Engineering the Response to Vascular Injury
Divergent Effects of Deregulated E2F1 Expression on Vascular Smooth Muscle Cells and Endothelial Cells Result in Endothelial Recovery and Inhibition of Neointimal Growth

David A. Goukassian, Raj Kishore, Kevin Krasinski, Christine Dolan, Corinne Luedemann, Young-sup Yoon, Marianne Kearney, Allison Hanley, Hong Ma, Takayuki Asahara, Jeffrey M. Isner,† Douglas W. Losordo

Abstract—Tumor necrosis factor-α (TNF-α) is expressed locally in the vessel wall after angioplasty and induces growth arrest and apoptosis in endothelial cells (ECs), thereby delaying reendothelialization. Prior studies have shown that direct antagonism of TNF-α, using a systemically administered soluble receptor, can enhance endothelial recovery and reduce neointimal thickening. These studies have also shown that downregulation of the transcription factor E2F1 was a key mechanism of TNF’s effect on ECs. We now show that Ad-E2F1 overexpression at sites of balloon injury accelerates functional endothelial recovery, consistent with the prior in vitro findings. Moreover these studies also reveal divergent effects of TNF-α and overexpression of E2F1 on ECs versus VSMCs. TNF-α exposure of VSMCs had no affect on proliferation or apoptosis, in contrast to the effect seen in ECs. In Ad-E2F1–transduced VSMCs, however, TNF-α–induced marked apoptosis in contrast to the survival effect seen in ECs. Finally, these studies suggest that differential activation of NF-κB may play a key role in mediating these opposing effects. Nuclear translocation and transcriptional activity of NF-κB was markedly attenuated in Ad-E2F1–transduced VSMCs, whereas it remained active in similarly treated ECs when the cells were exposed to TNF-α. These studies reveal that overexpression of Ad-E2F1 primes VSMCs to TNF-α–induced apoptosis. Furthermore, E2F1 potentiates VSMC death by blocking antiapoptotic signaling pathway through inhibition of NF-κB activation. The divergent responses of VSMCs and ECs to E2F1 overexpression provide unique therapeutic possibilities: simultaneously targeting the cell cycle of two different cell types, within same tissue microenvironment resulting in opposite and biologically complimentary effects. (Circ Res. 2003;93:162-169.)

Key Words: apoptosis ■ vascular smooth muscle cells ■ E2F1 ■ tumor necrosis factor-α ■ nuclear factor-κB

Vascular injury stimulates proliferation and migration of vascular smooth muscle cells (VSMCs), which accumulate in the intima of the injured site. In response to vascular injury, proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), are produced by activated macrophages as well as by VSMCs themselves.1 We have previously shown that TNF-α induces apoptosis in proliferating endothelial cells (ECs) via a mechanism involving repression of E2F1 activity. Adenovirus-mediated restoration of E2F1 activity rescued ECs from TNF-α–induced cell cycle arrest and apoptosis.2 Recently, we also showed that in vivo blockade of TNF-α accelerates functional endothelial recovery and inhibits neointimal lesion formation after balloon angioplasty.3 These findings indicate that TNF-α expressed locally at sites of balloon arterial injury mediates, at least in part, a delay in endothelial recovery and the associated neointimal lesion formation. These findings also imply that repression of E2F1 activity is a crucial pathway in TNF-α–induced inhibition of endothelial recovery.

To determine if overexpression of E2F1 in vivo would result in restoration of endothelial recovery similar to the documented in vitro effect, we performed an investigation using the rat carotid injury model in which E2F1 was constitutively overexpressed using the same adenoviral vector used for the in vitro studies. These studies revealed acceleration of endothelial recovery consistent with the survival effect shown in vitro. Moreover, these studies also showed attenuation of VSMCs in the neointima. Accordingly,
we performed a series of in vitro studies to determine the mechanism of the apparently divergent effect of deregulated E2F1 expression on VSMCs and ECs in the setting of TNF-α exposure. In the present study, we show that E2F1 induces VSMCs apoptosis via activation of caspase-dependent pathways. In addition, we also show that E2F1 induces VSMCs apoptosis via downregulation of NF-κB signaling pathway.

