Reversal of GATA-6 Downregulation Promotes Smooth Muscle Differentiation and Inhibits Intimal Hyperplasia in Balloon-Injured Rat Carotid Artery

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Abstract—The GATA-6 transcription factor is expressed in quiescent vascular smooth muscle cells (VSMCs) in culture, and levels of its transcript are rapidly downregulated on mitogen stimulation. In this study, we demonstrate that the GATA-6 transcript, protein, and DNA-binding activity are downregulated in rat carotid arteries on balloon injury. Downregulation was detected at 1 and 3 days after injury and recovered by 7 days. To assess the role of GATA-6 downregulation in injury-induced vascular lesion formation, adenoviral vectors were used to express wild-type human GATA-6 cDNA (Ad-GATA6) or an inactive mutant cDNA that lacks a portion of the zinc-finger domain (Ad-GATA6\DeltaZF). Adenovirus-mediated GATA-6 gene transfer to the vessel wall after balloon injury partially restored the levels of GATA-6 protein and DNA-binding activity to before injury levels. The local delivery of Ad-GATA6 but not Ad-GATA6\DeltaZF inhibited lesion formation by 46% relative to saline control and 50% relative to a control adenovirus that expressed lacZ. Local delivery of Ad-GATA6 also reversed changes in the expression patterns of smooth muscle myosin heavy chain, smooth muscle α-actin, calponin, vinculin, metavinculin, and proliferating cell nuclear antigen that are associated with injury-induced VSMC phenotypic modulation. These data indicate that the injury-induced downregulation of GATA-6 is an essential feature of VSMC phenotypic modulation that contributes to vessel lesion formation. (Circ Res. 1999;84:647-654.)

Key Words: gene expression ■ adenovirus ■ restenosis ■ muscle, smooth ■ genes

Vascular injury provokes the proliferation of medial VSMCs, which migrate to the intima and produce extracellular matrix.1 These processes are believed to contribute to restenotic lesions that occur after balloon angioplasty and are of particular importance in the development of lesions within stents2 and bypass grafts.3 Several studies have shown that overexpression of cell-cycle inhibitory genes can inhibit intimal lesion formation in animal models of vascular injury, which indicates that this approach may have use in the treatment of fibroproliferative diseases of the vessel wall.4 However, little is known about the endogenous regulators of VSMC phenotype that control the transition from a quiescent, differentiated cell under normal conditions to a proliferative, dedifferentiated cell in the presence of pathological stimuli.

The GATA family of transcriptional regulatory proteins includes 6 vertebrate genes, which each contain a highly conserved zinc-finger DNA-binding domain that interacts with DNA regulatory elements that contain a consensus A/T GATA A/G (WGATAR) or related sequence. The vertebrate related family members, GATA-6 is likely to regulate the differentiation state of VSMCs in vivo.
Previously, we reported on GATA-6, the primary structure of the human protein, localized the chromosomal location to 18q11.1–18q11.2 and described the tissue-restricted transcription pattern in human tissues. We also demonstrated that GATA-6 is expressed in quiescent rat and human VSMCs in culture and that mitogen activation of these cultures led to a rapid and transient downregulation of expression with kinetics similar to that exhibited by gas and gudd genes. These data suggested that GATA-6 might function to coordinate the expression of VSMC-contractile genes with genes required for cell-cycle progression. Recently, we also demonstrated that plasmid-mediated overexpression of GATA-6 inhibits mitogen-induced cell cycle activity in VSMCs and fibroblasts in vitro. Therefore, GATA-6 is an excellent candidate for a lineage-restricted regulatory protein that can regulate VSMC phenotype in proliferative lesions of the vessel wall.

