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21. The reprecipitation assay was as follows: Washed immunoprecipitates (50 μl of packed beads) were disrupted by incubation for 10 min at 95°C in 50 μl of 1% SDS–1% β-mercaptoethanol. Samples were then allowed to cool to room temperature and the supernatant transferred to a clean microfuge tube. Twenty microliters of 0.77 M iodoacetamide was added to give a twofold molar excess of iodoacetamide over β-mercaptoethanol, and incubation was continued for 30 min at room temperature. One milliliter of lysis buffer was then added to each sample and allowed to incubate for an additional 10 min at room temperature. Each sample was split into two equal aliquots, and 50 μl of a 50% slurry of protein A–Sepharose in phosphate-buffered saline was added to each aliquot. Either 0.4 μl of ZAP-70 antiserum or control antiserum was added to each aliquot, and immunoprecipitation was allowed to proceed overnight. Immunoprecipitates were washed two times with lysis buffer, eluted with reducing sample buffer at 95°C for 5 min, and analyzed by protein immunoblotting.
22. Parallel analyses of cell lysates with the use of CD3ε antibody immunoprecipitation followed by ZAP-70 antibody blotting, and ZAP-70 antibody immunoprecipitation followed by phosphotyrosine blotting, have shown, as expected, that the amount of ZAP-70 coprecipitated with the TCR is proportional to the amount of phosphorylated ζ and, further, that the stoichiometry of ZAP-70 association with these phosphorylated ζ chains is roughly similar in agonist- and antagonist-stimulated cells. Therefore, the failure to detect phosphorylated ZAP-70 in ZAP-70 antibody immunoprecipitates from lysates of antagonist-stimulated cells (Fig. 2, top) cannot be accounted for by the existence of only a very small amount of the total ZAP-70 associated with pζ in these cells, given the amounts of pζ in the two conditions.
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29. The authors wish to especially thank L. Racioppi for his work in establishing the 3C6 antagonist system and for his helpful criticisms during this project, J. Sloan-Lancaster for helpful discussions and for sharing data before publication, and J. Bolen for ZAP antibodies.

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Cytostatic Gene Therapy for Vascular Proliferative Disorders with a Constitutively Active Form of the Retinoblastoma Gene Product

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Vascular smooth muscle cell (SMC) proliferation in response to injury is an important etiologic factor in vascular proliferative disorders such as atherosclerosis and restenosis after balloon angioplasty. The retinoblastoma gene product (Rb) is present in the unphosphorylated and active form in quiescent primary arterial SMCs, but is rapidly inactivated by phosphorylation in response to growth factor stimulation in vitro. A replication-defective adenovirus encoding a nonphosphorylatable, constitutively active form of Rb was constructed. Infection of cultured primary rat aortic SMCs with this virus inhibited growth factor–stimulated cell proliferation in vitro. Localized arterial infection with the virus at the time of balloon angioplasty significantly reduced SMC proliferation and neointima formation in both the rat carotid and porcine femoral artery models of restenosis. These results demonstrate the role of Rb in regulating vascular SMC proliferation and suggest a gene therapy approach for vascular proliferative disorders associated with arterial injury.

The arterial wall is a complex multicellular structure and is important in the regulation of inflammation, coagulation, and regional blood flow (*1*). Vascular SMCs are located predominantly in the arterial tunica media and are important regulators of vascular tone and blood pressure. These cells are normally maintained in a nonproliferative state in vivo (*1*, *2*). Arterial injury results in the migration of SMCs into the intimal layer of the arterial wall, where they proliferate and synthesize extracellular matrix components. This neointimal SMC proliferative response has been implicated in the pathogenesis of atherosclerosis (*2*). In addition, arterial injury after percutaneous balloon angioplasty of the coronary arteries results in neointimal SMC proliferation and clinically significant restenosis in 30 to 50% of patients (*3*). Many growth factors induce the proliferation of vascular SMCs in vitro and in vivo (*3*, *4*). This redundancy in growth factor signaling pathways has led to the suggestion that effective cytostatic therapies for vascular proliferative disorders should target nuclear cell cycle regulatory pathways rather than more proximal signal transduction molecules (*5*).

The Rb protein inhibits cell cycle progression in many mammalian cell types (*6*, *7*). For example, in resting (G_0) peripheral blood T cells, Rb is unphosphorylated and, in this state, binds to and inactivates a set of cellular transcription factors, including E2F and Elf-1, that are required for cell cycle progression (*8*). After T cell activation, Rb is rapidly phosphorylated and the Rb-E2F and Rb–Elf-1 complexes are thereby disrupted (*8*, *9*). The release of these transcription factors is associated with progression through the G_1/S checkpoint of the cell cycle and subsequent T cell proliferation. Given its important role in regulating cell cycle progression, we reasoned that Rb might also be an important regulator of vascular SMC proliferation.

