Catalytic Oligodeoxynucleotides Define a Key Regulatory Role for Early Growth Response Factor-1 in the Porcine Model of Coronary In-Stent Restenosis

Harry C. Lowe, Roger G. Fahmy, Mary M. Kavurma, Andrew Baker, Colin N. Chesterman, Levon M. Khachigian

Abstract—Early growth response factor-1 (Egr-1) controls the expression of a growing number of genes involved in the pathogenesis of atherosclerosis and postangioplasty restenosis. Egr-1 is activated by diverse proatherogenic stimuli. As such, this transcription factor represents a key molecular target in efforts to control vascular lesion formation in humans. In this study, we have generated DNAzymes targeting specific sequences in human EGR-1 mRNA. These molecules cleave in vitro transcribed EGR-1 mRNA efficiently at preselected sites, inhibit EGR-1 protein expression in human aortic smooth muscle cells, block serum-inducible cell proliferation, and abrogate cellular regrowth after mechanical injury in vitro. These DNAzymes also selectively inhibit EGR-1 expression and proliferation of porcine arterial smooth muscle cells and reduce intimal thickening after stenting pig coronary arteries in vivo. These findings demonstrate that endoluminally delivered DNAzymes targeting EGR-1 may serve as inhibitors of in-stent restenosis. (Circ Res. 2001;89:670-677.)

Key Words: catalytic DNA ■ transcription factors ■ early growth response factor-1 ■ stenting ■ gene therapy

The capacity to selectively target specific mRNA sequences with catalytic molecules composed of DNA provides immense potential to broaden our understanding of the roles of specific mediators in normal and pathologic settings. Catalytic DNA can be used to cleave the phosphodiester linkage between virtually any unpaired purine and paired pyrimidine, selectivity being conferred by the nucleotide sequences of the hybridizing arms. These next-generation antisense oligonucleotides are extremely specific and easy to synthesize and have low toxicity, because they do not require phosphorothioate or other backbone modifications to confer nuclease resistance. DNAzyme biotechnology has practical therapeutic implications as a new category of gene-suppression agents in pathophysiological settings.

Stenting of coronary atherosclerotic lesions has revolutionized the treatment of cardiovascular disease in the last 5 years, after two landmark trials demonstrating a reduction in restenosis relative to coronary balloon angioplasty in comparable vessels.1,2 Stents prevent elastic recoil of the artery via their ability to provide a structural scaffold on the interior of the vessel.3 Stenting has now virtually replaced angioplasty4 to the extent that in-stent restenosis has now itself become a significant problem.5 Lesions of in-stent restenosis arise mainly from vascular smooth muscle cell (SMC)6 proliferation in response to mechanical injury imparted to the vessel by stenting.6 This culminates in the thickening of the intima within the rigid stent. Brachytherapy may help reduce in-stent restenosis,7,8 although a recent study indicates that radioactive stents may simply delay rather than prevent in-stent neointimal proliferation.9 To date there is only one example of a catalytic nucleic acid that has been used in efforts to inhibit SMC proliferation and in-stent restenosis in pig coronary arteries. Frimerman et al10 locally delivered hammerhead ribozymes targeting the cell-cycle regulatory protein proliferating cell nuclear antigen (PCNA) and observed a 28% reduction of in-stent restenosis. These ribozymes, despite their chimeric structure, were extremely labile to nuclease degradation.

The promoters of many genes whose products stimulate SMC proliferation, migration, and matrix synthesis contain binding sites for the immediate-early gene and transcription factor early growth response factor-1 (Egr-1, also known as NGFI-A, TIS8, and krox24).11,12 Egr-1 is rapidly activated by acute mechanical injury in vitro and in vivo and numerous other proatherogenic agonists and environmental stimuli, such as growth factors, hormones, and fluid biomechanical forces.12 In humans, FGF-2, a potent trigger of Egr-1 expression,13 is released locally in the vicinity of human atherosclerotic plaques after coronary stenting.14 EGR-1 immunoreactivity has recently been localized to SMCs and other cell...
types within atheromatous lesions. Together, these observations suggest that EGR-1 may play a regulatory role in the vascular response to injury in humans. The aim of this study was to generate novel DNAzymes targeting human EGR-1 and demonstrate their ability to inhibit human SMC proliferation, regrowth in response to injury in vitro, and in-stent restenosis in porcine coronary arteries after stenting and endoluminal delivery.

