

c-Jun Regulates Vascular Smooth Muscle Cell Growth and Neointima Formation after Arterial Injury

INHIBITION BY A NOVEL DNA ENZYME TARGETING c-Jun*

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Neointima formation is a characteristic feature of common vascular pathologies, such as atherosclerosis and post-angioplasty restenosis, and involves smooth muscle cell proliferation. Determination of whether the bZIP transcription factor c-Jun plays a direct regulatory role in arterial lesion formation, or indeed in other disease, has been hampered by the lack of a potent and specific pharmacological inhibitor. c-Jun is poorly expressed in the uninjured artery wall and transiently induced following arterial injury in animal models. Here we generated a gene-specific DNAzyme-targeting c-Jun. We show that c-Jun protein is expressed in human atherosclerotic lesions. Dz13, a catalytically active c-Jun DNAzyme, cleaved c-Jun RNA and inhibited inducible c-Jun protein expression in vascular smooth muscle cells. Dz13 blocked vascular smooth muscle cell proliferation with potency exceeding its exact non-catalytic antisense oligodeoxynucleotide equivalent. Moreover, Dz13 abrogated smooth muscle cell repair following scraping injury *in vitro* and intimal thickening in injured rat carotid arteries *in vivo*. These studies demonstrate the positive influence on neointima formation by c-Jun and the therapeutic potential of a DNAzyme controlling its expression.

The initiating event in the pathogenesis of atherosclerosis and restenosis following angioplasty is injury to cells in the artery wall (1). Injury stimulates signaling and transcriptional pathways in vascular smooth muscle cells, stimulating their migration and proliferation, and the eventual formation of a neointima. c-Jun, a prototypical member of the basic region-leucine zipper protein family, is transiently induced following arterial injury in animal model (2–4). c-Jun forms both homodimers and heterodimers with other bZIP proteins to form the AP-1 transcription factor. While investigations over the last decade have linked AP-1 with proliferation, tumorigenesis and apoptosis, AP-1 has also been implicated

in tumor suppression and cell differentiation (5). Recent reports indicate that c-Jun NH₂-terminal kinase/stress-activated protein kinase (JNK),¹ an upstream activator of c-Jun and numerous other transcription factors, is expressed by smooth muscle cells in human and rabbit atherosclerotic plaques (6, 7) and that dominant negative JNK inhibits neointima formation after balloon injury (8). c-Jun, however, has not been localized in human atherosclerotic lesions nor has it been shown to play a role in arterial repair after injury. Investigation of the precise regulatory role of c-Jun in the injured artery wall, or indeed in other pathologic settings, has been hampered by the lack of a specific pharmacological inhibitor.

DNAzymes represent a new class of gene-targeting agent with specificity conferred by the sequence of nucleotides in the two arms flanking a catalytic core, with advantages over ribozymes of substrate specificity and stability (9, 10). DNAzymes specific for c-Jun would be useful as molecular determinants of c-Jun biological function. To date, neither c-Jun nor indeed any other Jun family member has been targeted using catalytic nucleic acid strategies.

Here, we demonstrate that sequence-specific DNAzyme targeting c-Jun cleaves c-Jun RNA and inhibits inducible c-Jun protein expression and proliferation of vascular smooth muscle cells, with potency exceeding its exact non-catalytic antisense oligodeoxynucleotide equivalent. Moreover, the DNAzyme abrogates smooth muscle cell repair after injury *in vitro* and neointima formation in rat carotid arteries *in vivo*. These findings demonstrate the regulatory role of c-Jun in neointima formation in the injured artery wall.

EXPERIMENTAL PROCEDURES

DNAzymes, in Vitro Transcript and Cleavage Experiments— DNAzymes were synthesized by Oligos Etc. with a 3'-3'-linked inverted T and purified by high performance liquid chromatography. A ³²P-labeled 668-nt c-Jun RNA transcript was prepared by *in vitro* transcription (using T7 polymerase) of pBluescript containing the insert, cut previously with *Xba*I. Reactions were performed in a total volume of 20 μ l containing 10 mM MgCl₂, 5 mM Tris, pH 7.5, 150 mM NaCl, 0.5 pmol of *in vitro* transcribed substrate, and 10 pmol of DNAzyme, unless dose-dependent cleavage experiments were performed. Reactions were allowed to proceed for various times at 37 °C and quenched by transferring an aliquot to tubes containing formamide loading buffer. Samples were run on 12% denaturing polyacrylamide gels and autoradiographed overnight at -80 °C.