To study the expression and regulation of Rb in vascular SMCs, we arrested cultured primary rat aortic SMCs in the G_0/G_1 phase of the cell cycle by incubating them in serum-free medium for 96 hours and then induced the cells to proliferate by exposing them to 10% fetal bovine serum (FBS). The expression and phosphorylation of Rb were assessed by immunoblot analysis of whole cell extracts. After serum withdrawal, >85% of the cultured SMCs were arrested in G_0 or G_1 of the cell cycle as assayed by propidium iodide staining and fluorescence-activated cell sorting analysis (*10*). These quiescent cells contained exclusively unphosphorylated Rb (*10*). Serum stimulation of the cells was associated with their progression into the S phase of the cell cycle, and the concomitant, progressive phosphorylation of Rb during the first 24 hours after stimulation (*10*).

To test directly the role of Rb in regulating cell cycle progression in SMCs, we constructed a replication-defective adenovirus vector, AdΔRb (*11*), that encodes a nonphosphorylatable, constitutively active form of human Rb (HAΔRb) containing a 10–amino acid, NH_2-terminal epitope tag from the influenza hemagglutinin molecule (*12*). In this vector, transcription of the Rb gene is controlled by the cellular elongation

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factor–1α gene promoter (*13*) and the 4F2 heavy chain (*14*) gene enhancer (*15*). The constitutively active HAΔRb protein has been shown previously to inhibit E2F- and Elf-1–dependent transcription in P19 cells (*12*) and T cells (*8*), respectively. In an initial series of experiments, we demonstrated that the replication-defective adenovirus could be used to transduce >90% of cultured primary rat aortic SMCs in vitro (*10*), and that infection of these cells with AdΔRb, but not with the Ad*lacZ* control virus (*11*, *16*), resulted in the expression of a 107-kD protein that reacted with both anti-hemagglutinin and anti-Rb antibodies as detected by immunoblot analysis (*10*).

To determine the effect of overexpression of HAΔRb on growth factor–stimulated SMC proliferation in vitro, we exposed quiescent rat aortic SMCs to AdΔRb [20 plaque-forming units (PFU) per cell] and then stimulated the cells to proliferate by incubating them in medium containing 10% FBS (*17*). Control cultures were infected with Ad*lacZ* or were left uninfected before serum stimulation. Serum induced rapid proliferation of the uninfected or Ad*lacZ*-infected SMCs (Fig. 1A); during the first 48 hours after stimulation, these cells underwent approximately two doublings. In contrast, infection with AdΔRb inhibited SMC proliferation by >90% (Fig. 1A). Both the Ad*lacZ*- and AdΔRb-infected cells were >97% viable at the end of the experiment as determined by trypan blue exclusion (*10*). Thus, the lack of proliferation of AdΔRb-infected cells represented cell cycle arrest as opposed to cell death.

Serum stimulation of both uninfected and Ad*lacZ*-infected SMCs was associated with marked increases in [^{3}H]thymidine incorporation during the first 24 to 48 hours (Fig. 1B). In contrast, infection with AdΔRb inhibited [^{3}H]thymidine incorporation by >90% in these cells, consistent with the hypothesis that overexpression of HAΔRb arrests SMC proliferation before entry into the S phase of the cell cycle.

Two established animal models of restenosis were used to determine if AdΔRb could inhibit vascular SMC proliferation after arterial injury in vivo. The rat carotid artery injury model represents a well-characterized, reproducible vascular proliferative disorder that is dependent on SMC migration and proliferation (*5*, *18*). Balloon angioplasty of the porcine femoral artery produces a neointimal lesion that has been used as a model of human vascular proliferative disease (*19*, *20*). Both the size and structural organization of the porcine vessel closely resemble those of human coronary arteries (*19*, *20*).

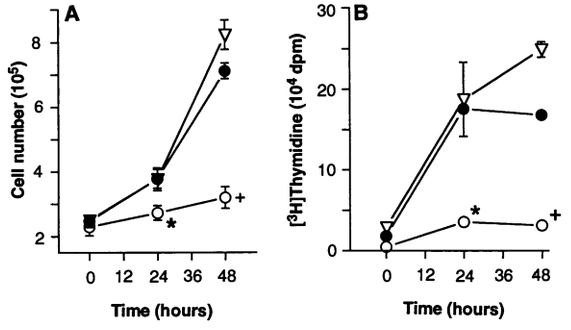
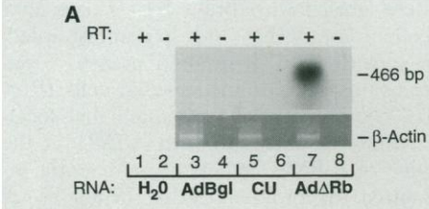