Materials and Methods

Rat Carotid Balloon Injury Model
Balloon carotid injury was performed in Sprague-Dawley rats (Charles River Labs, Wilmington, Mass) as previously described. After denuding injury, Ad-E2F1 or Ad-β-gal was infused into the isolated carotid segment as described (see online data supplement). All animal protocols were approved by the Institutional Animal Care and Use Committee at the St Elizabeth’s Medical Center.

Mouse Carotid Balloon Injury Model
To further examine the role of E2F1 in reendothelialization after balloon injury, we also performed a series of experiments in a mouse model of carotid injury. The mouse carotid injury model, modified in our laboratory from previous methods was used.

Evaluation of Reendothelialization
Reendothelialization was assessed by staining with 0.5% Evans blue dye (Sigma Chemical Co). To verify that the Evans blue stain accurately depicted the presence or absence of endothelium, sections of completely or partially reendothelialized carotid arteries (based on Evans blue appearance) were stained with antibodies to CD31, BS1 Lectin, β-gal, SM-α-Actin, and Factor VIII.

Evaluation of Recovery of Endothelial Function
In order to determine if functional recovery of the endothelium was accelerated by Ad-E2F1 treatment, the production of nitric oxide by excised arterial segments was measured using the Greiss reaction.

Evaluation of Intimal Hyperplasia
Neointimal thickening was evaluated by measuring the total area of neointima in longitudinal sections of elastic-trichrome stained arteries. The area of the media was also measured and the intima/media ratio was calculated.

Evaluation of Proliferation in Injured Arteries
Bromodeoxyuridine (BrdU) (30 mg/kg) (Amersham) was administered by intraperitoneal injection every 12 hours for 48 hours before animal euthanasia (4 injections total). Rat carotid artery segments to be used for immunohistochemistry were perfusion fixed as previously described. Sections of completely or partially reendothelialized carotid arteries (based on Evans blue appearance) were stained with antibodies to CD31, BS1 Lectin, β-gal, SM-α-Actin, and Factor VIII.

FACS Analysis
Subconfluent VSMCs were synchronized with medium containing 0.5% FBS for 48 hours. Cells were infected with Ad-E2F1 and Ad-β-gal while they were serum-synchronized. Six hours after transduction, cells were released from quiescence by replacing medium with 10% serum containing or not 40 ng/mL TNF-α and harvested at indicated times. Cells were then processed for labeling with FITC-conjugated anti-active caspase 3 antibody (Transduction Laboratories). A second set of similarly treated cells were processed for the detection of apoptosis (annexin V) using commercially available Vibriante Kit (Molecular Probes). Cells were labeled and then fixed according to manufacturer recommendations and analyzed using a FACSscan (Becton Dickinson) flow cytometer.

Western Blot Analysis
VSMCs synchronized for 2 days were stimulated with medium containing 10% FBS. Six hours before serum stimulation and TNF-α addition, BAEcs and VSMCs were infected with 30 MOI of either Ad-E2F1 or Ad-β-gal. Cells were harvested 0.5 and 2.5 hours after serum/TNF-α stimulation (6.5 and 8.5 hours after AdE2F1 transduction), and E2F1 expression was assessed by Western blot analysis. For evaluation of IκBα degradation, VSMCs and ECs were treated as described above for FACs analysis. At designated time points, cells were lysed and processed for Western blot analysis using anti–IκBα antibodies (both, 1:200 dilution).

Confocal Microscopy
Six hours before TNF-α exposure, VSMCs were infected with 30 MOI of either Ad-E2F1 or Ad-β-gal. For immunofluorescent studies, control and Ad-E2F1-treated and Ad-β-gal–treated VSMCs and HUVECs were serum stimulated in the presence or absence of TNF-α for 30 minutes. Cell monolayers were washed twice with 1×PBS/1%BSA then fixed with ice-cold methanol-acetone (1:1) at −20°C for 10 minutes and air-dried. Next, fixed cells on the chamber slides were reacted with NF-κB (p65) rabbit polyclonal antibody (1:50) for 1 hour at room temperature. After washing with PBS, cells were incubated for 45 minutes with FITC-conjugated secondary antibodies (1:100) (Santa Cruz). Chambers were then removed and slides were mounted with glass cover slips containing 10 μL of Vectashield (Vector, UK).