In this study, we examined the regulation and function of GATA-6 during the proliferative response of VSMCs to acute injury. Balloon injury of adult rat carotid arteries results in rapid VSMC loss due to apoptotic cell death followed by the temporally and spatially coordinated initiation of the cell cycle in the remaining VSMCs. VSMCs that proliferate then migrate to the lumen and form a neointimal lesion by 2 weeks. In this study, we show that GATA-6 is downregulated during the peak of proliferative activity after balloon injury. To determine the functional significance of this downregulation, adenovirus-mediated GATA-6 gene transfer to balloon-injured rat carotid arteries was performed, which resulted in restoration of GATA-6 expression. Although before injury levels were not achieved, vessels transduced with the GATA-6–encoding adenovirus displayed a higher degree of VSMC differentiation and a reduced level of intimal hyperplasia. These data suggest that GATA-6 controls VSMC phenotype in vivo and that suppression of this factor may contribute to the pathogenesis of VSMC-derived lesions.

Materials and Methods

Cell Lines

Rat primary VSMCs (passage<5) were obtained by enzymatic digestion of the media from the thoracic aorta of male Sprague-Dawley rats and maintained in DMEM with 15% FBS. Synchronous populations of quiescent cells were obtained by placing cultures in media that contained 0.5% FBS for 48 to 72 hours. VSMCs were infected with adenovirus at the indicated multiplicity of infection (MOI) for 12 hours, followed by virus removal and incubation with DMEM that contained 10% FBS for 48 to 72 hours. NIH3T3 and 10.1 fibroblasts were prepared in low-mitogen media for an additional 12 hours. Growth media (15% FBS) was then added for 24 hours, and cells were harvested by trypsinization, fixed, and stained with propidium iodide. The cell-cycle profile was determined with a Beckton Dickinson Vantage flow cytometer and Lysis II cell cycle analysis software.

Adenoviral Vector Construction

A cDNA encoding the full-length human GATA-6 protein was isolated from pCDNA-GATA-6wt19 and inserted into the KpnI-XbaI sites of pACCMV.pLPa, which contains a CMV promoter/enhancer, SV40 polyA tract, and the Ad5 adenoviral sequences required for homologous recombination to generate the plasmid pACCMV.pLPa-GATA-6wt. A cDNA that contained a deletion in the zinc-finger region, which results in expression of a protein that lacks codons 244 to 306, was subcloned similarly into pACCMV.pLPa to generate pACCMV.pLPa-GATA-6-ΔZF. Each plasmid and pJM17 was cotransfected into 293 cells, which allowed for homologous recombination. The E1/E3-deleted adenovirus constructs that contained GATA-6wt (Ad-GATA6) or GATA-6-ΔZF (Ad-GATA6-ΔZF) were purified from isolated plaques, identified by polymerase chain reaction (PCR) and immunoblotting analysis, and amplified in 293 cells. The viral preparations used for both in vitro and in vivo studies were purified by CsCl gradient ultracentrifugation and dialyzed against buffer, which contained 10 mmol/L Tris-HCl and 10% glycerol. The titer of recombinant adenoviruses was determined by plaque assay on 293 cells as described previously and expressed as plaque-forming units (PFU)/mL. The replication-defective adenoviral construct Ad-βgal expresses β-galactosidase from the CMV promoter.

**Rat Model of Balloon Injury and Adenoviral Infection**

The rat carotid artery balloon-injury model was based on a model described by Clowes et al. Adult male Sprague-Dawley rats that weighed 400 to 600 g were subjected to a distending, deendothelializing injury with an inflated 2F Fogarty embolectomy catheter inserted through the external carotid artery. The injured segment of the artery was then incubated in the absence or presence of adenovirus (1.0×10⁹ PFU) in a total volume of 100 μL saline for 20 minutes. Rats were killed at 1, 3, or 14 days after injury with an intraperitoneal injection of pentobarbital sodium (100 mg/kg). The balloon-injured segment of the artery from the proximal edge of the omohyoid muscle to the carotid bifurcation was perfused with saline and dissected. The tissue was then fixed with 100% methanol and embedded in paraffin. Histological images of 4 μm/L cross sections from the center of the injured segment were hematoxylin-eosin or elastic-trichrome stained and used to determine the intimal, medial, and luminal areas by quantitative morphometric analysis with a computerized sketching program (MACMEASURE version 1.9). Three sections from each artery were evaluated by an investigator who was blinded to the identities of the treatment groups. Experimental protocol was approved by the Institutional Animal Care and Use Committee of St. Elizabeth’s Medical Center and complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