Fig. 1. Effect of AdΔRb infection on cultured primary rat aortic SMC proliferation. (**A**) Effect of AdΔRb infection on SMC number. Replicate cultures of quiescent, serum-starved rat aortic SMCs were infected with AdΔRb (○) or Ad*lacZ* (●), or were left uninfected (▽), and then stimulated to proliferate by incubation in 10% FBS (*17*). Cells were harvested 0, 24, and 48 hours after serum stimulation and cell numbers were determined with a hemocytometer. Cell viability, as assessed by trypan blue exclusion, was >97% in all cultures at the end of the experiment (*10*). Data are means ± SEM (n = 3). (**B**) Effect of AdΔRb infection on SMC DNA synthesis. Replicate cultures of quiescent, serum-starved primary rat aortic SMCs were infected with either AdΔRb or Ad*lacZ*, or were left uninfected, and then stimulated with serum as in (A). Cells were labeled for 4 hours with [^{3}H]thymidine and harvested 0, 24, and 48 hours after stimulation (*17*). Data are means ± SEM (n = 3). $^*P < 0.01$, $^+P < 0.001$ versus uninfected and Ad*lacZ*-infected cells.



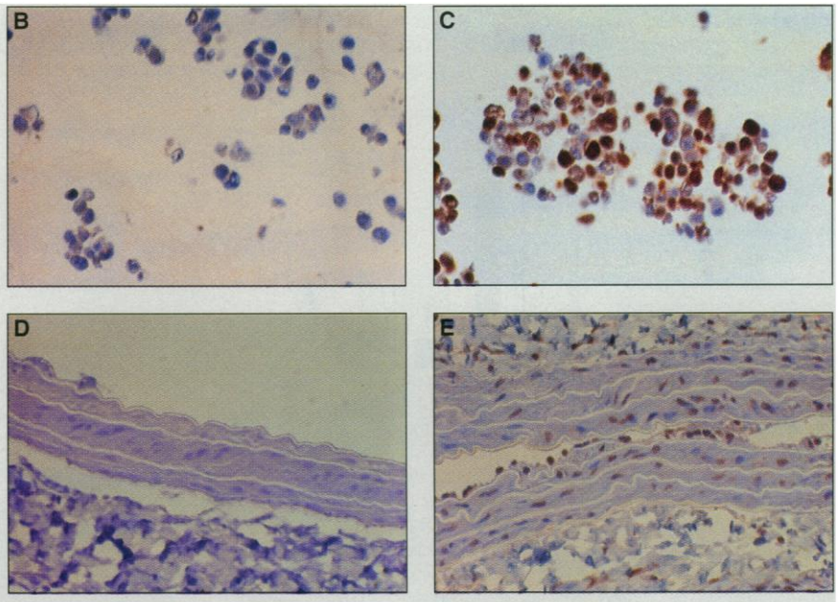


Fig. 2. HAΔRb expression in balloon-injured rat carotid arteries in vivo after local infection with AdΔRb. (**A**) RT-PCR analysis. The left carotid arteries of adult Sprague-Dawley rats were injured with a balloon catheter (*18*) and infected with either AdBgl (a replication-defective control adenovirus that does not encode a recombinant protein) (*22*) or AdΔRb (*11*). Five days after infection, these vessels and the contralateral uninfected carotid arteries (CU) were assayed for the recombinant HAΔRb mRNA by RT-PCR and Southern blot analysis (*23*). Each RNA sample was subjected to first-strand complementary DNA synthesis in the presence (+) or absence (−) of RT, followed by PCR. Negative control reactions contained water instead of arterial RNA (H_2O). A 466–base pair (bp) band corresponding to the expected size of the PCR product was detected only in the AdΔRb-infected carotid arteries (upper panel) and only in reactions that included RT. The quality of the PCR assay was ensured by subjecting all samples in parallel to RT-PCR with oligonucleotide primers complementary to mouse β-actin mRNA. A 934-bp band corresponding to the expected size of the amplified β-actin PCR product was observed in each of the RNA reactions that included RT (lower panel). (**B** through **E**) Immunohistochemical analysis of HAΔRb expression in cultured cells and rat carotid arteries after infection with AdΔRb (*24*). (B and C) Human 293 cells were infected with 20 PFU of the AdBgl control virus (B) or AdΔRb (C) per cell and stained with a mAb to HA. (D and E) The left carotid arteries of adult Sprague-Dawley rats were injured by dilatation with a balloon catheter (*18*) and infected with 2×10^9 PFU of either AdBgl (D) or AdΔRb (E) for 5 min. Five days after injury and infection, carotid arteries were fixed in situ and sections were stained with a mAb to HA.

