Conditional Expression of a Dominant-Negative c-Myb in Vascular Smooth Muscle Cells Inhibits Arterial Remodeling After Injury

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Abstract—Inhibiting activity of the c-Myb transcription factor attenuates G1 to S phase cell cycle transitions in vascular smooth muscle cells (SMCs) in vitro. To determine the effects of arterial SMC-specific expression of a dominant-negative c-Myb molecule (Myb-Engrailed) on vascular remodeling in vivo, we performed carotid artery wire-denudation in 2 independent lines of binary transgenic mice with SM22α promoter-defined Doxycycline-suppressible expression of Myb-Engrailed. Adult mice with arterial SMC-specific expression of Myb-Engrailed were overtly normal in appearance and did not display any changes in cardiovascular structure or physiology. However, bromodeoxyuridine-defined arterial SMC proliferation, neointima formation, medial hyperplasia, and arterial remodeling were markedly decreased in mice expressing arterial SMC-restricted Myb-Engrailed after arterial injury. These data suggest that c-Myb activity in arterial SMCs is not essential for arterial structure or function during development, but is involved in the proliferation of arterial SMCs as occurs in vascular pathology, and that the expression of a dominant-negative c-Myb can dramatically reduce adverse arterial remodeling in an in vivo model of restenosis. As such, this model represents a novel tissue-specific strategy for the potential gene therapy of diseases characterized by arterial SMC proliferation. (Circ Res. 2003;92:314-321.)

Key Words: dominant-negative c-Myb | conditional transgenic mouse | SM22α | restenosis | vascular smooth muscle cell proliferation

In both conduit and resistance arteries, smooth muscle cells (SMCs) underlying a healthy endothelium are normally ordered into discrete layers by elastic laminae and exist in a predominantly quiescent and contractile state. However, physical disruption or dysfunction of the endothelium and subsequent changes in arterial structure and/or flow, as occurs after angioplasty, atherosclerosis, or plaque rupture, may transform arterial SMCs into a migratory and proliferative phenotype. Transformed SMCs influence the balance between positive and negative arterial remodeling and define the extent of luminal stenosis and arterial perfusion in vascular disease.

c-Myb is a helix-turn-helix transcription factor involved in growth and differentiation of diverse cell lineages, including regulation of the G1 to S phase cell cycle transition in vascular SMCs. Previous studies have shown that treatment of cultured SMCs with an antisense c-Myb oligonucleotide (AS-Myb) was sufficient to prevent progression through cell cycle, causing arrest before the G1-S checkpoint. To determine whether c-Myb inhibition was a feasible therapeutic strategy for restenosis, the efficacy of AS-Myb treatment in arterial injury has been examined by several investigators. In one such study, rats treated with Pluronic gel containing AS-Myb at the time of balloon angioplasty had a decrease in neointima formation at the site of gene therapy as compared with untreated rats or those treated with gel containing sense, scrambled, or no oligonucleotides. However, the specific mechanism mediating this effect has been widely debated. Notwithstanding the controversy surrounding AS-Myb, other preliminary studies supporting a strategy of c-Myb-directed therapy include the use of an anti-c-Myb antibody in a human leukemia cell line, an adenovirus-delivered ribozyme directing c-Myb mRNA degradation in vascular SMCs, and our previous studies using dominant-negative c-Myb mutants in vascular SMCs and fibroblasts in vitro. c-Myb is composed of a N-terminal DNA binding motif, a transactivation domain, and a C-terminal negative regulatory Leucine-zipper. The dominant-negative molecule Myb-Engrailed (MEn) contains the c-Myb DNA binding domain.
fused to the repressor domain of the Drosophila Engrailed transcription factor. Expression of MEn in immortalized SMCs, embryonic vascular SMCs, or fibroblasts resulted in decreased c-Myb–dependent promoter-reporter activity, and attenuated cell cycle progression and proliferation.

Because the regulation and activities of c-Myb are complex, and may be influenced by interactions with tissue-specific factors, and/or DNA sequence binding competition with other Myb isoforms (A-Myb, B-Myb), the precise spatial and temporal regulation of c-Myb may have important implications. Additionally, because c-Myb homozygous knockout mice are embryonic lethal, the production of a conditional and tissue-specific model of dominant-negative c-Myb expression would facilitate studies on the role of c-Myb in the development and pathophysiology of arterial SMCs. Indeed, data generated by such a model may be informative in the design of cell-specific gene therapies for vascular disease and facilitate a better understanding of the necessary roles of c-Myb in adult tissue.

