Vascular Injury Induces Posttranscriptional Regulation of the Id3 Gene
Cloning of a Novel Id3 Isoform Expressed During Vascular Lesion Formation in Rat and Human Atherosclerosis

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Abstract—The molecular mechanisms that regulate the proliferation of smooth muscle cells (SMCs) of the vasculature in response to injury are poorly understood. Members of the inhibitor of DNA binding (Id) class of helix-loop-helix transcription factors are known to regulate the growth of a variety of cell types; however, the expression of the various Id genes in SMCs and in vascular lesions has not been examined. In the present study, the yeast 2-hybrid system was used to clone Id genes from a cultured rat aortic SMC library. By use of ubiquitous E proteins as bait, Id3 and a novel isoform of Id3 (Id3a) were cloned. Id3a is the product of alternative splicing of the Id3 gene, resulting in inclusion of a 115-bp “coding intron,” which encodes a unique 29–amino acid carboxyl terminus for the Id3a protein. Unlike Id3, Id3a mRNA was not detected in the normal rat carotid artery. However, after balloon injury, Id3a was abundantly expressed throughout the neointimal layer. In addition, mRNA of the human homologue of Id3a (Id3L) was detected in human carotid atherosclerotic plaques. Adenovirus-mediated overexpression of these Id3 isoforms in cultured rat aortic SMCs revealed that infection of SMCs with an adenovirus overexpressing Id3a (in contrast to Id3) resulted in a significant decrease in cell number versus AdLacZ-infected cells. DNA fragmentation analysis suggested that this decrease in SMC viability was due to increased apoptotic activity in cells infected with adenovirus overexpressing Id3a. These results provide evidence that alternative splicing of the Id3 gene may represent an important mechanism by which neointimal SMC growth is attenuated during vascular lesion formation. (Arterioscler Thromb Vasc Biol. 2001;21:752-758.)

Key Words: smooth muscle cells ■ helix-loop-helix factors ■ atherosclerosis ■ vascular injury ■ alternative splicing

The phenotype of smooth muscle cells (SMCs) within atherosclerotic and restenotic lesions in humans and lesions generated after vascular injury in experimental animals is modulated relative to normal medial SMCs. This modulation is characterized by loss of myofilaments, formation of a large endoplasmic reticulum and the Golgi complex, migration into the neointima, and extracellular matrix secretion. In addition, this phenotypic modulation is accompanied by alterations in growth factor receptor expression and growth responsiveness, resulting in enhanced proliferation. The proliferation of intimal SMCs leads to encroachment on the vessel lumen. Thus, the identification of factors that enhance and inhibit the growth of SMCs is important for understanding and potentially intervening in the SMC response that leads to vascular lesion formation.

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The inhibitor of DNA binding (Id) class of helix-loop-helix (HLH) transcription factors is known to regulate growth in a variety non-SMC types. There are 4 known Id genes (Id1 to Id4), and the rat homologues of 3 have been cloned (Id1 to Id3). Previous studies have determined that the expression of various Id genes is downregulated when cells terminally differentiate, and overexpression of Id impairs differentiation. Additionally, serum induces the expression of human Id1 and Id2 mRNA in human diploid fibroblasts, and antisense oligomers complementary to Id1 and Id2 inhibit entry into the S phase of the cell cycle. Furthermore, overexpression of Id1 enhances growth in fibroblasts. Although the Id proteins appear to be redundant in their enhancement of cell growth, recent data provide evidence that the different Id proteins may be involved in the regulation of distinct pathways. Additionally, it appears that the Id factors may also be involved in the regulation of pathways leading to programmed cell death.