**Cell-Cycle Analysis**

Primary cultures of rat VSMCs were infected at an MOI of 300 PFU per cell with Ad-βGal, Ad-GATA6ΔZF, or Ad-GATA6 for 12 hours, after which the virus was removed and cultures were incubated in low-mitogen media for an additional 12 hours. Growth media was then added for 24 hours, and cells were harvested by trypsinization, fixed, and stained with propidium iodide. Quiescent cells were harvested after 72 hours in low-mitogen media without infection. DNA content was analyzed by flow cytometry, and the cell-cycle profile was determined with a Beckton Dickinson Vantage flow cytometer and Lysis II cell cycle analysis software.

**Immunoblot Analysis**

Cells were infected with the indicated adenoviruses at an MOI of 100 (NIH3T3 and 10.1 cells) or 300 (rat VSMCs) as described above. Whole cell extracts from rat carotid arteries and cultured cells were prepared in ice-cold lysis buffer (1% NP-40, 9.1 mmol/L dibasic sodium phosphate, 1.7 mmol/L monobasic sodium phosphate, 150 mmol/L NaCl, 1 mmol/L DTT, 0.5 mmol/L PMSF, 2 μg/mL aprotinin, 2 μg/mL leupeptin, 1 mmol/L sodium orthovanadate) and then centrifuged. Arterial extracts were prepared from a pool of 4 or 5 arteries with a homogenizer (Tissumizer Mark II; Tekmar Co). Immunoblot analyses used rabbit polyclonal antibody against human GATA-6 protein, as described previously, mouse monoclonal anti–villin antibody (PCNA; Signet Laboratories, Dedham, Mass), rabbit polyclonal anti-Cdk4 (C22, Santa Cruz, Calif), mouse monoclonal anti–smooth muscle myosin (hSMM-V), mouse monoclonal anticalponin (hCP), mouse monoclonal antivin (hVIN-1; Sigma) and mouse monoclonal anti–a-tubulin (Calbiochem). Arterial segments were harvested at 24 hours, 72 hours, 7 days, and 14 days after balloon injury and treatment, and contrat-
eral carotid arteries were taken as uninjured control arteries. Western immunoblot analyses were preformed on 10 to 75 μg of carotid artery extract protein, which depended on signal intensity as described previously. All immunoblot experiments were performed on at least 3 separate occasions.

**Electrophoretic Mobility Shift Assays**

Whole-cell lysates were prepared from rat carotid arteries, NIH3T3 cells, or 10.1 fibroblasts that were uninfected or infected with the indicated adenoviral construct in ice-cold lysis buffer (50 mmol/L Hepes, pH 7.8; 420 mmol/L KCl; 0.1 mmol/L EDTA, pH 8.0; 5 mmol/L MgCl2; 20% glycerol; 1 mmol/L DTT; 0.5 mmol/L PMSF; 2 μg/mL aprotinin; 2 μg/mL leupeptin; and 1 mmol/L sodium orthovanaadate) by subjecting tissue or cells to 3 cycles of freezing and thawing followed by centrifugation. Double-stranded oligonucleotide probes and competitor DNAs were synthesized to correspond to the extensively characterized GATA consensus site in the α-globin promoter. The coding strand sequence for each probe was GATA-5′-GATCTCCCGACACTGAAAGATTCCCTG-3′ and GATA mt 5′-GATCTCCGGCACAATCTAGTCTAGATTTCCCTG-3′. The consensus and mutated GATA-binding sites are underlined.

