

New DNA enzyme targeting Egr-1 mRNA inhibits vascular smooth muscle proliferation and regrowth after injury

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Early growth response factor-1 (Egr-1) binds to the promoters of many genes whose products influence cell movement and replication in the artery wall. Here we targeted Egr-1 using a new class of DNA-based enzyme that specifically cleaved Egr-1 mRNA, blocked induction of Egr-1 protein, and inhibited cell proliferation and wound repair in culture. The DNA enzyme also inhibited Egr-1 induction and neointima formation after balloon injury to the rat carotid artery wall. These findings demonstrate the utility of DNA enzymes as biological tools to delineate the specific functions of a given gene, and implicate catalytic nucleic acid molecules composed entirely of DNA as potential therapeutic agents.

Ribozymes are ribonucleic acid (RNA) molecules that selectively bind to an RNA substrate by Watson–Crick base-pairing and cleave phosphodiester bonds^{1–3}. This property has been successfully exploited to inhibit gene expression in various cell types (review, ref. 4). However, the utility of ribozymes as biologic and therapeutic tools has been limited by their susceptibility to chemical and enzymatic degradation^{5,6} and restricted target site specificity. Chimeric ribozymes containing deoxyribonucleic acid (DNA) or phosphorothioate linkages have been generated to overcome sensitivity to degradation, but these ribozymes are expensive to synthesize and tend to degrade in serum. Antisense phosphorothioated oligodeoxynucleotides (ODNs) are more resistant to nucleolytic cleavage, but these molecules lack catalytic activity. A new generation of catalytic nucleic acid composed entirely of DNA has been developed using an *in vitro* selection strategy⁷. These magnesium-dependent moieties can potentially cleave RNA at any purine–pyrimidine junction⁷ and offer greater substrate specificity than hammerhead ribozymes⁸. Despite the therapeutic promise of DNA enzymes, their ability to influence biological responsiveness has not been determined at a cellular or molecular level.

Smooth muscle cells (SMCs) are important cellular components of atherosclerotic and post-angioplasty restenotic lesions^{9,10}. SMC migration and proliferation are essential events in the pathogenesis of these vascular disorders^{11,12}. The promoter regions of many genes that encode mitogenic and migratory factors expressed by SMCs in these lesions¹³ contain nucleotide recognition elements for the transcriptional regulator Egr-1 (ref. 14). Egr-1 (also known as NGFI-A, zif268, tis8, krox24 and cef5; ref. 15) is not expressed in the unmanipulated artery wall, but is rapidly activated by mechanical injury¹⁴. It is also induced in vascular endothelial cells and/or SMCs exposed to fluid biomechanical forces^{16,17} and many other pathophysiologically relevant agonists^{18,19}. Therefore, this pleiotropic transcription factor seemed an ideal target for the development and evaluation of sequence-specific DNA enzymes in SMCs.

DNA enzyme ED5 cleaves synthetic and transcribed Egr-1 mRNA

We designed a DNA enzyme to target specifically the translational start site AUG, at positions 816–817, in rat Egr-1 messenger RNA (Fig. 1a), a region that has low relative free energy²⁰. The two seven-nucleotide arms flanking the 15-nucleotide catalytic domain in the original DNA enzyme design⁷ were extended by two nucleotides per arm for greater target specificity. For resistance to 3'-to-5' exonuclease digestion, the 3' terminus of the molecule was capped with an inverted 3'-3'-linked thymidine (Fig. 1a). The nucleotide sequence in each arm of ED5 was scrambled (SCR) without altering the catalytic domain to produce the DNA enzyme ED5SCR (Fig. 1a).

To determine whether ED5 cleaved RNA, we synthesized a substrate composed of 23 nucleotides, corresponding to nucleotides 805–827 of rat Egr-1 mRNA (Fig. 1a). ED5 cleaved this 23-amino-acid peptide (labeled with ³²P at the 5' end) within 10 minutes (Fig. 1b). The 12-amino-acid product (Fig. 1b) is consistent with the length between the A816–U817 junction and the 5' (radiola-

Table 1 DNA enzyme target sites in mRNA

Gene	Accession number	Best homology over 18 nucleotides in arms (%)	
		ED5	hED5
Rat Egr-1	M18416	100	84.2
Human EGR-1	X52541	84.2	100
Murine Sp1	AF022363	66.7	66.7
Human c-Fos	K00650	66.7	66.7
Murine c-Fos	X06769	61.1	66.7
Human Sp1	AF044026	38.9	28.9

Similarity between the 18-nucleotide arms of ED5 or hED5 and the mRNA of rat Egr-1 or human EGR-1 and other transcription factors, expressed as a percentage. Target sequences: ED5 in Egr-1 (rat) mRNA, 5'-807-ACGUCCGGAUGGCAGCGG-825-3'; hED5 in EGR-1 (human), 5'-262-UCGUCCAGGAUGGCCGCGG-280-3' (bold, mismatches between rat and human sequences). Data obtained by a gap best-fit search in ANGIS using sequences derived from Genbank and EMBL. Rat sequences for Sp1 and c-Fos have not been reported.

