-galactosidase gene (Ad.lacZ) at 1×10^{10} pfu/ml concentration, or PBS vehicle alone was delivered intraluminally via a 24-gauge Angiocath at a pressure sufficient to gently but firmly distend the superficial femoral artery for 20 minutes. After that time, the solution was removed and the vessel was flushed briefly by controlled removal of the proximal clamp, followed by ligation of the great saphenous artery and restoration of flow via the popliteal and genu suprema arteries. Ties on the side branches were used as landmarks for the recording of the location of injury and vector delivery, which were of similar extent. The wound was closed in two layers with absorbable suture, and the animals were allowed to recover under supervision. After recovery, animals were allowed standard chow and water ad libitum. All suture materials were a generous gift from the United States Surgical Corporation.

Adenoviral vectors. The Ad.ASbFGF recombinant adenovirus was constructed by replacement of the β-galactosidase cDNA with a 1.1 kb rat bFGF cDNA (in the antisense orientation) in the pAd.CMV *lac Z* shuttle vector, followed by homologous recombination with the E1, E3 deleted, human adenovirus serotype 5 mutant dl7001 in human embryonal kidney 293 cells, as previously described.[16] Rat and rabbit bFGF is approximately 90% homologous.[17]

The control virus for all experiments was a replication-deficient recombinant adenoviral vector encoding the bacterial β-galactosidase reporter gene (gift of Dr. James Wilson, University of Pennsylvania).[18] In addition, PBS vehicle alone was used as a second control.

Tissue preparation. Fourteen days after balloon injury the animals were deeply anesthetized as described above, and 2.5 ml of 5.0% Evans blue (Sigma, St. Louis) was administered intravenously 20 minutes before death. By staining deendothelialized vessels a deep blue, the adequacy of the balloon injury was confirmed in all animals studied. The aorta and vena cava were then exposed via a midline incision, and the aorta was cannulated. The animal was then exsanguinated via an incision in the vena cava followed by anterograde perfusion via the aorta with lactated Ringer's solution at 120 to 150 mm Hg pressure until the effluent was clear. This was immediately followed by perfusion fixation with 10% neutral buffered formalin at the same pressure until the hindlimbs were stiff. Segments of superficial femoral arteries that were both deendothelialized and in the regions exposed to treatment solution were excised and immersion-fixed in 10% neutral buffered formalin for at least 24 hours. Thereafter, three segments were taken from each vessel and were paraffin-embedded, sectioned, and stained with hematoxylin and eosin. Cross-sections of each segment were examined under light microscopy, and quantitation of the intimal thickness, medial thickness, and intima/media (I/M) thickness ratio at eight circumferential points around each section was obtained by computerized planimetry using Summa Sketch II Plus from Summagraphics Corp. (Seymour, Conn.) and BioQuant System IV software from R+M Biometrics, Inc. (Nashville, Tenn.). Mean values for each artery were calculated.

In vivo quantitation of proliferation; 5-bromo-2-deoxyuridine (BrdU) labeling. To study proliferation in vivo, rabbits received an intramuscular dose of BrdU (50 mg/kg; Boehringer Mannheim Corp., Indianapolis) at 19 hours, 8

hours, and 1 hour before death. BrdU, a thymidine analog, is taken up by proliferating cells and can be visualized by immunohistochemical techniques. Arterial and small intestinal segments (positive control) were paraffin-embedded, sectioned, and incubated with antibody to BrdU at 10 μg/ml concentration. BrdU incorporation into cellular DNA was detected as previously described[19] using an avidin-peroxide complex (Vector Laboratories, Burlingame, Calif.) and 3,3 diaminobenzidine substrate (Sigma). Slides were counterstained with hematoxylin to enable counting of the total number of cells in each compartment. The proliferative index was calculated as the percent BrdU-positive cells in the media of vessels 4 days after balloon injury and in the intima and media of vessels harvested 2 weeks after injury. Means from Ad.ASbFGF-treated and Ad.lacZ-treated controls were compared.