Electrophoretic mobility shift assays (EMSAs) were performed as described previously. Supershift assays were performed with rabbit polyclonal anti–GATA-6 antibody or preimmune serum. All EMSA experiments were performed on at least 3 separate occasions.

**RNA Isolation and Reverse Transcription–PCR**

**Analysis on Balloon-Injured Rat Carotid Arteries**

Individual arteries were isolated from each rat and immediately frozen in liquid nitrogen. For each sample, the tissue was transferred to a 50-mL polypropylene tube that contained 2 mL of TRI reagent (Gibco/BRL). Under standard conditions, each sample contained 0.5 μg of RNA was purified by phenol-chloroform extraction, precipitated by a 100-fold molar excess of unlabeled consensus oligonucleotide probe that corresponded to the GATA site derived from a mouse α-globin promoter (Figure 2A). This nucleoprotein complex was not detected when the DNA-binding mixture contained a 100-fold molar excess of unlabeled consensus oligonucleotide probe, whereas in a parallel reaction, a 100-fold molar excess of probe that contained a mutation in the GATA consensus sequence was unable to compete for complex formation. The presence of GATA-6 protein in the GATA consensus sequence was unable to compete for complex formation. The presence of GATA-6 protein markedly decreased at 1 and 3 days after injury but recovered to preinjury levels by 7 days and was maintained at 14 days (Figure 1).

**Results**

**Levels of GATA-6 Gene Products and DNA Binding Activity Are Decreased After Injury in Rat Carotid Arteries**

To investigate the regulation of GATA-6 during VSMC proliferation in vivo, adult male rats were subjected to a balloon injury with a catheter inserted through the external carotid artery. At various times after injury, protein extracts or RNA were prepared from isolated arteries and control contralateral uninjured arteries and samples were analyzed for GATA-6 levels. Immunoblot analyses were performed with a specific antiserum against GATA-6 to assess changes in GATA-6 protein expression during the proliferative response. The expression of GATA-6 protein markedly decreased at 1 and 3 days after injury but recovered to preinjury levels by 7 days and was maintained at 14 days (Figure 1). Little or no change in α-tubulin levels was detected in these extracts, which indicated equal protein loading.

EMSAs were performed to examine GATA-6 DNA-binding activity in whole cell extracts prepared from injured and uninjured rat carotid arteries. With the use of extracts prepared from an uninjured artery, a diffuse nucleoprotein complex was detected with a radiolabeled duplex oligonucleotide probe that corresponded to the GATA site derived from a mouse α-globin promoter (Figure 2A). This nucleoprotein complex was not detected when the DNA-binding mixture contained a 100-fold molar excess of unlabeled consensus oligonucleotide probe, whereas in a parallel reaction, a 100-fold molar excess of probe that contained a mutation in the GATA consensus sequence was unable to compete for complex formation. The presence of GATA-6 protein in the nucleoprotein complex was demonstrated by the ability to supershift complex with anti–GATA-6 antibodies raised to regions of the GATA-6 protein that were not homologous to the other GATA proteins (Figure 2B). Inclusion of preimmune serum did not alter the nucleoprotein complex. Collectively, these data demonstrate that GATA-6 DNA-binding activity can be detected in extracts prepared from rat carotid arteries.
GATA-6 DNA-binding activity was decreased in extracts prepared from arteries at 1 or 3 days after injury compared with uninjured vessels, but DNA-binding activity recovered to preinjury levels at 7 and 14 days after injury (Figure 2C). The changes in GATA-6 DNA-binding activity parallel closely the changes in GATA-6 protein levels (Figure 1). The same EMSA results were obtained with GATA consensus DNA sequences from the mouse α-globin promoter (Figure 2), the GATA/CEF-1 binding site from the murine cardiac troponin C gene that binds GATA-6, \(^{15}\) or the GATA consensus binding site from the human T-cell receptor δ gene \(^{27}\) (data not shown).