Smooth Muscle Cell Culture, Proliferation, and Wounding Assays— Smooth muscle cells derived from human and porcine coronary arteries were obtained from Cell Applications, Inc. (San Diego, CA) and cultured in Waymouth's medium, pH 7.4, containing 10% fetal bovine

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¹ The abbreviations used are: JNK, c-Jun NH₂-terminal kinase/stress-activated protein kinase; nt, nucleotide.

serum, 50 µg/ml streptomycin, and 50 IU/ml penicillin at 37 °C in a humidified atmosphere of 5% CO₂. In all *in vitro* experiments, smooth muscle cells were not used beyond passage 7. Transfections were performed in smooth muscle cells 6 h after the change of medium to serum-free and again at the time of serum stimulation 24 h after the start of arrest, using FuGENE 6 according to the manufacturer's instructions (Roche Molecular Biochemicals). In proliferation assays, growth-arrested smooth muscle cells in 96-well plates (Nunc-InterMed) were transfected with the indicated concentration of DNAzyme or oligonucleotide, then exposed to 5% fetal bovine serum at 37 °C for 72 h. The cells were trypsinized and the suspension quantitated in an automated Coulter counter. In wounding assays, confluent smooth muscle cells in chamber slides (Nunc-InterMed) transfected with 0.5 µM DNAzyme were injured by scraping with a sterile toothpick. Cells were treated with mitomycin C (Sigma) (20 µM) for 2 h prior to injury to block proliferation. Seventy-two h after injury, the cells were washed with phosphate-buffered saline, pH 7.4, fixed with formaldehyde, and stained with hematoxylin and eosin.

Western Blot and Immunohistochemical Detection—Western immunoblot, and immunohistochemical analysis on human carotid endarterectomy specimens, were performed using rabbit polyclonal anti-peptide antibodies targeting c-Jun and Sp1 (Santa Cruz Biotechnology) as described elsewhere (11).

Common Carotid Injury and Evaluation of Neointima Formation—Sprague-Dawley rats (450 g males) were anesthetized using ketamine (60 mg/kg, intraperitoneal) and xylazine (8 mg/kg, intraperitoneal). The left common and external carotid arteries were exposed via a midline neck incision, and a ligature was applied to the external carotid proximal to the bifurcation. Two-hundred µl (at 4 °C) containing DNAzyme (750 µg), of FuGENE6 (30 µl), MgCl₂ (1 mM), and P127 Pluronic gel (BASF) was applied around the vessel, 6 h prior to and again at the time of ligation. The solution gelled after contact with the vessel at 37 °C. The incision was sutured and the rats allowed to recover. Animals were sacrificed 21 days after injury by lethal injection of ketamine/xylazine and perfusion fixed with 10% (v:v) formaldehyde at 120 mm Hg. Carotids were placed in 10% formaldehyde, embedded in 3% (w:v) agarose, fixed in paraffin, and sectioned 1000 µm from the tie. Neointimal and medial areas in 5-µm sections stained with hematoxylin and eosin were determined morphometrically and expressed as a mean ratio per group of six rats.

RESULTS AND DISCUSSION

Localization of c-Jun in Human Atherosclerotic Lesion—Fig. 1 demonstrates c-Jun expression by smooth muscle cells in the human atheromatous lesion. c-Jun is poorly, if at all, expressed by smooth muscle cells in the normal media. In contrast, the zinc finger transcription factor Sp1 is expressed in both the intima and media (Fig. 1).

Dz13 Cleaves c-Jun RNA and Blocks Inducible c-Jun Expression in Vascular Smooth Muscle Cells—Seven DNAzymes (Fig. 2A), bearing two nine-nucleotide hybridizing arms and a single 15-nt catalytic motif ("10–23" (6)) targeting various regions of low free energy (12), were evaluated for their capacity to cleave ³²P-labeled *in vitro* transcribed c-Jun RNA. The seven DNAzymes and c-Jun transcript were first resolved by denaturing electrophoresis to ensure structural integrity (Fig. 2B). The 668-nt c-Jun transcript was cleaved by DNAzymes Dz10, Dz12, Dz13, Dz14 and Dz15, but not by Dz9 and Dz11, within 1 h at 37 °C under physiological conditions (Fig. 2B). One of the active DNAzymes, Dz13, targeting the G¹³¹¹U junction (where the translational start site in human c-Jun mRNA is located at A¹²⁶¹UG), cleaved the transcript within 15 min in both a time-dependent (Fig. 2C, upper panel) and dose-dependent (Fig. 2C, lower panel) manner, generating 474- and 194-nt products. DNAzyme Dz13scr, in which the hybridizing arms of Dz13 were scrambled without disturbing the integrity of the catalytic domain, failed to cleave the substrate at any time or stoichiometric ratio (Fig. 2C). To demonstrate Dz13 inhibition of endogenous c-Jun in primary human arterial smooth muscle cells, we performed Western blot analysis on growth-quiescent cells previously transfected with 0.5 µM Dz13 or Dz13scr and exposed to serum for 2 h at 37 °C. Serum-inducible c-Jun immunoreac-

