B-Myb Represses Elastin Gene Expression in Aortic Smooth Muscle Cells*

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B-Myb represses collagen gene transcription in vascular smooth muscle cells (SMCs) in vitro and in vivo. Here we sought to determine whether elastin is similarly repressed by B-Myb. Levels of tropoelastin mRNA and protein were lower in aortas and isolated SMCs of adult transgenic mice expressing the human B-myb gene, driven by the basal cytomegalovirus promoter, compared with age-matched wild type (WT) animals. However, the vessel wall architecture and levels of insoluble elastin revealed no differences. Since elastin deposition occurs early in development, microarray analysis was performed using nontransgenic mice. Aortic levels of tropoelastin mRNA were low during embryonal growth and increased substantially in neonates, whereas B-myb levels varied inversely. Tropoelastin mRNA expression in aortas of 6-day-old neonatal transgenic and WT animals was comparable. Recently, we demonstrated that cyclin A-Cdk2 prevents B-Myb-mediated repression of collagen promoter activity. Cyclin A2 levels were higher in neonatal versus adult WT or transgenic mouse aortas. Ectopic cyclin A expression reversed the ability of B-Myb to repress elastin gene promoter activity in adult SMCs. These results demonstrate for the first time that B-Myb represses SMC elastin gene expression and that cyclin A plays a role in the developmental regulation of elastin gene expression in the aorta. Furthermore, the findings provide additional insight into the mechanism of B-myb-mediated resistance to femoral artery injury.

The B-myb gene was isolated based on its homology with c-myb in its DNA binding region (~90% homology) (1). The mRNA, which is ~3.3–3.5 kb, codes for a B-Myb protein of ~700 amino acids that migrates at ~93 kDa (2, 3). The consensus Myb binding site (MBS)1 is (C/T)AACNG (4). B-Myb forms complexes of comparatively lower stability showing less tolerance for binding site variations compared with c-Myb or A-Myb (5). B-Myb was also found to be able to regulate several reporter constructs without MBS sequences, including the DNA polymerase α promoter, the fibroblast growth factor-4 promoter, and its own promoter (6–9). B-Myb expression is low in early G1 and is induced in late G1 and S phases in many cell types, including vascular smooth muscle cells (SMCs) (3, 10, 11). Work by several investigators has shown that B-Myb functions as either a repressor or activator of gene transcription in a cell type- and promoter-specific fashion (3, 6, 7, 12–16). When B-Myb functions as an activator, it has been found to regulate genes that promote entry into S phase (17, 18). Furthermore, its activity is greatly enhanced by phosphorylation by cyclin A-Cdk2 (19, 20). We showed that B-Myb displays a strong negative regulatory activity on MBS element-driven reporter activity and on type I and type V collagen gene promoters in vascular SMCs in culture (3, 13). Furthermore, B-Myb mediates the decrease in type I collagen gene transcription induced by basic fibroblast growth factor (21). Interestingly, B-Myb does not induce proliferation of bovine SMCs (22), and cyclin A dramatically reduces its ability to repress collagen gene expression (23, 24). Consistent with our observations, B-Myb inhibits c-Myb-mediated transactivation of the α2(I) collagen promoter in scleroderma fibroblasts (25) and represses the α1(I) collagen gene transcription via interaction with Sp1 and CBF factors (26).

SMCs are the major cellular constituents of the medial layer of an artery and are responsible for the synthesis and deposition of connective tissue proteins, including elastin, that maintain vascular tone in the adult blood vessel (27–29). Elastin is one of the major structural proteins of large arteries, contributing the physical properties of extensibility and elastic recoil. Elastin is synthesized as a soluble monomeric precursor called tropoelastin, which has an apparent molecular mass of 62–75 kDa, depending on animal species and isoform (30, 31). Tropoelastin is subsequently secreted from the SMC and assembled into a highly stable, insoluble, polymeric structure in the extracellular matrix through covalent cross-links derived from lysine residues (32, 33). Elastic fibers are arranged into concentric rings of elastic lamellae and SMCs. Elastic lamellae provide the resilience that enables arteries to absorb hemodynamic stress of cardiac systole and to release this energy in the form of sustained blood pressure during diastole. The number of lamellar units appears to be species-specific, fixed, and genetically predetermined (34).