There is evidence that Id proteins are expressed in cultured SMCs. However, the specific Id proteins expressed in proliferating SMCs and in vascular lesions are unknown. To
identify Id proteins expressed in SMCs, we used the yeast 2-hybrid system to clone dimerization partners of ubiquitously expressed E proteins from a cultured rat aortic SMC library. In the present study, we report on the cloning of rat Id3 and a unique isoform of the rat Id3 protein (called Id3a). Id3a is generated via alternative splicing of the Id3 gene, resulting in the inclusion of a "coding intron." This coding intron encodes a unique 29-amino acid carboxyl terminus for the Id3a protein. Id3 and Id3a have differential mRNA expression patterns in the normal and balloon-injured rat common carotid artery. Additionally, the human homologue of Id3a (Id3L) mRNA is detected in human atherosclerotic lesions. We demonstrate that in contrast to Id3, infection of cultured SMCs with an adenosivirus overexpressing Id3a results in a significant decrease in cell number. The decrease in cell viability that results from ectopic overexpression of Id3a in SMCs appears to be due to increased apoptotic activity in these cells. These data provide evidence that posttranscriptional regulation of the Id3 gene represents a mechanism by which SMC growth and viability can be rapidly modulated in the developing neointima. The expression of Id3 isoforms in the neointima and in advanced atherosclerotic plaques suggests a role for these Id forms in the regulation of the balance between SMC growth and death during vascular lesion formation.

Methods

**cDNA Library**

Early-passaged cultured rat aortic SMCs were grown in DMEM:F-12 (1:1) with 10% FBS, 0.2 mg/mL glutamine, 10 U penicillin, and 10 μg/mL streptomycin per milliliter (DF10, GIBCO-BRL). The cells were harvested, frozen in liquid nitrogen, and shipped to Clontech for RNA extraction and custom library construction. The resultant cDNAs were inserted into the EcoRI site of the activation domain vector pGAD10[leu]+.

**Yeast 2-Hybrid Library Screening**

The Matchmaker Two-Hybrid System (Clontech) was used for library screening. The bait plasmid pBW202 was constructed by subcloning nucleotides 1231 to 1851 of the Pan-1cDNA into the Smal-BamHI site of the binding domain vector pGBT9[trp]+. The bait plasmid pBW206 was constructed by subcloning nucleotides 1831 to 2499 of the REBo cDNA into the EcoRI-SalI site of pGBT9[trp]+. The constructs were sequenced to verify orientation and protein coding frame. With pBW202 used as bait, 8 million colonies were screened. Positive colonies were identified by β-galactosidase filter assay. Plasmid DNA was extracted from the positive colonies and electroporated into the yeast strain HB101 to eliminate the pGBT9 plasmid. The bait plasmid pBW202 was constructed by subcloning nucleotides 1831 to 2499 of the REBo cDNA into the EcoRI-SalI site of pGBT9[trp]+. The constructs were sequenced to verify orientation and protein coding frame. With pBW202 used as bait, 10 million colonies were screened. During a subsequent round of screening with pBW206 used as bait, 8 million colonies were screened. Positive colonies were identified by β-galactosidase filter assay. Plasmid DNA was extracted from the positive colonies and electroporated into the Escherichia coli strain HB101 to eliminate the pGBT9 plasmid. The remaining plasmid DNA was extracted (SMC cDNA—containing GAD10 plasmid) from HB101, restriction-digested, and gel-electrophoresed. The resultant banding patterns were compared with eliminate identical clones before further characterization. Both plasmids (pGBT9 bait and pGAD–SMC cDNA) were retransformed into yeast, and a colony-lift β-galactosidase filter assay was performed to confirm dimerization. True positive clones were identified and sequenced.

**Cell Culture**

Rat aortic SMCs were grown to confluence in DF10, and then the medium was changed to a defined serum-free medium containing DMEM:F-12 (1:1) with 10 U/mL penicillin, 10 μg/mL streptomycin, 35 μg/mL ascorbic acid, 5 μg/mL transferrin, 2.85 μg/mL insulin, and 6.25 ng/mL selenium (all from Sigma Chemical Co). The cells were maintained in serum-free medium for 5 days and then stimulated with 10% FBS. The cells were harvested at 0, 4, 8, 12, and 24 hours after serum stimulation.

**Rat Carotid Injury Model**

The rat carotid endothelial denudation model was performed with the use of male 300-g Sprague-Dawley rats (n = 18, Harlan Laboratories, Indianapolis, Ind) as described elsewhere. At 1 (n = 5), 6 (n = 5), 14 (n = 4), and 28 (n = 4) days after the initial injury, the rats were given an overdose of intraperitoneal ketamine/xylazine. A midline sternotomy was performed, and the animals were pressure-perfused with 4% paraformaldehyde in cold PBS. The injured left and control right carotid arteries were removed and postfixed in 4% paraformaldehyde for 2 hours, dehydrated in a graded alcohol series, and paraffin-embedded for thin sectioning.