We and others have previously demonstrated that replication-defective adenovirus vectors can be used to program high levels of expression of reporter genes, such as the alkaline phosphatase gene or *lacZ*, in a large number of medial vascular SMCs in the balloon-injured rat carotid, rabbit coronary, and porcine femoral arteries (*20–22*). To demonstrate in vivo gene transfer of HAΔRb after AdΔRb infection, we injured rat carotid arteries by balloon angioplasty and then exposed them for 5 min to 2×10^9 PFU of AdΔRb (*23*). Five days after infection, carotid arteries were assayed for HAΔRb expression by both reverse transcriptase–polymerase chain reaction (RT-PCR) analysis (*23*) and immunohistochemistry with a monoclonal antibody (mAb) specific for the hemagglutinin (HA) epitope tag (*24*). HAΔRb RNA was detected by RT-PCR in the AdΔRb-infected carotid arteries, but not in the control AdBgl virus–infected or contralateral uninfected carotid arteries (Fig. 2A). In addition, >70% of the medial vascular SMCs in the AdΔRb-infected arteries contained HAΔRb protein localized in the nucleus, as detected by dark brown nuclear staining with the anti-HA mAb (Fig. 2E). No nuclear staining was seen in AdBgl-infected carotid arteries (Fig. 2D). Moreover, similar nuclear staining was observed in >90% of cultured human 293 cells infected with AdΔRb (*24*) (Fig. 2C) but was not apparent in 293 cells infected with the AdBgl control virus (Fig. 2B). These experiments thus demonstrated efficient transfer of the HAΔRb gene into medial vascular SMCs as a result of AdΔRb infection immediately after balloon injury.

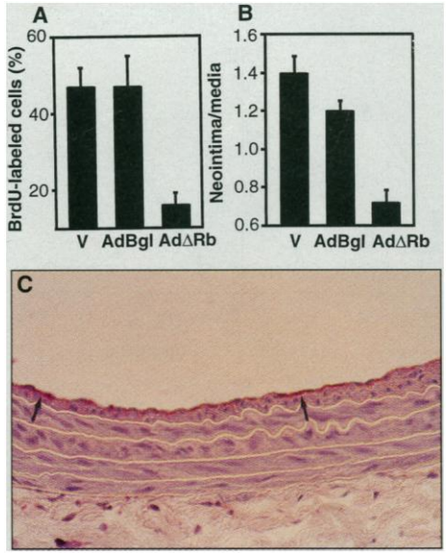


Fig. 3. Inhibition of neointima formation by AdΔRb infection after balloon injury of the rat carotid artery. (**A**) Effect of local expression of HAΔRb on vascular SMC DNA synthesis. Rat carotid arteries were injured with a balloon catheter (*18*) and exposed to either vehicle (V) (n = 5), the AdBgl control virus (n = 4), or AdΔRb (n = 5) (*25*). Animals were injected subcutaneously four times at 12-hour intervals with BrdU beginning 24 hours after carotid artery injury. Three days after injury, the carotid arteries were removed and stained with a mAb specific for BrdU (*25*). Total nuclei and BrdU-positive nuclei were counted in the media of the injured vessel walls. Values are means ± SEM. (**B**) Effect of local expression of HAΔRb on neointima formation in balloon-injured rat carotid arteries. Immediately after balloon injury (*18*), rat carotid arteries were exposed to vehicle (n = 9), or 2×10^9 PFU of AdBgl (n = 10) or AdΔRb (n = 10) (*26*). Twenty days after injury, the carotid arteries were removed and the cross-sectional areas of the neointima and media were measured by digital planimetry (*26*) in representative sections of injured vessels. Values are means ± SEM. (**C**) Reconstitution of the endothelial cell layer in AdΔRb-infected rat carotid arteries. Rat carotid arteries were injured with a balloon catheter and infected with AdΔRb as in (B). Twenty days after injury, the arteries were removed and stained with a mAb to von Willebrand factor (*24*). In all experiments, slides were coded and all determinations were performed by an observer blinded to the experimental conditions.