During the development of the aorta, synthesis of elastin occurs very early, and its expression is regulated, in part, at the transcriptional level (35). Once the artery has been fully formed, SMCs differentiate into a contractile phenotype in which they normally remain (36). In culture, vascular SMCs exhibit a synthetic phenotype, and an inverse relationship exists between cell proliferative state and matrix deposition (36). In exponential growth, cultured SMCs synthesize low levels of matrix proteins, and expression of matrix genes is induced

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‡ The abbreviations used are: MBS, Myb binding site; SMC, smooth muscle cell; CMV, cytomegalovirus; DMEM, Dulbecco’s modified Eagle’s medium; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT, reverse transcription; WT, wild type; CAT, chloramphenicol acetyltransferase; CREB, cAMP-response element-binding protein.

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when SMCs reach confluence or are deprived of serum growth factors (37–39). Specifically, it has been shown that the expression of elastin varies inversely with the growth rate of SMCs (40–42). Confluent primary chick vascular SMCs displayed a dramatic increase in elastin mRNA levels upon serum deprivation, and when serum was added back to the cultures, cell proliferation was induced, and elastin mRNA levels dropped (43). Similarly, it was shown that types I, III, and V/IX collagen mRNA levels were relatively low when SMCs were subconfluent and growing exponentially, and levels increased as cells become confluent (37, 43–45) or upon serum starvation, which renders SMCs quiescent (39, 46). Recently, to explore the role of B-Myb on collagen gene expression in vascular SMCs in vivo, we studied the effects of overexpression of the human B-myb gene in a transgenic mouse model. We observed a reduction in type I and type V collagen expression in adult animals and a reduction in neointima formation upon vascular injury (23). Here, these transgenic mice are characterized with respect to elastin gene expression. A substantial reduction in tropoelastin mRNA expression is observed in aortas as well as isolated aortic SMCs from adult B-myb transgenic versus age-matched wild type (WT) animals, although measurements of insoluble elastin and numbers of elastic layers in the vessel wall revealed no differences between WT and transgenic animals. Importantly, elastin mRNA levels are not reduced in neonatal transgenic mice, apparently due to high cyclin A expression, which ablates B-Myb-mediated repression of the elastin promoter. Thus, these findings help to elucidate the mechanism of B-Myb-mediated resistance to vascular injury that we reported recently (23) and implicate cyclin A and B-Myb in developmental regulation of expression of elastin, a key structural component of the vessel wall.

**EXPERIMENTAL PROCEDURES**

Isolation and Culture of SMCs—FVB mice overexpressing human B-myb (2300-bp human B-myb cDNA driven by the basal cytomegalovirus (CMV) promoter) in the aorta were generated as described previously (23). Murine SMCs were prepared as described previously (23). Briefly, the aortas of 3-month-old female CMV-B-myb transgenic or WT FVB mice (Taxonix Farms) were removed from the aortic arch to the diaphragm, and samples (100–200 mg) were frozen and homogenized in liquid nitrogen and then homogenized in radioimmunoprecipitation buffer (10 mmTris-HCl, pH 7.5, 150 mmNaCl, 1% Nonidet P-40, 0.1% SDS, 1% sodium sarcosyl, 0.2 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 1 μM dithiothreitol, 3 μg/ml aprotinin) using a Polytron homogenizer (Kinematica GmbH). Following incubation on ice, the homogenate was sheared either by sonication for 5–10 s or by passing the lysate 20 times through a 23-gauge and then a 25-gauge needle. The debris was removed by centrifugation at 13,000 rpm in an Eppendorf centrifuge for 30 min at 4 °C. Protein concentration was determined using the Bio-Rad DC protein assay (Bio-Rad). Proteins were resolved in 10% polyacrylamide–SDS gels and subjected to immunoblotting, as described previously (50). A goat antibody against rat tropoelastin (RA75) was purchased from Elastin Products. The antibodies against human B-myb (sc-724) and β-actin were purchased from Santa Cruz Biotechnology, Inc. and Sigma, respectively. The rabbit polyclonal cyclin A antibody (A5) was purchased from Lab Vision.

