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Growth Suppression of Human Coronary Vascular Smooth Muscle Cells by Gene Transfer of the Transcription Factor E2F-1

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Background—The transcription factor E2F-1 promotes S-phase entry and death in transformed cells and primary cardiomyocytes. We tested the hypothesis that overexpression of E2F-1 forces growth-arrested human coronary vascular smooth muscle cells (VSMCs) to enter the S phase, undergo apoptosis, and thereby regulate VSMC growth.

Methods and Results—Early-passage (≤5 passages) coronary VSMCs were transduced at an MOI of 100 with a recombinant adenovirus encoding human E2F-1. Early E2F-1 expression was observed by immunohistochemistry as early as 6 to 8 hours after exposure of the VSMCs to Ad.E2F-1 but not to the control vector Ad.RR. When cells were kept in growth-arrest medium, 40% of Ad.E2F-1–treated VSMCs entered the S phase by 96 hours, whereas the percentage remained <5% in Ad.RR-treated cells. Transition to the S phase in the E2F-1–transduced VSMCs was followed by apoptosis, as reflected by chromatin condensation, membrane blebbing, cell detachment, and loss of mitochondrial membrane integrity. E2F-1 overexpression resulted in positive dUTP nick end-labeling mediated by terminal deoxynucleotidyl transferase, associated with a robust increase in caspase 3–like activity. Four days after infection with Ad.E2F-1, the fraction of hypodiploid VSMCs in subG1 increased to 75%. At 7 days, gene transfer of E2F-1 had completely suppressed the growth of VSMCs, whereas the number of Ad.RR-infected cells had increased >8 times.

Conclusions—Overexpression of the transcription factor E2F-1 regulates growth of human coronary VSMCs by forcing the cells to enter the S phase and then to die. Cell death appears to involve caspase 3–like activity, which, in the VSMCs, is markedly increased by overexpression of E2F-1. (Circulation. 2001;103:407-414.)

Key Words: cells ■ genes ■ muscle, smooth ■ apoptosis ■ restenosis

Vascular injury during percutaneous revascularization interventions stimulates proliferation and migration of vascular smooth muscle cells (VSMCs), which accumulate in the intima of the injured vessel site.1,2 Gene transfer strategies that have attempted to reduce postinjury intimal hyperplasia have focused on the transfer of gene products that block cell cycle initiation or progression or induce apoptosis in VSMCs.3,5

E2F transcription factors function as initiators of cell growth in multicellular organisms and are tightly regulated by binding to members of the Rb family of pocket proteins. On phosphorylation of Rb by cyclin-dependent kinases, members of the E2F family are released and (with the exception of the transcriptional repressor E2F-6) transactivate gene expression of proteins that promote transition to the S phase and DNA replication, followed, in general, by completion of the cell cycle with mitosis of the cell.6,7

Early studies have concentrated on the growth-promoting properties of the E2F family of transcription factors and their putative role as oncogenes.8,9 In the vascular system, the introduction into balloon-injured rat carotid arteries of double-stranded DNA containing the consensus sequence that binds E2F with high affinity functioned as an E2F decoy and inhibited in vivo VSMC proliferation and formation of postinjury neointima.10 These studies, carried out before full recognition of the diversity of the E2F family, have promoted the notion that E2F transcription factors serve as universal initiators of cell proliferation and growth. More recently, however, mice with an inactivated E2F-1 gene were generated and, unexpectedly, exhibited features of hyperproliferation, suggesting that E2F-1 may function in vivo as a growth regulator and tumor suppressor gene.11,12 Further studies demonstrated that overexpression of E2F-1 in transformed rat fibroblasts,13 neonatal and postmitotic adult rat ventricular myocytes,14 and tumor cells15,16 promoted S-phase entry, followed by apoptosis of the cells. Because E2F-1 may have divergent growth-regulatory functions, depending on the tissue type, developmental stage,
and coexistence of dormant oncogenes, we studied the effect of overexpressed E2F-1 on early-passage human coronary VSMCs. We found that in human coronary VSMCs, E2F-1 induces caspase 3–like activity, initiates apoptosis, and markedly suppresses proliferation of these cells. Overexpressed E2F-1 in VSMCs appears to regulate growth by forcing vascular smooth muscle to enter the S phase and then to die.

**Methods**

**Preparation of Recombinant Adenoviral Vectors**

Construction of the recombinant adenovirus encoding human E2F-1 has been described. In brief, the full-length human E2F-1 cDNA, cloned from ML-1 cells, was inserted into the shuttle plasmid pXCCL-1 containing the human cytomegalovirus promoter and the bovine growth hormone polyadenylation signal. Recombinants were generated by cotransfection into 293 cells of pXCCL-1-E2F-1 and pJM17. Replication-defective adenovirus containing a human cytomegalovirus promoter expression cassette but no foreign gene (Ad.RR) was used as a control vector.

