B-Myb Represses Vascular Smooth Muscle Cell Collagen Gene Expression and Inhibits Neointima Formation After Arterial Injury

Claudia S. Hofmann, Christopher P. Sullivan, Hao-Yuan Jiang, Phillip J. Stone, Paul Toselli, Ernane D. Reis, Igor Chereshnev, Barbara M. Schreiber, Gail E. Sonenshein

Objectives—The function of B-Myb, a negative regulator of vascular smooth muscle cell (SMC) matrix gene transcription, was analyzed in the vasculature.

Methods and Results—Mice were generated in which the human B-myb gene was driven by the basal cytomegalovirus promoter, and 3 founders were identified. Mice appeared to develop normally, and human B-myb was expressed in the aortas. Total B-Myb levels were elevated in aortas of adult transgenic versus wild-type (WT) animals and varied inversely with α1(1) collagen mRNA expression. However, neonatal WT and transgenic aortas displayed comparable levels of α1(1) collagen mRNA, likely resulting from elevated levels of cyclin A, which ablated repression by B-Myb. Aortic SMCs from adult transgenic animals displayed decreased α1(1) collagen mRNA levels. To examine the role of B-Myb after vascular injury, animals were subjected to femoral artery denudation, which induces SMC-rich lesion formation. A dramatic reduction in neointima formation and luminal narrowing was observed in arteries of B-myb transgenic versus WT mice 4 weeks after injury.

Conclusions—Data indicate that B-Myb, which inhibits matrix gene expression in the adult vessel wall, reduces neointima formation after vascular injury. (Arterioscler Thromb Vasc Biol. 2004;24:1-6.)

Key Words: Myb • collagen • cyclin A • aorta • femoral artery

Smooth muscle cells (SMCs), the major cellular constituents of the medial layer of an artery, are responsible for synthesis and deposition of connective tissue proteins (including types I and V collagen, elastin, and proteoglycans) during artery development. After the artery is formed, SMCs differentiate into a contractile phenotype. During atherosclerosis development, a response to vascular injury is elicited. After monocyte invasion, SMCs migrate to the intima and dedifferentiate to a synthetic phenotype, displaying modest rounds of proliferation followed by matrix synthesis. Deposition of matrix proteins, lipids, and minerals results in atherosclerotic plaque formation. Rupture of the fibrous cap of plaque with resultant exposure of thrombogenic subendothelial plaque constituents is the critical event that leads to thromboembolic complications in atherosclerotic coronary and carotid artery disease.

Atherosclerotic lesions are frequently treated by balloon angioplasty and stent placement. However, reoccurrence of arterial narrowing at the site of balloon angioplasty, termed restenosis, occurs in 30% to 50% of patients. Acute disruption of the protective endothelial lining at the site of angioplasty appears to trigger excessive SMC hyperplastic responses, extracellular matrix deposition, and local vessel remodeling.

Cultured vascular SMCs from adult animals exhibit predominantly a synthetic phenotype, expressing genes encoding types I, III, and V/XI collagen at confluence or when deprived of serum growth factors, whereas during exponential growth, only low levels of matrix proteins are produced. Furthermore, basic fibroblast growth factor (bFGF), a potent inducer of SMC proliferation, decreased α1(1) and α2(V) collagen gene expression at the transcriptional level in bovine SMCs. Overall, an inverse relationship exists between the proliferative state of the adult SMC and matrix synthesis. Recently, we implicated B-myb, a member of the myb gene family, in repression of matrix gene expression in vascular SMCs. The B-myb gene was isolated on the basis of its homology with c-myb in its DNA-binding region and encodes a 3.3- to 3.5-kb mRNA and a 704-aa protein. The consensus Myb-binding site (MBS) is (C/T)AACNG. B-Myb also regulates promoters without MBS sequences. B-myb expression is linked tightly with proliferation, with mRNA and protein levels increasing in late G1 and S phase. Although B-Myb promotes G1/S phase transition in some

