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A DNA enzyme targeting Egr-1 inhibits rat vascular smooth muscle cell proliferation by down-regulation of cyclin D1 and TGF-β1

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Abstract

We have demonstrated that a synthetic DNA enzyme targeting early growth response factor-1 (Egr-1) can inhibit neointimal hyperplasia following vascular injury. However, the detailed mechanism of this inhibition is not known. Thus, the objective of the present study was to further investigate potential inhibitory mechanisms. Catalytic DNA (ED5) and scrambled control DNA enzyme (ED5SCR) were synthesized and transfected into primary cultures of rat vascular smooth muscle cells (VSMCs). VSMC proliferation and DNA synthesis were analyzed by the MTT method and BrdU staining, respectively. Egr-1, TGF-β1, p53, p21, Bax, and cyclin D1 expression was detected by RT-PCR and Western blot. Apoptosis and cell cycle assays were performed by FACS. Green fluorescence could be seen localized in the cytoplasm of 70.6 ± 1.52 and 72 ± 2.73% VSMCs 24 h after transfection of FITC-labeled ED5 and ED5SCR, respectively. We found that transfection with ED5 significantly inhibited cultured VSMC proliferation *in vitro* after 24, 48, and 72 h of serum stimulation, and also effectively decreased the uptake of BrdU by VSMC. ED5 specifically reduced serum-induced Egr-1 expression in VSMCs, further down-regulated the expression of cyclin D1 and TGF-β1, and arrested the cells at G_0/G_1, inhibiting entry into the S phase. FACS analysis indicated that there was no significant difference in the rate of apoptosis between ED5- and ED5SCR-transfected cells. Thus, ED5 can specifically inhibit Egr-1 expression, and probably inhibits VSMC proliferation by down-regulating the expressions of cyclin D1 and TGF-β1. However, ED5 has no effect on VSMC apoptosis.

Key words: DNA enzyme; Egr-1; VSMC; Proliferation; Apoptosis

Introduction

Although atherogenesis, percutaneous transluminal coronary angioplasty, and the subsequent anastomotic restenosis of the vasculature is a complicated pathological process involving multiple factors (1-3), the over-production of extracellular matrix by smooth muscle cells of the injured vessel, their hyperproliferation and their migration to the intima play a key role in lesion formation (4). Local endogenous early growth response factor-1 (Egr-1) is rapidly expressed and activated following vascular injury (5). Egr-1, an immediate-early gene product and zinc finger transcription factor, regulates the expression of multiple proliferation-associated genes (6,7), and Egr-1 levels closely correlate with cell proliferation (8). Deoxyribozyme (DNA enzyme, DRz) is a small single-stranded DNA fragment with enzymatic activity that cleaves specific RNA strands thereby regulating protein expression. In a previous study, we synthesized a specific DRz targeting rat Egr-1 mRNA (DNA enzyme/10-23 DRz, ED5), and transfected it into the rat carotid following balloon injury. We found that ED5 could inhibit neointimal hyperplasia (9) although the detailed mechanism underlying the inhibition was unclear. In the present study, we further analyzed the ED5-transfected vascular smooth muscle cells (VSMCs) by measuring the levels of Egr-1, TGF-β1, p53, p21, Bax, and cyclin D1 expression, changes in the cell cycle, apoptosis, cell proliferation, and DNA synthesis.

Material and Methods

VSMC isolation and primary culture

Rat primary aortic VSMCs were isolated as described previously (10). Briefly, Wistar rats (120 g, purchased from the Experimental Center, China Medical University) were sacrificed with excess anesthesia. The intima was peeled off from the aorta and the media stripped away. The medial layer was cut into approximately 1-mm^2 squares, which were

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transferred to a 35-mm plate and barely covered with DMEM supplemented with 20% FBS (Gibco-BRL, USA). The tissue blocks were cultured at 37°C in a humid atmosphere of 5% CO_2. After 2 weeks, cells that had migrated onto the tissue culture dish were collected by trypsinization and subcultured successively; cells were used from the fourth to the eighth passage. Culture homogeneity was confirmed by staining for smooth muscle actin and was confirmed when staining exceeded 98% (Figure 1).