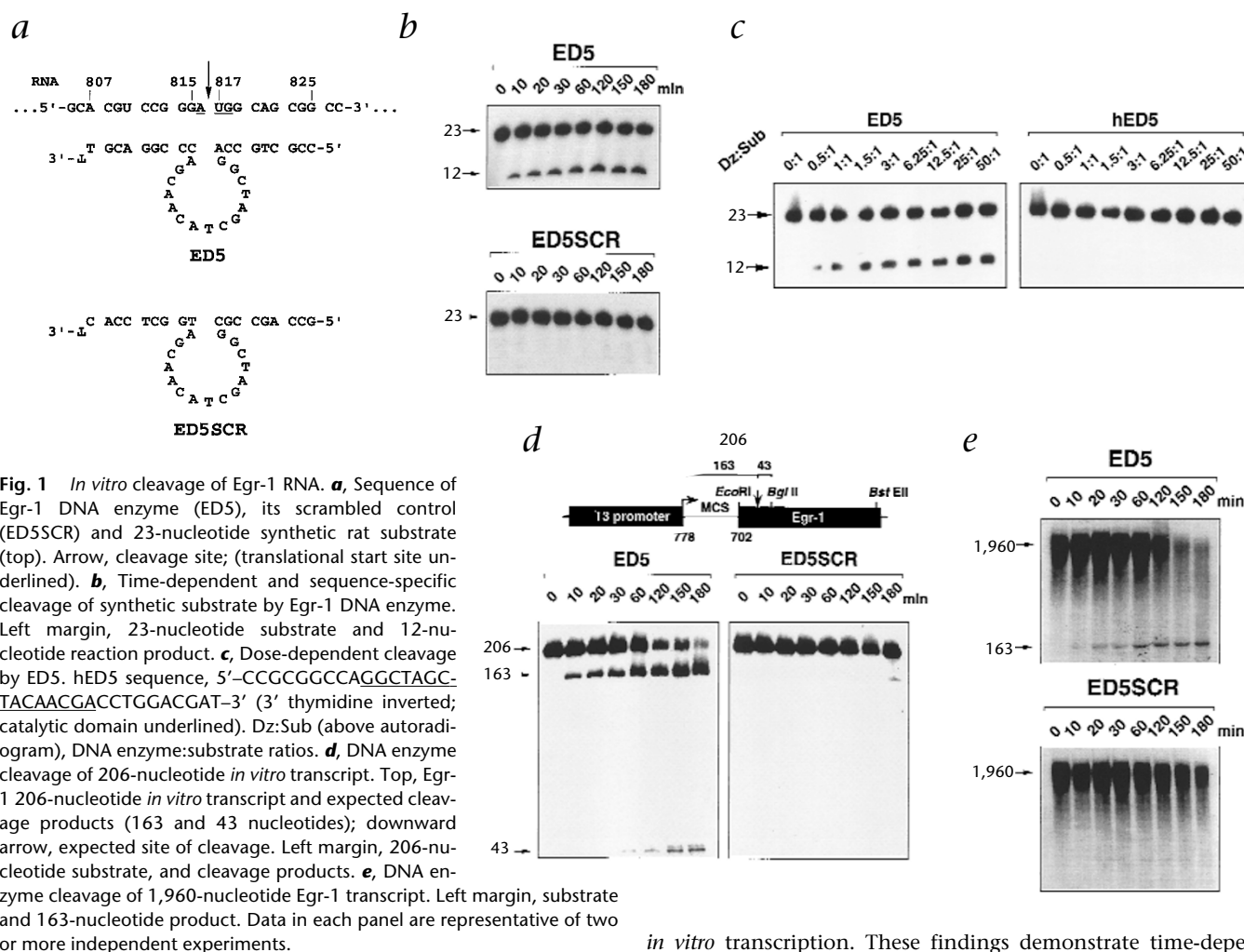


Fig. 1 *In vitro* cleavage of Egr-1 RNA. **a**, Sequence of Egr-1 DNA enzyme (ED5), its scrambled control (ED5SCR) and 23-nucleotide synthetic rat substrate (top). Arrow, cleavage site; (translational start site underlined). **b**, Time-dependent and sequence-specific cleavage of synthetic substrate by Egr-1 DNA enzyme. Left margin, 23-nucleotide substrate and 12-nucleotide reaction product. **c**, Dose-dependent cleavage by ED5. hED5 sequence, 5'-CCGCGGCCAGGCTAGC-TACAACGACCTGGACGAT-3' (3' thymidine inverted; catalytic domain underlined). Dz:Sub (above autoradiogram), DNA enzyme:substrate ratios. **d**, DNA enzyme cleavage of 206-nucleotide *in vitro* transcript. Top, Egr-1 206-nucleotide *in vitro* transcript and expected cleavage products (163 and 43 nucleotides); downward arrow, expected site of cleavage. Left margin, 206-nucleotide substrate, and cleavage products. **e**, DNA enzyme cleavage of 1,960-nucleotide Egr-1 transcript. Left margin, substrate and 163-nucleotide product. Data in each panel are representative of two or more independent experiments.