Figure 2. GATA-6 DNA-binding activity in rat carotid arteries is transiently decreased after balloon injury. A, Whole-cell extract from pools, uninjured arteries was incubated with \(^{32P}\)-labeled probe to the GATA consensus sequence from the α-globin promoter. The indicated binding reactions contained a 100-fold molar excess of nonlabeled consensus GATA-6 probe (WT) or a 100-fold molar excess of probe, which contained a mutation in the GATA sequence (MT). Nucleoprotein complexes were detected by EMSA. B, This nucleoprotein complex was diminished and supershifted by incubation with anti–GATA-6 antibody (G6) but not with preimmune serum (PI). C, GATA-6 DNA-binding activity is downregulated at 24 and 72 hours after balloon injury. Whole-cell extracts from pools of 4 or 5 uninjured (U) or injured arteries at the indicated time points were assayed by EMSA.

Previous work in cultured VSMCs indicated that steady-state GATA-6 transcript levels decreased after mitogen-activated cell proliferation. \(^{16}\) To investigate this regulation in vivo, transcript levels of GATA-6 and GAPDH (as a control) were measured in balloon-injured and uninjured rat carotid arteries by a semiquantitative RT-PCR assay under conditions that ensured a linear accumulation of signal with respect to input RNA and cycle number. An advantage of this RT-PCR approach is that RNA from individual arteries could be analyzed, which allowed a statistical analysis. The results confirmed the Western blotting experiments, which showed that GATA-6 transcript levels (relative to the control GAPDH mRNA) decreased significantly 3 days after balloon injury (Figure 3A). At day 3, transcript levels decreased nearly 3-fold (0.37±0.12 relative to 1.0 for uninjured arteries; Figure 3B). These results are consistent with a primary effect on the GATA-6 gene at the mRNA level after vessel injury in vivo.

Construction and Characterization of Replication-Defective Adenoviral Vectors Expressing GATA-6 cDNAs

Because the GATA-6 gene is regulated at the transcript level after stimulation of VSMC proliferation, it was reasonable to consider whether forced transcription to restore levels might inhibit intimal hyperplasia in the rat model of balloon injury. For this purpose, replication-defective adenoviral vectors were constructed to express GATA-6 (Ad-GATA6) or a mutant non-DNA-binding GATA-6 (Ad-GATA6ZF) that lacks a large portion of the zinc-finger DNA-binding domain (Figure 4A). In preliminary cell culture experiments, Western immunoblot analysis of NIH3T3 cells revealed prominent immunoactive bands that migrate with an apparent molecular weight of 52 kDa in the Ad-GATA6–infected cells and 46 kDa in the Ad-GATA6ZF–infected cells, which is consistent with the predicted molecular weight of the wild-type and mutant GATA-6 proteins (Figure 4B).

To confirm the expected activity of the wild-type and mutant proteins, whole-cell lysates from NIH3T3 cells infected with Ad-GATA6 were used in gel mobility shift experiments, which showed a significant increase in the predominant nucleoprotein complex (Figure 4C, arrowhead) relative to lysates from uninfected cells (the NIH3T3 lysates contain low levels of an endogenous GATA-binding activity). This complex was competed specifically with excess unlabeled target site probe but not by the mutant probe (Figure 4C). The inclusion of specific anti–GATA-6 antibodies gave a supershifted complex, whereas preimmune sera had no effect. No increase in specific binding activity was detected in lysates of NIH3T3 cells infected with Ad-GATA6ZF or the control virus that expresses β-galactosidase, Ad-βgal. Therefore, with respect to DNA-binding activity, these data show that
Ad-GATA6 expresses functional GATA-6 protein, whereas Ad-GATA6\(\Delta\)ZF does not.