**Human Tissue Preparation**

Freshly excised human carotid plaques (n = 6) were collected at the time of carotid endarterectomy in accordance with institution human tissue use protocols. The samples were fixed for 2 hours in 4% paraformaldehyde in PBS, dehydrated in a graded alcohol-xylene series, and mounted in paraffin for thin sectioning. Serial sections from each sample were examined for expression of Id3L mRNA as well as smooth muscle α-actin protein.

**In Situ Hybridization**

The polymerase chain reaction (PCR; Gene-amp, Perkin-Elmer) was used to synthesize a 175-bp fragment corresponding to nucleotides 270 to 444 of rat Id3a from a full-length cDNA. This region of the rat Id3a mRNA includes the 115-bp insert as well as 30 bp of the flanking sequence on either side of the insert. A similar strategy was used to produce a 150-bp fragment corresponding to nucleotides 386 to 535 of the 3′ untranslated region of rat Id3 and Id3a. A human Id3L riboprobe was constructed by using PCR to amplify a 137-bp fragment corresponding to the 107-bp insert of human Id3L and 15 bp of the flanking sequence on each side of the insert from human male genomic DNA (Promega Corp). These fragments were subcloned into the pGEM-t vector (Promega Corp), and digoxigenin-labeled riboprobes were generated. Probe specificity was confirmed by use of RNA dot blot hybridization. In addition, the specific activity of the Id3 and Id3a probes for their corresponding targets was determined by Northern hybridization. The binding activities of the Id3 and Id3a probes were determined to be equivalent with the use of this assay (data not shown).

Thin sections of rat and human tissue were fixed to Probe-on Plus slides (Fisher Scientific), dried at 37°C for 4 hours, deparaffinized in xylene, and rehydrated in a graded alcohol series. The sections were fixed in cold 4% paraformaldehyde in diethyl pyrocarbonate–treated PBS for 15 minutes at room temperature. The sections were then washed with 0.5× SSC and treated with proteinase K (10 μg/mL, Sigma) for 15 minutes at 37°C. Prehybridization was carried out at 42°C for 1 hour in buffer containing 55% formamide, 0.5 μg/mL yeast tRNA, 0.25 μg/mL salmon sperm DNA, 10% dextran sulfate, 0.5 μg/mL heparin, 2× SSC, and 1× Denhardt’s solution. Digoxigenin-labeled probes in hybridization buffer (500 ng/mL) were denatured at 80°C for 5 minutes, cooled on ice, and incubated with the tissue sections overnight at 50°C. After hybridization, the slides were washed twice in 1× SSC for 10 minutes each, twice in 0.5× SSC for 10 minutes each, and twice in Tris-buffered saline for 10 minutes each. The sections were blocked with 5% BSA in Tris-buffered saline (TBS) for 1 hour at room temperature. The sections were then incubated with alkaline phosphatase–conjugated anti-digoxigenin antibody (Boehringer-Mannheim) diluted 1:500 in 5% BSA in TBS for 4 hours at room temperature. Sections were then washed twice in TBS for 10 minutes each, washed once in 100 mmol/L Tris (pH 9.5), 100 mmol/L NaCl, and 50 mmol/L MgCl2 for 10 minutes, and incubated with color substrate solution (nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate in dimethylformamide. Boehringer-Mannheim) with 2.5 mmol/L levamisole (Sigma) overnight at 4°C. The colorization reaction was quenched with 25 mmol/L Tris (pH 8.0)/2 mmol/L EDTA, the sections were counterstained with 0.15% Fast Green FCF (Fisher Scientific).
are expressed in rat aortic SMCs (data not shown). The clones were subsequently sequenced, and all encoded the above-mentioned specific nucleotides of Pan-1 and REB were used because they contain the HLH motif. Arrows bracket the coding intron that distinguishes Id3a from Id3. Stop codons of Id3 and Id3a are indicated by bold type. Arrows bracket retained intron of Id3a. The unique carboxyl terminus of Id3a is highlighted in green; the carboxyl terminus of Id3 is highlighted in red.