ED5 synthesis and transfection

The catalytic DNA, ED5, and scrambled control DNA enzyme, ED5SCR, were synthesized by Takara (China). The sequences were as follows: ED5 5'-CCGCTGCCAGG CTAGCTACAACGACCCGGACGT-3'; ED5SCR 5'-GCC AGCCGCGGCTAGCTACAACGATGGCTCCAC-3'. The underlined regions at both ends are the oligonucleotide recognition sequences and the middle sequences represent the conserved catalytic zone. The 5' and 3' termini of the oligonucleotides were protected from exonucleases by a phosphorothioate linkage. The 5' terminus was labeled with fluorescein isothiocyanate (FITC) to identify gene distribution under fluorescence microscopy. VSMCs at 70% confluence were cultured in serum-free medium for 30 h and then transfected with 0.1 µM ED5 or ED5SCR using FuGENE6 in the dark for 18 h. The medium was then replaced with DMEM supplemented with 10% FBS containing 0.1 µM ED5 or ED5SCR using FuGENE6 and incubated for 24 h. The medium was discarded and 2 mL PBS was added. Transfection efficiency was determined using an inverted fluorescent microscope (Olympus, Japan).

Table 1. PCR primers used in the present study.

	Primer sequence	Size
	5'→3'	
Egr-1	CAGTCGTAGTGACCACCTTACCA	449 bp
	AGGTTGCTGTCATGTCTGAAAGAC	
TGF-β1	CTGAACCAAGGAGACGGAATACA	462 bp
	CAAAGCCTGCGGCACG	
p53	GTGGCCTCTGTCATCTTCCG	292 bp
	CCGTCACCATCAGAGCAACG	
p21	TCCGATCCTGGTGATG	442 bp
	TCTGTTAGGCTGGTCTGC	
Bax	GACACCTGAGCTGACCTTGG	311 bp
	GAGGAAGTCCAGTGTCCAC	
Cyclin D1	CTGACACCAATCTCCTCAACGAC	409 bp
	GCGGCCAGGTTCCACTTGAGC	
GAPDH	ACCACAGTCCATGCCATCAC	452 bp
	TCCACCACCCTGTTGCTGTA	

GAPDH = internal control.

MTT assay

VSMC proliferation was assayed using a colorimetric (MTT) assay kit (Amersco, USA). Approximately 5000 cells/well were plated onto a 96-well plate and incubated with 0.1 µM ED5 or ED5SCR using FuGENE6 for various times. Four hours before the end of the incubation, 100 µg MTT was added to each well. MTT was then removed and the cells were solubilized with 150 µL dimethyl sulfoxide. The absorbance of each well was measured using a microculture plate reader at 490 nm. The cell inhibition ratio (IR) was expressed using the following equation: IR = (absorbance in control - absorbance in treated groups / absorbance in control) x 100%.

Apoptosis and cell cycle assay

Apoptosis was measured by staining cells with a combination of fluoresceinated (FITC) annexin V and propidium iodide (PI) as described previously. Briefly, after transfection for 72 h, the VSMCs were resuspended in 100 µL binding buffer (140 mM NaCl, 2.5 mM $CaCl_2$, 10 mM HEPES, pH 7.4). Next, 5 µL annexin V-FITC and 10 µL PI (20 µg/mL; Jingmei, China) were added and incubated for 15 min at room temperature in the dark. Data were acquired with a flow cytometer (Becton Dickinson, USA). PI-stained cells were analyzed with a FACSCalibur flow cytometer (Becton Dickinson) for cell cycle analysis. The cell proliferation index (CPI) was calculated using the following equation: CPI = $((S + G_2/M) / (G_0/G_1 + S + G_2/M)) \times 100\%$.

RT-PCR

Total RNA was isolated using a Takara RNA PCR kit according to manufacturer instructions. The PCR primers were designed by Takara (China) and are shown in Table 1. GAPDH was amplified as an internal control. The amplification was performed with 30 cycles, which was the optimal condition for linearity and permitted semiquantitative analysis of signal strength (temperature profile: 93°C for 30 s, 55°C for 30 s, 72°C for 1 min). PCR products were separated by 1.5% agarose gel electrophoresis and stained with DNAgreen (Bioteke Co., China). The target bands were analyzed densitometrically with a gel image analysis system (Chemi Imager 5500, Alpha Innotech, USA).

Western blot analysis

Aliquots of cell lysates containing 50 µg protein were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose filters. The filters were blocked with TBS-T buffer containing 5% skim milk, incubated with antibodies (1:300) against Egr-1, TGF-β1, p53, p21, Bax, cyclin D1 (Santa Cruz Biotechnology, USA) and β-actin (Sigma, USA) overnight, followed by the addition

of a horseradish peroxidase-linked anti-mouse or rabbit IgG (Maixin Biological Company, China) and binding was visualized following incubation with a DAB solution (Maixin Biological Company). β-actin expression was used as an internal control to normalize protein content. Bands were scanned using a GS-800 Calibrated Densitometer (Bio-Rad, USA) and results were quantified using Quantity One software (Bio-Rad).