**Materials and Methods**

Details of in vitro transcript generation and cleavage experiments,16–18 cleavage kinetics of EGR-1 mRNA, cell culture, and DNAzyme transfection, Western blot analysis, in vitro SMC injury,19 cell proliferation assay, and the porcine in-stent restenosis model10,20–25 can be obtained in the online data supplement available at http://www.circresaha.org.

**Results**

**DNAzymes Targeting Human EGR-1 Cleave In Vitro Transcribed EGR-1 mRNA**

We generated a series of DNAzymes (DzA to DzG) targeting various purine-pyrimidine junctions in human EGR-1 mRNA (Figure 1A). Cleavage of an in vitro transcribed 388-nucleotide-long 32P-labeled human EGR-1 transcript spanning the translational start site by several of the DNAzymes (DzA, DzB, and DzC) produced fragments of expected size as early as 5 minutes and proceeded in a time-dependent manner (Figure 1B, left). In contrast, no reaction products were apparent using DzE (Figure 1B, right). ED5,17 targeting rat Egr-1 also failed to cleave the human substrate (data not shown), demonstrating the limitation of ED5 as a catalytic inhibitor of human EGR-1. A fourth DNAzyme, DzF, also cleaved the human EGR-1 mRNA (Figure 1C, left), whereas a size-matched counterpart of DzF, DzFscr, bearing an active catalytic domain flanked by arms with scrambled sequence, had no effect (Figure 1C, left). The DNAzyme cleaved its substrate over a wide range of molar ratios spanning 0.5 to 50 molecules of DNAzyme to 1-molecule substrate (Figure 1C, right). Single-turnover kinetic analysis of DzA and DzF with the 388-nucleotide RNA substrate revealed similar catalytic efficiencies (k_{cat}/k_{m}) of 3.42×10^3 (mol/L)^{-1} · min^{-1} and 1.90×10^3 (mol/L)^{-1} · min^{-1}, respectively (Figure 1D). k_{cat}/k_{m} values of 8.68×10^3 (mol/L)^{-1} · min^{-1} and 7.77×10^3 (mol/L)^{-1} · min^{-1} for DzA and DzF, respectively, after multiple turnover kinetic analysis using a 23-nucleotide synthetic RNA substrate (data not shown) indicate superior catalytic efficiencies comparable with other DNAzymes of the 10 to 23 sub-type.16,26,27 These data, taken together, demonstrate sequence-, time-, and dose-dependent DNAzyme cleavage of human EGR-1 mRNA.

**DNAzymes Inhibit Production of EGR-1 Protein in Human Vascular SMCs**

We next determined the effect of DzA and DzF on the serum-inducible expression of EGR-1. Western blot analysis demonstrated that EGR-1 protein is poorly expressed in growth-quiescent primary human aortic SMCs but is activated within 1 hour of exposure to serum. The induction of EGR-1 was virtually abrogated by previous exposure to DzA and DzF (0.4 µmol/L). In contrast, neither the noncleaving DNAzyme (DzE, Figure 2, top) nor the scrambled DNAzyme (DzFscr, Figure 2, bottom) had any inhibitory effect on the induction of EGR-1. Reprobing the blot with antibodies directed to the structurally related zinc finger transcription factor Sp1 revealed no inhibitory effect of DNAzyme on levels of this constitutively expressed nuclear protein (Figure 2, top).