RNA Isolation and Gene Microarray Analysis—Aortas were removed from C57BL6/6 embryos at embryonic day 12, 14, 16, and 18, postnatal days 0 (representing the first 24 h of life), 7, 10, 14, 21, 30, and 60, and 5.5 months of age. Total RNA was extracted from each sample of pooled aortas (10–14 aortas per time point) using a modified guanidinium/phenol extraction method. RNA quality and purity was evaluated with the use of the RNA 600 Nano Labchip (Agilent, Palo Alto, CA) and Agilent 2100 bioanalyzer per the manufacturer’s protocol (Agilent). cRNA target and gene chip hybridization were performed by the Multiplexed Gene Analysis Core Facility of the Siteman Cancer Institute at the Washington University in St. Louis School of Medicine. A duplicate of the P7 time point was taken and analyzed, which indicated that the variability was minimal. The MU74Av2 chips were scanned, and the intensities of the images were analyzed by the GeneChip Analysis Suite software (Affymetrix). All chip intensities were scaled by the software to an average of 1500 units. Statistical analysis was performed using a t-test to test if the difference between their maximum and minimum raw average difference values over the time series was less than 300. Details of the array procedures, sample selection and preparation, and data analysis can be found as described.2

Transient Transfection Assays—Bovine aortic SMCs were plated at 60 × 10^4 cells/well in 6-well dishes and transfected the following day using Lipofectamine (2.4–3.2 μg of DNA total in 5 μl of Lipofectamine reagent) according to the manufacturer’s instructions (Invitrogen). The following vectors were employed: elastin 2.2-CAT and elastin 0.5-CAT, which contain 2.2 and 0.5 kb of the proximal region of the human elastin promoter linked to a chloramphenicol acetyltransferase (CAT) reporter (kindly provided by J. Rosenbloom, University of Pennsylvania). A plasmid DNA containing the B-myb expression vector (3); and pCMVCycA, a human cyclin A expression vector, generously provided by Jim Xiao (Boston University School of Medicine, Boston, MA). Cells were harvested 72 h after transfection, and CAT activity was analyzed as described (21).

Verhoef and Van Gieson Staining—Age-matched 5-week-old to 2-month-old B-myb transgenic and WT mice were perfused with 5 ml of 1% paraformaldehyde followed by 10 ml of 4% paraformaldehyde, as described previously (23). The aortas were dissected out, fixed overnight in 4% paraformaldehyde, dehydrated, and embedded in paraffin. Five-μm cross-sections were prepared and subjected to Verhoef

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and Van Gieson staining with the Accustain Elastic Stain kit (catalog no. HT25-A, Sigma) according to the manufacturer's instructions. Layers of elastic lamellae were analyzed by counting the stained sections of the aortas.

**Measurement of Total Insoluble Aortic Elastin Protein**—To measure the levels of insoluble elastin, aortas from age-matched adult (3-month-old) B-myb transgenic and WT mice were dissected out, weighed, homogenized, and incubated in 0.1 N NaOH at 95 °C for 45 min to isolate insoluble elastin. The insoluble residue was subjected to acid hydrolysis and amino acid analysis (Beckman model 6300 with System Gold software) to determine the quantity of the elastin (32–55). The amount of elastin was calculated as the sum of the amino acids (in nmol) multiplied by the average amino acid mass of 85 ng/nmol. Nonelastin protein in the aorta was measured by combining the supernatant from the 0.1 N NaOH incubation with an equal volume of 12 N HCl followed by hydrolysis and amino acid analysis, as described above. Protein was estimated by multiplying the sum of the amino acids (in nmol) by 100 ng/nmol. The mean value for the elastin content of the aortas normalized by total protein of the WT group of mice (n = 6) was compared with those for Line 2, 4, and 16 transgenic mice using the Bonferroni/Dunn procedure for the analysis of variance.