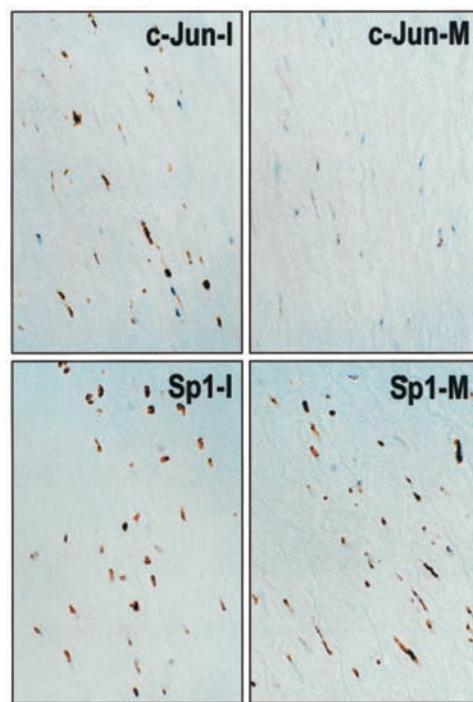


FIG. 1. c-Jun and Sp1 expression in human atherosclerotic lesions. Immunohistochemical staining for c-Jun and Sp1 in 5-µm sections of human carotid atherosclerotic lesions. The intima and media are indicated in the figure; L denotes lumen. Staining is representative of three independent samples.

tivity (39 kDa) was strongly inhibited by Dz13, whereas its scrambled counterpart has no effect (Fig. 2D).

Dz13 Blocks Vascular Smooth Muscle Cell Proliferation—We next determined the influence of Dz13 and the panel of c-Jun DNAzymes on the growth of primary vascular smooth muscle cells derived from human and porcine arteries. The Dz13 target site in c-Jun RNA is conserved between human, pig, and rat except for a single C nt at position 1319, which is an A in pig and rat c-Jun RNA (Fig. 3A, upper and middle panels). DNAzyme catalytic efficiency is largely unaffected by substitution of a single pyrimidine nt in the substrate with a purine (13), as in this case. Dz13 (0.5 µM) completely blocked serum-inducible proliferation in both cell types (Fig. 3, B and C) and was the most potent of the entire DNAzyme panel. Dz13 inhibition was dose-dependent and detectable at concentrations as low as 100 nM (Fig. 3D). In contrast, Dz13scr failed to inhibit smooth muscle cell proliferation (Fig. 3B), consistent with its inability to affect serum-inducible c-Jun protein (Fig. 2D). Surprisingly, some DNAzymes (Dz9, Dz11, Dz15) stimulated proliferation beyond the effect of serum alone (Fig. 3, B and C). Additionally, Dz10, which cleaved the c-Jun transcript as effectively as Dz13 (Fig. 2B), failed to modulate smooth muscle cell proliferation in either cell type, unlike Dz13 (Fig. 3, B and C). To demonstrate greater potency of the c-Jun DNAzyme compared with its exact antisense oligonucleotide counterpart, we generated As13 which, like Dz13, comprises a phosphodiester backbone and a 3'-3'-linked inverted T, but has no catalytic core (Fig. 3A). As13 produced dose-dependent inhibition, however, Dz13 was twice as potent an inhibitor (Fig. 3D).

Dz13 Inhibits Vascular Smooth Muscle Cell Repair after Injury in Vitro and Neointima Formation in Rat Carotid Artery—Smooth muscle cell regrowth at the wound edge following mechanical scraping in an *in vitro* model (14) was abolished by the presence of 0.5 µM Dz13 (Fig. 4), whereas