beled) end of the substrate (Fig. 1a). In contrast, ED5SCR had no effect (Fig. 1b). Neither the human equivalent of ED5 (hED5)(Fig. 1c) nor ED5SCR (data not shown) was able to cleave the rat substrate over a wide range of stoichiometric ratios. hED5 differs from rat ED5 by 3 of 18 nucleotides in its hybridizing arms (Table 1). ED5 also cleaved larger ³²P-labeled fragments of Egr-1 mRNA (206 nucleotides, Fig. 1d; 1,960 nucleotides, Fig. 1e) prepared by

in vitro transcription. These findings demonstrate time-dependent (Fig. 1b, d and e), dose-dependent (Fig. 1c) and sequence-specific (Fig. 1b–e) cleavage by ED5.

ED5 inhibits induction of endogenous Egr-1 mRNA and protein

To determine the effect of ED5 on endogenous Egr-1 mRNA, we exposed growth-quiescent SMCs to DNA enzyme before stimulating them with serum. Northern blot and densitometric analyses showed that 0.1 μM ED5 inhibited serum-inducible steady-state Egr-1 mRNA levels by 55% (Fig. 2a), whereas ED5SCR had no effect (Fig. 2a). Western blot analysis demonstrated that ED5 also blocked Egr-1 synthesis at the protein level (Fig. 2b). In contrast, neither ED5SCR nor EDC, which has a catalytic domain flanked by nonsense arms, had any influence on the induction of Egr-1 (Fig. 2b). ED5 failed to affect levels of the constitutively expressed, structurally related zinc-finger protein Sp1 (Fig. 2b). It was also

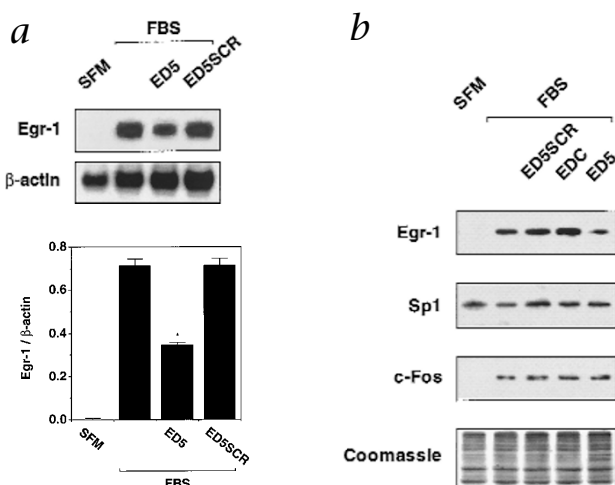


Fig. 2 Egr-1 DNA enzyme inhibits the induction of Egr-1 mRNA and protein by serum. **a**, Northern blot analysis for Egr-1 mRNA; the blot was 'stripped' and reprobed for β-actin. Autoradiograms were analyzed by scanning densitometry; vertical axis, Egr-1 band intensity as a fraction of β-actin band intensity (mean and s.e.m.). Data are representative of two independent experiments. *, $P < 0.05$, compared with control (FBS alone); Student's paired *t*-test. SFM, serum-free medium. **b**, Western blot analysis with antibodies against Egr-1, Sp1 or c-Fos. Bottom (Coomassie blue-stained gel), uniform amounts of protein were loaded per lane. EDC sequence, 5'-CGC-CATTAGGCTAGCTACAACGACCTAGTGAT-3' (3' thymidine inverted; catalytic domain underlined). SFM, serum-free medium.

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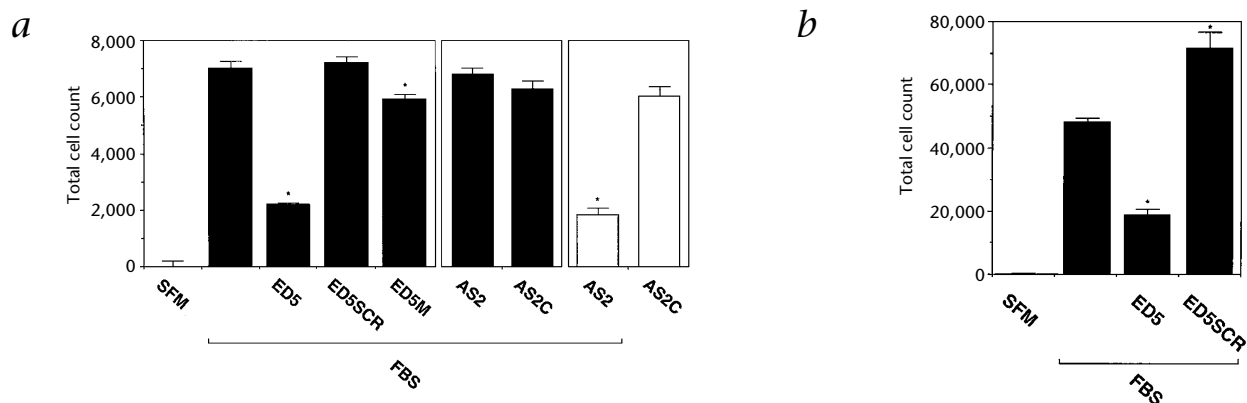