Received May 11, 2004; revision accepted July 2, 2004.
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Arterioscler Thromb Vasc Biol is available at http://www.atvbaha.org
DOI: 10.1161/01.ATV.0000139010.71779.f3
cells, it does not induce proliferation of bovine SMCs and fails to cooperate with c-Myc to promote entry into S phase, unlike c-Myb and A-Myb. B-Myb functions as either a repressor or an activator of transcription in a cell-type and promoter-specific fashion. We demonstrated that B-Myb is a strong negative regulator of MBS-driven reporter activity and matrix gene promoters in cultured adult vascular SMCs. B-Myb repressed α1(I) and α2(I) collagen promoter activity and decreased bFGF-induced type I collagen gene transcription. Whereas phosphorylation of B-Myb by cyclin A enhanced its ability to transactivate, cyclin A greatly reduced the ability of B-Myb to repress matrix gene expression. B-Myb also inhibited c-Myb-mediated transactivation of the α2(I) collagen promoter in scleroderma fibroblasts, and repressed the α1(I) collagen gene via interaction with Sp1 and CCAAT-binding factor (CBF) factors. Here, the hypothesis that B-Myb regulates SMC matrix gene expression in vivo was tested. A mouse model was generated in which the human B-myb gene was driven by the basal cytomegalovirus (CMV) promoter, which has been shown to express most highly in cells that are infected by the virus, such as the SMC. Aortas of adult transgenic animals displayed decreased type I collagen mRNA expression. Unchallenged mice appeared to develop normally, apparently because of elevated cyclin A expression in developing animals, leading to comparable levels of α1(I) collagen mRNA in neonatal transgenic and wild-type (WT) mice. Isolated SMCs from adult animals displayed reduced α1(I) collagen gene expression. Interestingly, adult transgenic animals subjected to femoral artery injury showed a dramatic reduction in neointima formation and matrix deposition compared with WT mice.

**Materials and Methods**

A detailed Materials and Methods section is available online at http://atvb.ahajournals.org.

**Results**

**Characterization of the CMV–B-myb Mouse**

To generate a mouse model in which B-myb is overexpressed in aortic SMCs, a construct was used containing full-length human B-myb cDNA (pCEP4–B-myb) driven by the basal CMV promoter, which expresses most highly in cells normally infected by the virus, such as vascular SMCs. Data demonstrate that the clone used to generate transgenic mice repressed collagen promoter activity in cultured SMCs and that 3 founder lines (lines 2, 4, and 16) overexpressing B-Myb were generated (Figure 1A and Figure I, available online at http://atvb.ahajournals.org). Western blot analysis revealed that expression of B-Myb was greatest in line 16, followed by line 4 and then line 2 (Figure 1A; and data not shown).

**Type I Collagen mRNA Expression, But Not Protein Deposition, Is Downregulated in Adult Transgenic Mouse Aortas**

To compare α1(I) collagen steady-state mRNA levels in aortas of WT and B-myb mice, RNA was isolated from 7 to 12 pooled aortas of 6- to 10-week-old mice, and Northern blot analysis revealed that expression of B-Myb was greatest in line 16, followed by line 4 and then line 2 (Figure 1A; and data not shown).

Figure 1. The aorta of adult but not neonatal transgenic mice display reduced α1(I) collagen mRNA levels. A, Immunoblot analysis. Whole-cell protein extracts (30–50 μg samples) from individual aortas of 6-week-old WT (n = 3) and transgenic line 16 mice (n = 5) prepared in radioimmunoprecipitation assay buffer were subjected to immunoblot analysis for levels of B-Myb (top) and α-actin as a loading control (bottom). B, Northern blot analysis. Total RNA (12-μg samples), extracted from pooled (7–12) adult WT or transgenic line 2, 4, or 16 mouse aortas, was subjected to Northern blot analysis for α1(I) collagen and GAPDH mRNA levels. Densitometry was performed, and α1(I) collagen values were normalized to GAPDH. Values for the percentage relative to the WT signal are given below each lane. C, Quadruplicate experiments were analyzed as in B; data represent the mean ± SD. Data were compared with WT by one group Student t test; asterisk indicates a statistically significant difference (P < 0.05). D, Graphic representation of the expression of B-Myb protein versus α1(I) collagen mRNA. E, Total RNA was extracted from a pool of aortas (7–12) from neonatal WT or line 16 transgenic mice (6 days old) and samples (12 μg) subjected to Northern blot analysis for levels of α1(I) collagen and GAPDH mRNA.
analysis was performed (Figure 1B). Two bands of α1(I) collagen were detected resulting from alternative polyadenylation, as seen previously. To test for RNA integrity and equal loading, the blot was hybridized to a glyceraldehyde phosphate dehydrogenase (GAPDH) probe (Figure 1B), and some minor variability was seen. Densitometry was performed on α1(I) collagen and GAPDH mRNA levels, and normalized values are reported as a percentage of the WT value (Figure 1B). The α1(I) collagen mRNA expression levels in lines 4 and 16 were 77.0% and 50.4% relative to the WT, respectively, whereas in line 2, levels were essentially identical to those in the WT mice. The average of 4 independent experiments show that lines 2, 4, and 16 displayed 69.0% (±31.2%), 51.4% (±18.1%), and 46.5% (±17.5%) of WT levels, respectively (Figure 1C). These data indicate that lines 4 and 16 display a statistically significant difference in α1(I) collagen mRNA expression versus WT mice. When values for collagen mRNA were plotted against levels of B-Myb protein (Figure 1D), an inverse correlation was observed, consistent with the hypothesis that B-Myb represses expression of the COL1A1 gene within the aorta in vivo.