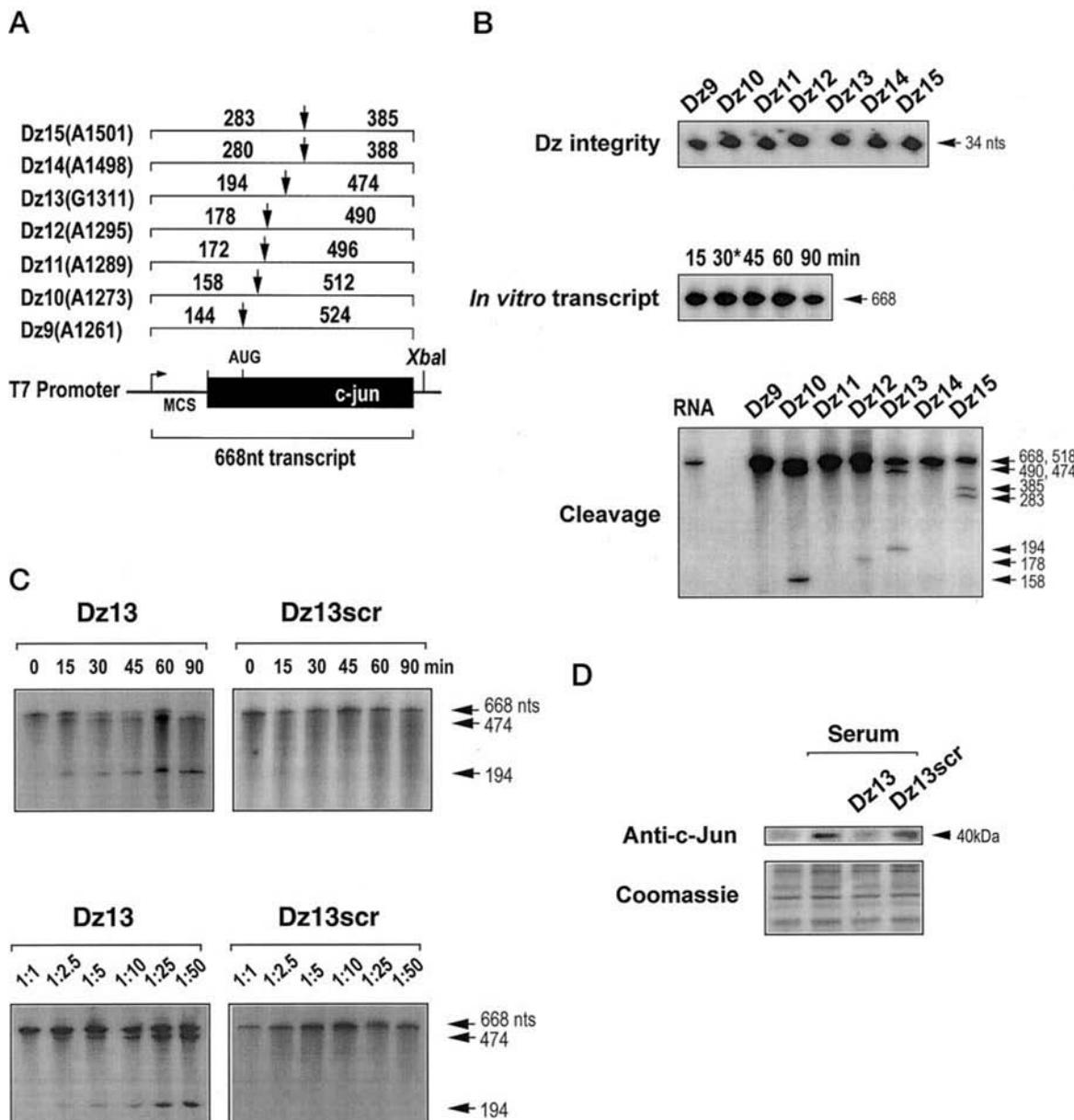


FIG. 2. Cleavage of *in vitro* transcribed c-Jun RNA and inhibition of c-Jun induction by c-Jun DNAzymes. *A*, representation of DNAzyme cleavage sites (arrows) in c-Jun RNA and sizes of expected products. The specific purine hosting the 3' cleavage is indicated for each candidate DNAzyme. Numbering is based on the human c-Jun complete *cds* (GenBank™ accession number J04111, NID g186624). The expression vector used for the of T7 RNA polymerase-dependent generation of c-Jun RNA is indicated. *B*, integrity analysis of DNAzyme (34 nt) (*upper panel*) and 668-nt c-Jun RNA (*middle panel*) and panning for nucleolytic activity of candidate DNAzymes after 1 h at 37 °C (*lower panel*). DNAzyme integrity was determined by 5'-end labeling with [γ -³²P]dATP prior to resolution on 12% denaturing polyacrylamide gels. Transcript integrity was determined by random labeling with [α -³²P]UTP prior to resolution on 12% denaturing polyacrylamide gels. The figure shows the 668-nt transcript after the reaction was allowed to proceed for the times indicated. Subsequent experiments used the 30-min run-off. *C*, time and dose dependence of Dz13 cleavage of c-Jun RNA. The 474- and 194-nt products are indicated. *D*, Western blot analysis for c-Jun protein. Extracts of smooth muscle cells (10 μ g) transfected with 0.5 μ M DNAzyme (Dz13 or Dz13scr) were assessed for c-Jun immunoreactivity using rabbit polyclonal anti-peptide antibodies (Santa Cruz Biotechnology). The Coomassie Blue-stained gel shows unbiased loading.

repair in the presence of Dz13scr was not different from wells without oligonucleotide (Fig. 4 and data not shown). Since smooth muscle cell proliferation and repair are processes negatively regulated by Dz13, we next determined whether the c-Jun DNAzyme could influence intimal thickening after ligation injury to rat carotid arteries. Neointima formation 3 weeks after injury, and local administration of Dz13scr was not significantly different from that observed in the vehicle alone group (Fig. 5, *A* and *B*). However, intimal thickening was suppressed by Dz13 of the order of 60% (Fig. 5, *A* and *B*). Immunohistochemical analysis revealed that Dz13 blocked the induction of c-Jun immunoreactivity in the smooth mus-

cle cells of the arterial media, whereas Dz13scr had no effect (Fig. 5*C*). In contrast, neither DNAzyme had any influence on levels of Sp1 (Fig. 5*C*). Together, these data demonstrate a crucial role for c-Jun in smooth muscle cell proliferation, wound repair, and neointima formation.