**RESULTS**

**Elastin mRNA Levels Are Reduced in the Aorta of Adult Transgenic B-myb Mice**—Recently, we demonstrated that B-Myb represses type I collagen gene expression in the aorta and isolated aortic SMCs from adult transgenic mice expressing human B-myb (23). Thus, it was of interest to evaluate whether B-Myb affects the expression of elastin, another matrix gene important for the architecture of the vessel wall. In the previous report, the characterization of three B-myb transgenic mouse lines (Lines 2, 4, and 16) was described. It was demonstrated that the level of expression of B-Myb was greatest in Line 16, followed by Line 4, with the least amount of B-Myb in Line 2. RNA was isolated from pooled aortas of the three 6–10-week-old B-myb transgenic mouse lines as well as from age-matched WT mouse aortas. As in the previous report, Northern blot analysis was performed for elastin gene expression, and the data were normalized for the levels of GAPDH. Using the average of six independent experiments with age-matched animals, Lines 2, 4, and 16 displayed 40.6 ± 23.0%, 38.4 ± 12.6%, and 21.4 ± 17.1% of WT normalized elastin mRNA levels, respectively (Fig. 1A). These data indicate that there is a statistically significant decrease in elastin mRNA expression in aortas from all of the lines versus WT mice. The relationship between the levels of expression of B-Myb protein and elastin mRNA was determined, using previously reported values for the levels of B-Myb protein in the aortas of the three transgenic mouse lines (29), specifically 1.6-, 2.5-, and 3.2-fold overexpression of B-Myb, respectively (Fig. 1B). These data suggest that elastin is another matrix gene target of repression by B-Myb in the aorta.

**Soluble Elastin Levels Are Reduced in the Aorta of Adult Transgenic Mice**—To evaluate the effects of B-Myb on intracellular tropoelastin levels, the two transgenic lines that expressed the highest levels of B-Myb were selected for study. Total soluble protein extracts were prepared from individual aortas from WT and transgenic Line 16 and 4 mice and subjected to immunoblot analysis for soluble tropoelastin protein, as well as for β-actin, as control to normalize for differences in loading (Fig. 2, A and B, respectively). Tropoelastin was substantially reduced in three of three aortas from transgenic Line 16 tested as compared with the four WT samples, whereas the aortas from transgenic Line 4 displayed a reduction compared with the WT samples that was less pronounced than Line 16. The average of three similar independent experiments showed that normalized tropoelastin levels were significantly diminished (i.e. 58.8 ± 16.6 and 43.6 ± 15.0% of WT levels in Lines 4 and 16, respectively), consistent with the relative increases in B-Myb expression levels (Fig. 2C). Thus, tropoelastin protein levels are reduced in the aortas of the transgenic mouse lines, compatible with the overall drop in elastin mRNA levels.

**Elastin mRNA Levels Are Reduced in Aortic SMCs from Adult Transgenic B-myb Mice**—To further assess the effects of B-Myb on elastin gene expression, SMCs were prepared from aortas of adult WT and transgenic Line 2, 4, and 16 animals. Total RNA was isolated, and RT-PCR analysis with primers specific for the transgene construct confirmed overexpression of human B-myb in all three lines (23) (data not shown). To monitor elastin gene expression, the aortic SMCs from transgenic and WT mice were plated at 40% confluence, and RNA was isolated after 72 h. Samples were subjected to Northern blot analysis, and elastin mRNA levels, analyzed by densitometry, were normalized relative to ethidium bromide staining of the gel. Compared with the WT, elastin mRNA levels were very low in SMCs from Lines 2 and 16 as well as in Line 4 (Fig. 3A, left and right panels, respectively). Levels of elastin mRNA in Lines 2 and 16 were reduced to 5.3 and 3.4% of WT levels, and levels in Line 4 were reduced to 13.6% of WT. Elastin and B-myb mRNA levels were averaged from five independent experiments for Lines 2 and 16 with SMCs from three separate preparations (Fig. 3B). A statistically significant reduction of elastin mRNA expression was observed in the SMCs, such that Lines 2 and 16 displayed 30.1 ± 34.2 and 28.7 ± 25.9% of WT elastin levels, respectively (Fig. 3B). B-myb mRNA levels were elevated 2.5 ± 1.4- and 3.4 ± 1.7-fold in Lines 2 and 16, respectively (Fig. 3C), similar to the in vivo pattern of B-myb expression where the highest levels were also observed in Line 16. Note that statistical analysis of this data indicates that B-myb expression is significantly higher in Line 16 versus WT.
but due to variability in the data, the difference between Line 2 and WT does not quite achieve significance (p = 0.057). Thus, elastin gene expression is dramatically reduced in SMCs isolated from aortas of adult B-myb transgenic compared with WT mice.

Analysis of Insoluble Elastin Content and Elastic Layering—
We next analyzed the levels of insoluble elastin in aortas of WT and transgenic mice. Aortas of six WT and four transgenic animals from each of Lines 2, 4, and 16 were removed from the aortic arch to the diaphragm, stripped of adventitia, and subjected to amino acid analysis as described under “Experimental Procedures.” The amount of insoluble elastin protein present in the aortic samples was expressed as a percentage of total protein. No differences in the amount of elastin/total protein were detected in the WT versus transgenic mice (26.8 ± 6.0, 31.6 ± 0.7, 24.9 ± 3.4, and 26.9 ± 2.0% (S.D.) for WT and Lines 2, 4, and 16, respectively).