Previously, we reported that plasmid-encoded GATA-6 inhibits cell growth. Thus, FACS analysis was performed to test whether the GATA-6-expressing adenoviral vectors also inhibit cell cycle activity (Table). Quiescent VSMCs were transduced with adenoviral constructs and stimulated with high-mitogen media. Ad-GATA6 transduced cells were arrested in G0/G1 phases of the cell cycle, comparable to uninfected quiescent cells. In contrast, FACS analyses revealed that mitogen-stimulated cells transduced with Ad-GATA6\(\Delta\)ZF or Ad-\(\beta\)Gal were comparable to mock-infected, mitogen-stimulated cells. Infection with Ad-GATA6 also led to a corresponding decrease in the proportion of cells in S phase compared with Ad-GATA6\(\Delta\)ZF, Ad-\(\beta\)Gal, or mock-infected cells. These data demonstrate that adenovirus-mediated overexpression of GATA-6 inhibits cell growth in vitro but the GATA-6 mutant lacking the zinc-finger domain does not.

### Ad-GATA6 Inhibits Neointima Formation in Balloon-Injured Carotid Arteries

To test the activity of GATA-6 in vivo, the adenoviral constructs were used to deliver wild-type and mutant GATA-6 locally to balloon-injured rat carotid arteries. Immediately after denudation with a balloon catheter, the vessel wall was exposed to adenoviral solution (1.0 \(\times\) 10\(^9\) PFU) for 20 minutes. Arteries were harvested 1 or 3 days afterward to examine protein expression by immunoblot analysis or EMSA or at 2 weeks to assess intimal hyperplasia. Immunoblot analysis revealed that local delivery of Ad-GATA6 partially restores GATA-6 protein levels at the 3-day postinjury time point (Figure 5A). Similar results were also observed with extracts prepared from vessels at 1 day after injury (not shown). Gel mobility shift experiments revealed that the injury-induced decrease in GATA-6 DNA-binding activity levels was partially reversed when assessed at day 3 (Figure 5B) or day 1 (not shown) postinjury. Therefore, adenovirus-mediated GATA-6 gene transfer results in restoration of GATA-6 expression, although at lower levels relative to uninjured vessels.

Immunoblot analysis revealed that expression of PCNA, a marker of VSMC proliferation, was suppressed in extracts prepared from Ad-GATA6–infected vessels, relative to saline injected controls (Figure 5A). Analyses of PCNA staining of histological sections at 3 days after injury revealed that the expression of PCNA-positive cells in Ad-GATA6–treated
vessels was reduced by a factor of 3.2 relative to the saline control ($P \leq 0.01$), whereas the Ad-βGal– and Ad-GATA6DZF–treated vessels displayed 14% more (not statistically significant) or 9% fewer (not statistically significant) PCNA-positive cells, respectively, than the saline control (data not shown). Therefore, similar to its antiproliferative function in vitro, forced expression of GATA-6 appears to inhibit cell growth in the vessel wall.

Western immunoblot analyses were performed on injured vessels to examine the effects of constitutive GATA-6 expression on markers of smooth muscle cell differentiation. Smooth muscle myosin heavy chain (SM MHC), smooth muscle α-actin (SM α-actin), and calponin expression and the ratio of metavinculin to vinculin expression are indicative of smooth muscle cell differentiation in developing vessels and vascular disease.28 As expected, vascular injury downregulated the expression of SM MHC, SM α-actin, and calponin, whereas the expression of the cyclin-dependent kinase cdk4 did not change (Figure 6). Injury also increased the ratio of vinculin to metavinculin expression, which is consistent with reports that this ratio is indicative of VSMC differentiation in adult human tissues.29,30 Infection of vessels with Ad-GATA6 at the time of injury attenuated the downregulation of SM MHC, SM α-actin, and calponin. In contrast, vinculin was reduced to near preinjury levels by the partial restoration of GATA-6 expression. These data suggest that GATA-6 regulates the differentiated phenotype of VSMCs under conditions that induce intimal lesions.