Arterial neointima formation has previously been inhibited by phosphorothioate-linked antisense oligonucleotides directed against certain transcription factors and cell cycle regulatory molecules, including the p65 subunit of NF- κ B, c-Myb, c-Myc, and Cdc2 kinase/proliferating-cell nuclear antigen (15–19). By directly comparing a phosphodiester-linked DNAzyme with an antisense oligonucleotide targeting the

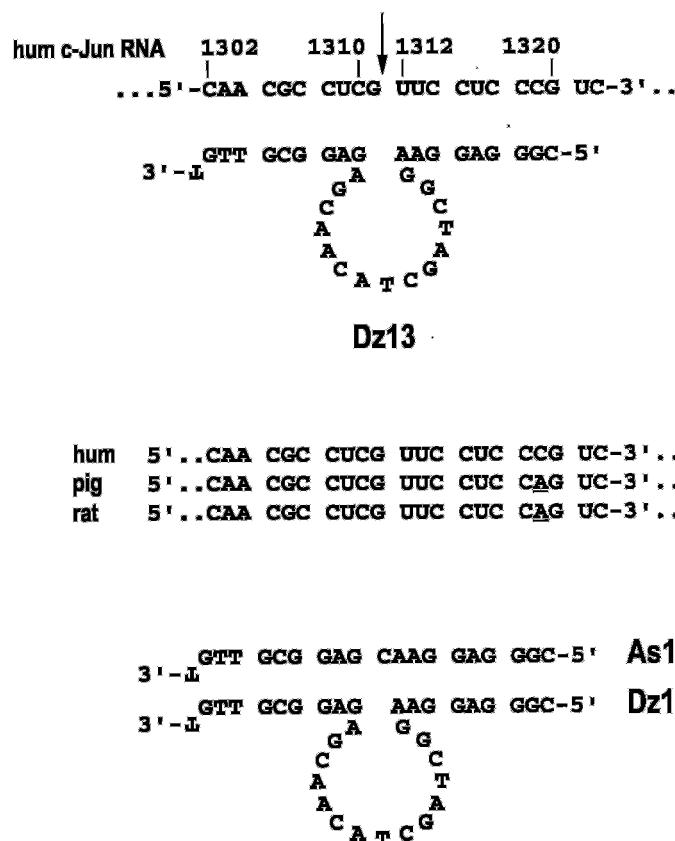
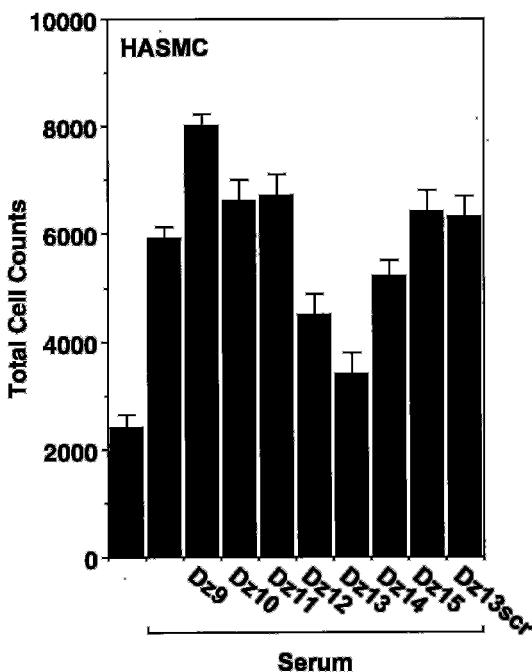
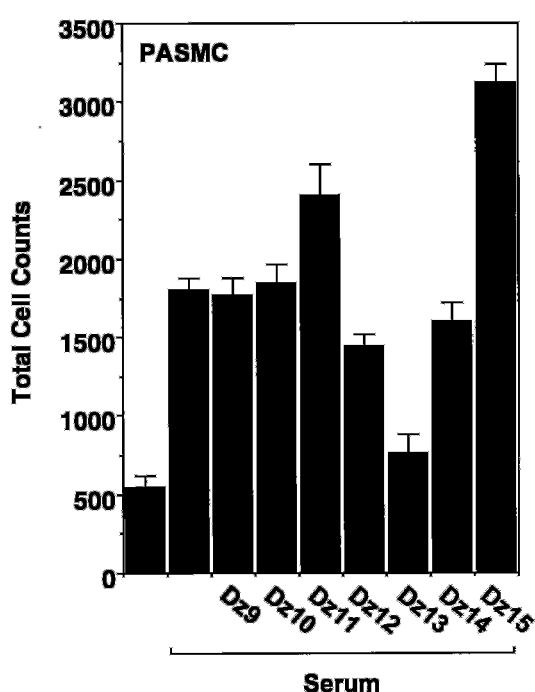
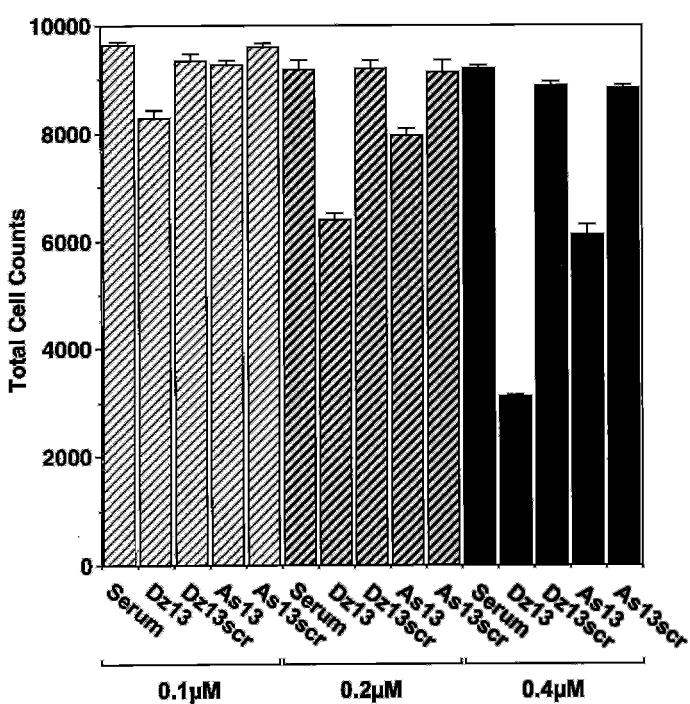
A**B****C****D**