Transient Transfection Assays
Transient transfection assays, using a luciferase reporter containing the NF-κB cis-acting enhancer element (Clontech), were performed to evaluate NF-κB–dependent transcriptional activation. BAEcs and E19P cells were co-transfected with NF-κB reporter and control pSVAPAP alkaline phosphatase (AP) with Superfect reagent according to the manufacturer guidelines (QIAGEN). After 16 hours of incubation at 37°C, cells were washed with PBS, trypsinized, pooled, and seeded. After 24 hours, cells were serum-starvation synchronized for 48 hours. Six hours before serum stimulation and TNF-α addition, cells were infected with Ad-E2F1 and Ad-β-gal. Cells were harvested at indicated times, lysed with 100 μL reporter lysis buffer (Promega) and processed for measurement of luciferase activity as described. Luciferase activity was normalized relative to the level of (AP) activity produced from co-transfected pSVAPAP plasmid as described previously.
Deregulated E2F1 expression accelerates endothelial recovery after balloon angioplasty. A, Compared with control, Ad-β-gal–transduced arteries, at 1 week and 2 weeks after injury, overexpression of E2F1 accelerates reendothelialization (ReEndo) measured as absolute area reendothelialized. B, Nitric oxide production was measured 1 week after injury. C, Wire injury was performed in 20 E2F1−/− and 20 wild-type 129 mice (genetic background of the E2F1−/−) After 7 days, Evans Blue solution was infused, and the arteries were harvested with care taken to identify the injured vessel segment according to anatomic landmarks. Quantification of ReEndo by an investigator blinded to the genotype of the animals revealed no difference in ReEndo in animals with wild-type or null expression of E2F1.

Statistical Analysis

To evaluate statistical significance of differences for cell proliferation and apoptosis studies between experimental groups, ANOVA with Fisher PLSD analysis were performed using StatView statistical program (SAS Inc). Statistical significance was assigned when \( P<0.05 \).

An expanded Materials and Methods section is available in the online data supplement at http://www.circresaha.org.

**Results**

Ectopic E2F1 Overexpression Accelerates Functional Endothelial Recovery After Balloon Injury

Quantification of endothelial recovery was performed by measuring the Evans blue–stained region and comparing it to the total area of injury based on standard anatomic landmarks on the excised rat carotid arteries. At both 1 and 2 weeks after injury, endothelial recovery was greater in arteries transduced with Ad-E2F1 versus Ad-β-gal (% injured area reendothelialized at 1 week, E2F1=57.0±3.1% versus β-gal=36.9±5.4%, \( P<0.03 \); at 2 weeks, E2F1=86.2±2.6% versus β-gal=68.6±3.4%, \( P<0.03 \) (Figure 1A). The improved anatomic recovery of the endothelium was accompanied by functional endothelial recovery as documented by increased NO production in E2F1 versus control treated arteries; 2.07±0.17 versus 1.02±0.51 nmol/L per mm2 per 15 minutes; \( P<0.0001 \) (Figure 1B). The role of E2F1 in endothelial recovery was further explored by comparing endothelial recovery in wild-type (WT) and E2F1−/− mice, revealing no difference in the rate of reendothelialization (Figure 1C). This finding is compatible with functional silencing of E2F1 in WT mice at sites of arterial injury and is consistent with our hypothesis that TNF represses E2F1 activity.

Deregulated E2F1 Expression Reduces Neointimal Thickening and Decreases Neointimal VSMC Proliferation After Balloon Injury

In contrast to the findings of increased endothelial recovery,2 VSMC proliferation was adversely affected at sites of balloon injury by overexpression of E2F1. The intima/media ratio was reduced significantly in arteries in which E2F1 was overexpressed (\( P<0.0057 \)) (Figure 2A), and this was associated with a decrease in medial cellularity noted at early time points. BrdU labeling revealed evidence of decreased proliferative activity in arteries in which E2F1 was overexpressed (Figure 2B). Thus, in contrast to endothelial cells, in which deregulated E2F1 expression in the setting of TNF-α exposure rescued ECs from apoptosis and cell cycle arrest, in VSMC E2F1 overexpression appeared to have the opposite effect. To further investigate this possibility, a series of in vitro investigations were performed.