**EGR-1 DNAzymes Block Human SMC Regrowth After Injury**

In a well-established in vitro model of wounding,13 DzA (0.4 µmol/L) inhibited SMC regrowth into the denuded zone by ~50% (Figure 3). Even greater inhibition (80%) was observed using DzF (Figure 3). In contrast, equivalent concentrations of DzFscr or ED5scr (ie, ED5 with scrambled arms) failed to modulate this process (Figure 3). Within the confines of this model, these data support the notion that EGR-1 plays a positive regulatory role in the reparative response of human SMCs to mechanical injury.

**Human SMC Proliferation Is Inhibited by EGR-1 DNAzymes**

We next assessed the capacity of the various EGR-1 DNAzymes to inhibit human SMC replication. At 0.3 µmol/L, DzF inhibited serum-inducible proliferation by ~50% (Figure 4A), whereas DzB, DzC, ED5scr, and, surprisingly, DzA had no effect (Figure 4A). However, at 0.4 µmol/L, DzF inhibited SMC proliferation virtually by 100%, whereas DzFscr, DzB, DzC, DzE, and ED5scr failed to attenuate the mitogenic response (Figure 4A). Interestingly, at this concentration (0.4 µmol/L), inhibition by DzA was apparent (Figure 4A). These findings show that DzF can inhibit cell growth at concentrations that DzA cannot. DzF is thus a more potent inhibitor of human SMC proliferation than DzA.

To determine whether the inhibitory effect of DzF on SMC proliferation was reversible, culture medium containing serum and DzF was replaced with medium containing serum alone. Twenty-four hours after continuous exposure to DNAzyme, cell numbers in the DzFscr group, as expected, exceeded those of the DzF group (Figure 4B, compare columns 2 and 1). When the cells were then incubated with fresh medium without DNAzyme, the population of cells in the DzFscr group continued to increase over the next 54 hours (Figure 4B, compare columns 4 and 2). In contrast, cell replication in the DzF group did not change over the same time course (Figure 4B, compare columns 3 and 1). These findings demonstrate sequence-specific, long-lived, and irreversible inhibition by DzF.

**Induction of EGR-1 Protein and Proliferation of Porcine Vascular SMCs Is Inhibited by EGR-1 DNAzymes**

Before evaluating the effect of these DNAzymes on intimal thickening in a porcine model of arterial injury, we first determined the influence of these molecules on levels of Egr-1 in growth-quiescent primary pig aortic SMCs. DzA and DzF both inhibited serum-inducible Egr-1 protein expression at 0.4 µmol/L, although the latter DNAzyme produced...
greater inhibition (Figure 5A). In contrast, ED5scr and DzG had no inhibitory effect. Neither DzF nor DzA could down-regulate levels of serum-inducible Egr-2 (Figure 5A) or c-Jun (Figure 5A) or affect constitutive levels of Sp1 (Figure 5A). Both DNAzymes also failed to affect levels of proliferating cell nuclear antigen (PCNA) or p53 (data not shown). These data indicate gene-specific inhibition by two EGR-1 DNAzymes.

DzA and DzF blocked serum-inducible porcine aortic SMC proliferation at 0.4 μmol/L. At this concentration, DzF completely inhibited cell replication (Figure 5A), whereas DzA inhibited by 75% (Figure 5B). Porcine vascular SMC proliferation was not significantly inhibited by DzE, DzG, or ED5scr (Figure 5B).

Figure 2. DzA and DzF inhibit EGR-1 protein expression in human aortic SMCs. Subconfluent growth-quiescent SMCs were transfected twice with DNAzyme and incubated in medium containing 5% FBS for 1 hour at 37°C. Western blot analysis was performed with cell lysates (5 to 10 μg), polyclonal antipeptide EGR-1 or Sp1 antibodies, and chemiluminescent detection. M, markers are indicated on the right of each figure. The Coomassie Blue-stained gel shows unbiased loading.