Finally, restoration of GATA-6 expression had a significant effect on lesion formation at 2 weeks after injury. Saline-treated arteries and Ad-βGal–infected arteries had neointima/media (I/M) area ratios of 1.4±0.14 and 1.3±0.13, respectively. In contrast, arteries infected with Ad-GATA6 had an I/M ratio of 0.70±0.13 ($P < 0.01$), which represented a 46% to 50% reduction relative to saline and Ad-βGal–infected arteries (Figure 7). Local delivery of Ad-GATA6ΔZF did not significantly inhibit neointima formation (I/M ratio of 1.2±0.13). Collectively, these data indicate that relatively low levels of GATA-6 expression in the vessel wall can inhibit injury-induced stenosis in the rat carotid model.

**Discussion**

The VSMCs of the normal adult vessel wall are quiescent and display characteristics of a differentiated phenotype. After injury and growth factor release, VSMCs regress to a less differentiated state and proliferate. This process has been

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**Figure 5.** Ad-GATA6 partially restores GATA-6 protein expression and DNA-binding activity to preinjury levels in balloon-injured rat carotid arteries. Balloon-injured rat carotid arteries were incubated with Ad-GATA6 or saline for 20 minutes immediately after injury. Whole-cell extracts were prepared from pools of 4 to 5 unjured or injured and treated vessels. A, Immunoblot of analysis of GATA-6, PCNA, and tubulin protein expression in extracts prepared from uninjured vessels or 3 day after injury vessels that were infected with Ad-GATA6 or mock-infected (saline), B, EMSA of whole cell extracts prepared from uninjured vessels, mock-infected vessels (saline) at 1 and 3 days after injury, and Ad-GATA6-infected vessels at 3 days after injury.

**Figure 6.** Ad-GATA6 promotes expression of VSMC-specific proteins in balloon-injured rat carotid arteries. Whole-cell lysates were prepared from pools of 4 to 5 uninjured or injured and treated vessels. Extract protein (10 to 15 μg per lane) was analyzed for expression of SM MHC, SM α-actin, calponin, metavinculin, vinculin, and cd k4 protein expression by immunoblot analysis. Vessels harvested at 1 day after injury were infected with Ad-GATA-6 or mock-infected (saline) at the time of injury.

**Figure 7.** Ad-GATA6 inhibits neointima formation in the rat carotid balloon-injured arteries. A, Representative hematoxylin-eosin stained cross section in rat carotid artery 14 days after balloon injury and adenovirus infection (Ad-βGal or Ad-GATA6). Arrows indicate elastic-lamina stain. B, Histological images from the artery cross sections were projected on a digitizing board and quantitative assessments of intimal and medial area were assessed for saline-, Ad-GATA6-, Ad-GATA6ΔZF-, and Ad-βgal–treated arteries at 14 days after injury. I/M is shown as mean±SEM. I/M was significantly reduced in Ad-GATA6(WT)-transduced arteries ($P < 0.01$) but not in Ad-GATA6ΔZF– or Ad-βgal–treated arteries compared with saline-treated controls.
referred to as phenotypic modulation, and phenotypically modified smooth muscle cells are a hallmark of atherosclerotic and restenotic lesions in humans. To understand the process that coordinates differentiation and cell cycle activity during VSMC phenotypic modulation, several studies have examined the role of smooth muscle transcription factors that are regulated by mitogen activation. Previous work has documented that GATA-6 is the predominant GATA factor expressed in VSMCs. In cultured VSMCs, GATA-6 transcript levels are rapidly and transiently decreased after mitogen stimulation.

In this study, we examined the role of GATA-6 in the genesis of a proliferative lesion in the blood vessel wall. GATA-6 regulation and function was examined in balloon-injured rat carotid arteries, a well-characterized model that induces robust VSMC proliferation and produces a lesion that partially occludes the lumen of the vessel. Downregulation of the GATA-6 protein was noted at 1 and 3 days after injury, which corresponds to a peak of proliferative activity in medial VSMCs. Levels of GATA-6 protein returned to normal after 7 days, and this level of expression was maintained at 14 days after injury. At these later time points, medial smooth muscle cell proliferation has ceased, whereas smooth muscle cells at the luminal edge of the lesion continue to proliferate.

