Suppression of Smooth Muscle Cell Proliferation by a c-myc RNA-cleaving Deoxyribozyme*

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A small catalytic DNA molecule targeting c-myc RNA was found to be a potent inhibitor of smooth muscle cell (SMC) proliferation. The catalytic domain of this molecule was based on that previously derived by in vitro selection (Santoro, S. W., and Joyce, G. F. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 4262–4266) and is known as the “10-23” general purpose RNA-cleaving deoxyribozyme. In addition to inhibiting SMC proliferation at low concentration, this molecule (targeting the translation initiation region of c-myc RNA) was found to efficiently cleave its full-length substrate in vitro and down-regulate c-myc gene expression in smooth muscle cells. The serum nuclease stability of this molecule was enhanced without substantial loss of kinetic efficiency by inclusion of a 3'-3'-internucleotide inversion at the 3'-terminal. The extent of SMC suppression was found to be influenced by the length of the substrate binding arms. This correlated to some extent with catalytic activity in both the short substrate under multiple turnover conditions and the full-length substrate under single turnover conditions, with the 9 + 9 base arm molecule producing the greatest activity.

Restenosis is a major complication following angioplasty, occurring in 30–60% of patients (7, 8). It is considered to be caused predominantly by vascular smooth muscle cell (SMC) proliferation after angioplasty. A variety of oncoproteins, such as c-myc, c-fos, and c-myb, have been found to be involved in SMC proliferation and migration as well as deposition of extracellular matrix associated with post-vascular injury (9–11). These genes provide attractive targets for the prevention of restenosis by therapeutic agents that can specifically and locally suppress their expression in vivo. In this study we explore the use of synthetic deoxyribozymes targeted to c-myc RNA as potential therapy for restenosis. The anti-c-myc deoxyribozyme designed for this purpose was found to cleave efficiently its substrate RNA and mediate suppression of SMC proliferation with a concomitant reduction of c-MYC protein in transfected SMCs. We therefore demonstrate the potential of catalytic DNA as a new class of genetic therapeutic agents.

EXPERIMENTAL PROCEDURES

Deoxyribozyme Synthesis—All the oligonucleotides were made by Oligo Etc. (Wilsonville, OR) and purified by gel electrophoresis for in vitro cleavage studies and by high pressure liquid chromatography for cell-based assays.

In Vitro Cleavage and Kinetic Analysis—The efficacy of deoxyribozymes in vitro was determined by measuring the rate of RNA cleavage under multiple turnover conditions. For these experiments a range of substrate concentrations was used such that [S] ≥10-fold excess over [E] which was fixed at 200 pM. The deoxyribozyme oligonucleotide and a 32P-labeled synthetic RNA substrate were pre-equilibrated separately for 10 min at 37 °C in 50 mM Tris-HCl, pH 7.5, 10 mM MgCl2, and 0.01% SDS. At time 0 the reaction was initiated by mixing the deoxyribozyme and substrate together. The reaction progress was then followed by the analysis of aliquots taken sequentially at various time points and quenched in 90% formamide, 20 mM EDTA, and loading dye. The product fragments and unreacted substrate in these samples were resolved by electrophoresis on a 16% denaturing polyacrylamide gel. The extent of reaction at each time point was determined by densitometry of the gel image produced through a PhosphorImager (Molecular Dynamics). The values for kobs (derived from the slope of these time course experiments) were used to generate a line of best fit in a modified Eadie-Hofstee plot (kobs versus kcat/[S]). In this expression the values for kcat and kM are given by negative slope of the regression line and the y intercept, respectively.

For cleavage of full-length c-myc RNA, substrate RNA (1.5 kilobase pairs) was transcribed from a pGEM7-Zf(+) vector in the presence of α32P-UTP with an RNA transcription kit (Promega). Cleavage was carried out at 37 °C in a 10-μl volume containing 10 mM MgCl2, 50 mM Tris-HCl, pH 7.5, 10 mM substrate RNA, and 50 nM deoxyribozyme oligonucleotide. Reaction was stopped at 60 min by adding equal volume of formamide loading buffer with EDTA, and the mixtures were then analyzed on a 6% denaturing polyacrylamide gel.