Evaluation of gene transfer using the *lac Z* reporter gene. In a subset of animals killed 4 days after balloon injury, the superficial femoral arteries were harvested before perfusion fixation. Segments of artery from a site known to have been both balloon-injured and exposed to treatment solution were excised, briefly washed in PBS, and frozen in OCT compound (Miles Inc., Elkhart, Ind.) in a 2-methylbutane/dry ice bath. Samples were then thawed, rinsed in PBS, and fixed in 0.5% glutaraldehyde for 15 minutes, then washed twice for 10 minutes each with 1 mmol/L $MgCl_2$ in PBS at room temperature. The samples were then incubated in 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) chromagen solution containing X-gal (1 mg/ml; Boehringer Mannheim), 5 mmol/L $K_3Fe(CN)_6$, 5 mmol/L $K_4Fe(CN)_6$, and 1 mmol/L $MgCl_2$ in PBS for 30 to 60 minutes at 37° C. The X-gal chromagen stains the β-galactosidase product of the *lac Z* gene blue and confirms gene transfer and protein production. Gross sections were examined en face under a Leitz dissecting photomicroscope (Leica Inc., Malvern, Pa.), and similar segments were paraffin-embedded, cross-sectioned, and counterstained with nuclear-fast red for photomicroscopic examination.

Evaluation of apoptosis. Identification of apoptotic cells in the vascular wall was performed by terminal deoxynucleotidyl transferase (TdT)-mediated 2'-deoxy-uridine-5'-triphosphate nick end labeling (TUNEL). This procedure involves the incorporation of labeled nucleotides to DNA strand breaks in situ, allowing visualization of DNA fragmentation characteristic of apoptosis by fluorescence microscopy. Paraffin-embedded, formalin-fixed cross-sections were heated to 60° C for 10 minutes, deparaffinized, and gradually rehydrated using decreasing concentrations of ethanol. Sections were then incubated in 20 μg/ml proteinase K (Boehringer Mannheim) for 30 minutes at 37° C. Samples were then washed twice in PBS and incubated in TUNEL reaction buffer containing fluorescein-isothiocyanate (FITC)-uTP and TdT according to the manufacturer's instructions (Boehringer Mannheim). After a 60-minute incubation with the TUNEL reaction mixture at 37° C in the dark, sections were rinsed three times in PBS and counterstained by brief exposure to 1 μg/ml concentration propidium iodide (Sigma) and PBS rinsing. Samples from Ad.ASbFGF-treated and Ad.lacZ-treated vessels 4 days and 2 weeks after balloon injury were immediately examined by indirect fluorescence microscopy.

Data analysis. Results are reported as mean ± SD. Mean values among Ad.ASbFGF, Ad.lacZ, and PBS treatment groups were compared using analysis of variance. Differences in mean values between individual groups were compared using a two-tailed Student's unpaired *t* test and were confirmed by pairwise comparison using the Student-Newman-Keuls Method (SigmaStat software, Jandel Scientific, San Rafael, Calif.). Differences were considered significant for p values less than 0.05. All experiments were performed in a blinded fashion such that the balloon injury was performed before random selection of the treatment agent. Planimetry was performed in blinded fashion, as well, and was confirmed by a blinded observer.

RESULTS

Evaluation of *lac Z* transgene expression. Multiple discontinuous segments were obtained from superficial femoral arteries 4 days after balloon injury and vector delivery. After processing with X-gal chromagen as described above, vessels treated with Ad.lacZ at a concentration of 1×10^{10} pfu/ml demonstrated deep blue staining across the full extent of each segment studied (Fig. 1, *A*), indicating successful gene transfer along the length of the treated vessel. A sharp demarcation at the border of treated and nontreated areas is noted, as well. To determine the depth of gene delivery within the vascular wall, X-gal–stained arterial segments were cross-sectioned, counterstained, and viewed under a light microscope. These sections revealed greater than 90% gene transfer efficiency with delivery through the full thickness of the vessel media and occasional staining within the adventitia (Fig. 1, *B*).