Bromodeoxyuridine (BrdU) incorporation

To measure BrdU incorporation, cells were seeded on coverslips and, at the indicated times, 10 µM BrdU (Sigma) was added to the medium for 24 h. Cells were then harvested and fixed in ice-cold 75% ethanol, treated with 2 N HCl, neutralized with 0.1 M borax, washed with PBS containing 0.05% bovine serum albumin, and incubated with anti-BrdU antibody (Sigma). BrdU incorporated into the cell nuclei was visualized by the avidin-biotin-peroxidase complex method using DAB. The signal intensity within a given area was quantified from digital images using Metamorph software (Universal Imaging Corporation, USA).

Statistical analyses

Experiments were repeated three to five times. Unless otherwise indicated, data are reported as means ± SD. Data were analyzed by one-way analysis of variance followed by the least significant difference (LSD) method for multiple comparisons. Differences were considered to be significant if $P < 0.05$. All analyses were performed using the SPSS 13.0 software.

Results

Transfection efficiency

The FITC-labeled ED5 and ED5SCR were readily detectable with the fluorescence microscope apparently within the cytoplasm and, to a lesser extent, within the nucleus as a result of endocytosis. Green fluorescence could be seen localized in the cytoplasm of 70.6 ± 1.52 and 72 ± 2.73% VSMCs 24 h after transfection of FITC-labeled ED5 and ED5SCR, respectively.

ED5 inhibits Egr-1 and TGF-β1 expression

Under serum-free conditions, very little Egr-1 and TGF-β1 mRNA expression and no Egr-1 protein expression were detected in VSMCs (Figures 2A and 3A). After 1 h of serum stimulation, Egr-1 was expressed extensively and its levels began to decline after 4 h of serum stimulation. Egr-1 mRNA and protein expression levels in ED5-transfected cells were significantly lower than in either ED5SCR-transfected or control cells at all times ($P < 0.05$; Figures 2B and 3B). After 1 h of serum stimulation, TGF-β1 began to increase and peaked after 24 h of stimulation. TGF-β1 mRNA and protein expression levels in ED5-transfected cells were significantly lower than in ED5SCR-transfected or control cells at all times ($P < 0.05$; Figures 2B and 3B). Egr-1 and TGF-β1 expression levels did not differ significantly between ED5SCR-transfected and control cells.

ED5 inhibits cell proliferation and DNA synthesis

As shown in Figure 4, ED5-transfected cell proliferation was significantly inhibited compared to the proliferation of ED5SCR-transfected and control cells ($P < 0.05$). This inhibition occurred in a time-dependent manner. The rate of BrdU incorporation into ED5-transfected cells was also significantly decreased ($P < 0.01$; Figure 5). However, there was no significant difference in the degree of cell proliferation or DNA synthesis between ED5SCR-transfected and control cells.

ED5 caused G_0/G_1 phase arrest by down-regulating cyclin D1

Under serum-free conditions, 89.99 ± 2.96% of VSMCs were at the G_0/G_1 stage, and only 1.62 ± 0.11% were at the S stage. After 24 h of serum stimulation the proportion of cells in the S stage was significantly increased to 13.16%, and the proportion of cells in the G_0/G_1 stage was decreased to 81.84%. However, after ED5 treatment, the cells in the G_0/G_1 stage were significantly increased and the cells at S + G_2/M were significantly decreased. There was no difference between ED5SCR-transfected and control in cell cycle stage after any treatment (Table 2). The CPI