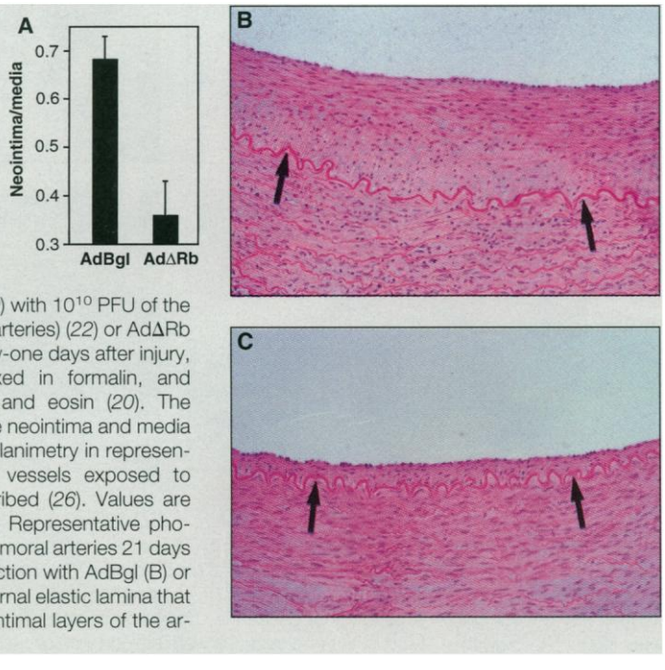


Fig. 4. Inhibition of neointima formation by AdΔRb infection after balloon angioplasty in a porcine femoral artery injury model. (**A**) The iliofemoral arteries of domestic Yorkshire pigs were injured for 5 min by balloon angioplasty as described (*20*) and infected for 20 min via a double balloon catheter (*20*) with 10^{10} PFU of the control AdBgl virus (n = 8 arteries) (*22*) or AdΔRb (n = 8 arteries) (*11*). Twenty-one days after injury, arteries were excised, fixed in formalin, and stained with hematoxylin and eosin (*20*). The cross-sectional areas of the neointima and media were measured by digital planimetry in representative sections of injured vessels exposed to AdBgl or AdΔRb as described (*26*). Values are means ± SEM. (**B** and **C**) Representative photomicrographs of porcine femoral arteries 21 days after balloon injury and infection with AdBgl (B) or AdΔRb (C). Arrows, the internal elastic lamina that separates the medial and intimal layers of the arterial wall.

To determine the effects of HAΔRb expression on restenosis, we subjected rat carotid arteries to balloon angioplasty and immediately exposed them to 2×10^9 PFU of either AdΔRb or the control AdBgl virus (*25, 26*). A third set of arteries was treated with vehicle alone. Two assays were used to measure SMC proliferation in vivo. First, to determine directly the numbers of proliferating medial SMCs, we labeled AdΔRb-infected and control arteries with 5′-bromodeoxyuridine (BrdU) 1 to 3 days after balloon injury (*5, 25, 27*). Control experiments with balloon-injured uninfected arteries demonstrated that medial SMC proliferation was maximal within 4 days after injury, with as many as 40% of the medial SMCs labeled with BrdU (*10*). As expected, neointimal SMC proliferation showed a slightly delayed time course, with ~90% of the neointimal SMCs staining with BrdU 6 days after balloon injury (*10*). Approximately 45% of the medial SMCs in the vehicle-treated or AdBgl-infected arteries were labeled with BrdU 1 to 3 days after balloon injury (Fig. 3A). In contrast, infection with AdΔRb resulted in a 67% decrease in BrdU-stained medial cells ($P <$ 0.006). These results suggested that localized arterial infection with AdΔRb at the time of balloon angioplasty efficiently inhibited the proliferation of medial SMCs before these cells had migrated into the neointima.

In a second series of experiments, restenosis, as determined by the neointima-to-media area ratio (*5, 18, 19, 27*), was measured 20 days after balloon injury in the rat carotid model. Uninfected and AdBgl-infected arteries showed ratios of 1.4 ± 0.1 and 1.2 ± 0.1, respectively (Fig. 3B). In contrast, the AdΔRb-infected arteries showed a 42% decrease in the neointima/media area ratio (0.7 ± 0.1, $P < 0.001$) relative to AdBgl-infected controls and a 50% decrease relative to uninfected control arteries. Thus, overexpression of HAΔRb after adenovirus-mediated in vivo gene transfer at the time of injury resulted in significant reductions in both vascular SMC proliferation and restenosis in the rat carotid artery model of balloon angioplasty. To determine the effects of AdΔRb infection on the endothelial cell

layer of carotid arteries after balloon angioplasty, we removed the arteries 20 days after injury and stained them with a mAb to von Willebrand factor (an endothelial cell marker) (Fig. 3C) (*24*). An intact endothelial cell layer was demonstrated by the red staining lining the luminal surface of the artery. This efficient reconstitution of the endothelial cell layer in the AdΔRb-infected, injured arterial segment may reflect the transient nature of adenovirus-mediated recombinant gene expression in vivo (*20, 22*) or the repopulation of the injured vessel segment by endothelial cells that had migrated from arterial sites located outside of the adenovirus-infected segment.