E2F1 Cooperates With TNF-α to Inhibit VSMC Cell Cycle

Ad-E2F1 transduction of BAECs and VSMCs led to the time-dependent increase in the level of E2F1 protein that was confirmed by Western blot analysis (Figure 3A). More importantly, exogenous E2F1 protein levels were comparable to wild-type (WT) and E2F1−/− mice, revealing no difference in the number of VSMCs. To assess the distribution of cells at different phases of the cell cycle, FACS analysis of VSMCs stained with propidium iodide was used. No significant difference in the cell cycle distribution of cells was observed in serum-stimulated, TNF-α−, and combined TNF-α/Ad-β-gal–treated cells (Figure 3C). In contrast, compared with TNF-α treatment alone, the number of cells in sub-G1/G0 in combined TNF-α/Ad-E2F1–treated cells rose to 8%, a more than 3-fold increase (\( P<0.03 \)). In addition, Ad-E2F1 treatment significantly inhibited entry of cells into G1/M phase of

*Figures A, B, and C*
but could not be identified in Ad-E2F1–infected arteries (B and C) and treated cells, annexin V positivity was markedly increased in VSMCs that were treated with TNF-α/Ad-E2F1 (P<0.001 at 18 hours; Figure 4C). The decrease in VSMC viability resulting from E2F1 overexpression was in sharp contrast to the enhanced survival seen in similarly treated ECs. To better understand the potential mechanisms responsible for this effect, we examined the activity of NF-κB, a transcription factor known to play an essential role in TNF-α-mediated cell survival pathways. TUNEL staining for apoptosis in vivo yielded very low rates of positivity in both Ad-E2F1– and Ad-β-gal–treated animals. Others have documented dramatic onset and rapid decrease of apoptosis within the first few hours after balloon injury. In our studies, we concentrated on later time points and could detect in vivo a significant rate of apoptosis. Indeed, given the transient nature of apoptosis in vivo, a significant rate sustained for any period of time would ultimately emaciate the medial layer. This suggests that the effects of TNF-α and E2F1 on proliferation may predominate in vivo.

**Overexpression of E2F1 Induces Apoptosis in TNF-α–Exposed VSMCs**

Flow cytometric analysis using antibodies to active caspase 3 revealed a marked increase in apoptosis in VSMCs in which E2F1 was overexpressed. Eighteen hours after treatment, 39±9% of cells treated with TNF-α/Ad-E2F1 were stained positively for active caspase 3 (Figures 4A and 4B). In comparison, only 13±8%, 3.5±2%, and 6±3.8% of serum–, TNF-α–, and TNF-α/Ad-β-gal–treated cells, respectively, were stained positive with active caspase 3 (P<0.02, TNF-α/Ad-E2F1 versus all other treatments). These findings suggest that the reduction in cell number documented in vivo and in vitro was not solely due to the inhibition of proliferation but was also the result of E2F1-mediated effects on VSMC viability. These findings were further substantiated by annexin V staining for apoptotic cells. Compared with TNF-α– and TNF-α/Ad-β-gal–treated cells, annexin V positivity was markedly increased in VSMCs that were treated with TNF-α/Ad-E2F1 (P<0.001 at 18 hours; Figure 4C). The decrease in VSMC viability resulting from E2F1 overexpression was in sharp contrast to the enhanced survival seen in similarly treated ECs. To better understand the potential mechanisms responsible for this effect, we examined the activity of NF-κB, a transcription factor known to play an essential role in TNF-α-mediated cell survival pathways. TUNEL staining for apoptosis in vivo yielded very low rates of positivity in both Ad-E2F1– and Ad-β-gal–treated animals. Others have documented dramatic onset and rapid decrease of apoptosis within the first few hours after balloon injury.

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**Overexpression of E2F1 Abrogates NF-κB Nuclear Translocation in TNF-α–Exposed VSMCs**

Nuclear translocation of NF-κB (p65) was studied at a single-cell level by confocal microscopy in VSMCs and ECs. In quiescent HUVECs and VSMCs, distribution of NF-κB was predominantly cytoplasmic (Figures 5a and 5f, respectively). Serum stimulation did not alter NF-κB cellular localization in HUVECs, whereas in serum-stimulated VSMCs (30 minutes after serum addition), localization of NF-κB was predominantly nuclear (95±5%) (Figures 5b and 5g). Stimulation of HUVECs and VSMCs with TNF-α for 30 minutes resulted in a marked nuclear translocation of NF-κB in 100±0% of both cell types (Figures 5c and 5h). After combined TNF-α/Ad-E2F1 treatment of HUVECs for 30
minutes, NF-κB remained translocated to the nucleus in 100% of cells (Figure 5d). In contrast, nuclear translocation of NF-κB was completely abrogated when E2F1 was overexpressed in TNF-α-exposed VSMCs (Figure 5i). In both cell types, TNF-α/Ad-β-gal–treated cells manifested similar degrees of NF-κB nuclear translocation to serum only and/or serum/TNF-α–treated cells (Figures 5e and 5j).

