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Antisense Egr-1 RNA Driven by the CMV Promoter is an Inhibitor of Vascular Smooth Muscle Cell Proliferation and Regrowth After Injury

Roger G. Fahmy and Levon M. Khachigian*

Centre for Thrombosis and Vascular Research, The University of New South Wales and Department of Haematology, The Prince of Wales Hospital, Sydney

Abstract Smooth muscle cell (SMC) migration and proliferation are key events in the pathogenesis of atherosclerotic and post-angioplasty restenotic lesions. Mechanical injury to the artery wall induces the SMC expression of the zinc finger transcription factor, early growth response factor-1 (Egr-1). Egr-1 in turn can bind and activate the promoters of many genes, whose products influence vascular repair. Here, a 127-bp cDNA fragment corresponding to the 5′ region of murine Egr-1 mRNA was cloned into a CMV-driven expression vector, in the sense or antisense orientation. We demonstrate that antisense Egr-1 RNA inhibited rat vascular SMC proliferation, whereas the sense counterpart produced only a modest effect. By semi-quantitative reverse-transcription PCR, antisense Egr-1 RNA blocked serum-inducible Egr-1 mRNA expression. Western blot analysis demonstrated that antisense RNA overexpression inhibited Egr-1 protein synthesis, without affecting levels of the immediate early gene product, c-*fos*. Finally, antisense Egr-1 RNA overexpression inhibited SMC regrowth after mechanical injury in vitro. In contrast, sense Egr-1 RNA had no effect on SMC repair, Egr-1 mRNA expression or protein synthesis. Analysis of transfection efficiencies revealed that both CMV-driven constructs (sense and antisense) were taken up by the SMCs with equivalent efficiency. These findings provide the first demonstration of antisense RNA strategies targeting Egr-1 as inhibitors of Egr-1 and Egr-1-dependent cellular processes. The antisense RNA approach may be potentially useful in gene therapeutic efforts to control SMC growth in the injured artery wall. J. Cell. Biochem. 84: 575–582, 2002. © 2001 Wiley-Liss, Inc.

Key words: antisense RNA; Egr-1; smooth muscle cells; proliferation; injury

Smooth muscle cell (SMC) proliferation and migration are processes strongly implicated in the pathogenesis of restenosis following percutaneous transluminal coronary balloon angioplasty (PTCA) [Popma et al., 1991; Libby et al., 1992]. Restenosis occurs in approximately 30%–40% of patients after angioplasty and in between 20%–30% of patients following coronary stenting [Fischman et al., 1994; Serruys et al., 1994]. SMCs proliferating in these lesions are thought to produce and respond to growth factors and chemoattractants that act in an autocrine/paracrine manner [Ross, 1993, 1999], further propagating the growing lesion. The promoter region of many genes whose products stimulate SMC replication and migration [Khachigian and Collins, 1997], possess recognition elements for the zinc finger transcriptional factor and early growth response factor-1 (Egr-1) [Gashler and Sukhatme, 1995]. Egr-1 is an 80–82 kDa immediate-early gene product of the C_2H_2-zinc finger subclass co-expressed with many Egr-1-dependent genes in human atherosclerotic plaques [McCaffrey et al., 2000]. Egr-1 levels are elevated more than five-fold in human carotid lesions relative to adjacent normal tunica media [Du et al., 2000]. Egr-1 is activated by balloon injury to the artery wall prior to the expression of Egr-1-dependent genes and intimal hyperplasia [Kim et al., 1995; Khachigian et al., 1996; Santiago et al., 1999]. These findings suggest that Egr-1 may play a positive regulatory role in the pathogenesis of arterial lesions.

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*Correspondence to: Levon M. Khachigian, Ph.D., Centre for Thrombosis and Vascular Research, School of Pathology, The University of New South Wales, Sydney NSW 2052, Australia. E-mail: L.Khachigian@unsw.edu.au

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Pharmacologic strategies available for the treatment of restenosis are extremely limited at the present time, with anti-platelet strategies providing the most benefit [Topol et al., 1994]. Recent studies have reported the use of nucleic acid-based drugs in reducing restenosis in experimental animal models aimed at targeting and suppressing key regulatory genes in SMCs, and include DNAzymes [Santiago et al., 1999], chimeric ribozymes [Frimerman et al., 1999], and antisense phosphorothioated oligonucleotides [Morishita et al., 1993; Autieri et al., 1995; Villa et al., 1995; Pitsch et al., 1996; Bennett et al., 1997]. Vascular lesions make an ideal setting for gene targeting or overexpression strategies, by facilitating local delivery via catheters or stents, thus reducing possible systemic side effects.