Effects of EGR-1 DNAzymes on Coronary In-Stent Restenosis

We next assessed the ability of these DNAzymes to inhibit in-stent restenosis in the porcine coronary stent model.24,28
Thirty-two stents were successfully deployed in 26 animals (Figures 6A and 6B). Three animals died from procedural complications (ventricular fibrillation in one, stent thrombosis in the second, and refractory ischemia after drug delivery in the third). At the 30-day histological examination, we found that two stents (from the stent-only and DzA groups) had been unsuccessfully deployed and were consequently excluded from additional analysis. The Transport catheter we used bears 48\( \times \)250-\( \mu \)m-diameter pores, which facilitates delivery at low pressure, reducing the likelihood of trauma from the delivery procedure per se. Increased neointima formation has been reported by infusion of vehicle alone using comparable delivery techniques, suggesting that local balloon delivery can induce vessel trauma. However, infusion of vehicle into the vessel wall did not influence the course of in-stent neointimal thickening after 30 days (Figures 6C and 6D).

DzF inhibited intimal thickening by \( >40\% \) (Table; Figures 6C and 6D), whereas neither DzA nor DzE had any effect (Figures 6C and 6D). Interestingly, the failure of DzA to block vascular repair in this model is entirely consistent with the preceding in vitro data showing inferior inhibition of regrowth after injury (Figure 3) and proliferation in two cell types (Figures 4A and 5B) at any given concentration. These observations, taken together, demonstrate the therapeutic potential of DzF as an inhibitor of in-stent restenosis.

### Effect of DNAzyme on Neointimal Formation After Porcine Coronary Stenting

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Neointimal Area</th>
<th>Original Lumen Area</th>
<th>Lumen Area</th>
<th>Injury Score</th>
<th>Stenosis, %</th>
<th>Stenosis/Injury Score, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stent</td>
<td>16</td>
<td>2.5 (1.1)*</td>
<td>6.2 (2.3)*</td>
<td>3.6 (1.9)</td>
<td>1.6 (0.6)</td>
<td>43.8 (19.3)</td>
<td>28.1 (13.7)</td>
</tr>
<tr>
<td>Vehicle</td>
<td>6</td>
<td>1.5 (0.9)</td>
<td>4.0 (1.5)</td>
<td>2.4 (1.2)</td>
<td>1.6 (0.6)</td>
<td>40.6 (17.4)</td>
<td>25.2 (6.5)</td>
</tr>
<tr>
<td>DzF</td>
<td>12</td>
<td>1.5 (0.9)</td>
<td>4.7 (1.2)</td>
<td>3.1 (0.7)</td>
<td>1.7 (0.5)</td>
<td>30.5 (12.2)</td>
<td>17.6 (4.0)†</td>
</tr>
<tr>
<td>DzA</td>
<td>12</td>
<td>2.5 (0.7)‡</td>
<td>5.1 (1.7)</td>
<td>2.6 (1.7)</td>
<td>1.8 (0.3)</td>
<td>54.5 (24.3)*</td>
<td>29.5 (12.8)</td>
</tr>
<tr>
<td>DzE</td>
<td>8</td>
<td>1.8 (0.8)</td>
<td>3.8 (0.8)</td>
<td>2.0 (0.9)‡</td>
<td>1.7 (0.5)</td>
<td>47.2 (18.2)*</td>
<td>27.8 (8.3)</td>
</tr>
</tbody>
</table>

Areas expressed in mm\(^2\). Data are presented as mean±SD unless otherwise stated. Two sections were measured per vessel.

\(*P<0.05\) vs DzF; †\(P<0.01\) for DzF vs stent, DzA and \(P<0.05\) for DzF vs vehicle, DzE; ‡\(P<0.01\) vs DzF.