Overexpression of E2F1 Inhibits IkB-α Degradation in TNF-α–Exposed VSMCs
IkB-α degradation was studied by Western blot analysis in BAECs versus VSMCs in the setting of combined TNF-α/AdE2F1 treatment. Serum stimulation led to rapid degradation of IkB-α in VSMCs (15 minutes), whereas in serum-stimulated BAECs, IkB-α levels were comparable to levels of quiescent cells (Figure 6). Treatment with TNF-α alone or in combination with Ad-β-gal led to comparable IkB-α degradation in VSMCs, as well as in ECs; however, IkB-α degradation was more prominent in VSMCs. Interestingly, these two cell types responded differently to the combined TNF-α/AdE2F1 treatment. In VSMCs, E2F1 overexpression inhibited IkB-α degradation, thereby preventing NF-κB activation in VSMCs, whereas E2F1 overexpression led to the rapid IkB-α degradation (TNF-α and/or TNF-α/Ad-β-gal treatments) in ECs (Figure 6), suggesting a possible mechanism of divergent effects of E2F1 overexpression in VSMCs versus ECs.

Overexpression of E2F1 Abrogates NF-κB Transcriptional Activity in VSMCs but Remains Intact in ECs
To verify that the alterations in NF-κB translocation were associated with a functional change in NF-κB–mediated transcriptional activity, we performed a series of transient transfection assays using a luciferase reporter construct containing the NF-κB cis-acting enhancer element. VSMCs and ECs were co-transfected with the reporter construct and alkaline phosphatase control plasmid (pSVAPAP). As expected the lowest NF-κB activity was detected in serum-starved ECs and VSMCs (Figures 7A and 7B). Compared with quiescent, in serum-stimulated VSMCs, the NF-κB reporter activity increased 4-fold, whereas NF-κB activity was not changed in ECs on serum stimulation. In contrast, TNF-α exposure of proliferating ECs and VSMCs led to 6- and 4.5-fold increases (versus quiescent), respectively, in NF-κB transcriptional activity (Figures 7A and 7B). However, in VSMCs overexpressing E2F1, NF-κB activation was completely abolished (Figure 7A), whereas NF-κB transcriptional activities were preserved in ECs under similar conditions (Figure 7B). These findings suggest a divergence in the signaling pathways triggered by overexpression of E2F1 in VSMCs versus ECs.

Discussion
Radiation therapy is currently available for inhibition of restenosis in stented arteries. Although effective in reducing...
the incidence of recurrent neointimal proliferation, this strategy is compromised by delayed reendothelialization, which results in a significant incidence of late stent thrombosis. More recently, a great deal of enthusiasm has been generated by reports that rapamycin and other cell cycle inhibitors are capable of eliminating restenosis. Because this chemotherapy strategy shares with radiation therapy an approach that is not designed to enhance endothelial recovery, it would come as no surprise to learn of late events due to endothelial dysfunction. Indeed, recent reports indicate a distinct antien-
dothelial action of rapamycin. In contrast to these prior strategies, the present studies suggest that, by exploiting certain signaling pathways in ECs and VSMCs, it may be possible to develop an approach to restenosis that is capable of inhibiting neointimal thickening while simultaneously encouraging recovery of a functional endothelium.

In response to vascular injury, proinflammatory cytokines, such as TNF-α, are produced by activated macrophages as well as by VSMCs themselves. Locally released TNF-α can regulate gene expression, differentiation, growth, and apoptosis of ECs and VSMCs at sites of vascular injury. The clinical relevance of TNF-α expressed at sites of balloon injury was documented in our earlier studies showing acceleration of reendothelialization in injured vessels after TNF-α soluble receptor-mediated blocking of TNF-α. We have also previously shown that TNF-α inhibited E2F1 expression and activity in vitro, accompanied by cell cycle arrest and enhanced apoptosis in proliferating ECs. Overexpression of E2F1 restored EC proliferation and inhibited apoptosis despite TNF-α exposure. The present studies reveal that the in vitro survival effect of E2F1 on ECs translates in vivo into enhanced endothelial recovery at sites of balloon injury.
Moreover, and perhaps more important, they document a divergence in the TNF-α/E2F1 signaling pathways of ECs and VSMCs under similar external cues. Instead of augmenting VSMC survival and proliferation as it did in ECs, E2F1 overexpression induces apoptosis and inhibits VSMC cell cycle progression. These findings thus identify a unique therapeutic approach: simultaneously targeting the cell cycle of two different cell types, within the same tissue microenvironment, via a pathway which results in opposite and biologically complimentary effects.