FIG. 3. *c-Jun* DNAzyme inhibition of smooth muscle cell proliferation. *A*, schematic representation of *c-Jun* DNAzyme Dz13 and target site ($G^{1311}T$) in human *c-Jun* mRNA (upper panel); comparison of Dz13 target site in human, porcine, and rat *c-Jun* mRNA (middle panel); and comparison of As13 and Dz13 (lower panel). The translational start site of human *c-Jun* mRNA is located at $A^{1261}TG$. *B*, effect of *c-Jun* DNAzyme ($0.5 \mu M$) on serum-inducible primary human smooth muscle cell (HASMC) proliferation inhibited by Dz13. The sequence of Dz13scr is 5'-GCG ACG TGA GGC TAG CTA CAA CGA GTG GAG GAG X-3', where X is a 3'-3'-linked inverted T. *C*, serum-inducible porcine smooth muscle cell proliferation (PASMC) inhibited by $0.5 \mu M$ Dz13. *D*, human smooth muscle cell proliferation is inhibited by Dz13 and As13 in a dose-dependent manner. The concentrations of DNAzyme ($0.1\text{--}0.4 \mu M$) are indicated in the figure. The sequence of As13scr is 5'-GCG ACG TGA C GTG GAG GAG X-3', where X is a 3'-3'-linked inverted T. Dz13 inhibition at $0.1 \mu M$ is significant by Student's *t* test ($p < 0.05$) compared with control.

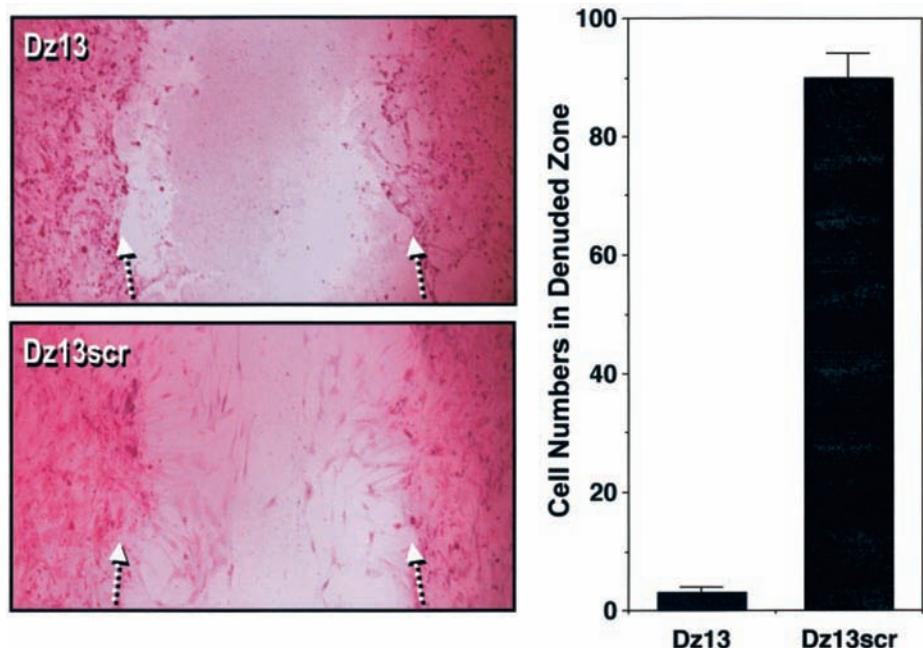


FIG. 4. c-Jun DNAzyme inhibition of smooth muscle cell repair. Smooth muscle cell regrowth in the denuded zone 3 days after scraping and transfection with 0.5 μ M Dz13 or Dz13scr. The cells were fixed and stained with hematoxylin and eosin prior to micrography.