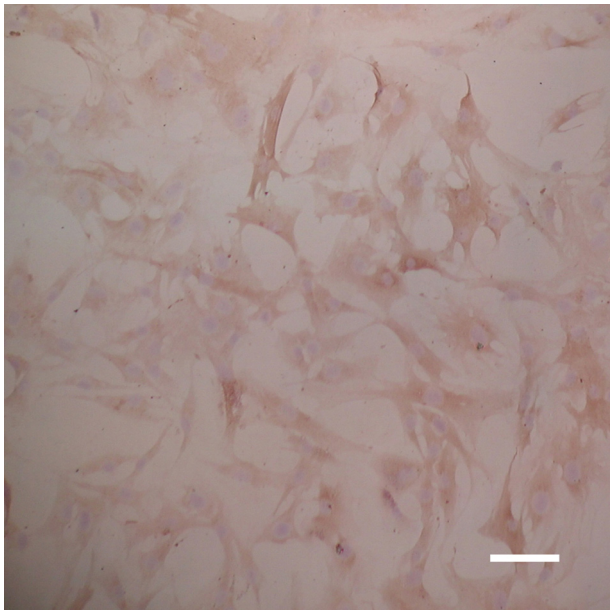

Figure 1. Immunochemical identification of vascular smooth muscle cells. The homogeneity of cultured cells was more than 98% as indicated by staining for smooth muscle actin using monoclonal rat-specific antibody obtained from Sigma (USA). Bar: 10 µm.

was significantly lower in ED5-transfected cells compared to ED5SCR-transfected and control cells at all times. We found that both cyclin D1 mRNA and protein expression in ED5-transfected cells was significantly decreased as well (Figure 6). These results indicated that ED5 may down-regulate cyclin D1 expression, arrest the cells at the G_0/G_1 phase, and block their entry into the S phase.

Apoptosis is not involved in ED5 inhibition

FACS analysis indicated that there was no significantly difference in the rate of apoptosis between ED5-transfected cells, ED5SCR-transfected cells and control cells (Figure 7I). ED5 did not induce necrosis of VSMC. Under serum-free conditions, only low levels of p53, p21, and Bax mRNA expression, and no protein expression, were detected in VSMCs (Figure 7II,III). After 1 h of serum stimulation, p53, p21, and Bax mRNA expression began to increase and peaked at 24 h. p53 and p21 protein expression was increased, but Bax protein was not detected. There was no significant difference in p53, p21, and Bax levels between ED5-transfected cells, ED5SCR-transfected cells, and control cells.

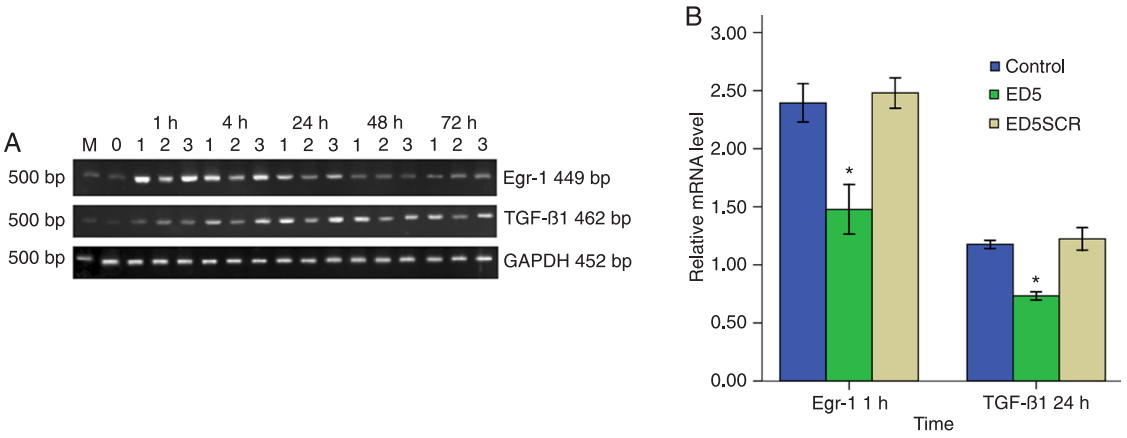

Figure 2. Representative expression of Egr-1 and TGF-β1 mRNA in cultured vascular smooth muscle cells (VSMC) in each group. *A*, DNA marker (M); serum-free VSMCs (lane 0); normal VSMCs as control (lane 1); VSMCs transfected with ED5 (lane 2); VSMCs transfected with ED5SCR (lane 3). Expression of Egr-1 and TGF-β1 mRNA was significantly inhibited following ED5 transfection. *B*, Quantitative data are shown in the graph. Comparison to normal VSMCs and ED5SCR-transfected cells showed that Egr-1 and TGF-β1 mRNA expression in ED5-transfected cells was significantly inhibited. GADPH is the internal control. *P < 0.01, compared to control and ED5SCR (one-way ANOVA).