E2F1, a member of the E2F family of transcription factors plays a major role in regulating a diverse array of cellular functions including gene expression, proliferation, differentiation, and apoptosis. These differential effects of E2F1, however, appear to be cell and stimulus specific. Differential regulation of cell proliferation and apoptosis by deregulated E2F1 in ECs and VSMCs shown in this study is supported by other studies reporting similar disparate effects of E2F1 on these cellular functions in different cell types. Overexpression of E2F1 either promoted S-phase entry and proliferation or apoptosis. Our finding that overexpression of E2F1 enhances VSMC apoptosis is also in agreement with a recent study in which E2F1 induced caspase 3-like activity and initiated apoptosis in coronary VSMCs. This study, however, differs from ours in the unique context of TNF-α exposure that mimics the in vivo arterial injury environment. Thus, our study demonstrates a negative, cooperative effect of TNF-α and E2F1 overexpression on VSMC proliferation and survival, because within the time frame of our experiments, TNF-α alone did not have a significant effect on VSMC survival. Additionally, no previous study has documented a biologically different response to TNF-α/E2F1 by two neighboring cell types within the same tissue.

Transmembrane signaling after TNF-α receptor binding triggers cellular apoptosis in some cancer cells and endothelial cells yet induces proliferation in normal diploid fibroblasts. Unresponsiveness of VSMCs to TNF-α seen in our study is supported by the evidence from other studies showing that TNF-α itself has little effect on the growth or apoptosis of VSMCs. The conclusion that E2F1 overexpression–mediated apoptosis in VSMCs results from a synergistic effect of E2F1/TNF-α is further strengthened by the data showing a significant increase in the activation of caspase 3, a marker for apoptotic cells, in Ad-E2F1–transduced VSMCs exposed to TNF-α (Figures 4A and 4B).

TNF-α signaling involves activation of various secondary messengers that in turn directly or indirectly lead to the activation of NF-κB, which then transcriptionally induces many genes. NF-κB has been also implicated in atherosclerosis because activated NF-κB is present in atherosclerotic lesions but not in normal tissues. In addition, in the rat model of arterial injury, NF-κB activity was induced at the time of rapid proliferation of VSMCs and neointima formation after balloon angioplasty. It is therefore conceivable that E2F1 overexpression–mediated inhibition of VSMC proliferation might reflect modulations in the NF-κB activity. Indeed, there is ample evidence suggesting E2F1-mediated inhibition of NF-κB and a consequential increase in apoptosis in various cell types. Mechanisms proposed for this inhibition, however, remain unclear. Tanaka et al showed that endogenous E2F1 competes with p50 for binding to p65 subunit of NF-κB and that this physical interaction of E2F1/p65 inhibits NF-κB transcriptional activities. It is noteworthy that VSMCs used in this study express almost undetectable level of endogenous E2F1 (Figure 3A). It is therefore not surprising that in TNF-α exposed, serum-stimulated VSMCs, we found no apparent apoptosis. Similarly, E2F1 has also been shown to interact with p50 subunit of NF-κB in Jurkat cells. Additionally, Phillips et al have shown that in Saos2 cells, E2F1-induced inhibition of NF-κB activity was mediated by the abrogation of TRAF-2 protein and inhibition of IkB kinase. And finally, Yoshimura et al showed that TNF-α/E2F1 signaling pathways of ECs and VSMCs are different.
have recently shown that NF-κB decoy inhibits neointimal formation in an animal model.

In the present study, we show that divergent E2F1 effects on TNF-α-exposed ECs and VSMCs exposed to TNF-α are, at least in part, dependent on cell-specific inhibition of NF-κB nuclear translocation and transcriptional activities in VSMCs. Inhibition of IkBα degradation in VSMCs suggests that NF-κB remains sequestered in the cytoplasm; an observation that parallels the results obtained in nuclear translocation and promoter activity studies. However, signaling events upstream of IkBα in E2F1/TNF-α-treated ECs and VSMCs leading to differential cellular response remain to be elucidated and are beyond the scope of this study. More detailed understanding of the mechanisms driving the cell cycle machinery in ECs versus VSMCs may yield important insights permitting the development of comprehensive therapies for restenosis prevention.