Viral stocks of Ad.E2F-1 and Ad.RR were propagated in 293 cells and purified as previously described. The concentration of infectious viral particles was determined in 293 cells by plaque assay. Viral preparations were found to be endotoxin-free (<0.125 EU/mL) when tested with a limulus amoebocyte lysate assay (BioWhittaker, Inc). When the lysate of VSMCs infected with Ad.E2F-1 at a multiplicity of infection (MOI, viral plaques/cell) of 100 to 500 was applied undiluted to 293 cells, no cytopathic effect or viral plaques were observed after 10 days.

**Cell Culture**

Passage-2 human coronary VSMCs were purchased from Cascade Biologics, Inc, and were not used after passage 5. For cell growth experiments, VSMCs were seeded in triplicate at a density of ~10^5/cm² and were growth-arrested for 60 hours in DMEM with added 0.1% BSA. Recombinant adenovirus was suspended in growth-arrest medium, and the cells were incubated for 6 hours with either Ad.E2F-1, Ad.RR, or growth-arrest medium alone (mock). After removal of the viral suspension, the cells were washed with DMEM and cultured in DMEM or Medium 231 (Cascade Biologics) supplemented with 10% FBS or the growth supplement SMGS (Cascade Biologics), respectively. At intervals, the cells were trypsinized and counted in a Coulter counter (Model Z1, Coulter Inc).

**E2F-1 Immunohistochemistry**

VSMCs were plated into chamber slides (Nalgen-Nunc Intl) and infected for 6 hours with Ad.E2F-1, Ad.RR, or mock, followed by removal of the virus suspension. The cells were washed with DMEM and fed fresh growth medium. At intervals beginning at the completion of the 6-hour infection, the cells were washed, fixed for 10 minutes at ~20°C in methanol/acetone 3:1, and immunostained for E2F-1 with a mouse monoclonal antibody recognizing human E2F-1 (Santa Cruz Biotechnology). Antibody binding was visualized with DAB, with a biotinylated secondary antibody and a streptavidin-biotin–horseradish peroxidase kit (Vector). PBS with 0.1% Triton X-100 was used to suspend the antibodies and for all washing steps. Cells were counterstained with Alcian blue/methyl green in PBS, dehydrated in ethanol, and coverslipped with Cytoseal 60 mounting medium (Stephens Scientific).

**Cell Cycle Analyses by DNA Flow Cytometry**

The coronary VSMCs were plated at ~3.5×10^5 cells/60-mm dish and growth-arrested for 60 hours, followed by 6-hour treatment with Ad.E2F-1, Ad.RR, or mock control. Cells were either stimulated with 10% FBS or kept in growth-arrest medium (see Results) and were harvested daily for cell cycle analysis by DNA flow cytometry, pooling detached cells and cells removed with trypsin.

Samples were centrifuged for 10 minutes at 1800 rpm and resuspended in 0.2 mL of PBS, followed by dropwise addition of 5 mL of ice-cold 85% ethanol with gentle vortexing. Fixed cells were stored at ~20°C. On the day of analysis, samples were centrifuged at 2500 rpm for 10 minutes and washed with PBS before resuspension in 400 μL of 100 μg/mL propidium iodide and 50 μg/mL
RNAse and incubation for 20 minutes at 37°C. At least 4 × 10^3 cells were analyzed on a Coulter EPICS Profile instrument (Coulter Inc). Histograms were analyzed with the Multicycle program from Phoe- nix Flow Systems.

Microscopic Evaluation of Cell Death

Cell morphology was evaluated by combined epifluorescence and differential interference contrast (DIC) microscopy. Briefly, the VSMCs were kept in serum-free medium for 60 hours, treated with Ad.E2F-1, Ad.RR, or growth-arrest medium alone, and stimulated by addition of DMEM with 10% FBS. Thirty hours after infection, the cells were incubated in the dark at 37°C with 10 μg/mL of Hoechst 33342 and 4-(4-(dimethylamino)styril)-N-methylpyridinium iodide (DASPMI) for staining of nuclear DNA and mitochondrial membranes, respectively. DNA staining and mitochondrial staining were detected with DAPI and FITC filter sets, respectively. All micrographs were digitally captured on a Zeiss Axioskop epifluorescence microscope with an Optronics DEI-750 CCD color camera with Adobe Premiere software (Adobe Systems), a TARGA 2000 video board (Truevision, Inc), and a PowerPC Macintosh 9500 (Apple Computer). Images were edited with Adobe Photoshop software (Adobe Systems).