Li et al. (56) observed that mice hemizygous for the elastin gene were viable and had a normal life span but higher than normal blood pressure. The aortas contained more elastic layers, each of thinner dimension than that of the WT mice. To determine whether the elastic layering of the B-myb transgenic animals differs from that of the WT mice, aortas ofagematched adult WT and transgenic Line 4 and 16 animals were isolated, fixed, and embedded. Sections were prepared from the same area near the arch of each aorta and subjected to Verhoeff and van Gieson staining (Fig. 4). There were no detectable differences in the number of elastin layers in WT versus transgenic animals. Thus, elastin deposition within aortas of adult B-myb transgenic and WT mice is comparable.

Elastin Expression Levels Are Not Reduced in Neonatal Animals—Since the bulk of elastin synthesis occurs early in development (57–60), and the transgenic mice appeared to develop normally with no detectable differences in deposition of elastin, it was of interest to study elastin expression earlier during development. To first assess the time course of elastin

![Figure 2](http://www.jbc.org/)

**FIG. 2.** Soluble tropoelastin levels are reduced in aortas of CMV-B-myb transgenic mice. A and B, protein extracts (30 μg) of individual aortas of 10-week-old WT and Line 4 and 16 B-myb transgenic mice were subjected to immunoblot analysis using antisera directed against either tropoelastin or β-actin, to control for equal loading. A, WT versus Line 16; B, WT versus Line 4. C, three independent experiments were performed as above, with age-matched 6–10-week old animals. Densitometry was performed, and values for tropoelastin normalized to those for β-actin were set at 100% for WT, and those for Lines 4 and 16 are expressed as a percentage of WT. Data represent the mean ± S.D. Data were compared with WT by one-tail, one-group Student’s t test; *, statistically significant difference, p < 0.05.

![Figure 3](http://www.jbc.org/)

**FIG. 3.** Elastin mRNA expression is reduced in aortic SMCs of adult B-myb transgenic mice. A, total RNA was extracted from exponentially growing aortic SMCs from adult WT and B-myb transgenic mice, as indicated, and samples (20 μg) subjected to Northern blot analysis for elastin (upper panels). RNA integrity and equal loading were confirmed by ethidium bromide (EtBr) staining (lower panels). B and C, five experiments were performed as in A for Lines 2 and 16 (using SMCs from three independent cell isolations), and the data are plotted for the expression of elastin (B) and B-myb mRNA (C) as a percentage of the WT (mean ± S.D.). Data were compared with WT by one-tail, one-group Student’s t test. *, statistically significant difference, p < 0.05.
mRNA expression, gene microarray analysis was performed. Aortic RNA was isolated from pools of 10–14 aortas from WT mice at days 12, 14, 16, and 18 of embryonal development and at birth as well as 4, 7, 10, 14, 21, 30, and 60 days and 5.5 months after birth. Affymetrix MU74Av2 chips were used to assess expression of elastin as well as B-myb RNA (Fig. 5). Elastin mRNA levels were relatively low during embryonic development and increased dramatically after birth, peaking between 7 and 14 days of age and then declining, consistent with previous analyses of developmental regulation of elastin gene expression in other species (61). Interestingly, B-myb mRNA expression was at its highest level in the embryos, at a time of active proliferation, consistent with previous studies linking its expression to the late G1 and S phases of the cell cycle in vascular SMCs (3). B-myb mRNA levels decreased through the late embryo stage, and levels were low at birth, when elastin mRNA levels began to increase. B-myb levels then decreased further in aortas of mice between 30 and 60 days after birth.