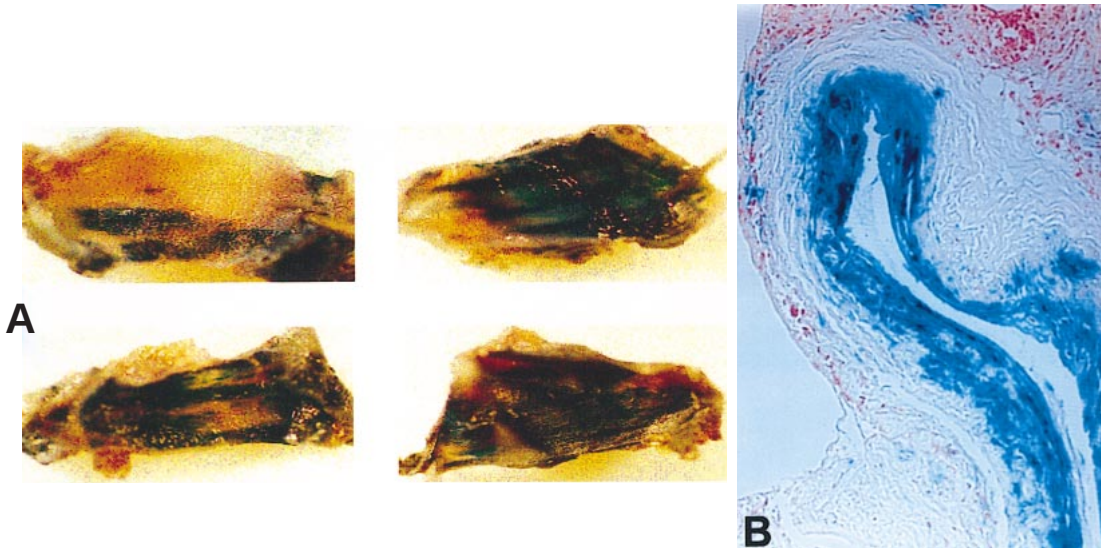

Fig. 1. A, En face views of luminal surface of multiple discontinuous segments of a single rabbit superficial femoral artery treated with Ad.lacZ at 1 × 10^{10} pfu/ml via 20 minute local intraluminal incubation and stained with X-gal chromagen 4 days after balloon injury and vector delivery. These images demonstrate successful gene delivery and protein production along the full length of the treated vessel. Note segment in upper left image that demonstrates demarcation between treated and nontreated zones. Original magnification, ×7.5. **B,** Cross-sectional photomicrograph of a segment of rabbit superficial femoral artery treated with Ad.lacZ at 1 × 10^{10} pfu/ml via 20 minute local intraluminal incubation and stained with X-gal chromagen 4 days after balloon injury and vector delivery. Segments were then embedded, sectioned, and counterstained with nuclear-fast red. This image demonstrates greater than 90% gene transfer efficiency with gene delivery through the thickness of the media. Original magnification, ×125.

Control vessel segments not treated with Ad.lacZ did not stain positively when treated with X-gal chromagen.

Limitation of intimal thickening via Ad.ASbFGF delivery. To determine the effects of Ad.ASbFGF delivery on the arterial response to injury, superficial femoral vessels (n = 25) were harvested from rabbits 14 days after balloon injury and delivery of either Ad.ASbFGF at 1 × 10^{10} pfu/ml, Ad.lacZ at 1 × 10^{10} pfu/ml, or PBS vehicle alone. Three segments per vessel from areas that were both injured and exposed to treatment solution were analyzed by light microscopy after fixation, cross-sectioning, and staining. The thickness of the intimal and medial layers were measured by computerized planimetry at eight points around the circumference of the cross section. Mean I/M thickness ratios were obtained for each vessel, and the treatment groups were compared (Fig. 2). Histomorphometric analysis revealed a significant limitation of intimal thickening in Ad.ASbFGF-treated arteries as compared with Ad.lacZ-treated and PBS-treated control specimens (I/M thickness ratios ± SD, 0.43 ± 0.22 vs 1.03 ± 0.28 for Ad.lacZ and 0.86 ± 0.19 for PBS; $p < 0.0001$ and $p = 0.004$, respectively). There was no significant difference in the I/M thickness ratios of Ad.lacZ-treated and PBS-treated vessels ($p = 0.27$; Fig. 3). Analysis of variance was used to compare mean values among the three treatment groups ($p < 0.0001$).