Terminal Deoxynucleotidyl Transferase–Mediated dUTP Nick End-Labeling

Terminal deoxynucleotidyl transferase (TdT)–mediated dUTP nick end-labeling (TUNEL) staining was carried out according to a recently modified procedure,20 in which TUNEL is performed immediately after the cells are labeled with trypan blue. The human VSMCs were infected at an MOI of 100 with Ad.E2F-1 or the Ad.RR-control and kept in either serum-supplemented or serum-free culture medium for an additional 44 hours. The VSMCs were then harvested and incubated for 2 minutes in trypan blue. After 2 washing steps in PBS, the cells were subjected to cytospin, and TUNEL staining was performed with a kit from Intergen. New fuchsin chromagen (Dako), a red horseradish peroxidase substrate, was used to visualize nick end-labeled DNA.

Caspase Fluorogenic Activity Assay

Human coronary VSMCs were plated at 1.2 × 10^6 cells/60-mm plate. After growth arrest as described above, the cells were treated for 6 hours with Ad.E2F-1, Ad.RR, or mock. After removal of the virus, the cells were cultured in serum-supplemented growth medium. At intervals, both detached and adherent cells were harvested in 200 μL ICE lysis buffer (12.5 mmol/L Tris [pH 7.0], 125 mmol/L EDTA, 5% glycerol), snap-frozen on dry ice, and stored at −80°C until analysis of all samples. On the day of analysis, samples were freeze-thawed 3 times, vortexed briefly, and centrifuged at 13 000 rpm for 10 minutes. The cleared supernatant was transferred to a new microcentrifuge tube for measurement of total protein and caspase 3–like activities. For measurement of caspase 3–like activity, 50 μL of the lysate from each sample was transferred in triplicate to a 96-well plate. Caspase 3–like activity was measured by monitoring the cleavage of the specific fluoroscent substrate, Ac-DEVD-AMC (Calbiochem-Novabiochem), over a period of 3 hours in a fluores- cent plate reader (PEBiosystems), as previously described.21 The rate
of fluorescence change, an average of 3 replicate measurements, was normalized to the protein concentration, as determined by BCA protein reagent (Pierce Chemical Corp). In some experiments, the cell lysates were incubated with the caspase 3 inhibitor Z-DEVD-FMK (Calbiochem-Novabiochem) before assessment of caspase activity.22

Results

Transduction of Human Coronary VSMCs by Ad E2F-1

Growth-arrested human coronary VSMCs were incubated at an MOI of 50 with Ad.E2F-1, Ad.RR, and mock control and after various time periods were processed for E2F-1 immunohistochemistry. Predominantly nuclear E2F-1 expression was detected as early as 6 to 8 hours after infection in the transduced VSMCs, whereas no staining was seen by 20 hours in cells treated with the Ad.RR control or mock (Figure 1). A faint signal of endogenous E2F-1 was detected by immunoblotting of the lysate from Ad.RR- and mock-treated VSMCs, when the immunoblots were exposed for several hours to a chemoluminescent enhancing solution (Amersham Pharmacia Biotech), greatly exceeding the recommended exposure time (data not shown).

Overexpression of E2F-1 Forces Serum-Deprived Coronary VSMCs to Enter the S Phase

After demonstrating relatively early overexpression of E2F-1 in the transduced VSMCs, we examined whether E2F-1 gene transfer promotes S-phase entry of VSMCs rendered quiescent by a 60-hour growth arrest. Serum-deprived coronary VSMCs were infected with Ad.E2F-1, Ad.RR, or mock control and kept in serum-free medium for an additional 4 days. DNA flow cytometry on the cells harvested daily demonstrated that despite prolonged serum starvation, transduction with E2F-1 promoted the transition from G1 to S in the VSMCs that had been made quiescent (Figure 2). In contrast, the percentage of VSMCs in the S phase remained consistently <5% in the control-treated cells.

S-Phase Entry of E2F-1–Transduced Coronary VSMCs Is Associated With Induction of Apoptosis

Within 24 to 36 hours after gene transfer of E2F-1 to VSMCs, we observed the development of apoptotic features, including membrane blebbing, cell shrinkage, and cleavage and condensation of chromatin (Figures 3 and 4). Chromatin condensation was apparent as intensified epifluorescence of nuclear fragments after staining with the intercalating DNA dye Hoechst 33342 (Figure 4B). In addition, E2F-1 overexpression resulted in mitochondrial membrane disarray, as visualized with DASPMI, a mitochondrial membrane–specific dye (Figure 4C). Ad.E2F-1–infected VSMCs studied 44 hours after infection stained positive for TUNEL, irrespective of the presence of serum before or after transduction of the cells (Figure 5).