In this study, we examined the feasibility of targeting Egr-1 by over-expressing antisense Egr-1 RNA in WKY12-22 cells, SMCs that are phenotypically similar to cells derived from arterial lesions [Seifert et al., 1984; Majesky et al., 1992; Lemire et al., 1994]. We demonstrate that antisense Egr-1 RNA inhibits Egr-1 mRNA expression and protein synthesis without affecting levels of a second immediate-early gene product, c-Fos. Moreover, we show in vitro that antisense Egr-1 RNA blocks SMC proliferation and regrowth after injury, two cellular processes in restenosis.

MATERIALS AND METHODS

Plasmid Constructs

A 127-bp cDNA fragment spanning the 179–306 nucleotide region of the murine Egr-1 transcript (M20157) was cloned into the *Apa*I/*HindIII* restriction sites of pcDNA 3.1 (−) (Invitrogen, Carlsbed) to generate a CMV-driven mEgr-1 sense strand construct. The same fragment was cloned into the *HindIII*/*Apa*I restriction sites of pcDNA 3.1 (+) (Invitrogen) to generate a CMV driven mEgr-1 antisense strand construct.

Cell Culture and Plasmid Transfection

WKY12-22 cells (a SMC line originally derived from the aortae of 2-week-old pup rats) [Seifert et al., 1984; Majesky et al., 1992; Lemire et al., 1994] were cultured in Waymouth's medium, pH 7.4, containing 10% fetal bovine serum (FBS), 10 μg/ml streptomycin and 10 U/ml penicillin at 37°C and 5% CO_2. Cells were passaged by washing in phosphate buffered saline (PBS) followed by trypsinization. Subconfluent SMCs (60%–70%) were growth arrested in serum free conditions for 6 h before being transfected with either antisense (A/S) or sense plasmid constructs (Invitrogen) using FuGENE6 (Roche Diagnostics GmbH, Mannheim Germany). Cells were transfected a second time in the presence of 5% FBS 18 h after the initial transfection. Following the second transfection, cells were incubated at 37°C.

Transfection Efficiency of Antisense and Sense Plasmid Constructs

Subconfluent SMCs were transfected in 100-mm tissue culture dishes. Four hours after the second transfection, in the presence of 5% serum, total DNA was extracted from the cells using TRIzol reagent (GIBCO BRL, Grand Island). Five hundred nanograms of total DNA was transformed into DH5α competent cells (GIBCO BRL) and plated out onto LB-ampicillin plates at different cell densities (40, 80, and 160 μl). Plates were incubated overnight in a dry incubator at 37°C and colony forming units (CFUs) counted per plate.

SMC Proliferation Assay

SMC were seeded into 96-well plates (TPP, Switzerland) (12,000 cells/well) in 10% FBS containing Waymouth's. Subconfluent SMCs were transfected with 200 ng of plasmid DNA. Following the second transfection in 5% FBS, cells were incubated for 18 h before being washed in PBS, trypsinised, and resuspended in 10 ml of isoton for counting. Cells were then counted using an automated Coulter counter (Coulter Z Series, Miami).

Egr-1 mRNA Analysis

Egr-1 mRNA was analysed by semi quantitative RT-PCR. Subconfluent SMCs were grown in 100-mm tissue culture plates (FALCON, Becton Dickinson, Franklin Lakes) and transfected with 18.75 μg of plasmid DNA. Total RNA was harvested using TRIzol reagent (GIBCO BRL), 1 h following second transfection in 5% FBS. Five μg of total RNA was reverse transcribed using SUPER SCRIPT RNase H^- Reverse Transcriptase (GIBCO BRL). Cycle-based PCR was used to semi quantitate Egr-1 and GAPDH mRNA expression. Each PCR reaction contained 1.5 mM $MgCl^{2+}$, 20 pmol of each primer, 0.1U (Egr-1), and 0.25U (GAPDH)