Investigation of mechanisms involved in Ad.ASbFGF-mediated reduction of intimal thickening response to balloon injury. At 4 days (n = 9) and 14 days (n = 8) after balloon injury, vessels were harvested from animals that received BrdU. Cross-sections of fixed and paraffin-embedded vessels were stained for BrdU and examined by light microscopy. BrdU-positive and BrdU-negative cells were counted in the media of entire sections at 4 days and from four high-power fields (over 400 cells per section counted) in the intima and media of sections harvested at 14 days after balloon injury and vector delivery. Results from vessels treated with Ad.ASbFGF at 1 × 10^{10} pfu/ml and Ad.lacZ at 1 × 10^{10} pfu/ml were compared. The results demonstrated a decline in the medial proliferation index

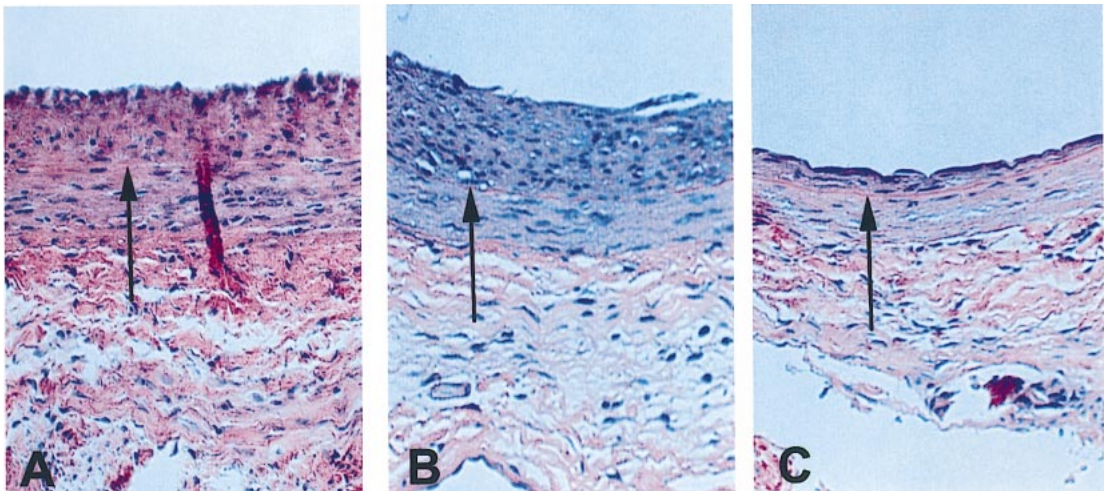

Fig. 2. Hematoxylin and eosin–stained photomicrographs of rabbit superficial femoral arteries 14 days after balloon injury. **A,** Treated with Ad.lacZ at 1×10^{10} pfu/ml. **B,** Treated with PBS vehicle alone. **C,** Treated with Ad.ASbFGF at 1×10^{10} pfu/ml. All treatment solutions were delivered as a local intraluminal incubation for 20 minutes, placed immediately after balloon injury and brief PBS irrigation. These photomicrographs demonstrate a marked inhibition of neointimal thickening in the Ad.ASbFGF-treated vessels. Original magnification, ×125.

from 4 days after injury to 14 days after injury in both the Ad.ASbFGF-treated group (from 23% at 4 days to 10% at 14 days) and in the Ad.lacZ-treated group (from 36% at 4 days to 10% at 14 days). There did not appear to be a statistically significant difference between the medial proliferation index at 4 days in the Ad.ASbFGF-treated group compared with the Ad.lacZ-treated group (23% vs 36%; $p = 0.15$). At 14 days after injury there does not appear to be a significant difference in the proliferation index between the Ad.ASbFGF-treated group and the Ad.lacZ-treated group in either the intima (21% vs 13%; $p = 0.35$) or the media (10% in both groups).