### Discussion

In the present study, we generated and characterized novel DNAzymes targeting human EGR-1, which specifically cleave EGR-1 mRNA and inhibit EGR-1 protein expression in vascular SMCs. These molecules block SMC replication and regrowth after acute physical injury and reduce in-stent restenosis. Histopathological studies suggest that SMC proliferation is an important contributor to the pathogenesis of in-stent restenosis. The present observations demonstrate the therapeutic potential of EGR-1 DNAzymes in this pathologic setting and provide the first in vivo proof-of-principle demonstration of the possible clinical utility of catalytic DNA-based molecules targeting a specific human transcription factor as adjuncts to stents.

DNAzymes represent an exciting new technology, with great potential for use in diverse pathologies. For example, biologically active DNAzymes have recently been generated to CCR5, Huntingtin, and bcr-abl. Common to the composition of all DNAzymes is gene specificity conferred by the two arms, which can vary in length, flanking a cation-dependent catalytic domain. Despite similar catalytic efficiencies for EGR-1 mRNA, DzF and DzA showed consistent differences in biological potency. At any given concentration, DzF inhibited SMC proliferation and wound repair more effectively than DzA. Similarly, DzF inhibited in-stent restenosis, whereas DzA had no effect. These data suggest that the performance of a given DNAzyme in the context of in vitro cleavage experiments has little bearing on its biological potency. This is additionally supported by our earlier demonstration that DzB and DzC cleave human EGR-1 substrate and yet have no inhibitory effect on SMC proliferation. These discrepancies may be explained by dif-
ferences in overall conformation between in vitro transcribed mRNA (which is often not the complete sequence) and endogenous cellular mRNA, influencing the accessibility of a given DNAzyme for its target site. In the selection of DNAzymes, therefore, the ability to cleave an mRNA substrate should be seen as a prerequisite, rather than a determinant, of in vivo efficacy.

A recent study indicates minimal neointimal hyperplasia in patients treated with stents coated with growth inhibitor Sirolimus,35 which binds its intracellular receptor, inducing p27 and cell-cycle arrest. Expression analysis of atherectomy samples from patients with symptomatic in-stent restenosis showed elevated levels of the Sirolimus receptor FK506BP12.36 These findings support the notion that proliferation is an important cellular process contributing to in-stent restenosis. The present study provides additional evidence that EGR-1 is a positive regulator in vascular SMC growth, both in vitro and in the pig coronary artery wall.

Figure 4. DzA and DzF inhibit human aortic SMC proliferation. A, Dose-dependent DNAzyme inhibition of cellular mitogenesis. Subconfluent growth-quiescent SMCs transfected with DNAzyme were exposed to 5% FBS at 37°C for 72 hours. Cells were trypsinized, and the suspensions were quantitated in an automated Coulter counter. B, Sustained DNAzyme inhibition of cellular proliferation. Twenty-four hours after transfection with DNAzyme in medium containing 5% FBS, cell populations in each cohort were quantitated. Fresh medium in which DNAzyme was absent was added to the remaining cells, and the suspensions were counted 54 hours later, 78 hours after transfection with DNAzyme.

Figure 5. DzA and DzF inhibit EGR-1 protein expression and proliferation of porcine aortic SMCs. A, EGR-1 DNAzymes inhibit EGR-1 but not EGR-2, c-Jun, or Sp1 expression. Subconfluent growth-quiescent porcine vascular SMCs were transfected twice with DNAzyme and incubated in medium containing 5% FBS for 1 hour at 37°C. Western blot analysis was performed with lysates (5 to 10 μg), antipeptide antibodies to EGR-1, EGR-2, c-Jun, or Sp1, and chemiluminescent detection. The Coomassie Blue–stained gel shows unbiased loading. B, Inhibition of serum-inducible porcine vascular SMC proliferation. Subconfluent growth-quiescent SMCs transfected with DNAzyme were exposed to 5% FBS at 37°C for 72 hours. Cells were trypsinized, and the suspensions were quantitated in an automated Coulter counter.