Accordingly, we created transgenic mice with conditional and arterial SMC-specific expression of MEn. SMtTA transgenic mice, which have SM22α promoter-defined expression of a tetracycline-responsive transcriptional activator (tTA), were crossed with transgenic mice that contain the tTA-responsive promoter; DBD, DNA Binding domain; En RD, Engrailed transcriptional repressor domain; and 9E10, c-Myc 9E10 epitope tag.

Materials and Methods

Transgenic Mouse Production

The Myb-Engrailed coding sequence, composed of the murine c-Myb DNA binding domain (aa 71-200), ligated in-frame to the Drosophila Engrailed transcriptional repressor domain (aa 2-98), and containing a c-Myc 9E10 epitope tag, was removed from pttMEnT and cloned into pBiG (Clontech Laboratories Inc, Palo Alto, Calif), to create pBiG:MEn. The bidirectional promoter of pBiG (pB,) allows tTA-regulated transcription of adjacent cassettes, namely β-galactosidase (β-gal) and MEn. An Asel/AseI fragment of pBiG:MEn (Figure 1) was then shuttled into pBluescript, linearized to remove flanking bacterial sequences, and used for transgenic mouse production as previously described. Animals used for generation of transgenic lines and subsequent backcrossing (C57BL/6) were purchased from Charles River Laboratories Inc, Wilmington, Mass.

RT-PCR

Total RNA extracted from whole aortae using the guanidinium method was treated with DNaseI (Invitrogen-Life Technologies) and used for first strand cDNA synthesis with random hexamers and Superscript II reverse transcriptase (Invitrogen-Life Technologies), as per manufacturer’s specifications. Hexameric primers were removed by filtration through a 5-kDa membrane (Millipore), and the first strand cDNA used for RT-PCR with Engrailed-specific primers: 5'-AGGACATAAGCAGCACCCTCACC-3' and 5'-TCTCCGAA-ACGATGCGCTCAGG-3'. Negative control reactions were performed excluding reverse transcriptase.

Immunohistochemistry

Tissues were harvested without fixation, washed in phosphate-buffered saline (PBS), frozen in tissue embedding medium on liquid nitrogen, and cut to a thickness of 6 μm. Sections were fixed in acetone, rehydrated, and then incubated for 5 minutes in 0.1% hydrogen peroxide/PBS. Slides were blocked using a Mouse-On-Mouse Immunodetection Kit (Vector Laboratories Inc), and incubated with a 1:200 dilution of anti-c-Myb (9E10 epitope) monoclonal antibody (Sigma Chemical Co) in 0.5% BSA/PBS. After PBS washes, slides were treated with a 1:100 dilution HRP-conjugated anti-mouse secondary antibody (Sigma Chemical Co), washed again with PBS, and treated with 3,3′-diaminobenzidine to assay peroxidase activity. Slides were counterstained with Hematoxylin, dehydrated, and sealed. Alternatively, a 1:100 dilution FITC-conjugated anti-mouse secondary antibody (Sigma Chemical Co) was also used for detection.

β-Gal Staining

Tissues were harvested, sectioned at 6 μm, and then incubated in filtered X-gal staining solution [0.2% X-gal, 100 mmol/L MgCl2, 5 mmol/L K3Fe(CN)6, 5 mmol/L K4Fe(CN)6, and 5 mmol/L K3Fe(CN)6] in PBS] for 4 hours as previously described. Images were acquired on a DMLB microscope (Leica Microsystems) with a Photometrics Cool-Snap digital camera (The Carsen Group) and analyzed using Scion Image Analysis software (Scion Co). β-Gal staining was quantified using 3 criteria: (1) number of positively stained nuclei/mm²; (2) mean density of β-gal–stained nuclei; and (3) the percent positive stained pixels above background within the media.