Deposition of fibrillar collagen in aortas of B-myb versus WT mice was examined. The adventitia was removed from 4 aortas per line, and samples were lyophilized, hydrolyzed, and subjected to amino acid analysis to determine the levels of insoluble collagen. Lines 2, 4, and 16 displayed 117.8% and 69.0% (±31.2%), 51.4% (±18.1%), and 46.5% (±17.5%) of WT levels, respectively. Thus, no significant differences in collagen deposition in aortas of WT and transgenic lines are observed.

Neonatal Transgenic and WT Mice Display Elevated Levels of Cyclin A and Comparable Collagen Gene Expression

Because vessel wall synthesis occurs early in development, collagen expression in neonatal animals was assessed. mRNA was isolated from pooled aortas of neonatal (6 days old) WT and line 16 mice, and Northern blot analysis was performed (Figure 1E). Levels of collagen mRNA normalized to GAPDH were no less in transgenic compared with WT animals (0.68 versus 0.5 for line 16 and WT, respectively), in contrast to the decreased expression in aortas of adult animals (Figure 1B). RT-PCR analysis confirmed overexpression of human B-myb in neonatal transgenic animals (data not shown). Thus, overexpression of B-myb does not reduce α1(I) collagen gene expression in neonatal mice.

We showed recently that the ability of B-Myb to function as a repressor of type V collagen promoter activity is abolished by cyclin A–regulated phosphorylation, so it was of interest to evaluate cyclin A expression in neonatal versus adult mouse aortas. The murine cyclin A subtype corresponding to human cyclin A is known as cyclin A2. To compare cyclin A2 levels in neonates and adults, RNA was isolated and Northern analysis performed using human cyclin A as probe (Figure 2A). Two bands that arise from alternative polyadenylation were detected. Although cyclin A2 expression was substantial in both WT and transgenic neonatal mice, levels were very low in aortas of adult animals. The
ability of cyclin A to alleviate B-Myb–mediated repression of the α1(I) collagen promoter was assessed using transient transfection analysis (Figure 2B). Bovine aortic SMCs were cotransfected with ColCAT3.6, a chloramphenicol acetyltransferase (CAT) reporter construct driven by a 3.5-kb upstream sequence of the human α1(I) promoter and 115 bp of the first exon, in the absence or presence of vectors expressing B-myb and cyclin A. B-Myb repressed α1(I) collagen-promoter activity to 8.3% of the control, and addition of 0.5 and 1 μg cyclin A re-established 79.6% and 105.1% of control activity, respectively. Thus, cyclin A alleviates B-myb–mediated repression of the α1(I) collagen promoter, similar to its effects on the α2(V) promoter.20 Because the bulk of matrix synthesis occurs early during development,25–27 the presence of cyclin A likely explains, at least in part, why no significant differences were observed in collagen deposition in the vessel wall of transgenic animals.

Isolated Vascular SMCs From Adult Animals Display Decreased Collagen Gene Expression

To determine whether collagen expression was downregulated in cultured aortic SMCs from adult transgenic animals, total RNA was isolated from aortic SMCs from 12-week-old WT and line 16 mice in exponential growth. Human B-myb expression was confirmed by RT-PCR analysis using the transgene specific primer pair (Figure 2C). In the presence of RT, a band corresponding to human B-myb was amplified selectively with RNA isolated from line 16, but not from WT mice, as expected. Analysis of β-actin confirmed integrity of the RNA samples. To determine the effects on collagen mRNA levels, RNA isolated from subconfluent cultures was subjected to Northern blot analysis for B-myb, α1(I) collagen, and GAPDH (Figure 2C). B-myb mRNA levels were higher (4.3-fold after normalization to GAPDH) in the vascular SMCs from the transgenic mice, whereas α1(I) collagen gene expression showed a decrease to 65.6% of WT levels. A comparable decrease was seen in synthesis of type I collagen during an 8-hour labeling period with 14C-proline (data not shown). Thus, aortic SMCs from transgenic animals display reduced type I collagen gene expression.