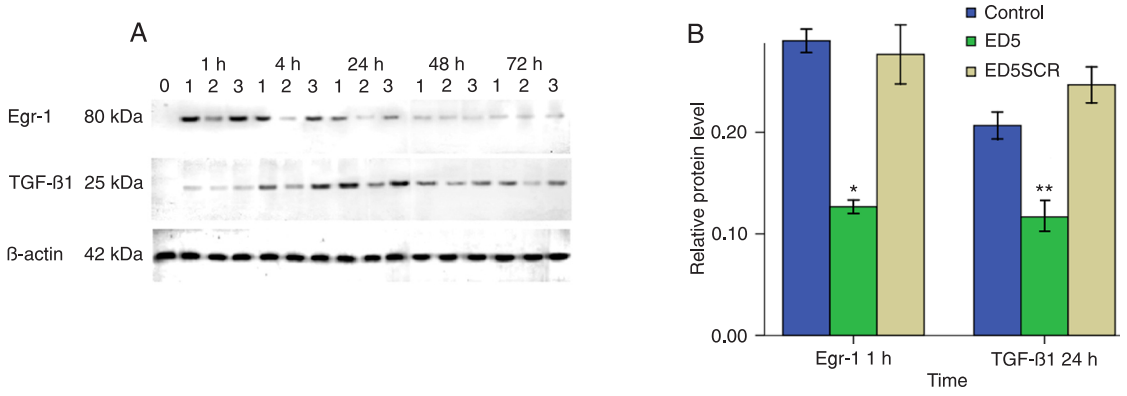

Figure 3. Time-lapse expression of Egr-1 and TGF-β1 protein levels in cultured vascular smooth muscle cells (VSMC) in each group. *A*, DNA marker (M); serum-free VSMCs (lane 0); normal VSMCs as control (lane 1); VSMCs transfected with ED5 (lane 2); VSMCs transfected with ED5SCR (lane 3). The expression of Egr-1 and TGF-β1 protein levels was significantly inhibited following ED5 transfection. *B*, Quantitative data are shown in the graph. Comparison to control and ED5SCR-transfected cells showed that Egr-1 and TGF-β1 protein expression in ED5-transfected cells was significantly inhibited. β-actin is the internal control. *P < 0.01, **P < 0.05, compared to control and ED5SCR (one-way ANOVA).

Discussion

Transcription factors are DNA-binding proteins that can bind to a specific sequence of the gene promoter to regulate gene expression. Recent studies (11,12) have found that some transcription factors can regulate the expression of several VSMC proliferation-associated genes, and thereby regulate VSMC proliferation. Egr-1 is a zinc finger transcription factor expressed at low basal levels or not expressed in normal vascular walls. However, following stimulation such as arterial injury, VSMCs and endothelial cell express Egr-1, which in turn promotes VSMC division, proliferation, and intimal hyperplasia (13,14). Consequently, interfering with the expression of the Egr-1 transcription factor gene as a method to inhibit VSMC proliferation and reduce intimal hyperplasia may provide a novel treatment for restenosis.

10-23 DRz, a DNA enzyme obtained by *in vitro* target screening (15), successfully hydrolyzes the phosphodiester bond binding free purine and paired pyrimidine. Because its active center is composed of a non-variable sequence, the recognition sequence at the two ends can vary depending on the sequence of the target substrate RNA. This enzyme can then theoretically cut the AUG loci located in any RNA sequence. Our previous study (9) demonstrated the speci-

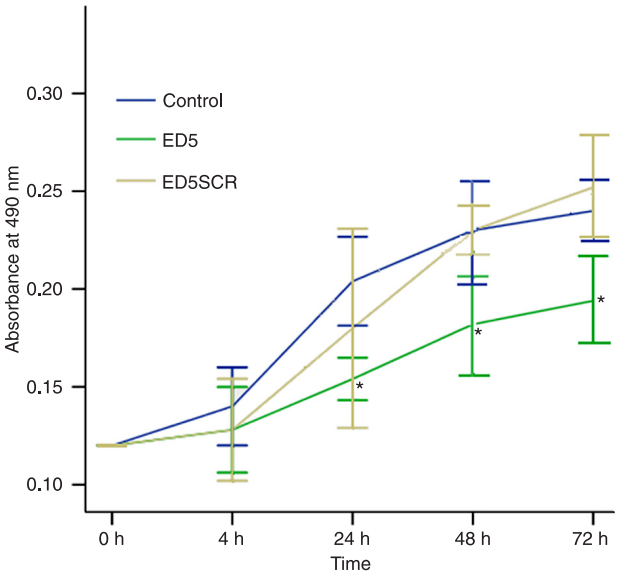

Figure 4. MTT cell proliferation assay. ED5-transfected cell proliferation was significantly inhibited following transfection for 24, 48, and 72 h. The cell inhibition ratio (IR) was expressed using the following equation: IR = (absorbance in control - absorbance in treated groups / absorbance in control) x 100%. *P < 0.05, compared to control and ED5SCR (one-way ANOVA).