Carotid Artery Injury

All animal experimentation was conducted in accordance with operating protocols approved by the Toronto General Hospital Animal Care Committee. Carotid artery injury was performed as previously described with the following modifications. Briefly, animals were anesthetized using intraperitoneal ketamine-HCl (100 mg/kg IP) xylazine-HCl (10 mg/kg, IP), and placed on a warming pad to regulate temperature. The primary bifurcation of the right common carotid artery was isolated after midline neck incision, and two ligatures were placed around the external branch. Next, the distal ligation was tied, and flow through the common carotid artery was temporarily occluded with a vascular clamp. An incision was made in the external carotid artery between the two ligatures, and a curved 350 μm polished copper wire introduced into the lumen. The wire was advanced past the primary bifurcation into the common carotid artery, and vessel systematically injured by simultaneously rotating the curved copper wire while passing along the vessel four times. The wire was removed, and the external carotid artery tied off.
proximal to the incision with the second ligature. The vascular clamp was next removed restoring flow through the common carotid artery. The skin was closed with a single suture, and animals were allowed to recover on a warming pad.

At 7 or 15 days after injury, PIPES-buffered (0.1 mol/L PIPES [pH 6.9], 2 mmol/L MgCl₂) 4% paraformaldehyde was used to fix the carotid artery in vivo at physiological pressure (100 mm Hg). Injured and control (uninjured) carotid arteries were harvested and imbedded in paraffin, or tissue-embedding medium on liquid nitrogen. Sections were cut from the proximal, middle, and distal thirds of the injured region of the common carotid artery. Adjacent sections were subjected to standard hematoxylin and eosin (H&E), Movat’s, Mason’s, and MEn (9E10) immunostaining. Two observers blinded to the identity of the specimens performed all staining and subsequent analyses.

**Arterial Morphometry**

Images were acquired as noted above for β-gal staining and analyzed by planimetry using Scion Image Analysis software (Scion Co). The mean lumenal, intimal, medial, and adventitial areas were obtained by averaging triplicate values at the proximal, middle, and distal thirds of the injured and sham-operated vessels. To normalize for vessels in which eccentric remodeling was observed, all mean areas were converted to mean diameter and thickness. Image analysis measurements were calibrated with a stage micrometer to obtain absolute dimensions.

**BrdU Incorporation Assay**

At the time of carotid injury, osmotic minipumps (No. 2002, Alzat Corp) loaded with BrdU set to deliver 25 mg/kg per day to the peritoneal cavity, were inserted through a small dorsal skin incision. Carotid arteries were harvested as above and the incorporation of BrdU assayed using a 1:200 monoclonal BrdU-specific antibody (Sigma Chemical Co), and a 1:100 FITC conjugated rabbit anti-BrdU antibody staining) are shown in representative carotid artery sections from MEn/SMtTA⁺ mice in the absence and presence of Doxycycline (+DOX; 1 mg/mL in drinking water) and a MEn/SMtTA⁻ littermate.

**Results**

**Creation of MEn Founder Mice**

Single cell pronuclei injections with the linearized pBIG:MEn construct (Figure 1) produced 7 transgene positive (MEn⁺) founders out of 64 live births. These founders faithfully transmitted the MEn transgene to offspring and did not exhibit any developmental or reproductive abnormalities.

**Characterization of Conditional, Tissue-Specific β-Gal and MEn Expression in MEn⁺/SMtTA⁺ Mice**

MEn mice were crossed with mice harboring the SMtTA, and the expression of tTA-responsive transgenes were examined in resulting progeny. β-Gal staining was done to survey nuclear-localized reporter activity in binary transgenic mice (MEn⁺/SMtTA⁻), in the presence and absence of 1 mg/mL doxycycline (DOX) administered ad libitum in drinking water, and nonbinary littermate controls (MEn⁺/SMtTA⁻, MEn⁻/SMtTA⁻, MEn⁻/SMtTA⁻). β-Gal activity was noted in vascular SMCs of aorta, carotid, mesentery, liver, lung, kidney, and spleen of MEn⁺/SMtTA⁻ mice from 2 of the 7 lines (220 MEn, 1986 MEn), which was reduced to undetectable levels by DOX treatment (Figure 2A). Lines with leaky (non-DOX-suppressible) β-gal expression entirely (3 of 7), or those lacking tTA-dependent β-gal expression entirely (3 of 7), were terminated without further analysis.