The time course of GATA-6 depletion and recovery was closely paralleled by changes in GATA-6 DNA-binding activity. Furthermore, GATA-6 mRNA levels also decreased and then recovered after balloon injury, which is consistent with the notion that the gene is regulated at the transcriptional level in balloon-injured vessels. This aspect is important because translational controls might preclude restoration of GATA-6 levels by gene transfer techniques. The time course of GATA-6 downregulation and recovery in injured carotid arteries was similar to that of Gax, another mitogen-regulated transcription factor in VSMCs that can inhibit cell growth.

To determine the functional significance of GATA-6 downregulation in vivo, replication-defective adenoviral constructs that expressed functional or nonfunctional GATA-6 cDNAs were constructed and used to deliver these transgenes locally to the sites of vascular injury. Adenovirus-mediated delivery of the wild-type GATA-6 gene inhibited intimal hyperplasia by 50%, relative to a control adenovirus that expressed lacZ, whereas adenovirus-mediated delivery of a mutated GATA-6 cDNA did not inhibit lesion formation. Note that although adenovirus-mediated GATA-6 gene transfer resulted in elevation of GATA-6 protein and DNA-binding activity at 1 and 3 days after injury, preinjury levels were not achieved. Therefore, physiological levels of GATA-6 appear to be critical to determining the size of the injury-induced lesion. In contrast, the efficacy of other gene products for inhibition of vascular lesion progression appears to require extensive overexpression.

GATA-6–mediated inhibition of intimal lesion formation probably results from the ability of this factor to promote VSMC differentiation and inhibit mitogen-activated cell-cycle activity. It has been shown previously that plasmid-mediated overexpression of GATA-6 can inhibit the proliferation of VSMCs and fibroblasts. In this study, we show that PCNA expression, a marker of VSMC proliferation, was reduced in the GATA-6-transduced vessels, which suggests that GATA-6 has antiproliferative properties in vivo. The importance of cell-cycle activity during intimal hyperplasia is underscored by the observation that injury-induced lesion formation is inhibited by molecules that can interfere with cell-cycle progression. For example, intimal hyperplasia is inhibited by antisense cdk2 oligonucleotides and by over-expression of both the wild-type and mutant forms of the retinoblastoma protein or the cdk inhibitor p21.

During VSMC phenotypic modulation, proliferation is accompanied by a reversion of VSMCs to a less differentiated, fibroblast-like state. Thus, it has been suggested that the same subset of transcription factors that regulate cell division might also be important in controlling the expression of tissue-specific genes associated with the differentiated state. In this study, we have shown that restoration of GATA-6 expression in balloon-denuded arteries attenuates the changes in SM MHC, SM α-actin, calponin, vinculin, and metavinculin expression that occur with injury-induced VSMC phenotypic modulation. Although direct transcriptional links between GATA-6 and these genes have not been demonstrated to date, many contain consensus WGA (T.C., K.W., unpublished observations, 1998). These data suggest that extracellular components present within the intact vessel wall are essential for GATA-6–induced expression of differentiation-specific genes.

Many diseases of the vessel wall are associated with the dedifferentiation and excessive proliferation of VSMCs. In this study, we demonstrated that the levels of GATA-6 transcript, protein, and DNA-binding activities are decreased in the vessel wall after acute injury and that lesion formation is inhibited by the partial restoration of GATA-6 expression. These data indicate that GATA-6 downregulation after injury is an essential feature of the proliferative response to vessel wall injury. Identification of GATA-6 as an endogenous regulator of pathological VSMC proliferation may provide insights about the molecular mechanisms that contribute to vascular lesions and may have implications for the development of new therapies to treat these diseases.

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References

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