In an individual cell, the development of apoptotic changes induced by overexpression of E2F-1 appeared to proceed in a relatively short time. Figure 6 shows the changes observed with video time-lapse microscopy in a single VSMC from a sample of cells infected with Ad.E2F-1. Surface blebbing and loss of membrane integrity with extrusion of cellular contents were nearly complete within 2 hours after the first changes were observed at 30 hours after infection (Figure 6: frame 1, 30 hours; frame 9, 32 hours).

To further evaluate the time course and magnitude of VSMC apoptosis induced by E2F-1, the cells were infected with Ad.E2F-1, followed by growth stimulation in 10% FBS. Using DNA flow cytometry on samples harvested at 24-hour intervals, we observed a dose-dependent induction of apoptosis by Ad.E2F-1, reflected by an increase in the hypodiploid cell population (subG1 cells) containing cleaved DNA and observed at an MOI of Ad.E2F-1 as low as 5 (Figure 7A).
With MOIs of 100 and 200, the fraction of apoptotic cells reached a plateau of 75% on day 4. With Ad.E2F-1 at an MOI of 10, only 17% of cells were in subG1 on day 3, whereas the subG1 fraction was <5% in control cells (Figure 7B).

Activation of Caspase 3 by E2F-1

We examined whether E2F-1 induced caspase 3–like activity in coronary VSMCs. With Ad.E2F-1 at an MOI of 100, caspase 3–like activity increased 2- and >6-fold compared with the Ad-RR at 24 and 30 hours after infection of the cells, respectively (Figure 8). In contrast, with an MOI of 10, caspase 3–like activity above baseline was detected only 30 hours after infection of the VSMCs with Ad.E2F-1. The E2F-1–induced increase in caspase 3–like activity was suppressed completely by addition to the VSMC lysate of the caspase–3 inhibitor Z-DEVD-FMK (Figure 8), indicating specificity of the caspase measurement.22

Growth Suppression of Coronary VSMCs After Adenovirus-Mediated E2F-1 Gene Transfer

After we had observed that E2F-1–transduced VSMCs undergo cell death at a rate commensurate with the amount of gene vector used, we assessed whether Ad.E2F-1–induced cell death translated into growth suppression of the VSMCs. Growth curves were established for the early-passage human coronary VSMCs after a 6-hour treatment with Ad.E2F-1, Ad.RR, or mock control. Daily cell counts demonstrate that gene transfer of E2F-1 suppressed VSMC proliferation after infection at an MOI as low as 10. Growth of the VSMCs was abolished after a single application of Ad.E2F-1 at an MOI of 100 (Figure 9).

Discussion

We report that overexpression of E2F-1 forces growth-arrested, early-passage human coronary VSMCs into the S phase, followed by apoptosis of the VSMCs. Overexpression of E2F-1 was detected as early as 6 to 8 hours after infection, and the growth of VSMCs infected with Ad.E2F-1 was markedly suppressed for up to 7 days. Another novel observation is that E2F-1 induces caspase 3–like activity in VSMCs. Both caspase activation and apoptosis correlated with the viral dose.

Induction of the S phase and apoptotic cell death after overexpression of exogenous E2F-1 has been observed previously in cardiac myocytes and tumor cells.14,16,23,24 In early-passage human coronary VSMCs, overexpressed E2F-1 led to cell detachment, cytoplasmic membrane blebbing, nuclear fragmentation, and loss of mitochondrial integrity, which, in combination with other markers, serve to distinguish apoptosis from inflammatory-necrotic modes of death.25,26 One of these is TUNEL.27 TUNEL, performed 44 hours after infection with Ad.E2F-1, was occasionally accompanied by VSMC uptake of trypan blue, indicating loss of cell membrane integrity. Thus, after a longer interval, E2F-1–induced apoptosis can progress to cell lysis, representative of the late stage of “aponecrosis,” combining features of apoptotic and necrotic death.26,28,29 In contrast, treatment of VSMCs with the empty control virus Ad.RR at an MOI >500 caused predominantly necrosis of cultured VSMCs, as judged by the presence of marked trypan blue positivity, extensive cell lysis, and sparse TUNEL-positivity (Shelat et al, unpublished observations, 2000).