To measure transgenic MEn expression, total aortic cellular RNA was subjected to RT-PCR specific for MEn and GAPDH control. MEn transcript was detected in aortae from MEn⁺/SMtTA⁺ mice, immunostaining was performed with a 9E10 epitope-specific antibody. Figure 2A
were then stained with H&E, Movat’s pentachrome, and Mason’s trichrome staining of carotid artery sections, 15 days after wire-injury, in MEn+/SMtTA+ mice with and without DOX. MEn+/SMtTA+ mice, and sham-operated vessels.

Figure 3. MEn expression inhibited neointima formation after carotid artery injury. H&E (Hematoxylin and eosin), Movat’s pentachrome, and Mason’s trichrome staining of carotid artery sections, 15 days after wire-injury, in MEn+/SMtTA+ mice with and without DOX. MEn+/SMtTA+ mice, and sham-operated vessels.

shows representative transgenic MEn protein expression, using either a peroxidase (brown staining) or FITC (green immunofluorescence) conjugated secondary, in aortic sections from a MEn+/SMtTA+ mouse and littermate control. MEn immunoreactivity was localized to arterial SMCs of MEn+/SMtTA+ mice and was absent in DOX-treated or nonbinary littermate control mice (n>3 each). These data demonstrate the DOX-suppressible and vascular SMC-specific localization of β-gal activity and transgenic MEn, in MEn+/SMtTA+ mice.

Characterization of the MEn+/SMtTA+ Phenotype
In crosses between heterozygous MEn+ and SMtTA+ mice, MEn+/SMtTA+ were produced with the expected frequency (25%) and were overtly normal in appearance. Careful histological examination of organs from binary transgenic MEn+/SMtTA+ mice displayed normal vascular structure and arterial SMC morphology within aorta, carotids, lung, liver, spleen, mesentery, and kidney (data not shown). Furthermore, MEn+/SMtTA+ mice did not display a significant difference in tail blood pressure using a piezoelectric device, or carotid blood pressure measured by in vivo Millar cannulation (data not shown). These data suggest that SM22α-directed expression of MEn in adult mice did not overtly alter vascular SMC development, gross structure, or physiology.

Response of MEn+/SMtTA+ Mice to Carotid Arterial Injury
To examine the effects of vascular SMC MEn expression on arterial remodeling in vivo, we performed wire denudation injury of the right common carotid artery from binary MEn+/SMtTA+ transgenic mice and nonbinary littermate controls, with and without DOX. At 15 days after injury, operated (right) and sham (left) carotid arteries were harvested and fixed at physiological pressure. These vessels were then stained with H&E, Movat’s, or Mason’s (Figure 3) for structural and morphological analysis. Injured carotid vessels from littermate control or DOX-treated MEn+/SMtTA+ mice displayed disordered elastic laminae, increased collagen staining (Mason’s: light blue staining), and increased neointimal cell accumulation, indicative of vascular reorganization in response to endothelial damage. Whereas injured carotid arteries from MEn+/SMtTA+ mice also displayed disrupted elastic laminae and an increase in extracellular collagen deposition, suggesting an equivalent vascular insult due to the injury protocol, these mice had reduced accumulation of neointimal cells within the internal elastic laminae.

Dimensions of the arterial tunica intima, media, and adventitia were quantified in sham and injured carotid arteries from MEn+/SMtTA+ mice and nonbinary transgenic littermates, with and without DOX. Although there was no difference in the dimensions of the adventitia between any of the experimental groups (data not shown), the intimal and medial dimensions of MEn+/SMtTA+ mice were significantly reduced as compared with those of DOX-treated MEn+/SMtTA+ or nonbinary transgenic littermates (Figure 4). Indeed, the intimal and medial dimensions of MEn+/SMtTA+ mice in the absence of DOX were not significantly different from those of sham-operated controls (line 220 MEn intima 3.3±1.1 μm; media 32.2±3.5; line 1986 MEn intima 4.5±1.3; media 30.5±4.4; sham intima 1.2±0.5; media 26.1±1.8; P=NS). Together these data suggest that there is reduced vascular wall remodeling after endothelial injury as a result of arterial SMC-specific expression of MEn.