SMC Proliferation Assay—Rat smooth muscle cells (SV40LT-SMC, ATCC CRL 182) were cultured at 33 °C with 5% CO2 in Dulbecco’s

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1 The abbreviations used are: SMC, smooth muscle cell; ODN, oligodeoxynucleotides; bp, base pair; FBS, fetal bovine serum; DMEM, Dulbecco’s modified Eagle’s medium; DOTAP, N1-[(-2,3-dioleoyloxy)propyl]N,N,N-trimethylamino-niummethyl sulfate.

Antisense and ribozyme technologies are major tools in gene inactivation approaches for human gene therapy (2–5). However, these molecules (both as native nucleic acids and in modified forms) are highly susceptible to enzymatic hydrolysis and have potential for side effects in a cellular environment, thus limiting their pharmaceutical applications in a direct delivery mode. Recently, a new class of catalytic molecules made of single-stranded DNA (deoxyribozyme or DNA enzyme) was obtained through in vitro selection (1, 6). One model denoted as the “10-23” deoxyribozyme was especially useful because of its ability to bind and cleave any single-stranded RNA at purine/pyrimidine junctions (1). This molecule is comprised of a catalytic domain of 15 deoxynucleotides, flanked by two substrate-recognition domains of seven to eight deoxynucleotides each. Analysis has shown that this deoxyribozyme can efficiently cleave its substrate RNA with a catalytic rate of ~0.1 min−1 and KM < 1 nM under simulated physiological conditions in vitro. Given the catalytic efficiency, relative stability, and economy of production of DNA, an assessment of the therapeutic potential of this deoxyribozyme through cellular biochemistry is warranted.
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modified Eagle’s medium supplemented with 10% calf serum and 200 
µg/ml G418. In the proliferation assay, smooth muscle cells were plated at 25,000 cells per well in a 6-well cluster plate and allowed to attach overnight. The following day, the cells were washed twice with PBS and then grown in 0.25% calf serum/DMEM for a period of 4 days at 33 °C. After 4 days, the media were replaced with 10% calf serum/DMEM, and the deoxyribozyme oligonucleotides were added as triplicate samples. Three days later, the cells are trypsinized and counted by a Coulter counter.

Deoxyribozyme Stability Analysis—Briefly, 150 µM unlabeled deoxyribozyme oligonucleotide was incubated in 100 µl of 100% human serum at 37 °C, and duplicate samples of 5 µl were removed at time points of 0, 2, 8, 24, 48, and 72 h. Immediately upon sampling 295 µl of Tris/EDTA was added to the 5-µl aliquot, and phenol/chloroform extraction was performed. All the samples from each time point were end-labeled with 32P-ATP and run directly on 16% polyacrylamide RNA sequence analyses of c-MYC protein and RNA.

Deoxyribozyme-mediated Inhibition of SMC Proliferation—Anti-c-myc deoxyribozyme activity was tested in vascular SV40LT smooth muscle cells (14). After growth arrest in 0.5% FBS/DMEM, serum-starved SMCs were released from Go by the addition of 10% calf serum/DMEM. These cells were simultaneously exposed to deoxyribozyme or control (the 9/9 arm deoxyribozyme with an inverted catalytic core sequence) oligonucleotides in a suspension containing DOTAP transfection reagent. Deoxyribozyme-mediated suppression of SMC proliferation was determined at 72 h post-treatment by quantitation of cell numbers. Each deoxyribozyme was shown to induce a 30–80% decrease in cell numbers compared with the control at 10 µM concentration (Fig. 6A). The effective concentration range of the most active molecule (Rs-6, 9/9 arms with 3 inverted base modification) was analyzed further in a dose-response assay. The results indicated that this deoxyribozyme could cause significant suppression of SMC growth at concentrations down to 50 nM (Fig. 6B). This cell proliferation profile was supported by the quantitative cyto logical evidence of replication given by the mitotic index (Table II), with deoxyribozyme Rs-6-treated cells showing no increase. This was in