Based on these profiles, it was of interest to determine whether elastin expression in B-myb transgenic versus WT mice differed in young animals, at a time when elastin expression is normally high and presumably contributing to its deposition in the developing vasculature. In this regard, neonatal animals at 6 days of age were selected to compare elastin gene expression. Total RNA was isolated from pooled aortas from neonatal WT and Line 16 mice, and Northern blot analysis was performed (Fig. 6A). The level of elastin mRNA in transgenic animals, normalized to GAPDH, was slightly elevated in Line 16 as compared with the WT (138% of WT levels), in contrast to the decreased expression in the aorta of the adult animals (Fig. 1A). To compare tropoelastin protein levels in the neonatal animals, immunoblot analysis was performed. No difference in aortic tropoelastin levels was observed between three samples of WT and two samples of Line 16 neonatal animals, in contrast to the reduction observed in the samples from adult Line 16 mice (Fig. 6B). It is also of note that the levels of tropoelastin protein in the neonatal mice are much higher than that of the adult mice, as might be predicted from the time course of mRNA expression shown by the gene microarray analysis (Fig. 5). Thus, overexpression of B-Myb does not lead to a reduction in elastin gene expression in the aortas of neonatal mice.

Cyclin A2 Levels Decrease with Age—Recently, we showed that cyclin A ablates the ability of B-Myb to function as a repressor of the promoters of the collagen α2(V) and α1(I) genes (23, 24). Moreover, the expression of cyclin A mRNA is reduced in adult (6-week) versus neonatal (6-day) transgenic and WT mice, as shown by Northern blot analysis (23). To further evaluate the expression of cyclin A2 protein levels, immunoblot analysis was performed on aortic extracts from neonatal and adult animals. A dramatic reduction in cyclin A2 protein expression was observed in the adult animals as compared with the neonatal mice (Fig. 6B), consistent with the previous mRNA analysis (23). Furthermore, the WT and transgenic animals express equivalent cyclin A2 protein levels as seen previously for its mRNA. When the data from the WT and transgenic animals were combined to assess normalized aortic cyclin A2 expression as a function of age, an average was obtained with the neonatal versus adult mice of 0.63 ± 0.29 versus 0.12 ± 0.01 (p = 0.01 using an unpaired Student’s t test). Thus, levels of cyclin A2 expression in neonatal animals decrease with age and are quite low in adult mice, suggesting a potential role for cyclin A2 in relief of B-Myb-mediated repression in neonatal, but not adult, B-Myb transgenic mice.

Cyclin A Alleviates B-Myb-mediated Repression of the Elastin Gene Promoter—To directly assess the ability of cyclin A to alleviate B-Myb-mediated repression of the elastin gene promoter, transient transfection analysis was performed in SMCs from adult animals (Fig. 7). Two elastin promoter-CAT reporter vectors, driven either by a 2.2- or 0.5-kb fragment of the human elastin promoter and first exon upstream of the AUG start codon (elastin 2.2-CAT or elastin 0.5-CAT) were used. Bovine aortic SMCs were co-transfected with the reporter constructs in the absence or presence of vectors expressing bovine B-Myb and an increasing dose (0, 0.2, 0.4, or 0.8 μg) of human cyclin A (equivalent of murine cyclin A2). The basal activity of the elastin 0.5-CAT vector was substantially higher than the elas-
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Fig. 6. Elastin expression in WT and B-Myb transgenic mice is equivalent in neonates, an age at which cyclin A2 expression is high. A, total RNA (12 μg/lane), extracted from pooled aortas (7–12 aortas) of neonatal WT and Line 16 B-Myb transgenic mice, was subjected to Northern blot analysis using probes for elastin and GAPDH. Densitometry was performed, elastin values were normalized to those for GAPDH, and data are presented below each lane. B, total protein extracts (20 μg) of individual aortas of neonatal and Line 16 B-Myb transgenic mice were subjected to immunoblot analysis using antisera directed against tropoelastin (lighter and darker exposures are shown), cyclin A2, and β-actin. The gel was stained with Coomassie Blue, and a region between ~110 and 40 kDa is shown as an additional control for equal loading. Densitometry was performed, and values for cyclin A2 were normalized to those given for β-actin.