Fig. 3 SMC proliferation is inhibited by Egr-1 DNA enzyme. **a**, Growth-arrested SMCs exposed to serum (FBS) and/or ODN for 3 d were resuspended and counted. Sequences: ED5C, 5'-CCGCTGCCAGGCTACCTACAACGAC-CCGGACGTT-3' (3' thymidine inverted; catalytic domain underlined; G-to-C mutation at position 6 in the catalytic core italicized); AS2, 5'-CTTGGCCGCTGCCAT-3'; AS2C, 5'-GCATTCTGCTGTCC-3'. ■, 0.1

μM ODN; □, 0.6 μM ODN. **b**, Effect of ED5 on pup SMC proliferation. Growth-arrested WKY12-22 cells exposed to serum (FBS) and/or DNA enzyme for 3 d were resuspended and counted. SFM, serum-free medium. Data are presented as mean and s.e.m. and are representative of two independent experiments in triplicate. *, $P < 0.05$, compared with ODN control, Student's paired t-test.

unable to block serum induction of the immediate-early gene product c-Fos (Fig. 2b), whose expression, like that of Egr-1, depends upon serum response elements in its promoter and phosphorylation by extracellular-signal-regulated kinase^{21,22}. These findings demonstrate the ability of ED5 to inhibit induction of Egr-1 mRNA and protein in a gene- and sequence-specific manner. The specificity of ED5 cleavage is consistent with loose sequence homology between the DNA enzyme target site in Egr-1 mRNA and other mRNA (Table 1).

ED5 inhibits smooth muscle cell replication

We incubated growth-quiescent SMCs with DNA enzyme, then exposed them to serum and assessed cell numbers after 3 days. ED5 at a concentration of 0.1 μM inhibited SMC proliferation stimulated by serum by 70% (Fig. 3a). In contrast, ED5SCR failed to influence SMC growth (Fig. 3a). ED5M, which has a mutation of G to C at position 6 in the catalytic core and inactivates the DNA enzyme²³, only weakly inhibited proliferation (Fig. 3a), probably as a consequence of non-enzymatic antisense mechanisms. AS2, a phosphorothioate-linked antisense ODN targeting the same region in Egr-1 mRNA as ED5, had no effect on SMC proliferation at an identical concentration (0.1 μM) (Fig. 3a). AS2 inhibition was similar to ED5 only if the concentration of ODN was increased by 600% (Fig. 3a), which indicates greater potency of the DNA enzyme. ED5 inhibition was not a consequence of cell death, as no change in morphology resulted and the proportion of cells incorporating trypan blue in the presence of serum was not influenced by exposure to DNA enzyme (data not shown). These findings are consistent with inhibition of G₁-to-S phase transition in other cell types by overexpression of dominant negative Egr-1 (ref. 24).

Cultured SMCs derived from the aortae of 2-week-old rats (WKY12-22) are morphologically and phenotypically similar to SMCs derived from the neointima of balloon-injured rat arteries^{25,26}. The epithelioid appearance of both WKY12-22 cells and neointimal cells contrasts with the elongated, bipolar nature of SMCs derived from normal quiescent media²⁷. WKY12-22 cells grow more rapidly than medial SMCs and overexpress a large number of growth-regulatory molecules²⁸ such as Egr-1²⁹, consis-

tent with a 'synthetic' phenotype^{26,30}. ED5 attenuated serum-inducible WKY12-22 proliferation by approximately 60% (Fig. 3b). ED5SCR had no inhibitory effect (Fig. 3b).

DNA enzymes resist degradation in serum and localize in nuclei

To ensure that differences between ED5 and ED5SCR did not arise from dissimilar intracellular localization, we labeled both DNA enzymes at the 5' end with fluorescein isothiocyanate (FITC) and incubated them with the cells for 24 hours before fluorescence microscopy. Both FITC-ED5 (Fig. 4a, center) and FITC-ED5SCR (Fig. 4a, bottom) localized almost exclusively in the nuclei of approximately 75% of cells; punctate fluorescence in the nucleus was independent of DNA enzyme sequence or autofluorescence (Fig. 4a). To eliminate the possibility that one