To demonstrate that the reduction in restenosis in the rat carotid artery model was not species- or model-dependent, we also tested the effects of AdΔRb infection in the porcine femoral artery model of restenosis. A double balloon catheter was used to infuse 10^{10} PFU of either AdBgl or AdΔRb into a localized segment of the femoral artery immediately after balloon angioplasty (*20*). Twenty-one days after balloon angioplasty, the AdBgl-infected arteries showed a neointima/media area ratio of 0.68 ± 0.05 (Fig. 4A). The neointima/media area ratio in AdΔRb-infected arteries (0.36 ± 0.07) was decreased by 47% ($P < 0.003$) relative to that of the AdBgl-infected controls. Thus, AdΔRb infection significantly reduced neointima formation in two different animal models of restenosis.

Previous studies have demonstrated inflammatory responses and clinical toxicity associated with the in vivo administration of replication-defective adenovirus vectors (*28*). However, in our experiments, localized arterial infection with AdΔRb did not result in increased vascular inflammation or cell necrosis relative to vehicle-treated or AdBgl-treated control arteries (Fig. 4, B and C). Moreover, serum electrolytes, liver function tests, complete blood counts, and clotting parameters were all normal in rats 20 days after intra-arterial infusions of AdΔRb (*10*). Pigs locally infected with AdΔRb showed a small decrease in serum phosphate relative to control, saline-treated animals (7.7 ± 0.4 mg/dL versus 8.8 ± 0.8 mg/dL, respectively); the reason for this decrease remains unclear. Necropsies of the AdΔRb-treated rats and pigs did not reveal significant organ inflammation or pathology (*10*). Thus, with the exception of mild hypophosphatemia, localized vascular infection with AdΔRb did not result in significant toxicity in two mammalian species.

We have demonstrated that adenovirus-mediated gene transfer of a constitutively active form of Rb is sufficient to inhibit vascular SMC proliferation and neointima formation significantly in two animal models of restenosis. Previous studies have suggested that antisense oligonucleotides directed to c-Myb or to proliferating cell nuclear antigen (PCNA) and Cdc2 transcripts also may be effective inhibitors of restenosis in vivo (*5*). In addition, three recent studies have demonstrated a reduction in restenosis as a result of in vivo transfer of the herpes simplex virus thymidine kinase (HSV TK) gene to the arterial wall followed by the systemic administration of ganciclovir (*20, 29*). The inhibition of restenosis produced by intravascular infection with AdΔRb in our study (50%) is comparable to that seen after intraluminal administration of antisense oligonucleotides to PCNA and Cdc2 transcripts (*5*) as well as to that observed after HSV TK gene transfer and systemic ganciclovir treatment in both the rat and pig models (*20, 29*). However, from a therapeutic standpoint, the use of localized AdΔRb infection has several advantages over antisense oligonucleotide approaches or cytotoxic therapies such as HSV TK gene transfer and systemic ganciclovir treatment. First, recent studies have suggested that the growth inhibitory effects of antisense oligonucleotides may, in many instances, reflect nonspecific activities of these agents, including their ability to cause degradation of multiple RNA species and their ability to bind to intracellular and cell surface proteins in a sequence-nonspecific manner (*30*). Some of these nonspecific activities may vary markedly between different preparations of the same oligonucleotide (*30*). In addition, it has been difficult to deliver antisense oligonucleotides stably and efficiently to the vessel wall with the use of percutaneous catheter-mediated approaches in the absence of viral vectors or extravascular biodegradable matrices (*5*). In this regard, it is important that we have demonstrated the efficacy of intraluminal AdΔRb infection in two animal models of restenosis in experiments performed in two laboratories with different batches of AdΔRb. Our observation that AdΔRb infection inhibits medial vascular SMC proliferation as early as 1 to 3 days after injury suggests that this virus can inhibit restenosis before the proliferating medial SMCs have migrated into the neointima of the injured arterial segment. Moreover, unlike cytotoxic protocols such as those involving the HSV TK gene (*20, 29*), cytostatic therapy with the Rb gene has the potential advantage of arresting cell cycle progression without inducing cell death or inflammation in the vessel wall. Of equal importance, our approach does not require systemic ganciclovir therapy, which has been associated with neutropenia, thrombocytopenia, and ventricular arrhythmias in humans (*31*).