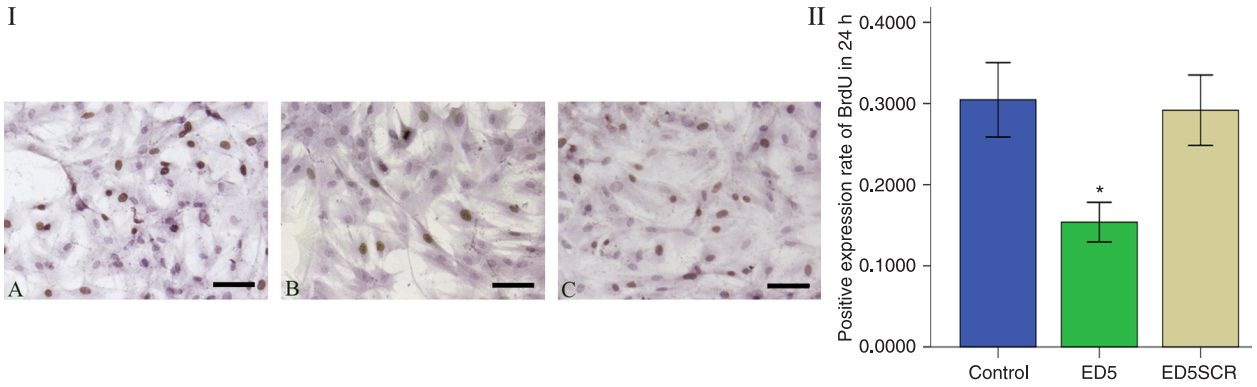

Figure 5. *I*, BrdU labeling of normal vascular smooth muscle cells (VSMC) as control (A), VSMCs transfected with ED5 (B), and VSMCs transfected with ED5SCR (C). DNA synthesis was significantly inhibited following ED5 transfection for 24 h. Bars: 10 µm. *II*, Quantitative data are shown in the graph. Comparison to control and ED5SCR-transfected cells showed that the rate of BrdU incorporation in ED5-transfected cells was significantly decreased (*P < 0.01, one-way ANOVA).

Table 2. Effects of ED5 transfection on the cell cycle of vascular smooth muscle cells (VSMC).

Group	24 h			48 h			72 h		
	G_0/G_1	S	G_2/M	G_0/G_1	S	G_2/M	G_0/G_1	S	G_2/M
Control	81.84 ± 1.20	13.16 ± 0.87	5.00 ± 0.44	81.2 ± 0.45	14.22 ± 0.73	4.58 ± 0.47	81.17 ± 1.68	16.70 ± 0.64	2.13 ± 0.05
ED5	91.14 ± 0.77*	4.19 ± 0.23*	4.80 ± 0.45	93.9 ± 0.08*	0.93 ± 0.05*	5.15 ± 0.14	96.85 ± 0.17*	0.28 ± 0.01*	2.54 ± 0.24
ED5SCR	81.80 ± 0.62	12.52 ± 0.38	5.67 ± 0.28	81.38 ± 1.08	13.86 ± 0.06	4.74 ± 0.12	81.25 ± 0.02	16.27 ± 0.18	2.48 ± 0.20

Data are reported as means ± SD of the percent of cells in each stage of the cell cycle. *P < 0.05 compared to control and ED5SCR-transfected cells with same cycle stage (LSD test).