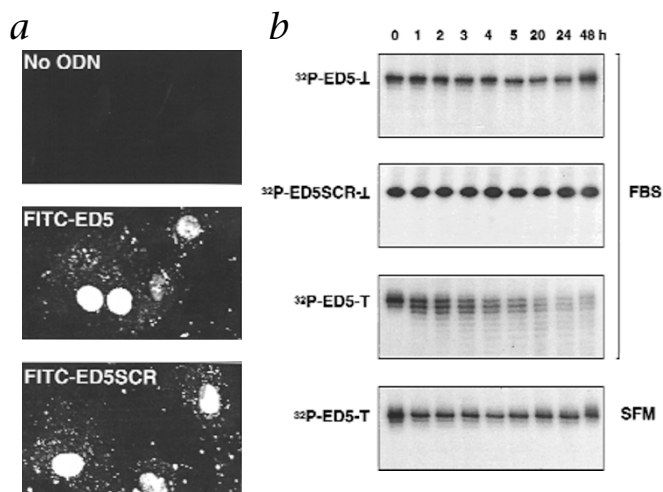


Fig. 4 Cellular localization and stability of Egr-1 DNA enzymes. **a**, ED5 and ED5SCR localize mainly in SMC nuclei. Growth-arrested SMC were transfected with DNA enzyme labeled with FITC at the 5' end, and fluorescence microscopy was done after 24 h at 37 °C. Original magnification, ×400. **b**, The 3' inverted thymidine confers resistance to nucleolytic degradation in serum. 32P-labeled ED5 or 32P-labeled ED5SCR with a 3'-thymidine in the correct (T) or inverted (L) orientation were incubated (times, above autoradiograms) in 5% FBS or serum-free medium (SFM).

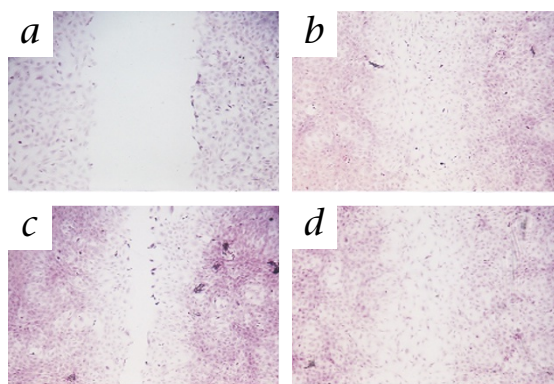


Fig. 5 Egr-1 DNA enzyme inhibits SMC repair after mechanical injury. **a**, SMC cultures immediately after scraping injury. **b**, SMC cultures 3 d after injury. **c** and **d**, SMC cultures 3 d after injury in the presence of ED5 (**c**) or its scrambled counterpart, ED5SCR (**d**). Original magnification, $\times 100$; hematoxylin and eosin staining. Data are representative of three independent experiments

DNA enzyme was more stable than the other, we labeled both DNA enzymes with ^{32}P and incubated them in culture medium for various times before electrophoresis. ^{32}P -labeled ED5 and ^{32}P -labeled ED5SCR each remained intact even after 48 hours of exposure to medium (Fig. 4b). In contrast, we detected degradation of ^{32}P -labeled ED5 with a thymidine in the correct orientation at the 3' end as early as 1 hour (Fig. 4b), yet it remained intact in serum-free medium even after 48 hours (Fig. 4b). These results indicate there is no difference in cellular localization between ED5 and ED5SCR, and demonstrate the importance of an inverted thymidine at the 3' end for resistance to nucleolytic cleavage by components in serum.

ED5 inhibits SMC regrowth after mechanical injury *in vitro*

Physical trauma imparted to SMCs in culture results in outward migration from the wound edge and proliferation in the denuded zone. We determined whether ED5 could modulate this response to injury by exposing growth-quiescent SMCs to DNA enzyme and mitomycin C, an inhibitor of proliferation³¹, before injuring the cells with a single scrape. Cultures in which DNA enzyme was absent repopulated the entire denuded zone within 3 days (Fig. 5a and b). ED5 inhibited this reparative response to injury (Fig. 5c) and prevented additional growth even after 6

days (data not shown). ED5SCR had no effect in this system (Fig. 5d), which further demonstrates the sequence-specific inhibition of SMC responsiveness by ED5.

ED5 inhibits induction of Egr-1 and neointima formation

We next investigated the ability of ED5 to inhibit arterial neointima formation, a combination of SMC migration and proliferation, after balloon injury to the inner wall of the rat carotid artery. This well-established model of mechanical injury³² maintains tissue perfusion during neointima formation. ED5 inhibited intimal thickening 14 days after injury by more than 50%, whereas neither ED5SCR nor the vehicle alone could attenuate this process (Fig. 6). ED5 also inhibited neointima formation in a second rat model, after permanent ligation of the carotid artery³³ (data not shown). We applied FITC-labeled ED5 or ED5SCR to the vessel in a manner identical to their unlabeled counterparts; fluorescence microscopy of cross-sectioned artery 3 hours after injury showed that each DNA enzyme localized to SMCs in the vascular media (data not shown). There was no fluorescence in the artery wall after application of the FITC moiety alone (data not shown). These findings demonstrate the ability of ED5 to suppress SMC accumulation in the vascular lumen in a sequence-specific manner.