Sections from the same vessels studied by BrdU incorporation were also evaluated by the TUNEL reaction to investigate the presence of apoptotic cells in the vessel walls after balloon injury and gene delivery. Increased numbers of apoptotic cells were found in the media of Ad.ASbFGF-treated vessels 4 days after injury as compared with vessels treated with Ad.lacZ (Fig. 4). By 2 weeks apoptotic cells are no longer detectable.

DISCUSSION

The choice of a rabbit femoral artery injury model to study the effects of *ASbFGF* gene delivery on the arterial response to injury was made for several reasons. There can be significant variability between species with respect to the efficacy of a particular intervention or agent on the intimal thickening response to vascular injury. Whereas Powell et al.[13] demonstrated an 80% reduction in intimal hyperplasia induced by balloon catheterization of rat carotid arteries by administration of the angiotensin-converting enzyme inhibitor, Cilazapril, this could not be reproduced by Hanson et al.[14] in baboons. In the Hanson study, baboons were administered sufficient doses of Cilazapril to almost completely inhibit plasma ACE activity, but no significant reduction in intimal thickening could be appreciated in a variety of vascular injury models. Although efficacy in rabbits does not guarantee success in primates or specifically in human beings, reproducibility in more than one animal model increases the likelihood that a particular approach might be suitable for clinical application.

Concern has been raised about the inflammatory reaction to the virus itself stimulating neointimal hyperplasia, particularly in rabbit femoral arteries,[15] thereby potentially limiting the efficacy of virally encoded therapeutic genes. Although we did not specifically examine the inflammatory reaction to viral delivery with regard to inflammatory markers, a significant inflammatory response could be seen surrounding the vessels treated with adenovirus 4 days after injury and vector delivery. The demonstrated efficacy of Ad.ASbFGF in reducing

intimal thickening in this model suggests that significant inflammatory reactivity to the virus is not limiting in this context.

There are important differences in the biologic properties and structure of different vessels that contribute to altering the response to injury, so it is important to study the mechanism of vascular injury at different sites. Because femoral artery angioplasty is currently a widespread clinical treatment method, studies of femoral artery injury mechanisms and potential interventions are indicated.

The major advantages of adenoviral gene delivery are that gene transfer and gene expression are highly efficient and that cellular proliferation is not required to produce transgene product.[20] In addition to the advantages of using adenoviral vectors for gene delivery, the disadvantages commonly reported do not seem to be as problematic for application to the vasculature as to other organ systems and tissues. The disadvantages of transient expression as a result of the immune response to viral antigens and the lack of chronic integration may be a relative advantage in the context of vascular injury because most major events that lead to intimal thickening have taken place by 2 weeks after injury.[21] In addition, the potential systemic toxicity of the virus can be averted by local delivery. Lastly, the vascular barrier that inhibits viral delivery to other target organs in nonvascular studies is the vascular target. We have shown that the vessel readily takes up virus and produces gene product after balloon injury and local intraluminal dwelling of virus solution. Other investigators have also demonstrated, in a variety of models of vascular injury, significant inhibition of neointimal thickening after transient gene delivery.[22]

Concern has been raised in the past as to the specificity of antisense-based therapies, particularly relating to the use of oligonucleotides.[23] We have addressed this by transfecting a full-length antisense sequence, which should have a very high specificity for its target. Consequently, unlike with oligonucleotides, there is no similar "missense" control. The most appropriate control remains a similar adenovirus carrying a commonly used reporter gene. In addition, we have previously shown in vitro that the provocation of cell death by *ASbFGF* can be prevented by exogenous bFGF and not by exogenous epidermal growth factor, further suggesting that the effects of *ASbFGF* are specific to bFGF production and function.