Egr-1 is activated after angioplasty in animal models as well as several other pathophysiologic stimuli, including growth factors, cytokines, and changes in fluid biomechanical forces.37 Egr-1, in turn, switches on the transcription of many genes whose expression positively influences SMC prolifer-
EGR-1 might therefore be considered a master switch, given its activation by diverse agonists and conditions and its capacity to trigger the production of multiple proatherogenic and restenotic genes in the injured artery wall.\textsuperscript{37} The present data demonstrate reduced intimal thickening in vessels treated with EGR-1 DNAzyme delivered at the time of injury. Because EGR-1, as an immediate-early gene, is rapidly and transiently expressed on injury, it is therefore imperative that in the clinical setting, DNAzymes targeting EGR-1 should also be delivered locally at the time of injury. Such therapy would be particularly valuable if inhibition of SMC proliferation persisted well after the agent had been delivered. Our findings demonstrate that replication does not reinitiate in cells transfected with DzF, even days after removal of the DNAzyme from the culture supernatant, consistent with the
superior stability 9-9 DNAzymes bearing a 3’-3’-linked inverted T to nucleolytic digestion in serum.17

The porcine model of coronary stenting used in the present study has several well-documented limitations.28,38 Porcine coronary arteries are normal, atheroma-free vessels, distinct from the atheromatous lesions treated clinically in humans.38 In the pig, in-stent restenosis is examined at 28 to 30 days, whereas in humans, restenosis is generally examined within at least 3 months.9,28 However, the response to stent-induced injury is similar between pig and human coronary arteries,28 and the pig is presently the most widely used large animal model of coronary stenting and local drug delivery.23,39,40

Two reported studies to date have demonstrated efficacy of locally delivered antisense molecules after porcine coronary angioplasty.22,41 Shi et al41 used a porous transcatheter balloon to deliver naked phosphorothioate oligonucleotides directed against c-myc. Gunn et al22 used the transport catheter to deliver phosphorothioate oligonucleotides directed to c-myc. Both achieved inhibition of neointimal formation after angioplasty.22,41 However, oligonucleotide inhibition of intimal thickening has not yet been demonstrated in humans. A preliminary report of antisense oligonucleotides targeting the transcription factor c-myc delivered by the transport catheter after stenting failed to show a benefit in a small group of patients.42 This lack of an observed effect may have been attributable to an inefficient delivery system, the type of stent used, an inadequate drug dose, or the inappropriateness of the target. The present study also used the transport catheter as the delivery device, which has its own limitations.21 One animal died of refractory ischemia immediately after delivery; likely the consequence of the 90-second inflation. However, although this technique appeared to cause acute, but rare, morbidity, there were no apparent longer-term sequelae, such as increased neointima formation, observed with other devices22,29 (Figures 6C and 6D). We limited total delivery balloon inflation time to a minimum (90 seconds), which is similar or less than the times used in analogous studies.10,22 Our use of intracoronary nitroglycerine,20 bretylium,43 heparin monitoring and additional bolus dosing based on activated clotting time (ACT) measurements,20 and pretreatment with clopidogrel in addition to aspirin44 likely contributed to lowered morbidity in relation to delivery. The morbidity rates in the present study are comparable to or lower than those described in other published studies.20,45

The amount of DNAzyme (1000 µg) used in the present study was based on an ex vivo dose-escalation study in which FITC-labeled DNAzyme was delivered into pig coronary artery wall using the transport catheter. Histological examination of the cross-sections after in vivo delivery of 1000 µg of FITC-DNAzyme by fluorescence microscopy revealed extensive localization within the intima (Figure 6B). It is, of course, possible that a more efficient local delivery system could result in greater inhibition by DzF. The recent development of DNA-eluting stents in pig coronary arteries40 provides one means of achieving this goal and demonstrates the potential for the therapeutic exploitation of DNAzymes and other demonstrably effective nucleic acid agents in human arteries.


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