Importantly, at the concentrations we have employed, DOX itself had no significant effect on the intimal and medial dimensions or remodeling of nonbinary transgenic littermates (Figure 4). These data suggest that the effect of DOX on binary transgenic mice was due to a tTA-mediated loss of MEn expression and not other distinct mechanisms of action.
Transgenic β-Gal and MEn Expression in SMCs of Injured Carotid Arteries

Given the potential downregulation of SM22α after vascular SMC injury,33 we examined both β-galactosidase and MEn immunostaining in carotid arteries from MEn+/SMtTA− mice after sham and injury procedures. β-Gal staining was detected in the media of injured vessels from MEn+/SMtTA− mice at days 7 and 15 after injury. Importantly, the number of β-gal–positive nuclei per mm² (MEn+/SMtTA− injury 96±10 versus sham 73±10), the intensity of nuclear β-gal staining (MEn+/SMtTA− injury 64±26 versus sham 100±26), or the percent positive pixels above background in the media (MEn+/SMtTA− injury 8.0±2.8 versus sham 12.7±1.9) did not differ from sham-operated vessels (P=NS for all comparisons) (Figure 5 and data not shown). Men-specific immunostaining was also detected in injured carotids from MEn+/SMtTA− mice at the same time points and was not significantly different from sham arteries (Figure 5 and data not shown). β-Gal or MEn expression was not detected in injured or sham carotid arteries from DOX-treated MEn+/SMtTA− mice or nonbinary littermate controls. These data highlight persistent and detectable levels of vascular SMC expression of both transgenes at days 7 and 15 after arterial injury in MEn+/SMtTA− mice.

Discussion

To explore the effects of inhibiting c-Myb–dependent genes on adult vascular SMC pathophysiology, we created a conditional binary transgenic mouse with SM22α promoter–directed expression of a dominant-negative c-Myb molecule (Myb-Engrailed). Specifically, we examined the efficacy of arterial SMC-specific Myb-Engrailed (MEn) expression in preventing adverse vascular remodeling in an in vivo model of restenosis. Two lines of binary transgenic MEn+/SMtTA− mice were generated with expression of MEn mRNA (RT-PCR) and protein (immunostaining) localized to vascular SMCs. Importantly, in both lines, MEn expression was reduced to undetectable levels with doxycycline treatment (DOX).

Vascular SMC–specific MEn-expressing mice did not exhibit any measurable difference in baseline vascular structure, morphology, or blood pressure. These data would suggest that the transcriptional activation of c-Myb–responsive promoters is not critical in the development and/or normal physiological function of vascular SMCs, or alternatively, that they may be compensated by redundant signaling pathways. Additionally, these results are consistent with data from c-Myb knockout mice, which succumb at embryonic day 13 to 15 due to a deficiency in the onset of adult hepatic hematopoiesis and resulting anemia, but display normal heart...
and vascular development. The current data are also interesting in light of the critical roles for c-Myb in hematopoiesis, colonic epithelial development, leukemia, and colon carcinoma, because they highlight the tissue-specific intricacies of c-Myb activity.

Because MEn is a potent transcriptional repressor and wild-type c-Myb negatively regulates certain genes (e.g., thrombospondin 2, N-ras, MRP14, c-erbB-2, 5-lipoxygenase, colony-stimulating factor-1 receptor), MEn-expressing mice may retain intact signaling at negatively responsive downstream genes relevant to vascular SMCs. Accordingly, we cannot rule out entirely the contributions of c-Myb in the development of vascular SMCs past embryonic day 13 to 15. Additionally, critical roles for c-Myb in the development of vascular SMCs may preclude the temporal window defined by the SM22α promoter and may not become apparent in c-Myb knockout mice due to their premature lethality. Nevertheless, our data suggest that acute arterial SMC-specific therapeutic strategies using SM22α-directed MEn would not have adverse effects on normal vascular structure or function in adults.

The response to vascular endothelial injury was dramatically blunted in MEn-expressing mice, compared with DOX-treated or littermate controls. Whereas MEn-expressing mice showed evidence of vascular injury, such as ruptured elastic laminae and increased extracellular collagen deposition, there was significantly less neointimal cell accumulation compared with injured vessels from control littermate or DOX-treated binary transgenic mice. These data demonstrate that vascular SMC-specific expression of MEn was sufficient to prevent intimal and medial thickening and limit remodeling after arterial injury.