To determine whether the effect of ED5 on neointima formation involved inhibition of the induction of Egr-1, we did immunoperoxidase staining of sections of injured rat carotid arteries treated with ED5 or ED5SCR using polyclonal antibodies directed against Egr-1. Inducible Egr-1 expression 1 hour after injury (as seen in rat arteries as mRNA; ref. 14) was inhibited by ED5 (Fig. 7), whereas neither ED5SCR nor vehicle alone had any effect (Fig. 7). In contrast, levels of Sp1 antigen in the artery wall were unaffected by DNA enzyme treatment (data not shown).

Catalytic nucleic acid molecules that recognize and cleave target RNA in an efficient and selective manner offer the promise of being valuable research tools in delineating the specific role(s) of individual genes in biochemistry and disease. Ribozymes targeting *MDR-1*, pleiotropin and HIV-1, in particular, have been useful *in vitro* in overcoming drug resistance³⁴, inhibiting tumor growth³⁵ and blocking viral replication³⁶, respectively. However, a principal limitation in the wider application of ribozymes has been the inherent instability of RNA-rich molecules. DNA enzymes offer several distinct advantages over ribozymes and other nucleic acid molecules as biocatalysts in gene therapy. DNA enzymes are simpler to design, have greater flexibility for selection

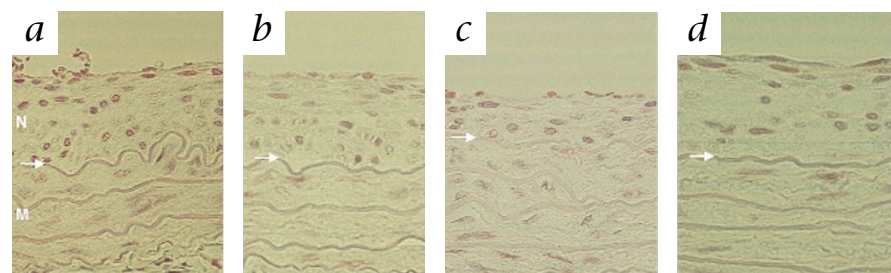


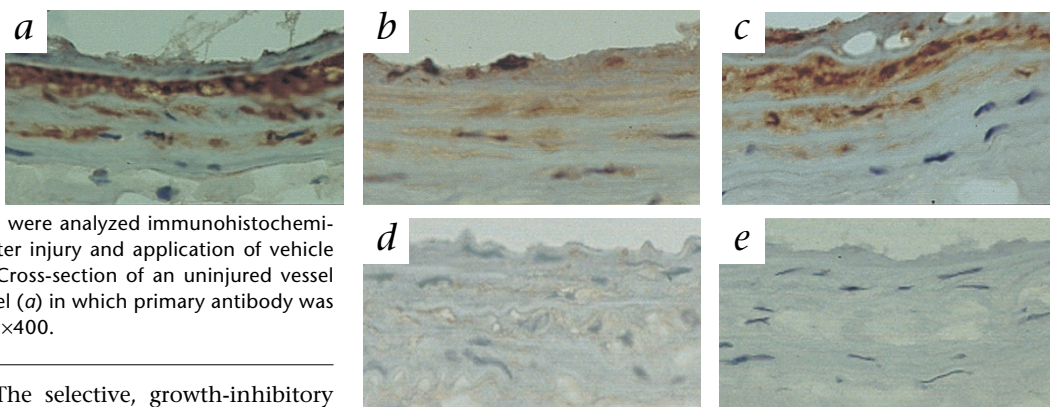
Fig. 6 Sequence-specific inhibition of neointima formation by Egr-1 DNA enzyme in the rat carotid artery. Cross-sections of carotid arteries showing neointima achieved 14 d after balloon catheter injury of the left common carotid artery, with DNA enzyme (500 μg) or vehicle alone applied 6 h before and immediately after balloon inflation. **a**, Balloon injury alone. **b–d**, Injury and adventitial application of vehicle (**b**), ED5 (**c**) or ED5SCR (**d**). Arrow, pre-injury intima; N, neointima; M, media. Original magnification, $\times 400$. Neointimal and medial areas of five consecutive sections per rat (five rats per group) were determined digitally and expressed as a neointima:media ratios per group: **a**, 0.71 ± 0.17 ; **b**, 0.72 ± 0.15 ; **c**, $0.31 \pm 0.11^*$; **d**, 0.62 ± 0.13 . *, $P < 0.05$ compared with all other groups (Wilcoxon rank sum test for unpaired data).

of cleavage sites⁸ and require lower cost for synthesis. In addition, DNA enzymes do not have phosphorothioate backbones, which confer additional negative charge onto the oligomer³⁷ and reduce binding affinity for target mRNA (ref. 38), increase binding avidity for serum and/or cellular proteins³⁸ and may be toxic³⁹. The sequence-specific nature of rat ED5 may preclude use of this particular DNA enzyme in other animals, given the subtle differences in Egr-1 mRNA sequences between species.