RESULTS

c-myc Gene Target—Suppression of c-myc gene expression has been shown to be a promising cytostatic strategy for the prevention of restenosis. The reduction of c-myc is thought to be effective in this condition by inhibiting cell cycle progression in the early stage of the disease (13–15). In this study an RNA-cleaving deoxyribozyme designed (using the 10-23 model, Fig. 1A) to target the c-myc translation initiation codon (Fig. 1B) was used. This site has been found to be an effective target for oligodeoxynucleotide (ODN)-mediated suppression of c-myc (15). The start codon in general has also been shown to be an amenable target for oligonucleotide-based gene inactivation (16).

In Vitro Characterization of Anti-c-myc Deoxyribozymes—Multiple turnover kinetics were used to examine the efficiency of deoxyribozyme-catalyzed cleavage of a short synthetic c-myc RNA sequence in vitro. Three modified deoxyribozymes and their unmodified controls with symmetrical 7-, 8-, and 9-base pair substrate binding arms (Fig. 2) were incubated with an excess of the 32P-labeled synthetic c-myc RNA. From the values for kobs, the kinetic parameters kM and kcat were determined (Fig. 3 and Table I).

The overall catalytic efficiency of each deoxyribozyme as measured by kcat/KM values displayed a significant amount of variability between the modified and unmodified species. In the short arm deoxyribozymes (7 + 7 bp) the inclusion of an inverted base modification produced a 3-fold decrease in the kcat/KM, i.e., a decrease in catalytic efficiency. In contrast to this negative effect on the cleavage activity, the relative efficiency of the long (9 + 9 bp) arm version was enhanced 10-fold by the presence of inverted base modification. The intermediate length (8 + 8 bp) binding arm deoxyribozyme was the least affected by modification, showing a 2-fold increase in the value of kcat/KM. The effect of the 3′-inverted terminal base was therefore different depending on the length of the substrate binding arms.

In Vitro Cleavage of Full-length c-myc mRNA—A full-length c-myc RNA was used to test further the deoxyribozyme cleavage of more biologically relevant sequence. Cleavage reactions were performed under single turnover conditions, with 10 nM long substrate (c-myc mRNA) and 50 nM deoxyribozyme in 10 mM MgCl2, pH 7.5, 37 °C. The results demonstrated that all the deoxyribozymes could effectively cleave c-myc mRNA to an extent of 20–50% (Fig. 4). The deoxyribozymes with longer arms cleaved substrates more efficiently, and 3′-inverted base modification decreased cleavage efficiency of the 7/7 arm deoxyribozyme but increased cleavage efficiency of the 9/9 arm deoxyribozyme. Interestingly, there was no difference in deoxyribozyme cleavage under conditions of either preheating the deoxyribozymes together with the c-myc RNA or no preheating. This indicated that the selected target site within the c-myc mRNA was very accessible in terms of RNA secondary structure in vitro.

Stability of Modified Deoxyribozymes—An assay was developed for examining deoxyribozyme oligonucleotide stability in 100% human AB serum. The results showed that the deoxyribozyme modified by a 3′-3′ inversion at the 3′ end had substantially greater stability in human serum (t1/2 = 20 h) compared with the unmodified deoxyribozymes that exhibited a half-life of <2 h (Fig. 5).