**Sequence of Id3a**
Id3a is 100% homologous to rat Id3 in the coding region except for 115 bp of retained intron sequence downstream from the HLH domain, which disrupts the carboxyl-terminal coding region of Id3 (Figure 1). The divergent region of Id3a was identified as an intronic sequence by comparison with the DNA sequence of the Id3 genomic clone.25 The nucleotides flanking the insert are homologous to donor-acceptor sites at intron-exon boundaries (GT-AG), suggesting that this is a retained coding intron. Retention of a coding intron resulting in an isoform with a unique carboxyl terminus has been previously described for rat Id1.26 The deduced amino acid sequence of Id3a results in a unique 29--amino acid carboxyl terminus. This deduced Id3a amino acid sequence was confirmed by mass spectrometric sequencing of bacterially expressed Id3a protein (data not shown). The carboxyl terminus of Id3a is 19 amino acids in length and lacks homology to the carboxyl terminus of Id3.

**Id3 and Id3a Are Differentially Expressed in the Vessel Wall After Vascular Injury**
By use of reverse transcription–PCR, we confirmed the expression of Id3a mRNA in cultured SMCs and in cultured endothelial cells and fibroblasts. Expression of Id3a in these cells was determined to be quite low compared with the expression of fully spliced Id3 (data not shown). To determine whether Id3a is expressed in vascular SMCs in vivo and whether this expression is altered after vascular injury, we examined Id3a mRNA expression after balloon endothelial

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**Immunohistochemistry**
Human carotid thin sections were rehydrated by use of a graded alcohol series. The sections were stained for smooth muscle α-actin by using the Vectastain Elite ABC kit (Vector Laboratories). Sections were blocked for 30 minutes in 2% horse serum, followed by incubation with a monoclonal anti–smooth muscle α-actin antibody (Boehringer-Mannheim) diluted 1:100 in 2% horse serum for 2 hours at room temperature. Slides were washed twice with PBS and incubated with a biotinylated anti-mouse antibody diluted 1:2000 in 2% horse serum for 1 hour at room temperature. Slides were then incubated for 30 minutes in 0.3% hydrogen peroxide followed by Vectastain Elite ABC reagent for 30 minutes at room temperature. Slides were washed twice with PBS and incubated with diamobenzidine peroxidase substrate (Sigma) for 2 minutes. Sections were counterstained with hematoxylin, dehydrated through a graded alcohol series, and mounted with the use of Permount (Fisher Scientific).

**Construction of AdId3 and AdId3a5/3**
Description of the Cre-lox–based adenoviral system is described elsewhere.20 Full-length Id3 and Id3a cDNAs were synthesized from rat SMC mRNA by using reverse transcription–PCR. An Id3a cDNA (Id3a5/3), which cannot be spliced, was constructed from the Id3a cDNA by site-directed mutation of the 5′ splice site and polypyrimidine tract as well as deletion of the 3′ consensus splice site of the Id3a insert. These constructs were subcloned into the vector pAdlox, which was to be used as the bait. The GBT9 domain (GBT9) vector, which was to be used as the bait. The Id3 and Id3a were chosen as bait sequences because they are known to interact with Id proteins and because we have demonstrated that they are expressed in rat aortic SMCs (data not shown). The
denudation of the rat common carotid artery with the use of in situ hybridization with an RNA riboprobe that was complementary to the 115-bp coding intron unique to Id3a mRNA. No Id3a mRNA was detected in uninjured rat carotid arteries (Figure 2A) or at 24 hours after balloon injury (Figure 2B). Six days after injury, Id3a mRNA was detected for the first time (Figure 2C) predominantly within the growing neointima and periluminal medial layers. Additionally, Id3a mRNA was detected in scattered cells of the adventitia. High-power magnification revealed that Id3a mRNA was cytoplasmic, providing evidence that Id3a mRNA is indeed exported from the nucleus for translation (Figure 2D; arrowheads indicate nuclei). This pattern of Id3a expression continued at 14 days (Figure 2E) and 28 days (Figure 2F) after injury. No staining was noted at any time point by use of the control sense Id3a probe (Figure 2G), confirming specificity of the signal.