In conclusion, our study demonstrates the precision with which DNA enzymes can be used to delineate specific functions of a given gene. Egr-1 plays a necessary and sufficient part in DNA synthesis, proliferation and wound repair and in the arterial response to

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Fig. 7 Immunoperoxidase detection of Egr-1 in balloon-injured carotid arteries treated with DNA enzyme. DNA enzyme (500 μ g) or vehicle alone was applied 6 h before and immediately after balloon inflation; rats were killed 1 h later. Sections 5 μ m in thickness were analyzed immunohistochemically. **a–c**, Cross-sections 1 h after injury and application of vehicle (**a**), ED5 (**b**) or ED5SCR (**c**). **d**, Cross-section of an uninjured vessel stained for Egr-1. **e**, Injured vessel (**d**) in which primary antibody was omitted. Original magnification, $\times 400$.



mechanical injury *in vivo*. The selective, growth-inhibitory properties of ED5 indicate that DNA enzymes may be useful in therapeutic strategies involving vascular disorders caused by inappropriate SMC growth.

Methods

ODN synthesis. DNA enzymes were synthesized commercially (Oligos Etc., Wilsonville, Oregon) with an inverted thymidine at the 3' position, unless otherwise indicated. Substrates in cleavage reactions were synthesized with no such modification. Where indicated, ODNs were labeled at the 5' end with γ^{32} P-dATP and T4 polynucleotide kinase (NEB). Unincorporated label was separated from radiolabeled species by centrifugation on Chromaspin-10 columns (Clontech, Palo Alto, California).

***In vitro* transcript and cleavage experiments.** A 32 P-labeled, 206-nucleotide Egr-1 RNA transcript was prepared by *in vitro* transcription (T3 polymerase) of plasmid construct pJDM8 (a gift from J. Milbrandt) cut before transcription with *Bgl*II. The 32 P-labeled, 1,960-nucleotide transcript was generated from the same vector, cut before transcription with *Bst*Ell. Reactions were done in a total volume of 20 μ l containing 10 mM $MgCl_2$, 5 mM Tris, pH 7.5, 150 mM NaCl, 4.8 pmol of *in vitro*-transcribed or synthetic RNA substrate and 60 pmol DNA enzyme (1:12.5, substrate:DNA enzyme ratio), unless otherwise indicated. Reactions were allowed to proceed at 37 °C and were 'quenched' by transfer of aliquots to tubes containing formamide loading buffer⁴⁰. Samples were separated by electrophoresis on 12% denaturing polyacrylamide gels and detected by autoradiography overnight at -80 °C.

Culture conditions and DNA enzyme transfection. Primary rat aortic SMCs were obtained from Cell Applications (Santa Cruz, California) and were grown in Waymouth's medium, pH 7.4, containing 10% fetal bovine serum (FBS), 50 μ g/ml streptomycin and 50 IU/ml penicillin at 37 °C in a humidified atmosphere of 5% CO_2 . SMCs were used in experiments between passages 3 and 7. Pup rat SMCs (WKY12-22) were a gift from S.M. Schwartz and C.C. Giachelli (Department of Pathology, University of Washington) and were grown in similar conditions. Subconfluent (60–70%) SMCs were incubated in serum-free medium for 6 h before being transfected with DNA enzyme or ODN (0.1 μ M, unless otherwise indicated) using Superfect (6 h exposure in serum-free medium) in accordance with manufacturer's instructions (Qiagen). At 24 h after the first change of medium to serum-free medium, cells were washed with phosphate-buffered saline (PBS), pH 7.4, before being transfected a second time in 5% FBS.

Northern blot analysis. Total RNA was isolated using the TRIzol reagent (Life Technologies) and 25 mg was separated by electrophoresis before being transferred to Hybond-N+ membranes (NEN). Prehybridization, hybridization with Egr-1 or β -actin cDNA labeled with α^{32} P-dCTP, and washing were done essentially as described⁴¹.

Western blot analysis. Growth-quiescent SMCs in 100 mm plates (Nunc, Copenhagen, Denmark) were transfected with ED5 or ED5SCR as described above and incubated with 5% FBS for 1 h. The cells were washed in cold PBS, pH 7.4, and were extracted in 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 5 mM EDTA, 10

μ g/ml leupeptin, 1% aprotinin and 2 mM PMSF. Protein samples were separated by 10% denaturing SDS-PAGE and electroblotted onto PVDF nylon membranes (NEN). Membranes were air-dried before being blocked with non-fat skim milk powder in PBS containing 0.05% Tween 20 (weight/volume). Membranes were incubated with rabbit antibodies against Egr-1 or Sp1 (Santa Cruz Biotechnology, Santa Cruz, California), diluted 1:1,000, then with HRP-linked mouse secondary antiserum against rabbit Ig, diluted 1:1,000. Where mouse monoclonal antibody against c-Fos (Santa Cruz Biotechnology, Santa Cruz, California) was used, detection was achieved with HRP-linked rabbit antibody against mouse Ig. Proteins were visualized by chemiluminescent detection (NEN).