Disruption of the bovine B-Myb expression clone substantially repressed CAT activity of both elastin promoter constructs compared with their respective controls. For the elastin 2.2-CAT vector, the addition of a low dose of cyclin A expression vector reversed the B-Myb-mediated inhibition, and addition of higher doses caused a substantial increase in elastin promoter activity. For the elastin 0.5-CAT promoter, the lower dose of cyclin A expression vector partially alleviated the B-Myb-mediated repression, but a somewhat higher dose of cyclin A was required to reverse the inhibition, and again 0.8 μg of cyclin A caused a stimulation over basal levels (~2.2-fold). We next tested whether excess B-Myb can reverse cyclin A-mediated activation of elastin promoter activity. SMCs were transfected with either the elastin 2.2-CAT or elastin 0.5-CAT vector, 0.2 μg of cyclin A expression vector, and an increasing dose of B-Myb expression vector (0, 0.4, 0.8, or 1.6 μg). Expression of cyclin A alone led to a substantial induction in activity of both elastin promoters, consistent with release of endogenous B-Myb-mediated repression in these young adult SMCs (Fig. 7B). Ectopic B-Myb expression caused a dose-dependent decrease in the cyclin A-mediated activation of elastin promoter activity, which returned to basal values when 0.4–0.8 μg of B-Myb expression vector were added. The addition of 1.6 μg of B-Myb expression vector resulted in activity that was lower than base line. Thus, cyclin A reversibly alleviates B-Myb-mediated repression of the elastin promoter, similar to its effects on the α1(I) and α2(V) promoters (23, 24). Since matrix protein production and deposition during vessel wall synthesis occurs early in development (57–60), the presence of high levels of cyclin A2 in embryonic and neonatal development probably explains, at least in part, why no significant differences were observed in elastin deposition in the vessel wall of the transgenic versus WT animals.

Fig. 7. Cyclin A ablates repression of the elastin promoter by B-Myb. A, bovine aortic SMC cultures, plated in triplicate at a density of 6 × 10⁴ cells in 6-well dishes, were transfected with 0.6 μg of elastin 2.2-CAT or elastin 0.5-CAT, as indicated, in the absence or presence of 0.8 μg of bovine B-Myb and 0, 0.2, 0.4, or 0.8 μg of human cyclin A expression vectors and pBluescript (to make up a total of 2.4 μg of DNA/well) using Lipofectamine reagent. After 72 h, CAT activity, normalized to total protein, was determined. B, bovine aortic SMC cultures, plated in duplicate at a density of 6 × 10⁴ cells in 6-well dishes, were transfected with 0.6 μg of elastin 2.2-CAT or elastin 0.5-CAT in the absence or presence of 0.2 μg of human cyclin A and 0-, 0.4-, 0.8-, or 1.6-μg bovine B-Myb expression vectors and pBluescript (to make up a total of 3.0 μg of DNA/well) using Lipofectamine reagent. After 48 h, CAT activity, normalized to total protein, was determined.

Discussion

Here, we identify elastin as a new target for B-Myb-mediated repression in vitro and in vivo and demonstrate that the ability of B-Myb to function as a repressor of elastin gene expression is alleviated by cyclin A2, which is expressed in a developmentally controlled fashion. In aortas of adult animals, levels of elastin mRNA and precursor tropoelastin protein were reduced in the B-Myb transgenic versus WT mice. Furthermore, elastin mRNA levels were similarly reduced in isolated aortic SMCs from the transgenic lines, and the elastin promoter was inhibited by B-Myb in transient transfection into adult bovine SMCs. Taken together, these results indicate that B-Myb can functionally repress elastin gene expression. Although it was initially surprising that there were no abnormalities in the vessel wall in the transgenic mice, a likely explanation was provided by the time course of elastin and B-Myb mRNA expression. Elastin mRNA levels are low in the embryo when B-Myb mRNA expression is highest and cells are rapidly proliferating. Following birth, elastin mRNA levels increase dramatically, whereas conversely B-Myb expression decreases. Furthermore, cyclin A2, which alleviates the ability of B-Myb to function as a transcriptional repressor, is expressed in the aortas of neonatal mice. When elastin mRNA levels were compared between transgenic and WT neonates, no differences were seen. In the adult animals, the level of cyclin A2 drops to almost below detection, whereas B-Myb remains essentially constant. Thus, a functional increase in B-Myb-mediated repression occurs only in the adult aorta of the transgenic animals. Importantly, mature, cross-linked elastin has remarkable longevity, with an estimated half-life of 40 years in humans...
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(62–64), and there is little appreciable synthesis of mature elastin in the adult aorta. The unaltered vessel structure in the transgenic mice can most likely be attributed to the levels of cyclin A2 in aortas of young mice, where it prevents B-Myb-mediated repression of matrix gene expression. Upon injury of the femoral artery, however, we have recently shown that B-Myb functions to inhibit neointima formation (23), suggesting that the inhibition of expression of matrix proteins such as collagen and elastin contributes to the decrease in lesion formation. Overall, these findings help to further elucidate the mechanism of resistance to vascular injury mediated by B-Myb.