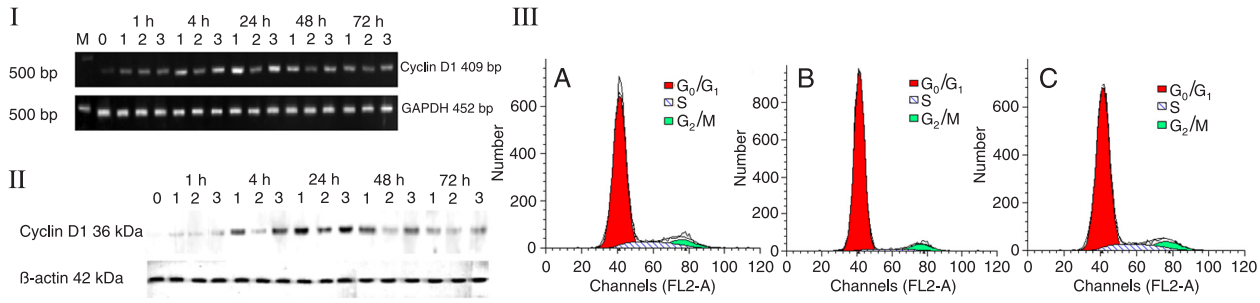

Figure 6. ED5 transfection induces G_0/G_1 arrest by down-regulating cyclin D1. ED5 (lane 2) treatment inhibited cyclin D1 mRNA (I) and protein (II) expression levels relative to the control (lane 1) and ED5SCR-transfected (lane 3) cells. The vascular smooth muscle cell (VSMC) cycle was arrested at the G_0/G_1 phase and entry into the S phase was blocked 48 h after 10% FBS stimulation as determined by FACS (III). *A*, Normal VSMCs as control; *B*, VSMCs transfected with ED5; *C*, VSMCs transfected with ED5SCR. GADPH and β-actin are internal controls.

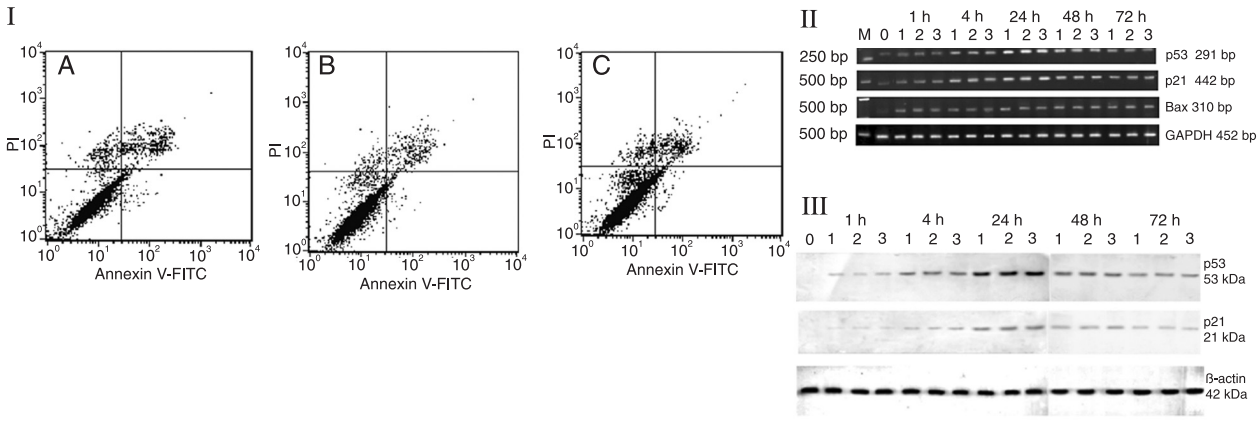

Figure 7. *I*, FACS analysis indicated that there was no significant difference in the rate of apoptosis between control cells (A), ED5-transfected cells (B) and ED5SCR-transfected cells (C) after transfection for 72 h. *II*, Under serum-free conditions (lane 0), only low levels of p53, p21, and Bax mRNA expression were observed. There was no significant difference in p53, p21, or Bax mRNA levels between control (lane 1), ED5-transfected (lane 2), and ED5SCR-transfected (lane 3) cells. *III*, Under serum-free conditions (lane 0), no protein expression was detected in VSMCs. There was no significant difference in p53 and p21 protein levels between control (lane 1), ED5-transfected (lane 2) and ED5SCR-transfected (lane 3) cells. GADPH and β-actin are internal controls.

ficity and efficacy of synthetic ED5 for inhibiting Egr-1 and reducing subsequent intimal hyperplasia. However, the exact mechanism of ED5 action and whether it is related to cell apoptosis was unclear. In the present study, a specific DNA enzyme targeting the mRNA of the transcription factor Egr-1 was synthesized to investigate a putative mechanism of rat VSMC proliferation and apoptosis.