B-Myb Protects Against Neointima Formation After Femoral Artery Injury

Although B-myb overexpression did not appear to affect normal vascular development, it was of interest to evaluate whether it might alter vascular remodeling or neointima formation after injury to the developed vasculature. A model of guidewire-induced denudation of the femoral artery was used. Northern blot analysis confirmed the decrease in α1(I) collagen mRNA levels in femoral artery RNA isolated from pools of 10 to 12 line 16 versus WT adult animals (46.9% normalized to GAPDH; data not shown). The response to injury was compared in femoral arteries of WT versus B-myb transgenic (line 16) mice (n=9). Four weeks after bilateral injury, injured and sham-operated arteries were harvested, fixed, and 5-μm sections were made throughout the entire guidewire-injured region of each artery. Every twentieth section was stained with Masson’s trichrome, and the images were quantified using Image-Pro Plus software. As shown previously,29 the typical lesion of a WT mouse displayed a large neointima rich in matrix (blue-green color) and SMCs (red color); however, these large lesions were not apparent in B-myb mice (Figure 3). The lesions with the largest neointima/media in each group are depicted in Figure 3. As expected, no lesion formation was evident in sham-injured arteries.

Image analysis of the lesions demonstrated that compared with WT mice, B-myb mice displayed a significant decrease in the area of the neointima (Table). Similarly, the ratio of the areas of the neointima and the medial layers and the percentage of luminal narrowing ([neointimal area/neointimal

<table>
<thead>
<tr>
<th>Measurement</th>
<th>WT</th>
<th>B-myb</th>
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<tbody>
<tr>
<td>Vessel area (μm²)</td>
<td>69 955±3934</td>
<td>61 213±6291</td>
</tr>
<tr>
<td>Lumenal area (μm²)</td>
<td>39 774±4423</td>
<td>48 029±5302</td>
</tr>
<tr>
<td>Medial area (μm²)</td>
<td>12 791±1189</td>
<td>10 979±551</td>
</tr>
<tr>
<td>Neointimal area (μm²)</td>
<td>17 389±4301</td>
<td>2204±1192t</td>
</tr>
<tr>
<td>Neointimal area/media</td>
<td>1.3±0.3</td>
<td>0.2±0.1t</td>
</tr>
<tr>
<td>Vessel perimeter (μm)</td>
<td>957±27</td>
<td>955±36</td>
</tr>
<tr>
<td>Lumenal perimeter (μm)</td>
<td>743±38</td>
<td>857±32t</td>
</tr>
<tr>
<td>Lumenal narrowing (%)</td>
<td>30.4±6.5</td>
<td>3.4±1.7t</td>
</tr>
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*Data are expressed as mean±SEM (n=9).
1WT and B-myb transgenic mice were significantly different (P<0.05) by 2-tailed unpaired Student t test.
area + lumenal area) \times 100]$ were significantly lowered in the B-\textit{myb} mice (Table; Figure 4). Some of the lesions were extremely small, and if one took a value of $>0.5$ to indicate a substantial neointima/media ratio, then 8 of the 9 injured arteries from the WT mice developed significant lesions, whereas only 2 of the 9 B-\textit{myb} mice did so. The data also show that there was no significant difference between the areas of the entire vessel, lumen, or media layers of the femoral arteries in WT versus B-\textit{myb} transgenic mice (Table). Likewise, the outer perimeters of the vessels (ie, the length of the external elastic laminae) were not significantly different in injured arteries of the 2 mouse lines. Finally, the lesions were rich in SMCs and negative for monocyte/macrophage invasion as judged by immunohistochemistry for \textit{\alpha}-actin, and MOMA-2, respectively (Figure II, available online at http://atvb.ahajournals.org). Thus, circulating monocytes are not likely to be responsible for the observed inhibition of lesion formation in B-\textit{myb} transgenic mice.

**Discussion**

Using a transgenic mouse model in which the human B-\textit{myb} cDNA was driven by the basal CMV promoter, this study demonstrates for the first time that B-\textit{Myb} leads to markedly reduced neointima formation after mechanical injury to the vasculature and to decreased \textit{\alpha}1(I) collagen mRNA expression in the aorta and femoral artery of adult animals. Three independent transgenic mouse lines were generated, all of which apparently developed and bred normally. An inverse relationship between levels of B-\textit{Myb} protein and expression of \textit{\alpha}1(I) collagen mRNA in the adult aorta was demonstrated. A decrease was also seen in \textit{\alpha}2(V) collagen mRNA levels in the aorta and in cultured aortic SMCs isolated from adult transgenic B-\textit{myb} mice (data not shown). Importantly, when a femoral artery model of endothelial denudation was used, a dramatic reduction in neointima formation was observed in arteries of transgenic versus WT mice 4 weeks after injury. After injury, the neointimal area and the ratio of the areas of the neointima and media were significantly reduced in transgenic animals to 12.7% and 15.4%, respectively, of the levels observed in WT injured mice. As expected, the lesions were rich in SMCs, whereas invading monocytes/macrophages were not detected. Furthermore, Masson’s trichrome staining was consistent with the lack of lesion formation and deposition of collagen and other matrix proteins. Thus, B-\textit{myb} overexpression inhibits neointima formation after vascular injury.