Acknowledgments
This study was supported in part by NIH grants (HL-53354, HL-60911, HL-63414, HL-63695, and HL-66957) and the Shaughnessy center for clinical genetics. This article is dedicated to Dr Jeffrey M. Isner who passed away on October 31, 2001. We would like to gratefully acknowledge his inspirational leadership. We gratefully acknowledge M. Neely and I. Johnson for their assistance in the preparation of this manuscript.

References
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Circ Res. 2003;93:162-169; originally published online June 26, 2003;
doi: 10.1161/01.RES.0000082980.94211.3A

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/93/2/162

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Supplemental Material

Detailed Methods for Carotid Balloon Injury, Adenoviral Delivery Procedures: Balloon carotid injury was performed in Sprague-Dawley rats as previously described. (1) After denuding injury Ad-E2F1 or Ad-β-gal were infused into the isolated carotid segment. Since the goal of these experiments was to infect endothelial cells at the border of the denuded arterial segment, great care was be taken to ensure that unperturbed segments of the carotid artery came into contact with the adenoviral solution. This was accomplished by applying a temporary silk ligature slightly proximal to the point of maximum balloon insertion, leaving an area of un-abraded endothelium. Similarly, the distal ligature on the internal carotid artery as placed slightly above the bifurcation allowing the adenoviral solution to come in contact with these protected endothelial cells. To document the transfection of EC in these border zones we harvested arteries 2 days after rat carotid balloon injury with infusion of Ad-Beta Gal into the isolated vessel segment. Tissue sections were cut from the uninjured portion of the vessel and immunostaining for Beta Gal and CD31 was performed. As shown in Fig. 2C Beta Gal expression is documented in cells along the inner luminal surface that also express the EC marker CD31, thus verifying the transfection of EC in the vessel adjacent to the site of injury. The procedure is shown schematically below.

Reference:
Remove temporary ligatures from Carotid; permanently ligate external Carotid, animal recovery

- Harvest artery with perfusion fixation.
- Stain for Beta-Gal expression and for endothelial identity
**RESULTS**

*Medial Cellularity in Ad-E2F1 vs. Ad-Beta-Gal transduced arteries:*
Seven days after balloon injury and adenoviral transduction arteries were harvested for analysis. Medial cellularity was quantified on H&E stained sections by counting the nuclei in 10 randomly selected high power fields from each artery. Medial cellularity was similar in both treatment groups. Ad-Beta Gal = 103.9±20.0 vs Ad-E2F1 = 96.2±21.1.

![Medial Cellularity](image-url)
**Transduction Efficiency In Vivo:**

To assess the efficiency of VSMC transduction in vivo arteries were harvested 2 days after balloon injury and adenoviral delivery. Ad-BetaGal transduced arteries were perfusion fixed, embedded and sectioned with alternate sections stained for BetaGal expression and expression of smooth muscle alpha actin (SM-α-actin). The number of positive cells in 10 random high power fields per artery were counted and the percentage of beta-gal expressing cells was calculated as a percentage of SM actin expressing cells in adjacent sections. Beta-gal was expressed in 67.3±6.4% of medial SMC.

To assess the efficiency of EC transduction in vivo arteries were harvested 2 days after balloon injury and adenoviral delivery. Ad-BetaGal transduced arteries were perfusion fixed, embedded longitudinally and sectioned with alternate sections stained for BetaGal expression and Isolectin B4 (endothelial marker). The number of positive cells in 20 random high power fields per artery were counted and the percentage of beta-gal expressing EC was calculated as a percentage of total number of visible nuclei in endothelial layer in adjacent sections. Beta-gal was expressed in 51.75±9.4% of intimal EC. **The efficiency of EC vs. VSMC transduction in vivo was similar**\( (p=NS)\).

The transduction pattern revealed site-specific gene transfer. Expression of transgene was uniformly distributed within the injured segment in the medial layer. The intact endothelium adjacent to the zone of injury also displayed a symmetrical pattern of Beta-Gal expression. In neither case did transgene expression extend beyond the isolated target segment of the carotid artery.