Neither conventional TUNEL staining nor the demonstration of internucleosomally cleaved DNA on gel electrophoresis is an absolute criterion for apoptosis, and, conversely, apoptosis may occur without the characteristic laddering pattern observed on electrophoretically separated DNA.29,30 To further substantiate the induction of apoptosis by E2F-1 and investigate whether overexpressed E2F-1 in coronary VSMCs activates the death cascade common to other apoptosis initiators, we examined caspase 3–like activity in VSMCs after transduction with E2F-1. Consistent with a recent report of activation-cleavage of the execution caspase,
caspase 3 (CPP32), in E2F-1–transduced glioma cells, we found that overexpression of E2F-1 in VSMCs induced a dose-dependent increase in caspase 3–like activity. Thus, in coronary VSMCs, as in tumor cells, overexpressed E2F-1 appears to activate common downstream effectors of apoptotic death. Of note, E2F-1 expression by immunohistochemistry was observed as early as 6 to 8 hours after VSMC infection with Ad.E2F-1, whereas activation of caspases and cell death became apparent only after 24 to 32 hours. This time lag argues for the requirement for the arrested VSMCs to reenter G1 and transit to the S phase before engaging in the apoptosis signaling and execution cascade.

How overexpressed E2F-1 initiates the apoptotic cascade is unclear and may be influenced, in principle, by the cell type, the presence of cycling versus growth-arrested cells, and the status of endogenous p53 and other proapoptotic genes.31 Cell cycle stage and p53 status, however, do not appear to pose absolute restraints on the ability of overexpressed E2F-1 to induce cell death. Indeed, apoptosis was observed in transduced coronary VSMCs whether the cells were maintained in...
continuous serum deprivation or allowed to cycle before and after infection with Ad.E2F-1. In addition, induction of cell death by E2F-1 was previously observed in p53−/− cardiac myocytes19 as well as in breast cancer, melanoma, and squamous cell carcinoma cells.16,23,24 Thus, our findings in VSMCs enhance the notion that E2F-1, if present in sufficient amounts, exerts proapoptotic effects in many transformed and nontransformed lineages. Of note, however, cell lines harboring the Harvey (H)-ras gene undergo tumorigenic transformation in the presence of overexpressed E2F-1,9,18 indicating that overexpressed E2F-1 may have profoundly different effects in the presence of dormant oncogenes. The tumorigenicity of E2F-1 in cells that do not harbor oncogenes has not been demonstrated conclusively.9

Whether endothelial cells constitute an exception to the proapoptotic effect of overexpressed E2F-1 remains to be confirmed. In a recent report, adenovirus-mediated E2F-1 overexpression was reported to protect cultured bovine endothelial cells from tumor necrosis factor (TNF-α)-mediated apoptosis.32 In preliminary studies, in contrast, we have observed that gene transfer of E2F-1 resulted in marked S-phase induction, apoptosis, and growth suppression of human umbilical vein endothelial cells (Zoldhelyi et al, unpublished observations, 1999). These observations suggest that the effects of E2F-1 may be modulated by the presence of cytokines (such as TNF-α) and growth factors, including insulin-like growth factor-1, which was reported to rescue cells from E2F-1–mediated death.33

Our observation of marked growth suppression of VSMCs by overexpressed E2F-1 requires reconciliation with those showing a similar effect by inhibiting E2F.10,34 This apparent paradox may be resolved by considering the mechanism of growth control exerted by these approaches. By using an oligonucleotide decoy strategy of binding all E2F transcription factors to a consensus sequence, Morishita et al and Mann et al appear to have achieved a favorable shift in the balance between inhibition of E2F transcription factors that promote (E2F-2 to E2F-4) and those that limit (E2F-1 and E2F-6) proliferation of cells. The endogenous levels of individual transcription factors are critical determinants of the response to an inhibitory decoy strategy but may have little relevance when a potent growth suppressor gene is overexpressed. Consistent with this hypothesis is the observation that endogenous levels of E2F-1 in coronary VSMC cultures were low (see Results).

In conclusion, our observations indicate that gene transfer of E2F-1 leads to S-phase entry of growth-arrested human coronary VSMCs, followed by dose-dependent caspase activation and apoptotic death. Given the critical role of VSMC proliferation, migration, and synthesis of extracellular matrix and growth factors, E2F-1–induced apoptosis may play a role in future strategies to prevent neointima formation after vascular injury. Potentially undesirable effects of E2F-1 gene transfer on cells other than VSMCs could be circumvented in vivo by the use of vectors with smooth muscle–specific promoters, such as SM22-α.35 Use of tissue-specific promoters and other strategies, including the use of vectors with extensive deletions of viral genes, may provide an opportunity to evaluate an E2F-1–based strategy of VSMC apoptosis for the prevention of postinjury vascular stenosis and vein graft disease.

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