Dysfunction of adult vessel endothelium causes a phenotypic switch in the normally quiescent, contractile SMCs. Mitogenic signals can promote this switch to the synthetic phenotype and migration to the intima, where the cells reintegrate production of matrix proteins. Microarray analysis of neointimal SMCs in primate bypass graft neointima showed that 6 of the 13 genes expressed more highly in the neointima than in the aorta encoded collagens, suggesting the importance of transcriptional regulation of collagen genes in formation of a collagenous lesion within the neointima.\textsuperscript{31} Interestingly, there is evidence that type I collagen promotes SMC migration in vitro.\textsuperscript{32,33} De novo synthesis of collagen has been shown to occur as an early response to injury,\textsuperscript{3} and may be required for normal migration of SMCs during arterial remodeling. After injury in transgenic mice, inhibition of type I collagen gene expression could affect 2 critical steps in the response to injury: migration and collagen deposition. Our studies do not exclude the possibility that B-\textit{Myb}–mediated effects on other genes may also play a role in the observed inhibition of neointima formation (including matrix synthesis or degrading enzymes that might alter the matrix metalloproteinase/tissue inhibitor of metalloproteinase balance). A phenotype similar to the B-\textit{myb} mouse was seen after overexpression of MMP 1, which has the capacity to degrade newly synthesized collagen.\textsuperscript{34} Thus, injury of the vessel wall in a situation in which there is reduced collagen synthesis or increased collagen degradation protects against lesion formation.

No differences in the vessel structure were observed in the unchallenged animal, and no change in collagen protein levels in the adult vessel wall was noted. Cyclin A levels were higher in aortas of the neonatal versus adult mice, and cyclin A reversed the B-\textit{Myb}–induced downregulation of the \textit{\alpha}1(I) collagen promoter (as shown previously for \textit{\alpha}2(V) collagen).\textsuperscript{30} This suggests that elevated cyclin A expression during development prevents B-\textit{Myb}–mediated repression of collagen gene expression. In addition, other post-translational modifications, coactivators, and corepressors that affect B-\textit{Myb} may also be subject to developmental regulation.\textsuperscript{14,35-37} Recent work by Baskar et al using the \textit{lacZ} reporter gene showed that CMV-driven expression is not as ubiquitous as originally thought. In developing embryos\textsuperscript{38} and in adult animals,\textsuperscript{23} high levels of CMV-driven \textit{lacZ} expression were noted specifically in tissues that are naturally targeted and infected by CMV in humans (eg, vascular SMCs). In contrast, \textit{lacZ} expression was not detectable in adult endothelial cells or in blood-borne cells (including peritoneal macrophages). Our data, which show that expression of B-\textit{myb} is evident in adult aortas and isolated vascular SMCs of the transgenic mice, are in agreement with these observations and suggest that B-\textit{Myb} overexpression in SMCs contributed to inhibition of lesion formation in transgenic mice. The guidewire-induced femoral artery injury in the FVB mouse produces an SMC-rich lesion without extensive monocyte/macrophage infiltration.\textsuperscript{29} Because the injury is performed on unaffected vessels, it does not recapitulate

\textbf{Figure 4.} The formation of a neointima and lumenal narrowing are reduced in B-\textit{myb} transgenic mice. Averages of the ratio of the areas of neointima and media (\textbullet, left axis) and percentage of lumenal narrowing (\textbullet, right axis; \textit{n}=9) are plotted.
exactly human restenosis, which occurs after balloon angioplasty or stenting of an atherosclerotic lesion; however, it may provide information on the SMC response to vascular injury. Overall, our findings suggest that vascular SMC expression of B-Myb protects against the formation of a neointima in response to vascular injury.

Acknowledgments

This work was supported by grants from the National Institutes of Health (PO1 HL13262; B.M.S., G.E.S.) and the American Heart Association (0256215T; B.M.S.). We thank K. Ravid for generously providing cloned DNA, and V. Verbitski for excellent technical assistance. We thank R. Romieu-Mouriez for comments on this study and B. Smith for help with Image Pro analysis.