Deoxyribozyme-mediated Inhibition of SMC Proliferation—Anti-c-myc deoxyribozyme activity was tested in vascular SV40LT smooth muscle cells (14). After growth arrest in 0.5% FBS/DMEM, serum-starved SMCs were released from Go by the addition of 10% calf serum/DMEM. These cells were simultaneously exposed to deoxyribozyme or control (the 9/9 arm deoxyribozyme with an inverted catalytic core sequence) oligonucleotides in a suspension containing DOTAP transfection reagent. Deoxyribozyme-mediated suppression of SMC proliferation was determined at 72 h post-treatment by quantitation of cell numbers. Each deoxyribozyme was shown to induce a 30–80% decrease in cell numbers compared with the control at 10 µM concentration (Fig. 6A). The effective concentration range of the most active molecule (Rs-6, 9/9 arms with 3′-inverted base modification) was analyzed further in a dose-response assay. The results indicated that this deoxyribozyme could cause significant suppression of SMC growth at concentrations down to 50 nM (Fig. 6B). This cell proliferation profile was supported by the quantitative cyto logical evidence of replication given by the mitotic index (Table II), with deoxyribozyme Rs-6-treated cells showing no increase. This was in
marked contrast to the untreated cells and those treated with the control deoxyribozyme, which displayed a 6–7-fold increase in mitotic index.

c-myc Expression in Deoxyribozyme-transfected SMCs—To demonstrate the biological activity of the anti-c-myc deoxyribozyme at the molecular level, the relative expression of c-MYC protein in deoxyribozyme-treated and untreated SMCs was determined by immunoprecipitation. Treatment of SMCs with the deoxyribozyme was found to reduce the synthesis of metabolically labeled c-MYC protein (40%) down to the level seen in the unstimulated cells (Fig. 7A). Incubation with the control oligonucleotide, however, had no effect on c-myc expression in SMCs. Northern analyses further confirmed this result, showing a similar level of reduction in the c-myc mRNA caused by the active deoxyribozyme in SMCs (Fig. 7B).

DISCUSSION

Deoxyribozyme Kinetics and Optimal Designs—The optimal length of the 10-23 deoxyribozyme substrate binding arms for achieving maximal target RNA cleavage in vitro depends on the target sequence composition. Out of three human immunodeficiency virus RNA sequences targeted in vitro (1), it was shown that the deoxyribozyme activity achieved with 7-bp...
arms in GC-rich target sequences (gag/pol) was not significantly improved by their extension to 8-bp arms (6). Where the target GC content was lower (in the case of the env and vpr), deoxyribozymes with 8-bp arms demonstrated substantially greater activity. The values of $K_M$ for each of these deoxyribozymes was found to correlate with the predicted stability of the DNA/RNA heteroduplex (17). In the case of the c-myc-

**Table II**

|                  | Mitotic Index $^a$ |
|------------------|---------------------|
| Unstimulated    | 0.5 ± 0.11          |
| DOTAP           | 1.9 ± 0.22          |
| Rs-6            | 0.3 ± 0.13          |
| Rs-8 (control)  | 2.2 ± 0.15          |

$^a$ Mitotic indices were assayed on the serum-stimulated SMCs with or without deoxyribozymes. The number of mitoses per 1000 cells was determined microscopically.

**Fig. 5.** Stability assay of the 3'-inverted deoxyribozyme in human serum. Deoxyribozyme oligonucleotides were incubated with AB-type human serum (Sigma). Samples were collected at different time points as indicated and labeled with $^{32}$P. The labeled oligonucleotides were analyzed on 16% polyacrylamide gel. Typical gel patterns were shown here for unmodified (top right) and 3'-inverted deoxyribozyme (bottom right).

**Fig. 6.** Testing of c-myc-cleaving deoxyribozymes in SV-LT-SMCs. A, growth-arrested SMCs were stimulated with 10% FBS/DMEM in the presence of 10 µM anti-c-myc deoxyribozyme oligonucleotides or 10 µM control oligonucleotide Rs-8 (same arm sequences as Rs-6, with an inverted catalytic core sequence) or liposome alone (DOTAP). The data are displayed as mean ± S.D. B, dose-response experiments for Rs-6 deoxyribozyme in SMCs. The data are expressed as percent inhibition calculated from $(1 - \text{Rs-6/Rs-8}) \times 100$.