The virally encoded *ASbFGF* RNA was designed to inhibit de novo synthesis of bFGF to study the contribution of ongoing bFGF production on the

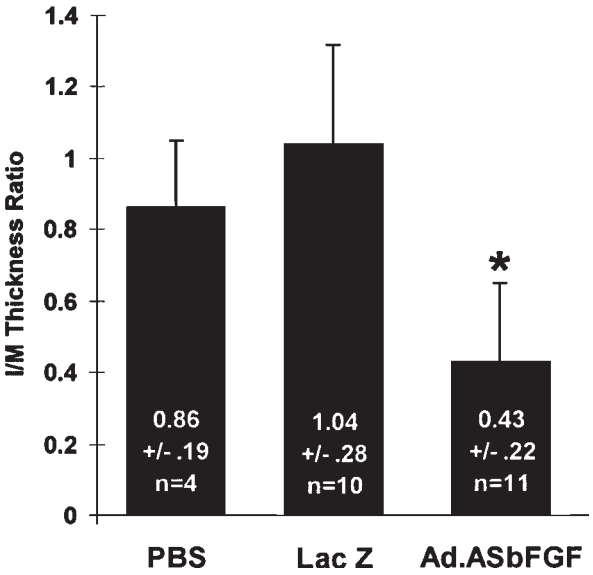

Fig. 3. I/M thickness ratios 14 days after balloon injury immediately followed by a 20-minute local incubation of treatment solution. Treatment groups included PBS vehicle alone, Ad.lacZ at 1×10^{10} pfu/ml, and Ad.ASbFGF at 1×10^{10} pfu/ml. Graph demonstrates significant reduction in intimal thickening in Ad.ASbFGF group compared with PBS and Ad.lacZ control groups ($p = 0.004$ and $p < 0.0001$, respectively). There was no significant difference in intimal thickening between the PBS and Ad.lacZ groups ($p = 0.27$).

arterial response to balloon injury. We have previously shown in the rat carotid artery injury model that Ad.ASbFGF inhibits intimal thickening in a dose-dependent fashion when administered by local intraluminal incubation immediately after balloon injury.[11] The current study documents the effects of Ad.ASbFGF in inhibiting intimal thickening in a second animal model and supports the concept that ongoing production of bFGF plays an important role in the arterial response to injury. It is important to note, however, that these effects are seen at the relatively early time point of 2 weeks after injury. Further studies are needed to determine whether these effects are indeed durable over the long term.

In attempting to elucidate the mechanisms involved in the limitation of intimal thickening associated with the intraluminal delivery of Ad.ASbFGF, proliferating cells were labeled by incorporation of BrdU. In addition, sections were evaluated by TUNEL reaction and fluorescence microscopy for biochemical evidence of apoptosis. Studies of proliferation and apoptosis were performed on tissue sec-