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Arterioscler Thromb Vasc Biol. published online July 15, 2004;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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SUPPLEMENTAL MATERIALS

Materials and Methods

CMV-B-myb Construct and Transgenic Mice

The 2,300 bp human B-myb cDNA was cloned into the Bam HI site of pCEP4 (Invitrogen) (pCEP4-B-myb). At the 3’-end of the B-myb sequence, a 500 bp Sma I fragment containing the SV40 T antigen intron and polyA sequence (from pSPE/SV40, kindly provided by K. Ravid, Boston University School of Medicine, Boston, MA) was inserted by blunt end ligation into an Hpa I site of the pCEP4 vector to enhance the efficiency of transgene expression (pCEP4-B-myb-IpA). The whole sequence including the CMV promoter was then released using Sal I on the 5’-end of the construct and Eco RV on the 3’-end of the construct (Fig. IA).

Following gel purification, the resulting 3800 bp fragment was used for the production of transgenic mice in the FVB strain of mice, as described previously, by the Transgenic Core Facility at Boston University School of Medicine. Potential founder mice were identified by Southern blot analysis for transgene integration (see below). Mice were housed in a 2-way
barrier at the Boston University School of Medicine Transgenic Core Facility in accordance with the regulations of the American Association for the Accreditation of Laboratory Animal Care.

**Southern and Northern Blot Analyses**

Genomic DNA was isolated from the tail of potential transgenic animals, samples (10 µg) digested with Bam HI, and subjected to Southern blot analysis using the full-length 2,300 bp B-myb cDNA fragment released with Bam HI, as a probe, as described previously.\(^1\) Total RNA was isolated from cultured cells by guanidinium isothiocyanate extraction followed by purification on CsCl density gradients, as described.\(^2\) The region from the aortic arch to the diaphragm was dissected out. For extraction of RNA, samples (100-200 mg) of aortic tissue were frozen in liquid nitrogen and then pulverized with a mortar and pestle. RNA was prepared using the Ultraspec-II RNA Isolation System (Biotecx Laboratories), and samples (12 µg) subjected to Northern blot analysis.\(^3\) RNA was stained with ethidium bromide to assess integrity and equal loading. Probes include human B-myb, \(\alpha_1(I)\) collagen cDNA,\(^4\) and glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA. Scanning densitometry was performed using KDS1D version 2.0 (Kodak). Data are expressed as arbitrary units of expression of collagen/GAPDH. For each Northern blot, expression of collagen/GAPDH in WT was set to 100% and others were expressed as a percentage of the WT. Data were compared to WT by one group Student's \(t\) test (\(p<0.05\)). Data from male and female didn’t differ and hence these values were analyzed together.

For Reverse Transcriptase (RT)-PCR, RNA was digested for 30 min at 37°C with RQ1 RNase-free DNase (Promega Corporation), and samples (5 µg) incubated in the presence or absence of SUPERSCRIP™ RNase H RT and 200 ng of random primers. PCR reactions were
performed using $1/10^{\text{th}}$ volume of the total RT reaction (2 µl) and *Taq* DNA polymerase, according to the manufacturer’s instructions (Invitrogen Life Technologies), except that 0.85 µCi of $\alpha$-$^{32}$P-dCTP and $\alpha$-$^{32}$P-dGTP were added. Annealing of the reaction was performed at 55°C. A primer pair from the 3’ end of the human *B-myb* cDNA at 2076 bp and at 368 bp within pCEP4 vector sequence adjacent to the 3’ end of the *B-myb* cDNA (5’-GAAGCCACTTCACGACACCT-3’ and 5’-TGGTTTGTCCAAACTCATCAA-3’) was employed, yielding a fragment of 350 bp. As control for RNA quality, the following β-actin primers were employed: 5’-ACCAGTTCCATGGATGACGATA-3’ and 5’-AGCTCATACTCTTCTCCAGGGAG-3’.

**Immunoblot Analysis**

For preparation of whole cell protein extracts, aortas were frozen in liquid nitrogen and homogenized using a Polytron homogenizer in RIPA buffer (10 mM Tris HCl pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 1% sodium sarcosyl, 0.2 mM phenylmethylsulfonylfluoride, 10 µg/ml leupeptin, 1mM dithiothreitol, 3 µg/ml aprotinin). Following incubation on ice for 30 min, the DNA was sheared by sonication for 5-10 seconds, and the debris was removed by centrifugation. Protein concentrations were determined using the Bio-Rad DC protein assay (Bio-Rad Laboratories). Proteins were resolved on 10% polyacrylamide-SDS gels and subjected to immunoblotting, as we have described previously. The antibodies against human B-Myb (sc-724) and α-actin were purchased from Santa Cruz Biotechnology and Sigma, respectively.