**Fig. 7.** c-myc expression in the deoxyribozyme-treated SMCs. A, c-MYC protein. Cells were labeled with $^{35}$S-methionine as described under "Experimental Procedures," and immunoprecipitation was performed to determine expression level of c-MYC protein in deoxyribozyme-treated SMCs. B, c-myc mRNA. SMCs were starved in a low serum medium (0.25% FBS) for 4 days and then subjected to stimulation and deoxyribozyme treatment in the medium containing 10% FBS and 2 µM oligonucleotides for 6 h. 10 µg total RNA from each treatment was loaded onto a denaturing agarose gel. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe was used as a loading control for the assay.
cleaving deoxyribozymes, we observed optimal cleavage efficiency in the unmodified versions with 8-bp arms. Both the 7- and 9-bp versions of the unmodified c-myc deoxyribozyme had lower overall efficiency according to their respective values for $k_{cat}/K_M$.

The kinetic profile of these three different length c-myc cleaving molecules was altered considerably by the inclusion of a 3′-terminal nucleotide inversion. This modification was applied to these molecules as a strategy for improving their stability in the presence of exonuclease activity in vitro (18). The influence of this DNA modification on the kinetics of c-myc RNA cleavage was particularly apparent in the short 7-bp arm deoxyribozyme. This molecule was substantially less efficient in terms of its value for $k_{cat}/K_M$ compared to the unmodified version. This reduction in catalytic efficiency was recovered and even enhanced by the addition of another two nucleotides in the 8-bp modified version. This indicated that the reduction of activity in the short deoxyribozyme was due to some disturbance of DNA-RNA interactions caused by the nucleotide inversion, which could be recovered by increasing the arm lengths to 8 bp. Further slight improvement in catalytic efficiency was found by increasing the arm lengths of the modified deoxyribozyme further to 9 bp. This was in contrast to the situation in the unmodified deoxyribozyme that demonstrated a sharp decline in activity between the versions with 8- and 9-bp arms.

These results demonstrated that 8 bp is the optimal arm length for c-myc RNA cleavage under these conditions by the unmodified DNA. To attain the optimal catalytic cleavage activity in the deoxyribozymes modified with a 3′-terminal inversion, it may be necessary to increase the length of each to 9 bp. The decline in catalytic efficiency seen in the unmodified deoxyribozyme with 9-bp arms was partially reflecting a reduction in enzyme turnover rate apparent as a lower value for $k_{cat}$. This was probably a result of an increase in the affinity of the enzyme for the products that slow down product dissociation. This reduction of activity was possibly avoided in the DNA modified by terminal base inversion, as a result of destabilization of the enzyme-product interactions.

Deoxyribozyme Suppression of SMC Proliferation—All six anti-c-myc deoxyribozymes tested were found to inhibit SMC growth after serum stimulation. The most effective of these deoxyribozymes (Rs-6) contained symmetrical 9-bp arms and was stabilized at the 3′-terminal with a 3′-3′ internucleotide linkage. This deoxyribozyme, with 80% suppression, compared favorably to the analogous phosphorothioate ODN (directed to the same site on the c-myc target) which demonstrated up to 70% suppression of SMC proliferation under similar conditions (15). The anti-proliferative activity of the deoxyribozyme Rs-6, however, was still significant down to a concentration of 50 nM. This dose-response range was much broader than that seen with the conventional antisense ODN, with a substantial deoxyribozyme effect maintained at concentrations approximately 20-fold lower. The reduction in SMC proliferation was further reflected in the quantitative cytological evidence, which showed fewer cells appearing in a state of mitosis in the treated cultures compared with the controls.