The detection of Id3a mRNA was localized predominantly to the neointima and inner medial layers after rat carotid injury, and this expression pattern was first noted at 6 days after injury. This pattern of expression contrasts with that of the fully spliced Id3. Expression of Id3 mRNA was detected by use of an antisense riboprobe that is complementary to a 150-bp region of the 3′ untranslated region common to Id3 and Id3a. There are no sequences that are unique to Id3; therefore, any positive staining with use of the Id3 probe could represent Id3 or Id3a. Given the fact that Id3a was not detected in the media of uninjured vessels or 1 day after injury with the Id3a-specific probe, the positive staining seen at these time points with use of the common probe represents Id3. Id3 mRNA was expressed diffusely throughout the media of the uninjured carotid artery (Figure 3A) and at 24 hours after balloon denudation (Figure 3B). Control sense Id3 probe was negative in uninjured vessels (Figure 3C, uninjured vessel), confirming signal specificity. To ensure that the presence of medial cell expression of Id3 does not reflect higher specific activity of the Id3 probe versus the Id3a probe for Id3a mRNA, we used Northern hybridization to confirm that the specific activity of the 2 probes for their corresponding target sequences was equivalent (data not shown). Interestingly, Id3 mRNA was not detected in the media at 6 to 28 days after injury (data not shown). It is not known whether the absence of Id3 mRNA expression at these time points represents downregulation of Id3 in medial SMCs or if Id3-positive SMCs noted at earlier time points subsequently migrated to the neointima at these later time points.

**Id3L Is Expressed in Human Carotid Atherosclerotic Lesions**

Results of in situ hybridization on injured rat carotid arteries revealed that Id3a mRNA was present in abundance in the cells constituting the neointima. Given the association of this Id3 isoform with a vascular neointimal lesion in an animal model, we were interested in the expression of the human homologue of this Id3 isoform (Id3L) in human atherosclerotic plaques. Like rat Id3a, human Id3L is the result of alternative splicing of the Id3 gene, resulting in the inclusion of a coding intron that results in a unique carboxyl terminus.

**Figure 2.** Expression of Id3a mRNA is upregulated in the rat common carotid artery after balloon injury. In situ hybridization used digoxigenin-labeled antisense Id3a riboprobe. Sections are counterstained with 0.15% Fast Green FCF. Arrows indicate internal elastic lamina. No Id3a mRNA is detected in uninjured vessels (A) or in vessels 24 hours after balloon injury (B). Six days after injury, Id3a mRNA is detected in the inner media and neointima, as well as in scattered areas of the adventitia (C). High-power magnification of 6-day neointima confirms cytoplasmic staining of Id3a mRNA (D; arrowheads indicate nuclei that are not stained). Diffuse neointimal staining of Id3a mRNA continues to be detected at day 14 (E) and day 28 (F) after injury. No signal is seen with use of the corresponding sense probe (G, 28 days).

**Figure 3.** Compared with expression of Id3a, expression of Id3 mRNA in the normal rat carotid artery and arteries after balloon injury differs. In situ hybridization used digoxigenin-labeled antisense Id3 riboprobe. Diffuse Id3 mRNA expression is noted in medial cells of the uninjured carotid artery (A) and in vessels 24 hours after balloon injury (B). No staining is seen with use of the corresponding sense probe (C, uninjured vessel).
Adenovirus-Mediated Overexpression of Id3a Inhibits SMC Growth

We were unable to test the effect of overexpression of Id3a in a stable transfection experiment, presumably because of splicing of the Id3a coding intron by these SMCs. Evidence for this was the fact that we were unable to detect Id3a protein in these stable lines by using a carboxyl-terminal Id3a monoclonal antibody but that we did detect significant overexpression of fully spliced Id3. Therefore, we used site-directed mutagenesis to create the Id3a cDNA (Id3a5/3), which contains an insert that cannot be spliced. Point mutations of the 5′ splice sites and the internal polypyrimidine tract as well as a deletion mutation of the 3′ splice site were required to make Id3a5/3. These mutations did not change the amino acid sequence of the Id3a protein. This construct was then used to construct an adenovirus overexpressing Id3a (AdId3a5/3). Additionally, we constructed an adenovirus vector overexpressing a full-length Id3 cDNA (AdId3). Protein overexpression in cells infected with AdId3 and AdId3a was confirmed by Western blotting (data not shown).