Assays of cell proliferation. Growth-quiescent SMCs in 96-well titer plates (Nunc, Copenhagen, Denmark) were transfected with DNA enzyme or ODN as described above, then exposed to 5% FBS at 37 °C for 72 h. The cells were rinsed with PBS, pH 7.4, and trypsinized, and the cells in suspension were counted using an automated Coulter counter.

Assessment of DNA enzyme stability. DNA enzymes were labeled at the 5' end with γ^{32} P-dATP and were separated from free label by spin-column centrifugation. Radiolabeled DNA enzymes were incubated in 5% FBS or serum-free medium at 37 °C. Aliquots of the reaction were 'quenched' by being transferred to tubes containing formamide loading buffer⁴⁰. Samples were separated by 12% denaturing SDS-PAGE and detected by autoradiography overnight at -80 °C.

SMC wounding assay. Confluent growth-quiescent SMCs in chamber slides (Nunc, Copenhagen, Denmark) were exposed to ED5 or ED5SCR for 18 h before being injured with a single scrape with a sterile toothpick. Cells were treated with 20 μ M mitomycin C (Sigma) for 2 h before injury^{31,42}. At 72 h after injury, the cells were washed with PBS, pH 7.4, fixed with formaldehyde, then stained with hematoxylin and eosin.

Rat carotid injury model. Adult male Sprague Dawley rats weighing 450–550 g were anesthetized with ketamine (60 mg/kg, intraperitoneally) and xylazine (8 mg/kg, intraperitoneally). The left common and external carotid arteries were exposed through a midline neck incision. A 2-french Fogarty balloon catheter (Baxter Healthcare, Deerfield, Illinois) was introduced through an arteriotomy to the external carotid, advanced into the common carotid, inflated to generate resistance and withdrawn three times in a manner that has been described³². After removal of the catheter, a ligature was applied to the external carotid proximal to the arteriotomy. A 200- μ l solution (at 4 °C) containing 500 μ g DNA enzyme (in DEPC-treated water), 30 μ l transfection agent, 1 mM $MgCl_2$ and P127 Pluronic gel (BASF, Noble Park, Australia) was applied around the vessel as described⁴³ 6 h before and again immediately after injury. This formula allowed the solution to form a gel after contact with the vessel at 37 °C. The incision was closed and the rats were allowed to recover. Rats were killed 14 d after injury by lethal injection of phenobarbitone, and were perfusion-fixed using 10% formaldehyde (volume/volume) at 120 mm Hg. Carotids were dissected from the rats, placed in 10% formaldehyde, cut into 2-mm lengths and embedded in 3% agarose (weight/volume) before paraffin fixation. Neointimal and medial areas in 5 μ m sections stained with hematoxylin and eosin were

determined digitally using a customized software package (Magellan, School of Anatomy, University of New South Wales)(ref. 44) and expressed as a mean ratio per group of five rats.

Immunohistochemical analysis for Egr-1. A second group of rats underwent balloon catheter injury and drug delivery in a manner identical to that described above, except these rats were killed 1 h after arterial injury. The carotids were dissected from the rats, washed with PBS, pH 7.4, placed in OCT (Miles Laboratories, Naperville, Illinois) and 'snap-frozen' in liquid nitrogen. Sections were air-dried, fixed in acetone for 15 min, then air-dried a second time. Immunohistochemical detection for Egr-1 used a modification of a described method⁴⁵. The sections were rinsed in PBS, pH 7.4, and incubated for 45 min with 100 μ l rabbit polyclonal antibodies raised against Egr-1 (Santa Cruz Biotechnology, Santa Cruz, California), diluted 1:100 in 0.5% BSA and PBS, pH 7.4. The sections were washed with 0.5% Tween 20 in PBS, pH 7.4, followed by PBS, pH 7.4, alone, and incubated for 30 min with 100 μ l biotinylated secondary antibody against rabbit (Vector Laboratories, Burlingame, California), diluted 1:300 in 0.5 % BSA. The sections were washed in PBS, pH 7.4 for 5 min before being incubated for 30 min in 100 μ l avidin-biotin complex (ABC, Vector Laboratories, Burlingame, California), diluted 1:100 in 0.5% BSA. After the sections were washed again in Tween and PBS as described above, antigen-antibody complexes were detected in 3 min using the diaminobenzidine (DAB) system. Sections were washed in PBS, pH 7.4, counterstained with hematoxylin for 20 s, dehydrated, cleared and mounted, then visualized by light microscopy.

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