**Transfection Conditions**

Subconfluent bovine SMCs (plated at 15x10^4 cells/35 mm dish) were transfected in triplicate using Lipofectamine (2.5 µg DNA total in 5 µl Lipofectamine Reagent), according to manufacturer’s instructions (Invitrogen Life Technologies). Cells were harvested, and subjected
to reporter assays as described.\textsuperscript{2} Collagen constructs employed were 804hCol1-luc, which consisted of a 804 bp proximal region of the human $\alpha_1$(I) collagen promoter linked to a luciferase reporter\textsuperscript{6} or ColCAT3.6, a CAT reporter construct driven by a 3.5 kb upstream sequence of the human $\alpha_1$(I) promoter and 115 bp of the first exon.\textsuperscript{2}

**Culture of SMCs**

Bovine aortic SMC explants were derived from the aortic arches of female calves and cultured as we described previously.\textsuperscript{7} For the generation of murine SMCs, the aortas of 10 three-month old female CMV-B-\textit{myb} transgenic or WT FVB mice (Taconic Farms) were removed from the aortic arch to the femoral bifurcation, stripped of adventitia, minced, and subjected to digestion with 0.5 $\mu$g/ml bacterial collagenase (Sigma type I) and 0.125 $\mu$g/ml porcine pancreatic elastase (Sigma type III) in Dulbecco’s modified Eagle Medium (DMEM) at 37\textdegree C. Resulting SMCs were collected by centrifugation at 400 g for 10 min at room temperature, and grown in DMEM supplemented with 10\% fetal bovine serum (FBS), nonessential amino acids, 1 mmol/L sodium pyruvate, 100 U/ml penicillin, 100 $\mu$g/ml streptomycin (Invitrogen Life Technologies). Second passage cultures, were subjected to immunohistochemistry with an antibody against SMC $\alpha$-actin, which demonstrated that contamination with endothelial cells and fibroblasts was negligible. Cells were used between passage 2 and 6.

**Measurement of Collagen in Aortas**

Collagen content of aortas (from the arch to the diaphragm) was determined by amino acid analysis (Beckman Model 6300 with System Gold software) of the hydroxyproline content of the supernatant material that resulted from treatment of the tissue with 0.1 N NaOH at 95\textdegree C.\textsuperscript{8} Calculations of collagen mass used an average amino acid residue weight of 92 MW and were
based on the estimation that there are 100 residues of hydroxyproline/1000 amino acid residues in collagen.\textsuperscript{8}

**Femoral Artery Injury**

Endothelial denudation and distal ligation of the femoral artery of 10-week old male WT and B-myb transgenic Line 16 mice (n=9) was carried out as described.\textsuperscript{9,10} After clamping the femoral artery proximally, an arteriotomy was made distal to the take-off of the epigastric artery, and a 0.25 mm angioplasty guidewire introduced. The clamp was removed, and the guidewire advanced 10 times to the aortic bifurcation. The wire was removed, the arteriotomy site ligated, and the incision closed. Five WT and 3 B-myb transgenic mice were subjected to sham injury, which included all of the procedures described except that no guidewire was introduced. Four weeks after bilateral femoral artery injury, mice were anaesthetized, and perfused with 4% formaldehyde. Hind limbs were excised, further fixed by immersion, decalcified as described,\textsuperscript{9,10} paraffin-embedded, and sectioned (5 µM). The entire injured region of the femoral artery (or corresponding region on sham-injured mice) was sectioned. Sequential sections at 100 µM intervals (every 20\textsuperscript{th} section) were subjected to analysis by Masson's trichrome staining [using a modification of the Accustain Trichrome Stains (Masson) kit from Sigma Diagnostics, as described previously\textsuperscript{11}]. Images of stained sections were digitized and analyzed using Image-Pro Plus software (Media Cybernetics) for computerized morphometry. The measurements include the area of the entire vessel (the region within the external elastic lamina), perimeter of the external elastic lamina, area of the lumen, perimeter of the lumen, area of the medial layer, area of the neointima, ratio of the areas of the neointima and medial layers, and percentage of luminal narrowing [(neointimal area/neointimal area + luminal area) x 100]. For statistical analyses of arteries with lesions, the region with the largest neointima/media ratios, indicative of the area
with the largest involvement, was chosen. For analysis of arteries with no lesions, the region with
the largest vessel area was selected. Data from injured arteries are expressed as mean ± SEM
using the values for the multiple injuries/group. Data on morphometric measurements from WT
and B-myb-expressing mice were compared using a 2-tailed unpaired Student's t test. Values of
p<0.05 were considered significantly different.