The current study represents the first demonstration of attenuating vascular restenosis in vivo using an engineered dominant-negative c-Myb molecule specifically targeted to a single cell type. Furthermore, unlike the confounding effects of AS-Myb, the consequences of MEn expression on vascular remodeling are specific, as these were abrogated by DOX and have been confirmed in two distinct MEn-expressing lines, negating the possibility of regional transgene effects. In addition, no measurable effect of DOX was observed in control littersmates, consistent with previous reports on the inability of this antibiotic to modulate a rabbit balloon-injury model of restenosis.

Given that c-Myb can be both a positive- and negative-acting transcription factor, the present results with MEn suggest that the role of c-Myb in vascular SMC proliferation involves the activation of c-Myb-responsive genes. However, we have not ruled out the possibility of a more complex disruption of negatively regulated c-Myb responsive genes that may also modulate vascular SMC transformation after injury.

Of note, there was no appreciable downregulation of MEn transgene expression in medial SMCs of injured carotid arteries. Whereas a previous report suggested that the SM22α protein was downregulated in medial vascular SMCs after endothelial injury, endogenous SM22α mRNA levels were increased. These observations are consistent with our data generated using the proximal 2.8-kb fragment of the SM22α promoter and suggest that this region is sufficient for sustained expression in vascular SMCs after initiation of the remodeling process. Additionally, although the induction of SM22α represents an early event that marks the differentiation of a pluripotent adventitial myofibroblast population that may also contribute to intimal-medial remodeling, we noted no induction of transgene-positive cells within the adventitia after vascular injury, suggesting that sequences mediating this effect are not contained within the proximal 2.8-kb region of the SM22α promoter.
of the SM22α promoter. Moreover, these properties of the SMtTA driver line should facilitate the examination of other molecules that have important implications for SMC pathology after arterial injury.

Importantly, transgenic mice with arterial SMC MEn expression had a significant decrease in the number of proliferating cells (BrdU-positive nuclei) within the vascular wall of injured blood vessels, suggesting that fewer SMCs from transgenic mice had entered S-phase of cell cycle. These data support previous in vitro findings, demonstrating a critical role for c-Myb in G0/G1 to S-phase cell cycle progression. Interestingly, because we noted robust medial but not adventitial transgene expression, and a concurrent reduction in proliferation, this would suggest that the source of transformed SMCs in this model is the media and not the adventitia. Additionally, our results demonstrate that inhibition of arterial SMC proliferation is sufficient to attenuate restenosis, and suggests that bone marrow stem cells do not play a major role in arterial proliferation. However, we cannot rule out a process that may be initiated by the differentiation of an adventitial or osteomedullary lineage that is then amplified within the medial SMC layer.

The reduction but not absence of interstitial collagen deposition seen in injured vessels from MEn+SMtTA− mice, and the dramatic reduction in the number of BrdU-positive nuclei in binary but not control transgenic littermates, suggests a reduction in the number of transformed migratory vascular SMCs. However, future studies will be required to determine whether MEn expression has any direct effects on genes involved in matrix homeostasis or cell motility. Furthermore, although the current findings support a role for c-Myb in promoting SMC proliferation after endothelial denudation, it remains to be tested whether this paradigm is a common feature of other vascular diseases (eg, atherosclerosis, pulmonary hypertension) where transformed vascular SMCs are also believed to be critical pathogenic participants.

In conclusion, the present study is the first report of a genetic model with conditional and vascular SMC-targeted c-Myb inhibition. Interestingly, this has no overt effect on vascular SMC development, arterial structure, or blood pressure, but a dramatic effect on neointima formation and arterial cell proliferation after arterial injury. The conditional nature of this system will facilitate future studies aimed at defining other effects associated with c-Myb activity, for example the contribution of arterial SMC proliferation to atherosclerosis in a permissive genetic background, and specific roles in other tissues by crossing MEn mice with other TTA driver lines. Finally, the finding that arterial SMC-specific delivery of a dominant-negative c-Myb prevents adverse vascular remodeling highlights potentially valuable strategies for the development of novel and precise gene therapies for human vascular disease.

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