The classical tumor suppressor genes, *p53* and *Rb*, have each been shown to play important roles in regulating cell cycle progression in several mammalian cell types (*5–7, 32*). Moreover, recent evidence suggests that p53-dependent G_1 arrest of cell cycle progression is mediated, at least in part, through Rb or Rb-like proteins (*33*). The inactivation of Rb or p53, either by mutation or by viral oncoproteins, results in unregulated proliferation and tumorigenesis in both animals and humans (*34, 35*). We have now demonstrated that Rb also plays a critical role in regulating the proliferation of vascular SMCs, both in response to growth factor stimulation in vitro and to injury in vivo. These results suggest significant parallels between the molecular mechanisms that underlie carcinogenesis and those responsible for human vascular proliferative disorders such as atherosclerosis and restenosis. In this regard, in vivo gene transfer of constitutively active negative regulators of the cell cycle into various cell types may prove efficacious for the treatment of a variety of human diseases associated with uncontrolled cellular proliferation.

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11. Adenovirus vectors were constructed by recombination in 293 cells between plasmid DNA and E1- and E3-deleted Ad5Sub360 adenovirus DNA digested with Xba I and Cla I, as described (*15, 22*). Recombinants were plaque-purified three times to avoid contamination with replication-competent virus. High-titer adenovirus stocks were prepared by infecting 293 cells with two to five plaque-forming units (PFU) of virus per cell. Viral lysates were purified by centrifugation in discontinuous CsCl gradients (*22*). Viral titers were determined from the absorbance at 260 nm of the purified virus preparation (1 absorbance unit = 10^{10} PFU/ml). AdΔRb encodes a hemagglutinin (HA) epitope–tagged, nonphosphorylatable mutant form of the human Rb gene product (HAΔRb) (*12*) under transcriptional control of the human elongation factor–1α gene promoter (*13*) and the human 4F2 heavy chain gene transcriptional enhancer (*14*). Ad*lacZ* contains the bacterial β-galactosidase (*lacZ*) gene under transcriptional control of