Taq DNA polymerase (Sigma, St. Louis, MO), 2 mM (Egr-1) and 5 mM (GAPDH) dNTPs, and 2 μl cDNA. Cycle based PCR was carried out using a GeneAmp PCR System 2400 (Perkin Elmer, Norwalk). PCR for Egr-1 expression consisted of a 2 min initial denaturing step at 94°C. Thermal cycling consisted of 1 min denaturing at 94°C, 1 min annealing at 50°C, and a 1 min extension at 72°C for 22 cycles. A final extension period at 72°C for 5 min was followed. Thermal cycling for the house keeping gene GAPDH consisted of 30 s denaturing at 94°C, 30 s annealing at 60°C and 2 min extension at 68°C for 15 cycles. Primer sequences were as follows: Egr-1, 5′-GCATGTAACCCG-GCCA-3′ (forward) and 5′-CCGAACGGGTCA-GAGAT-3′ (reverse); GAPDH, 5′-ACCACAG-TCCATGCCATCAC-3′ (forward) and 5′-TCCA-CCACCCTGTTGCTGTA-3′ (reverse). The expected sizes of the PCR products for Egr-1 and GAPDH were 163 and 451 bp, respectively.

Western Blot Analysis

Subconfluent SMCs were cultured in 100-mm tissue culture plates (FALCON) and transfected with 18.75 μg of plasmid. One hour after the second transfection in 5% FBS, cells were washed in PBS and total protein was extracted in 150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 1% sodium deoxycolate, 0.1% SDS, 1% Triton X-100, 5 mM EDTA, 10 μg/ml leupeptin, 1% aprotinin, and 2 mM PMSF. Five milligrams of protein sample was loaded onto a 10% denaturing SDS–PAGE and electroblotted onto a PVDF nylon membrane (Millipore, Bedfrod). Membranes were air dried before being blocked in 5% skim milk powder in PBS containing 0.05% Tween 20 (v/v). Membranes were primarily incubated with either rabbit polyclonal Egr-1 or c-Fos antibodies (Santa Cruz Biotechnology, Santa Cruz) at a concentration of 2 μg/ml. Memebranes were then incubated with horse radish peroxidase-linked swine anti rabbit IgG secondary antibody (Dako, Carpinteria). Proteins were visualized by chemiluminesent detection (NEN, Boston).

SMC Injury/Migration Assay

SMCs were grown to confluence in 8-well chamber slides (Nunc, Copenhagen Denmark) and transfected with 2.5 μg plasmid DNA. Following the second transfection in 5% FBS, injury was performed with a single scratch using a sterile toothpick. Eighteen hours after injury, chambers were replaced with fresh medium and incubated for 6 h when the cells were washed in PBS, fixed in 4% formaldehyde (v/v), then stained in hematoxylin and eosin prior to photomicroscopy.

RESULTS

Antisense Egr-1 RNA Downregulates Endogenous Egr-1 mRNA Expression in WKY12-22 Cells

Antisense inhibition of the expression of a target gene involves interaction of the antisense molecule with the mRNA of that gene, triggering cleavage of the duplex by RNase H thus inhibiting translation of the mRNA into functional protein. We used semi-quantitative RT-PCR to determine whether antisense Egr-1 RNA could influence the expression of Egr-1 mRNA in WKY12-22 cells by exposure to serum for 1 h. The RT-PCR had been optimised for semi-quantitative analysis for both Egr-1 and GAPDH by cycle-based amplification in pilot experiments. Antisense Egr-1 RNA markedly reduced levels of steady-state Egr-1 mRNA (Fig. 1). In contrast, sense Egr-1 RNA driven by the same CMV promoter did not influence Egr-1 mRNA expression. Transfection with either sense or antisense Egr-1 RNA had no effect on levels of GAPDH (Fig. 1).

Antisense Egr-1 RNA Inhibits Egr-1 Protein Synthesis in Serum-Stimulated WKY12-22 Cells

Having shown that antisense Egr-1 RNA inhibited Egr-1 mRNA expression, we next determined whether this strategy influenced Egr-1 at the level of protein. Western blot analysis revealed that antisense Egr-1 blocked the induction of Egr-1 protein in WKY12-22 cells by serum (Fig. 2). In contrast, overexpression of sense Egr-1 RNA had no inhibitory effect on Egr-1 protein, whose levels were comparable to those in untransfected cells incubated with serum (Fig. 2). Antisense Egr-1 RNA had no inhibitory affect on levels of the serum-inducible immediate early gene product, c-Fos (Fig. 2).