MTT was first employed in our study to evaluate the transfection efficiency; we found that transfection with ED5 significantly inhibited cultured rat VSMC proliferation *in vitro* after 24, 48, and 72 h of serum stimulation. We also found that cells transfected with ED5 could effectively inhibit the uptake of BrdU by VSMC; specifically, the number of cells in the DNA synthesis phase decreased, whereas the control transfection ED5SCR showed no such effects. These results indicate that ED5 likely affects entry into the cell cycle after specifically inhibiting Egr-1 expression. To explore this hypothesis, we employed FACS analysis, which revealed that, following transfection with ED5, the ratio of cells in the S phase and the cell proliferation index were markedly decreased, whereas the ratio of cells in the G_0/G_1 phase was increased. ED5 transfection prevented FBS-induced progression to the S phase from the G_0/G_1 phase. Control transfections did not show this pattern. Therefore, ED5 transfection not only affected cell cycle regulation, but specifically influenced the G_1/S phase transition.

To confirm this hypothesis, we further investigated the expression of cyclin D1, the G_1 phase apoptosis gene product p53, a p53 downstream gene cyclin-dependent kinase inhibitor $p21^{waf1/cip1}$, the apoptosis gene Bax and

the proliferation-associated gene TGF-β1. Following treatment with 0.1 µM ED5, there were no significant differences in p53, p21, or Bax mRNA relative to controls. However, cyclin D1 and TGF-β1 mRNA syntheses were significantly inhibited by ED5 treatment. Our findings are consistent with those of Fu et al. (16) using gene chip analysis of human vascular endothelial cells. Our results are also consistent with those of McCaffrey et al. (17) who performed a cDNA chip analysis on human carotid atherosclerotic plaques, and concluded that cyclin D1 is regulated by Egr-1. The cyclin D1 expression study reported here not only confirms the specific effect of ED5 on Egr-1, but also demonstrates that ED5 down-regulates cyclin D1 expression by inhibiting Egr-1 expression, resulting in cell cycle arrest at the G_0/G_1 phase, which therefore decreases the S + G_2/M ratio and further inhibits VSMC proliferation.

Previous studies have suggested that the enhancement of TGF-β1 activity is observed not only following stenosis lesions in animal models (18,19) but also in human vessels (20). A study of blood vessels following rat balloon injury demonstrated that the elevated expression levels of TGF-β1 could last for at least 2 weeks; importantly, administration of an antibody against TGF-β1 inhibited neointimal formation (21,22), suggesting that TGF-β1 plays an essential role in restenosis. The present results demonstrate that 1) Egr-1 and TGF-β1 have low expression levels under serum-free conditions, 2) expression of Egr-1 increases earlier than that of TGF-β1 following stimulation with 10% serum, and 3) levels of TGF-β1 decrease later than do Egr-1 levels following transfection with ED5. Taken together, these findings suggest that the expression and activation of TGF-β1 are likely to be regulated by Egr-1. This theory is consistent with the finding that Egr-1 bound to the specific recognition region adjacent to the TGF-β1 promoter, and induced TGF-β1 expression (23). The TGF-β1 promoter has two GC-rich Egr-1 binding sites; activated Egr-1 may directly interact with these sites and thereby regulate gene expression (24). In addition, TGF-β1 expression itself may serve as a stimulus to activate Egr-1, forming a positive feedback loop with an amplified cascade effect (25). These results may explain why ED5 down-regulates TGF-β1 and Egr-1 expression.

Nair et al. (26) reported that Egr-1 inhibits cancer cell growth and induces cell apoptosis by p53. However, Santiago et al. (5) found that the intimal hyperplasia triggered by Egr-1 is not a result of decreased cell apoptosis. Therefore, whether Egr-1 is related to cell apoptosis remains unclear. Our FACS results suggested that ED5 transfection had no effect on VSMC apoptosis or necrosis. In addition, transfection with ED5 did not result in any alterations in p53, p21, or Bax expression levels, suggesting that ED5 did not induce cell cycle arrest in the G_0/G_1 phase or VSMC apoptosis through a p53 or p21 pathway. These findings are consistent with those of Mnjoyan and colleagues (27) who demonstrated that serum-induced elevations of p53 have a negative-feedback regulatory effect on VSMC proliferation and division through regulation of p21 and Bax.

As a transcription factor, the protein product of the Egr-1 gene, either independently or by complexing with other nucleoproteins, can directly regulate the expression of target genes and ultimately influence cell proliferation. Targeting Egr-1 expression may be a novel therapeutic strategy to treat atherogenesis and secondary intimal hyperplasia.

Acknowledgments

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