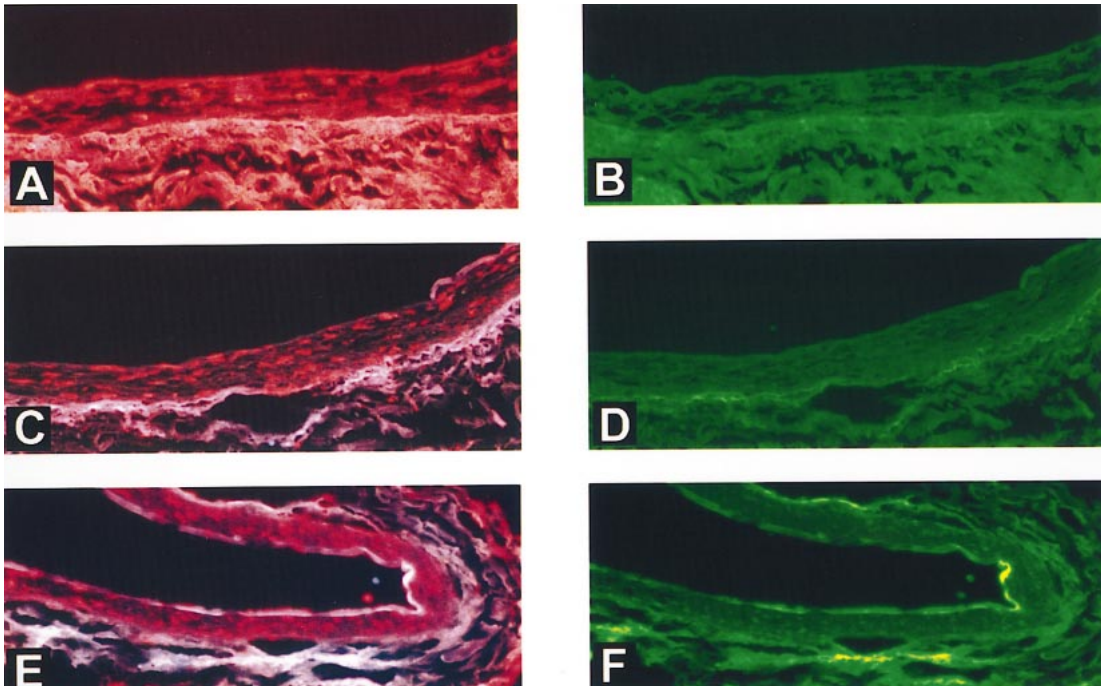

Fig. 4. Indirect fluorescence microscopy of rabbit superficial femoral artery segments 4 days after balloon injury, treated with either Ad.lacZ at 1×10^{10} pfu/ml or Ad.ASbFGF at 1×10^{10} pfu/ml by local intraluminal incubation immediately after injury. **A, C,** and **E,** Red emission demonstrates propidium iodide counterstained nuclei (visualizing all nuclei present) of Ad.lacZ-treated, negative control, and Ad.ASbFGF-treated vessels, respectively. **B, D,** and **F,** Green emission visualizes FITC-labeled nuclei of cells undergoing apoptosis of Ad.lacZ-treated, negative control, and Ad.ASbFGF-treated vessels, respectively. Images demonstrate an increase in apoptotic cells in media of Ad.ASbFGF-treated vessel **(F)** compared with Ad.lacZ-treated vessels or negative control. Negative controls were of an Ad.ASbFGF-treated vessel stained in parallel with other sections but incubated in the absence of terminal deoxynucleotidyl transferase (TdT) enzyme. Original magnification, ×125.

tions harvested from animals at both 4 days and 14 days after superficial femoral artery injury and treatment. No significant difference was demonstrated in the medial proliferation index of Ad.ASbFGF-treated and Ad.lacZ-treated vessels at 4 and 14 days after injury and in the intimal proliferative index at 14 days after injury. However, an increase in apoptosis was noted in the medial cells of Ad.ASbFGF-treated vessels 4 days after injury. There was minimal evidence of apoptosis in the vessels treated with Ad.lacZ at the same time point. By 2 weeks after balloon injury and gene delivery, there was no evidence of apoptosis in vessels of either treatment group. These data would suggest that the effect of Ad.ASbFGF gene delivery on the limitation of intimal thickening after balloon injury is not mediated by a significant inhibition of cellular proliferation, as seems to be the case when initial bFGF released by injured and dying cells is blocked by antibody,[3,6] but rather by induction of apoptosis and cellular demise. These findings are consistent with the in vitro data previously reported by Fox and Shanley[16] and suggest that sustained bFGF signaling is critical to the survival of vascular SMCs in vitro and in vivo. It would appear that loss of SMCs by apoptosis in the early phase leads to fewer cells actually proliferating (at a rate not significantly altered); therefore, there would be fewer cells depositing extracellular matrix, thereby limiting intimal thickening by 14 days after injury. Further studies to evaluate this theory are warranted.

CONCLUSION

Our data suggest that the use of Ad.ASbFGF gene transfer, designed to inhibit de novo bFGF synthesis after balloon injury, results in a significant

inhibition of neointimal formation. Continued bFGF synthesis may contribute to the arterial response to injury in rabbits by sustaining SMC survival in the neointima. Ad.ASbFGF gene transfer may be an effective strategy in limiting the intimal hyperplastic response after vascular reconstructive procedures.

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