Equivalent Uptake of Antisense and Sense Egr-1 Plasmids by WKY12-22 Cells

To ensure that differences in Egr-1 mRNA and protein expression in the antisense and sense groups were not due to different transfection efficiencies of these constructs, we transformed competent *E. coli* with DNA of

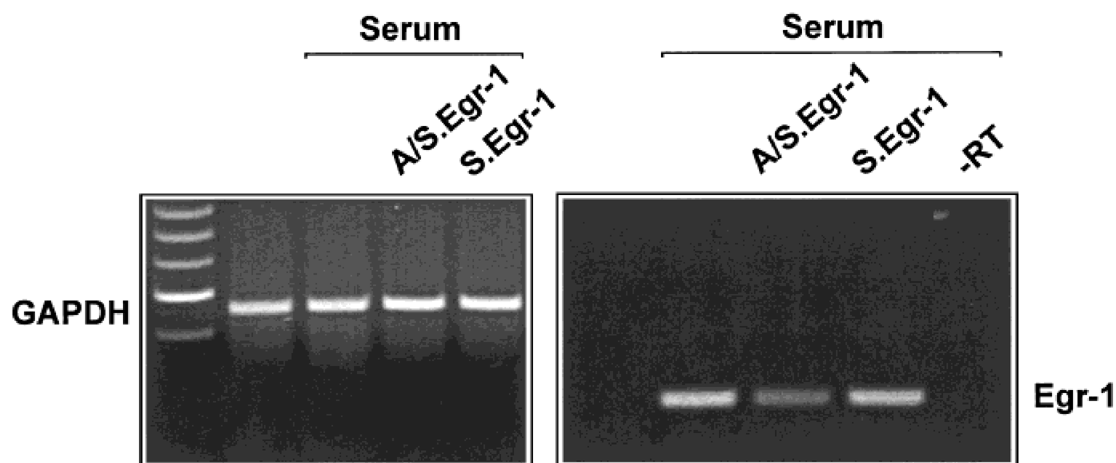

Fig. 1. Antisense Egr-1 RNA attenuates serum-inducible steady-state Egr-1 mRNA expression. WKY12-22 cells transfected with pCMV.A/S.Egr-1 (antisense) or pCMV.S.Egr-1 (sense) in 100-mm dishes were rendered growth-quiescent by incubation in serum-free medium for 24 h, then incubated with 5% serum for 1 h. Total RNA was isolated using TRIzol reagent, and semi-quantitative RT-PCR was performed for GAPDH or Egr-1. -RT denotes PCR amplification of template preparation in which RT was not included. The PCR reaction was resolved by electrophoresis on 1% agarose gels and stained with ethidium bromide prior to photography. The data is representative of two independent determinations.

WKY12-22 cells that had been transfected with either construct, and the resultant CFUs were quantitated in a blinded manner. Ampicillin resistance of the transformants on agar would be conferred by either plasmid. This analysis revealed no significant difference in the CFUs at any given plated volume (Fig. 3). In contrast, no CFUs were detectable in the untransfected group. These findings indicate that the inhibitory effect of antisense Egr-1 construct was not the consequence of preferential cellular uptake of one construct over another. In separate experiments using a comparable CMV promoter-driven plasmid driving green fluorescent protein expression, we determined that the efficiency of SMC transfection using FuGENE6 was 55%–60% (data not shown).

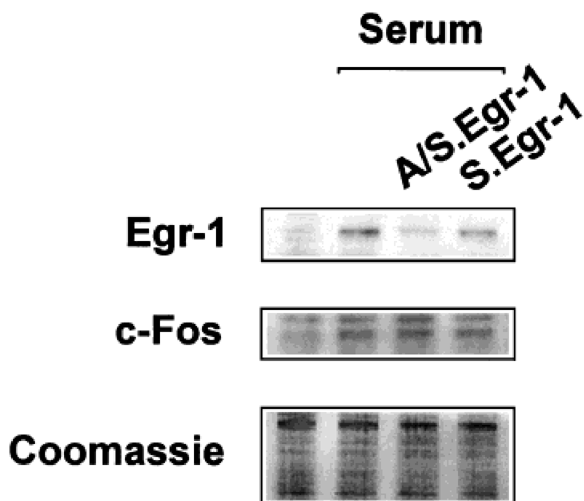

Fig. 2. Antisense Egr-1 RNA blocks serum-inducible Egr-1 protein expression. WKY12-22 cells transfected with pCMV.A/S.Egr-1 (antisense) or pCMV.S.Egr-1 (sense) in 100-mm dishes were rendered growth-quiescent by incubation in serum-free medium for 24 h, then incubated with 5% serum for 1 h. Cell extracts were assessed for Egr-1 and c-Fos immunoreactivity by Western blot analysis using polyclonal antibodies to Egr-1 or c-Fos, and chemiluminescence detection. The Coomassie Blue-stained gel indicates unbiased loading. The data is representative of two independent determinations.