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17. Primary rat aortic SMCs were isolated and grown as described [R. S. Blank, M. M. Thompson, G. K. Owens, *J. Cell Biol.* **107**, 299 (1988)]. SMCs from the third passage were placed in serum-free medium [50% Dulbecco's minimum essential medium (DMEM), 50% Ham's F-12, L-glutamine (292 mg/ml), insulin (5 mg/ml), transferrin (5 mg/ml), selenious acid (5 ng/ml)] for 48 hours and then infected with 20 PFU of Ad*lacZ* or AdΔRb per cell for 1 hour. Twenty-four hours after infection, the cells were stimulated to proliferate by incubation in medium comprising 45% DMEM, 45% Ham's F-12, and 10% FBS. For [^{3}H]thymidine assays, cultured SMCs were labeled for 4 hours with medium containing [*methyl*-^{3}H]thymidine (1 μCi/ml) (5 Ci/mmol; Amersham) as described [G. K. Owens, A. Loeb, D. Gordon, M. M. Thompson, *J. Cell Biol.* **102**, 343 (1986)]. [^{3}H]thymidine incorporation was determined with a Packard model 1900 TR liquid scintillation spectrophotometer.
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23. The left carotid arteries of adult Sprague-Dawley rats were injured by dilatation with a Fogarty catheter as described (*18*). After balloon injury, the instrumented carotid artery segments were isolated with microvascular clamps. A 24-gauge intravenous catheter was introduced into the lumen of each isolated segment and 2 × 10^9 PFU of either AdBgl or AdΔRb were instilled for a 5-min period. Five days after injury and infection, the rats were killed and the carotid arteries removed. RNA was prepared by the acid guanidinium-phenol method [P. Chomczynski, *Biotechniques* **15**, 532 (1993)]. To ensure that the RNA samples were free of DNA contamination, we subjected them to digestion with 10 U of deoxyribonuclease I for 30 min at 37°C. First-strand complementary DNA (cDNA) synthesis reactions were performed with 1 μg of RNA and a commercially-available kit (Perkin Elmer, Norwalk, CT) in the presence or absence of reverse transcriptase [B. A. Karpinski *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 4820 (1992)]. The polymerase chain reaction was performed as described previously (*22*) with primers specific for the HAΔRb cDNA [5′-AAGCTTCCCGGGGAATTCACCATGGGGTACCCATACGATGTTCCAGATTACG (sense) and 5′-ATAGCATTATCAACCTTGGTACTGG (antisense)] or the mouse β-actin cDNA [5′-GTGACGAGGCCCAGAGCAAGAG (sense) and 5′-AGGGGCCGGACTCATCGTACTC (antisense)]. Southern (DNA) blot analysis was performed with a ^{32}P-labeled probe corresponding to nucleotides 1 to 392 of the HAΔRb cDNA (*22*). All animal experimentation was performed in accordance with NIH guidelines in the A. J. Carlson Animal Research Facility of the University of Chicago.
24. Rat carotid arteries were removed 5 or 20 days after balloon injury and infection with AdBgl or AdΔRb and were embedded in paraffin. Human 293 cells were infected with AdBgl or AdΔRb (20 PFU per cell), and 24 hours later cells were fixed in 4% paraformaldehyde and cell pellets were embedded in paraffin. Paraffin was then removed from 5-μm-thick sections of the carotid arteries or cell pellets and the sections were hydrated and treated with H_2O_2 for 4 min at 42°C to inactivate endogenous peroxidases. Immunohistochemistry was performed with a Ventana ES-320 automated immunostainer (Ventana Medical Systems, Tucson, AZ). Slides were stained with a mAb to von Willebrand factor (Dako, Santa Barbara, CA) or a mAb (12CA5) to HA (Boehringer-Mannheim, Indianapolis, IN) for 32 min at 42°C. Slides were then exposed to biotinylated antibodies to mouse immunoglobulin G (Ventana). Slides stained for von Willebrand factor were finally treated with avidin-conjugated alkaline phosphatase and fast red–naphthol (Ventana), and those stained for HA were exposed to avidin-conjugated horseradish peroxidase and diaminobenzidine plus copper (Ventana) and lightly counterstained with hematoxylin.
25. Carotid arteries of adult rats were injured with a balloon catheter (*18*) and immediately infected with 2 × 10^9 PFU of either AdBgl or AdΔRb, or exposed to vehicle (Hepes-buffered saline) alone. Animals were injected subcutaneously with 5′-bromodeoxyuridine (25 mg per kilogram of body mass) at 12-hour intervals, starting 24 hours after injury, for a total of four doses. Carotid arteries were fixed in situ by intravascular administration of 4% paraformaldehyde, embedded in paraffin, and sectioned. Sections (5 μm thick) from which paraffin had been removed were treated with 3% H_2O_2 in methanol and permeablized by incubation in 0.4% pepsin and 3.3 M HCl. Treated sections were exposed to 1.5% horse serum to block nonspecific sites, and incubated sequentially with a 1:100 dilution of a mAb to 5′-bromodeoxyuridine (Becton-Dickinson, San Jose, CA), a 1:200 dilution of biotinylated horse antibodies to mouse immunoglobulin, and avidin-conjugated horseradish peroxidase (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA). Sections were finally treated with diaminobenzidine and counterstained with hematoxylin and eosin. At least 500 nuclei were quantitated in two sections from the balloon-injured and infected arterial segment from each animal. Statistical analyses were performed with Sigmaplot (Jandel Scientific, Corte Madera, CA).
26. Rat carotid arteries were injured with a balloon catheter (*18*) and treated with vehicle alone or infected with 2 × 10^9 PFU of AdBgl or AdΔRb. Twenty days after injury, carotid arteries were removed and tissue sections were stained with hematoxylin and eosin. Neointimal and medial boundaries were determined by digital planimetry of tissue sections with the MOCHA program (Jandel) on a Gateway 486 computer. The neointimal and medial cross-sectional areas were measured from six sections of each artery spanning the 1-cm site of balloon injury and infection, and the mean of these six determinations was used to calculate the neointimal-to-medial area ratio for each animal.
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Molecular Basis of the *cauliflower* Phenotype in *Arabidopsis*

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Genetic studies demonstrate that two *Arabidopsis* genes, *CAULIFLOWER* and *APETALA1*, encode partially redundant activities involved in the formation of floral meristems, the first step in the development of flowers. Isolation of the *CAULIFLOWER* gene from *Arabidopsis* reveals that it is closely related in sequence to *APETALA1*. Like *APETALA1*, *CAULIFLOWER* is expressed in young flower primordia and encodes a MADS-domain, indicating that it may function as a transcription factor. Analysis of the cultivated garden variety of cauliflower (*Brassica oleracea* var. *botrytis*) reveals that its *CAULIFLOWER* gene homolog is not functional, suggesting a molecular basis for one of the oldest recognized flower abnormalities.

In *Arabidopsis*, the genes that determine an early event of flower development, the specification of floral meristem identity, include *CAULIFLOWER* (*CAL*), *APETALA1* (*AP1*), and *LEAFY* (*LFY*) (*1*, *2*). In *ap1* single mutants, sepals are replaced by leaf-like organs, and petals generally fail to initiate (*3*). Axillary floral meristems arise at the base of these leaf-like organs, producing secondary flowers that resemble the phenotype of the primary *ap1* mutant flower (Fig. 1B). When the *ap1* mutation is combined with mutations in *CAL*, cells that would normally constitute a floral meristem instead behave as an inflorescence meristem, giving rise to additional meristems in a spiral phyllotaxy (*2*). The resulting *cauliflower* phenotype has an extensive proliferation of meristems at each position that in wild-type

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