We previously showed that B-Myb decreases endogenous α1(I) collagen mRNA levels (13, 21) and negatively regulates the activities of the α1(I) and α2(I) collagen promoters in a dose-dependent fashion in bovine SMCs in culture (3). Here, the data demonstrate that B-Myb also represses the elastin promoter and decreases endogenous elastin mRNA levels. In the aorta of the B-Myb transgenic mouse, the -fold reduction in the level of elastin mRNA was greater than the decrease in α1(I) collagen mRNA. Interestingly, the 2.2-kb sequence upstream of the elastin transcription start site has ~12 putative MBSs, two of which are within the 0.5-kb fragment, which was also regulated by the B-Myb expression vector. B-Myb can also regulate without binding DNA, via interaction with other factors (15, 65, 66). Interestingly, we found that the down-regulation of the α2(V) collagen promoter occurs through displacement of a transcriptional activator (13).

Recently, we compared lesion formation in adult B-Myb transgenic versus age-matched WT mice using a model in which the endothelium of the femoral artery is denuded by insertion of a guide wire. The data showed a dramatic reduction in neointima formation in the arteries of the B-Myb transgenic versus WT mice 4 weeks postinjury (23). Following 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 in the WT (FVB) mice 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. It is intriguing to speculate on the role that B-Myb-mediated repression of elastin gene expression played in the observed inhibition of neointima formation upon vascular injury of adult B-Myb transgenic mice. A model can be proposed based on the reported findings. Although B-Myb is ubiquitously expressed, the data suggest that it is developmentally regulated at both its level of expression and functional activity in the aorta. In particular, a correlation was noted between the drop in B-Myb mRNA levels and the induction of elastin gene expression in the WT mice. Therefore, it is possible that the decrease in B-Myb levels in the neonatal animals, in combination with the presence of cyclin A2, leads to a decline in the extent of B-Myb-mediated repression of elastin. Thus, any activator(s) present can now function to induce transcription of the elastin gene in the neonatal animals. In the adult animals, cyclin A2 levels drop, leading to renewed ability of B-Myb to repress gene transcription. Furthermore, the expression of the activator(s) may also be decreased given the sharp drop in the level of elastin gene expression seen in the aorta from the adult mice. In the transgenic animals, B-Myb is elevated ~1.6–3.2-fold in the three lines (Lines 2, 4, and 16) (23). Apparently, this increase is not sufficient to functionally overcome the block of repression by cyclin A2 in the neonatal mice; hence, the aortas in the neonatal transgenic and WT mice contain comparable levels of elastin mRNA, and vessel wall development is unaffected. In the adult animals, however, the lowered cyclin A2 levels contribute to the ability of B-Myb to function as a repressor, and the increased levels of B-Myb in the transgenic mice lead to decreased expression of elastin mRNA as well as to a drop in type I collagen mRNA and protein (23). This model provides a likely explanation for the dramatic difference in injury response discussed above (i.e. that the neointima formation upon vascular injury was substantially reduced in the transgenic B-Myb mice). Thus, the presence of overexpressed B-Myb in the transgenic mice only causes a detectable phenotypic change upon injury to the adult animals.

Previously, we observed that cyclin A-Cdk2 phosphorylation reduces the ability of B-Myb to repress the α2(V) and α1(I) collagen promoters in aortic SMCs in culture (23, 24). Here, these results are extended to show that cyclin A also alleviates B-Myb-mediated repression of the elastin promoter, indicating that the ability of cyclin A to counteract B-Myb-mediated repression might be a more general mechanism. Interestingly, the capability of B-Myb to function as a transcriptional activator was first shown to be enhanced by phosphorylation through cyclin A-Cdk2 complexes (20, 67, 68), suggesting a more general role of this cyclin to regulate B-Myb function. Last, one cannot exclude the possibility that other proteins that modulate B-Myb activity, such as CREB-binding protein, p100, poly- (ADP-ribose) polymerase, p107, N-CoR, and SMRT (19, 69–73), may also be subject to developmental regulation in the aorta. Overall, the present study supports a major role for B-Myb as a general regulator of extracellular matrix gene expression in the vessel wall.

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