Antisense Egr-1 RNA Inhibits Serum-Inducible WKY12-22 Proliferation

We next investigated the effect of antisense and sense Egr-1 on serum-inducible WKY12-22 proliferation. Cell populations were quantitated 18 h after exposure to serum. Antisense Egr-1 inhibited SMC replication virtually to completion, whereas only mild inhibition was evident in WKY12-22 cells expressing sense Egr-1 RNA (Fig. 4). Trypan Blue exclusion experiments and morphologic observations revealed that antisense Egr-1 RNA-mediated inhibition of SMC proliferation was not the consequence of toxicity (data not shown).

Antisense Egr-1 RNA Inhibits SMC Regrowth after Mechanical Injury

A denuded zone is produced when a confluent SMC population is injured by mechanical scraping. This is followed by outward SMC migration from the wound edge and proliferation into the denuded zone [Santiago et al., 1999]. To

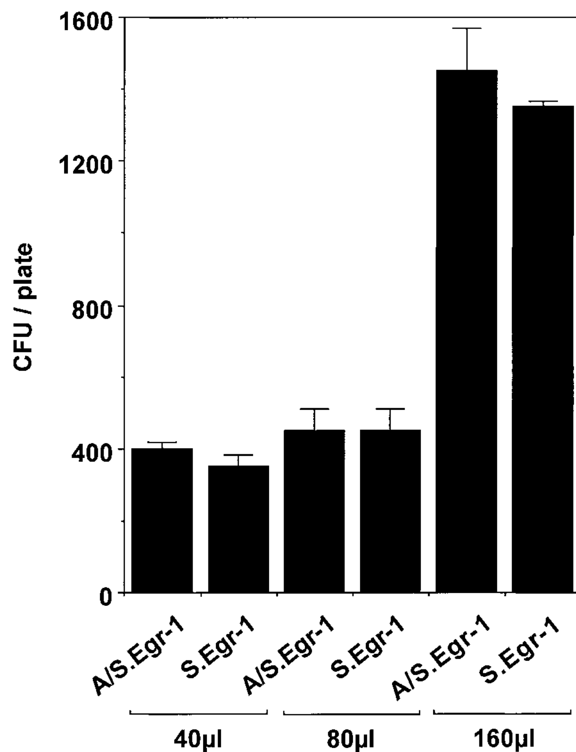

Fig. 3. Equivalent efficiencies of transfection of antisense and sense Egr-1 expression vectors. DNA extracts of WKY12-22 cells transfected with pCMV.A/S.Egr-1 (antisense) or pCMV.S.Egr-1 (sense) prepared in TRIzol were used to transform competent *E. coli.* The indicated volumes of suspension were spread onto LB-Amp plates and CFUs were counted after 24 h in a blinded manner. The data is representative of two independent determinations.