Cultured rat aortic SMCs were plated in equal numbers and infected with 5 MOI of either AdId3, AdId3a5/3, or AdLacZ. Twenty-four hours after infection, cells were replated on 96-well plates at a density of 5000 cells per well and counted at 3 and 5 days after replating. Results are averages of 3 repeats. *P<0.001 vs AdLacZ (LacZ).

Figure 5. Adenovirus overexpression of Id3a results in decreased cell number. Cultured rat aortic SMCs were plated at equal density and infected with 5 MOI of either AdId3, AdId3a5/3, or AdLacZ. Twenty-four hours after infection, cells were replated on 96-well plates at a density of 5000 cells per well and counted at 3 and 5 days after replating. Results are averages of 3 repeats. *P<0.001 vs AdLacZ (LacZ).

Decreased Viability of SMCs Overexpressing Id3a Is due to Increased Apoptotic Activity

The significant decrease in cell number in SMCs infected with AdId3a5/3 suggested that overexpression of Id3a may induce apoptosis in these cells. Therefore, we assayed SMCs infected with these adenoviral constructs for the presence of cytoplasmic histone-associated DNA fragmentation, a marker of apoptosis. SMCs were infected with 5 MOI of AdId3, AdId3a5/3, or AdLacZ. Forty-eight hours later, cells were

Figure 4. Id3L mRNA is expressed in human carotid atherosclerotic plaques. In situ hybridization of a representative human carotid endarterectomy specimen with use of digoxigenin-labeled antisense Id3L riboprobe reveals Id3L mRNA expression in discreet areas with the specimens (A). No staining is seen in a contiguous section of this specimen hybridized with the corresponding sense probe (B). Immunohistochemical staining of a contiguous section with a monoclonal antibody against smooth muscle α-actin (C, D [high power]) reveals positive diaminobenzidine (brown) staining in areas of Id3L mRNA staining (hematoxylin counterstain).

with respect to the parent protein. However, unlike the rest of the Id3 gene, the coding intron of Id3L is completely divergent from the coding intron in rat Id3a. We performed in situ hybridization on human carotid endarterectomy specimens. A total of 6 specimens were collected at surgical carotid endarterectomy. These plaques contain complex lesions composed of heterogeneous cellular material; however, Id3L mRNA was detected in multiple discrete areas of each of the plaque specimens by use of the antisense riboprobe; one such area of a representative specimen is depicted (Figure 4A). Sense probe on a contiguous section (thus representing the same area of plaque) confirmed the specificity of this signal (Figure 4B). By use of a monoclonal antibody against smooth muscle α-actin, it was determined that Id3L mRNA in this plaque was localized to the α-actin–positive regions of the vessel wall (Figure 4C and 4D; high power, brown staining). Thus, Id3L is expressed in human carotid atherosclerotic plaques and is localized to regions of plaque that contain abundant SMCs.
harvested, and cytoplasmic fractions were assayed for histone-associated DNA fragments by use of a colorimetric ELISA. Results are reported as absorbance at 405 nm (Figure 6). Cytoplasmic lysates from SMCs infected with AdId3a5/3 (versus AdLacZ-infected cells) yielded a 3-fold increase in optical density at 405 nm ($P<0.01$), providing evidence that the substantial decrease in viability of SMCs overexpressing Id3a reflects increased apoptotic activity in these cells.