same sequence in c-Jun mRNA, each of identical arm length and bearing a 3'-3'-inverted T, this study demonstrates for the first time superior inhibition by the former molecule at any given concentration. c-Jun DNAzymes could serve as new, more potent gene-specific tools to probe the precise function(s) of this transcription factor in a wide array of fundamental cellular processes.

All the c-Jun DNAzymes screened in this specification have targeted regions in the mRNA likely to be exposed, based on a Zukerian prediction of regions of low free energy in the mRNA (12), and preference for the 5'-end of the mRNA, where the translational apparatus attaches and moves along the chain.

The present study shows that Zuker analysis does not guarantee the efficacy of any given DNAzyme in intact cells, since only some, but not all, the DNAzyme sequences that cleave *in vitro* transcribed c-Jun mRNA could actually inhibit cell proliferation. This may be due (although not confined) to differences in conformation and site accessibility between *in vitro* transcribed mRNA and endogenous mRNA, DNAzyme transfection efficiency, the concentration of ions and other DNAzyme cofactors in the local cellular milieu, and the possible existence of DNA-binding proteins (such as growth factors, signaling molecules, etc.) having unintended preference for certain nucleotide sequences, thereby reducing the amount of bioavailable DNAzyme.

That c-Jun, or indeed any other given gene, is inducibly expressed in the artery wall following balloon angioplasty does not necessarily translate to it playing a positive regulatory role in transcription, proliferation, or neointima formation. For example, our own work shows that three transcriptional repressors (NAB2, GCF2, and YY1) are activated in vascular smooth muscle cells by mechanical injury *in vitro*, as well as in the rat artery wall. NAB2 directly binds the zinc finger transcription factor Egr-1 and represses Egr-1-mediated transcription (20). GCF2 is a potent repressor of the expression of PDGF-A, a well established mitogen for vascular smooth muscle cells, and inhibits smooth muscle cell proliferation (21). Similarly, YY1 overexpression blocks smooth muscle cell growth without affecting endothelial cell proliferation (22). Second, c-Jun can repress, as well as activate, transcription. c-Jun binds the corepressor TG-interacting factor to suppress Smad2 transcriptional activity

(23). c-Jun also blocks transforming growth factor β -mediated transcription by repressing the transcriptional activity of Smad3 (24). Finally, c-Jun can inhibit, as well as stimulate, proliferation. Using antisense oligonucleotides to c-jun, Kanatani and colleagues (25) demonstrated that inhibition of human monocyteid leukemia cell growth by tumor growth factor- β and dexamethasone is mediated by enhanced c-Jun expression. These oligonucleotides dose-dependently decrease the growth inhibitory effect of tumor growth factor- β and dexamethasone (25). Thus, strategies targeting c-Jun are not predictive of a specific molecular and cellular consequence.

Investigation of the precise regulatory role of c-Jun in the injured artery wall has been compromised because of the unavailability of a gene-specific inhibitor. Angiopeptin, a synthetic cyclic octapeptide analogue of somatostatin, inhibit the induction of c-jun and neointima formation after balloon injury to rabbit aortae (26). Angiopeptin, however, also blocks c-fos activation in the artery wall (26). Dominant negative JNK inhibits neointima formation 2–4 weeks after injury (8). However, JNK, as well as phosphorylating c-Jun (on Ser⁶³ and Ser⁷³) (27), also phosphorylates ATF-2, Elk-1, c-Myc, p53, and Bcl2 (28–31). Dominant negative c-Jun has not been used in efforts to inhibit intimal thickening, although this strategy has recently been found to inhibit low density lipoprotein induction of intercellular cell adhesion molecule expression in vascular cells (32). The present study exploits the emerging field of DNAzyme biotechnology as gene-specific tools by demonstrating that c-Jun plays a necessary and sufficient positive regulatory role in smooth muscle cell proliferation, regrowth, and neointima formation following injury. Additionally, since c-Jun has been implicated in the pathogenesis of other fibroproliferative-inflammatory processes, such as arthritis, neoplasia, acute lung injury, scarring, and UV-induced corneal damage and osteopetrosis (33–38); DNAzymes targeting c-Jun and other key regulatory molecules (39, 40) may, alone or in combination, be useful in efforts to inhibit proliferative vascular disease and other pathological processes.