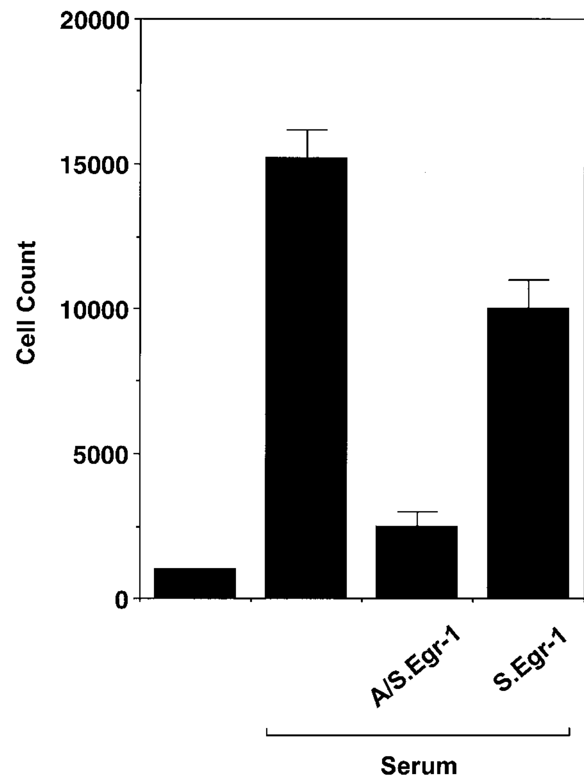

Fig. 4. Antisense Egr-1 RNA blocks serum-inducible WKY12-22 proliferation. WKY12-22 cells transfected with pCMV.A/S.Egr-1 (antisense) or pCMV.S.Egr-1 (sense) in 96-well plates were rendered growth-quiescent by incubation in serum-free medium for 24 h, then incubated with 5% serum for 18 h. The number of cells in each group was determined using an automated Coulter counter. The data is representative of two independent determinations.

determine whether antisense Egr-1 RNA could inhibit SMC repair after injury, we transfected confluent cells with either antisense or sense Egr-1 RNA prior to scraping. SMC regrowth from the wound edge was significantly inhibited by antisense Egr-1 RNA (Fig. 5), whereas regrowth in the sense Egr-1 RNA cohort was no different from the untransfected control group (Fig. 5). Taken together these data demonstrate that CMV promoter-driven antisense Egr-1 inhibits SMC replication and regrowth after injury 24 h after serum-induction, and the expression of Egr-1 mRNA and protein, but not that of a second immediate-early gene within hours of exposure to serum.

DISCUSSION

SMC proliferation and migration in response to mechanical injury to the artery wall are key processes contributing to intimal thickening and restenosis after balloon angioplasty. The lack of clinically-effective drugs able to inhibit the incidence of restenosis, which occurs in up to 40% of all patients, who undergo an angioplasty, provides an important challenge to vascular biologists concerned with the control of this adaptive cellular response to injury. Genes mediating SMC replication and migration have become targets for molecular intervention. One approach has been the application of antisense technology, wherein the oligonucleotide, ribozyme, DNAzyme, or complementary RNA, such as in the present study, binds to and prevents production of the endogenous protein [Jen and Gewirtz, 2000].

Previous findings have demonstrated that short synthetic antisense oligonucleotides possessing phosphorothioate backbones targeting genes such as c-*myc* [Bonnett et al., 1994; Bennett et al., 1997], c-*myb* [Simons et al., 1992; Villa et al., 1995; Pitsch et al., 1996],