**Discussion**

Despite the importance of SMC growth and death in vascular lesion formation, the molecular mechanisms that regulate these processes are poorly understood. The Id proteins are known to regulate growth and differentiation in a variety of cell types; however, their role in SMCs is unknown. The present study is the first to demonstrate that Id3 mRNA is expressed in the vessel wall. Unlike Id3, the newly cloned isoform of rat Id3 described in the present study (Id3a) is not expressed in vascular media but is abundantly expressed in the developing neointima after vascular injury. Interestingly, the rat Id3 and Id3a mRNAs are identical except for the inclusion of a 115-bp coding intron in Id3a located downstream from the HLH domain. This insert encodes a unique 29-amino acid carboxyl terminus of the Id3a protein. Of note, recent studies have shown that the C-terminus of Id3 is essential for its dominant-negative function of inhibiting specific gene expression. Although the overexpression of wild-type Id3 efficiently inhibited the MyoD-mediated activation of the muscle-specific creatine kinase reporter gene, a chimeric protein containing the N-terminal region of Id3 and the C-terminus of Id2 lost this inhibitory function. Conversely, although Id2 was also capable of inhibition of MyoD-mediated muscle-specific creatine kinase reporter activity, deletion of the C-terminus of Id2 did not affect this function. Consistent with these data that provide evidence for the importance of the C-terminus for Id3 function, we found that Id3 and Id3a have very different effects on cell growth and death. Although adenovirus-mediated overexpression of Id3 appears to have no effect on SMC growth, overexpression of Id3a is actually proapoptotic. The Id proteins have traditionally been thought of as growth promoting and have been associated with a highly proliferative state; however, several recent studies have implicated them in the induction of apoptosis. Norton and Atherton have demonstrated that overexpression of Id3 induces apoptosis in serum-starved fibroblasts. This effect appears to be associated with increased S phase entry, suggesting close coupling between G1 progression and apoptosis functions of the Id proteins. The fact that Id3 did not promote apoptosis in SMCs in the present study provides evidence that there may be cell-type specificity to the proapoptotic effect of Id3.

The pattern of apoptosis of SMCs in the rat carotid injury model has been studied extensively. After injury, medial SMCs undergo an early wave of apoptosis in the initial hours after balloon endothelial denudation, followed by a second increase in apoptosis that begins in the first week after balloon injury, peaks at day 9 after injury, and is limited to the neointima. The factors that regulate the balance between cellular growth and death within the evolving neointima are poorly understood. It is intriguing to note that the expression pattern of Id3a mRNA in the balloon-injured rat carotid artery is limited to the neointima and peaks between 6 and 14 days after endothelial denudation, inasmuch as this closely follows the spatial and temporal pattern of late SMC apoptosis in this model. The timing of Id3a mRNA expression in vivo coupled with the in vitro data demonstrating Id3a-induced SMC apoptosis suggests that alternative splicing of the Id3 gene resulting in expression of the Id3a protein limits neointimal formation via enhancement of SMC apoptosis. Although the expression of Id3a mRNA does not confirm the presence or functional importance of Id3a protein in the vessel wall, the in situ data demonstrating cytoplasmic expression of the Id3a mRNA (Figure 2D) provide evidence that unlike the rapid splicing of the wild-type Id3a cDNA in cultured rat aortic medial SMCs, the mature endogenous Id3a mRNA is not spliced by neointimal cells in vivo. Splicing of pre-mRNA occurs in the nucleus before mRNA transport to the cytoplasm. Thus, cytoplasmic expression of Id3a mRNA provides evidence that the mature Id3a mRNA has been exported from the nucleus for translation. Further work to confirm Id3a protein expression in vivo by use of a specific antibody is under way. Additionally, the high rate of cell loss noted in AdId3a5/3-infected SMCs may not reflect the effect of this protein when expressed at endogenous levels in cells of the neointima, inasmuch as adenovirus-mediated gene transfer likely results in much higher levels of transgene expression than are present in vivo.

The present study also provides evidence that vascular injury regulates the alternative splicing of Id3 mRNA and/or the stability of Id3a mRNA in the vasculature in vivo. Posttranscriptional modification of mRNA has the unique potential to result in quantitative and qualitative regulation of gene expression by generating different mRNAs from a single gene. It has previously been shown that vascular injury regulates the alternative splicing of other genes implicated in the modulation of the SMC phenotype, such as myosin heavy chain and fibronectin. Alternative splicing appears to be a mechanism by which physiological stimuli alter the expression patterns of specific genes during SMC phenotypic modulation. This mode of posttranscriptional regulation may be an important mechanism for controlling specific gene expression in vascular SMCs after vessel injury. In particular, posttranscriptional regulation of...
the Id3 gene may be an important mechanism for controlling the balance between cell growth and death in the developing neointima.

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References
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