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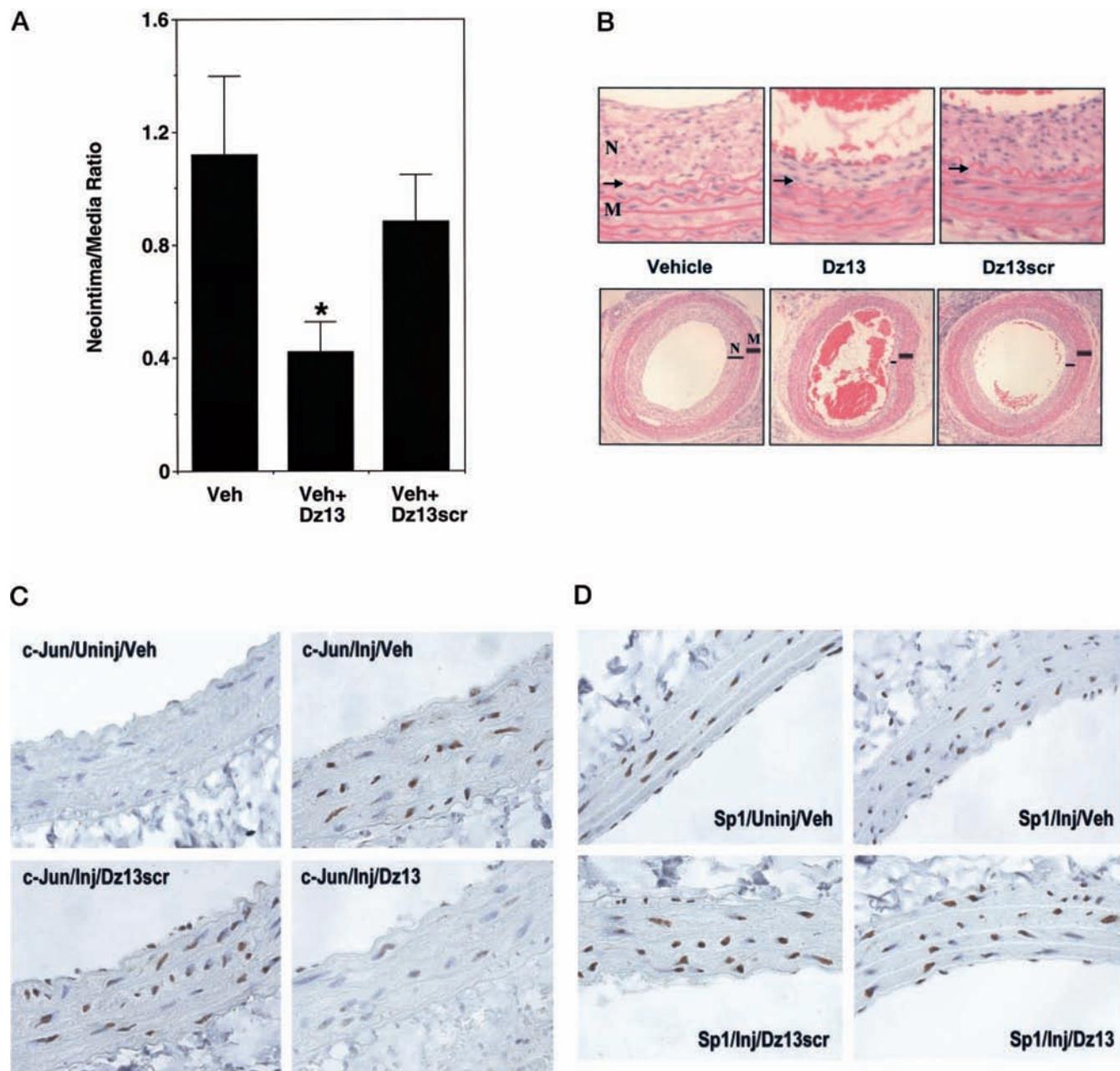


FIG. 5. Blockade of neointimal thickening in rat common carotid arteries. *A*, neointima/media ratios for each group (vehicle alone, vehicle containing Dz13, vehicle containing Dz13scr) 21 days after injury. * indicates $p < 0.05$ compared with vehicle and vehicle containing Dz13scr groups using Student's *t* test. The vehicle and vehicle containing Dz13scr groups were not statistically different. *B*, representative cross-sections stained with hematoxylin-eosin. *N* and single line denotes neointima; *M* and triple line denotes media; arrow denotes preinjured intima. Thrombosis was occasionally observed and not confined to any particular group. *C*, immunoperoxidase staining for c-Jun protein 6 h after arterial injury. *D*, immunoperoxidase staining for Sp1 6 h after arterial injury. DNazyme in vehicle (FuGENE 6, MgCl₂, phosphate-buffered saline, pH 7.4) was applied to the carotid in Pluronic gel (BASF) at the time of injury. Three weeks subsequently the arteries were perfusion-fixed and 5-μm sections taken for immunohistochemical and morphometric analysis.

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