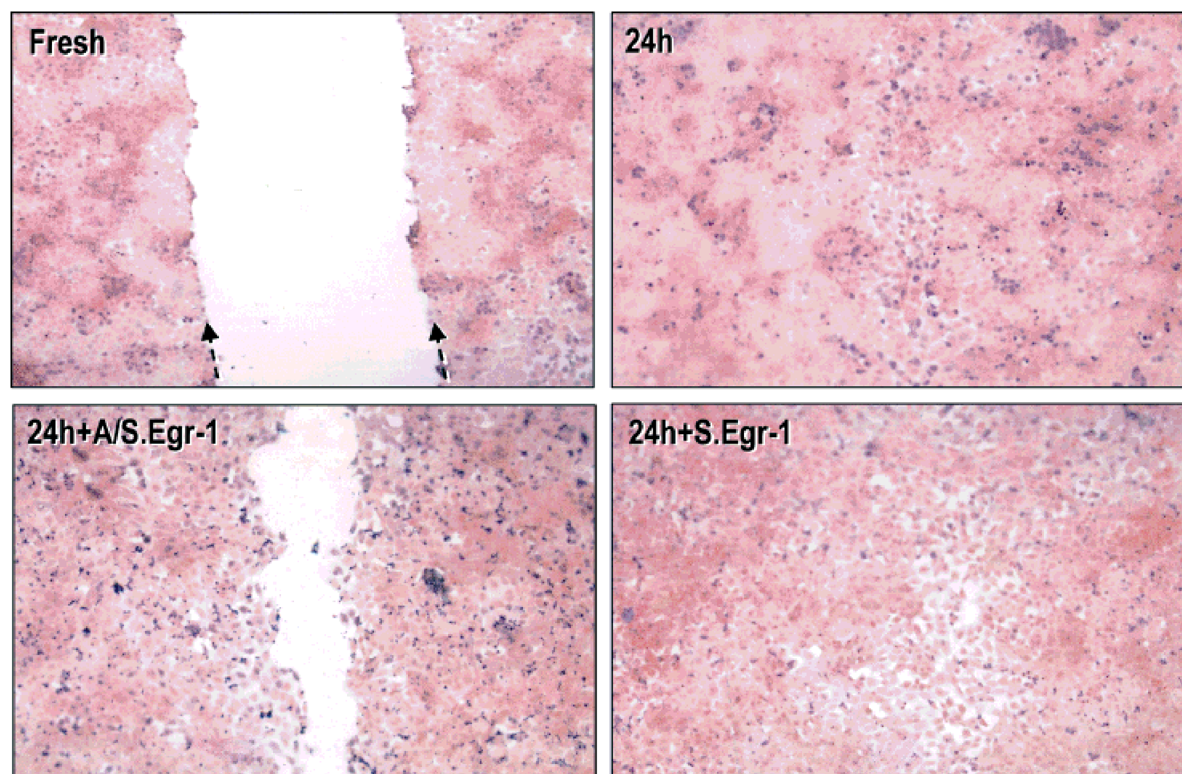

Fig. 5. Antisense Egr-1 RNA blocks serum-inducible WKY12-22 proliferation. WKY12-22 cells transfected with pCMV.A/S.Egr-1 (antisense) or pCMV.S.Egr-1 (sense) in chamber slides were grown to confluence and injured by the single scrape of a sterile toothpick. The cultures were left undisturbed for 24 h prior to washing, fixing, staining with haematoxylin and eosin, and photography. The data is representative of two independent determinations.

nuclear factor NF-kappaβ [Autieri et al., 1995], cdc2 and PCNA [Morishita et al., 1993], and PDGFR-beta receptor subunit [Sirois et al., 1997] reduced intimal thickening following insults to the vessel wall. Although substitution of the phosphodiester backbone of native DNA with phosphorothioate moieties increases resistance to nuclease digestion [Marcus-Sekura et al., 1987; Cazenave et al., 1989], the use of phosphorothioate-oligonucleotides as therapeutic tools in the treatment of restenosis is somewhat controversial because of potential toxicity and the propensity of some sequences to bind unintended proteins non-specifically [Guvakova et al., 1995; Shoeman et al., 1997]. Indeed, high concentrations of phosphorothioate-oligonucleotides can bind and inhibit the activity of DNA polymerases and RNase H [Gao et al., 1989, 1992], signaling molecules [Ho et al., 1991; Stein et al., 1993; Perez et al., 1994; Maltese et al., 1995; Stein, 1997], and growth factors [Yakubov et al., 1993; Guvakova et al., 1995]. In this study, we used the CMV promoter to generate antisense Egr-1 RNA in WKY12–22 cells, a SMC line with "synthetic" rather than the quiescent "contractile" phenotype characteristic of SMCs of arterial lesions [Seifert et al., 1984; Majesky et al., 1992; Lemire et al., 1994]. Egr-1 is weakly, if at all, expressed in the artery wall, but is strongly expressed in SMCs and endothelial cells in response to arterial injury [Khachigian et al., 1996; Silverman et al., 1997]. Using the antisense RNA approach, we demonstrate that the expression of Egr-1 mRNA and protein is suppressed, and that SMC proliferation and regrowth in response to injury is significantly inhibited. Analysis of transfection efficiency revealed that both antisense and sense Egr-1 constructs were taken up by the SMCs equivalently, demonstrating that the antisense effect was not due to differences in plasmid incorporation.

Plasmid DNA, such as that used in the present study, has been used as a means to deliver genes into the injured artery wall. For example, plasmids generating mutant Ras protein inhibited SMC hyperplasia after balloon injury [Indolfi et al., 1995]. Other studies have

used adenoviral vectors to transfer a urokinase receptor-targeted protease inhibitor [Quax et al., 2001], a ribozyme to c-*myb* [Macejak et al., 1999], a dominant negative H-*ras* [Ueno et al., 1997] and antisense basic fibroblast growth factor [Hanna et al., 1997], and demonstrated reduced intimal thickening. In this study we have shown for the first time that antisense Egr-1 RNA, driven using the CMV promoter, is a useful strategy for inhibiting Egr-1 expression, SMC regrowth and proliferation. As such, whether delivery is achieved by plasmid, viral, or other means, this study suggests that antisense RNA strategies targeting Egr-1 in SMCs may eventually be useful in the gene therapeutic management of restenosis.

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