**Immunohistochemistry**

Reagents were purchased from Vector Laboratories (Burlingame, CA), and used according to the
manufacturer’s instructions. Sections were rehydrated as we have described previously. Following
treatment with Antigen Unmasking Solution, endogenous peroxidase activity was
blocked with 3% hydrogen peroxide in Cadenza buffer (Thermo Shandon). Washed slides were
then blocked using Avidin Biotin Blocking Kit. For α-actin staining, sections were blocked,
incubated with mouse anti-α-actin (1:1000) and prepared for detection using the M.O.M.
immunodetection kit. For MOMA-2 staining, sections were blocked in 10% normal goat serum,
and then incubated overnight at 4°C in 1:150 dilution of primary rat anti-mouse MOMA-2
antibody (Serotec, Raleigh, NC), and then in biotinylated goat anti-rat (1:100) for 1 hour at 37°C.
For detection, ABC Elite kit and DAB substrate were employed, and sections were
counterstained with hematoxylin (Thermo Shandon).

**Results**

**Characterization of CMV-B-myb Mice**

To generate a mouse model in which B-myb is overexpressed in aortic SMCs, we employed a
construct containing the full length human B-myb cDNA (pCEP4-B-myb) driven by the basal
CMV promoter, which has been shown to promote expression particularly in cells normally
infected by the virus such as the vascular SMC.\textsuperscript{23} This construct was modified by insertion of an SV40 T antigen intron-polyA cassette to ensure more efficient expression \textit{in vivo} (Fig. IA). We first confirmed that the new clone (pCEP4-B-\textit{myb}-IpA) was able to repress collagen promoter activity in transient transfection analysis (Fig. IB). Bovine aortic SMCs were co-transfected with a luciferase construct driven by a 804 bp fragment of the human \(\alpha\textsubscript{1}(I)\) promoter and either 0, 0.8 or 1.7 \(\mu\text{g}\) pCEP4-B-\textit{myb}-IpA. The pCEP4-B-\textit{myb}-IpA clone repressed \(\alpha\textsubscript{1}(I)\) collagen promoter activity in a dose-dependent fashion to 41.3% and 17.3% of the control 72 h after transfection, indicating that the construct is functional in vascular SMCs. The DNA insert was then excised and utilized to generate transgenic mice in the FVB mouse strain. Three founder lines termed Lines 2, 4 and 16 successfully passed the transgene through the germline and their progeny were used for this study. Lines 2 and 16 had approximately 8-12 copies and Line 4 had approximately 4-6 copies of the transgene, as estimated by Southern blot analysis (data not shown). CMV-B-\textit{myb} transgenic mice of all founders developed and bred apparently normally.

To test for transgene expression, RNA was isolated from the mouse aortas, and subjected to radiolabeled RT-PCR analysis, in the presence or absence of RT, as a control for DNA contamination, using a transgene-specific primer pair that yields a 350 bp fragment (Fig. IA). Detection of a radiolabeled band at 350 bp confirmed the presence of the human B-\textit{myb} transgene RNA in Line 16 (Fig. IC), and in Lines 2 and 4 (data not shown). Minimal DNA contamination was evident, as judged by the reactions performed in the absence of RT (data not shown). Thus, human B-\textit{myb} transgene mRNA is expressed in the aortas.

\textbf{Lesions are Rich in SMCs and Show Minimal Monocytes/Macrophage Invasion}

To further characterize the lesions following femoral artery injury, immunohistochemistry was performed using antibodies selective for SMCs (\(\alpha\)-actin) or
monocytes/macrophages (MOMA-2) (Fig. II). The entire neointima in both the WT and transgenic animals stained α-actin positive, indicating the lesions were rich in SMCs. In contrast, no MOMA-2 positive staining was detected, indicating that substantial monocyte/macrophage invasion had not occurred in the B-myb or in WT animals.

References


Fig. 1. CMV-driven B-myb expression in transgenic mice.
(A) Diagram of the human B-myb cDNA construct employed for the generation of the transgenic mice. The positions of the primer set used in RT-PCR analysis (see Materials and Methods) are indicated by arrows. (B) SMC cultures, plated in triplicate at a density of 6 x 10^4 cells/35mm dish, were co-transfected with 0.8 µg 804hCol1-luc, and either 0, 0.8 or 1.7 µg pCEP4B-myb-IpA and pBluescript (to make up a total of 2.5 µg DNA) using Lipofectamine Reagent. After 72 h, luciferase activity, normalized to total protein, was determined. (C) Samples of aortic RNA (5 µg) were DNase treated and subjected to radiolabeled RT-PCR using transgene specific primers, and 0.85 µCi α-^32^P-dCTP and α-^32^P-dGTP in the absence (-) or presence (+) of RT. hB-myb, human B-myb.
Fig. II. WT lesions are rich in SMCs and monocyte/macrophage invasion is not evident. Immunohistochemistry was performed on sections of injured arteries from WT or B-myb transgenic Line 16 mice using antisera specific for SMC α-actin (left panels) or MOMA-2 (right panels).