The molecular basis of growth suppression by phosphorothioate-modified ODN at this c-myc site and other targets is frequently disputed. The uncertainty is partially due to the many reports of toxicity and nonspecific activity associated with these molecules. The specificity of antisense agents used to target the start codon of the c-myc target has been particularly difficult to define because of the presence of a 4-G motif (19). Phosphorothioate ODN’s (including the c-myc antisense) that contain this contiguous 4-G motif, also known as a “G-quartet,” have been shown to reduce growth of proliferating cells by a mechanism unrelated to their proposed hybridization-mediated anti-gene effect (19). This nonspecific effect on proliferation can be excluded from the mechanism of deoxyribozyme Rs-6 activity for a number of reasons. First, the deoxyribozyme Rs-6 did not contain any phosphorothioate linkage and was active at comparatively low concentrations (50 nM). More importantly, the biological activity of Rs-6 was compared with a control oligonucleotide (Rs-8) that was identical except for an inactivated catalytic domain. This oligonucleotide had very little effect in cells even at high concentrations (>10 μM) despite having the same arm sequence. In fact, it had less activity than expected given that it may be capable of inducing a conventional antisense effect. It is possible that this molecule is a poor substrate for RNAse H because of the non-pairing intervening sequence derived from the inactivated catalytic domain. Although unlikely, it is possible that the biological activity of Rs-6 was derived from some other form of nonspecific activity related directly to the structure of Rs-6. To address this issue, a control oligonucleotide that is catalytically inactivated by a point mutation rather than a full inversion could be employed.

Correlation between In Vitro and in Vivo Activity—The biological activity of the various anti-c-myc deoxyribozymes correlated surprisingly well with their activity in vitro, as observed through multiple turnover kinetics and cleavage efficiency on the full-length transcript. This is exemplified by the deoxyribozyme Rs-6 which not only exerted the greatest suppression of SMC proliferation but also had the greatest kinetic efficiency under multiple turnover conditions and catalyzed the most extensive cleavage of the full-length c-myc RNA transcript. Moreover, this deoxyribozyme was found to induce a 40% reduction in metabolically labeled c-MYC protein synthesis in serum-stimulated SMCs.

Another observation was the dramatic decrease in the biological activity in the unmodified 9-bp arm deoxyribozyme (Rs-5) compared with the 3′-3′ inversion-stabilized deoxyribozyme (Rs-6). This was argued showing the value of the 3′-terminal stabilizing chemistry in protecting the oligonucleotide from exonuclease digestion. However, this may also have been partially reflecting the difference in cleavage kinetics observed between these two deoxyribozymes at the molecular level. This is supported to some extent by the smaller difference in biological activity seen between the modified and unmodified deoxyribozymes with shorter pairing arm sequences (Rs1–2 and Rs3–4, Fig. 6A). The difference in kinetic efficiency between these modified and unmodified deoxyribozymes was also not as great as that for the 9-bp arm deoxyribozymes, particularly in terms of $k_{cat}$.

Potential Therapeutic Applications—This is the first report to indicate that deoxyribozyme technology could form the basis of a new class of therapy with potential advantages over ODN antisense and ribozymes. The 10-23 deoxyribozyme has the same general composition as conventional antisense agents and greater endogenous catalytic activity than that of comparable ribozymes. Our results show that the natural nuclease stability of DNA can be enhanced substantially by the simple reversal of the 3′-terminal base without compromising the kinetic efficiency of the deoxyribozymes. This report also demonstrates that deoxyribozymes are effective in a biological system at both the molecular and cellular level. We show that a deoxyribozyme can be a sequence-selective inhibitor of c-myc expression in SMCs. The results described in this report further confirm that c-myc gene activation plays a critical role in the process of SMC proliferation. This provides the basis for further studies assessing the therapeu-
tic role of anti-c-myc deoxyribozymes in vascular restenosis. In combination with an effective means of local delivery, the full potential of deoxyribozymes as therapeutic agent in restenosis should be realized.

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