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The antiproliferative activity of c-myb and c-myc antisense oligonucleotides in smooth muscle cells is caused by a nonantisense mechanism

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ABSTRACT **Smooth muscle cell (SMC) proliferation is thought to play a major role in vascular restenosis after angioplasty and is a serious complication of the procedure. Developing antisense (AS) oligonucleotides as therapeutics is attractive because of the potentially high specificity of binding to their targets, and several investigators have reported inhibition of SMC proliferation *in vitro* and *in vivo* by using AS strategies. We report here the results of our experiments on vascular SMCs using AS oligonucleotides directed toward c-myb and c-myc. We found that significant inhibition of SMC proliferation occurred with these specific AS sequences but that this inhibition was clearly not via a hybridization-dependent AS mechanism. Rather, inhibition was due to the presence of four contiguous guanosine residues in the oligonucleotide sequence. This was demonstrated *in vitro* in primary cultures of SMCs and in arteries *ex vivo*. The *ex vivo* model developed here provides a rapid and effective system in which to screen potential oligonucleotide drugs for restenosis. We have further explored the sequence requirements of this non-AS effect and determined that phosphorothioate oligonucleotides containing at least two sets of three or four consecutive guanosine residues inhibit SMC proliferation *in vitro* and *ex vivo*. These results suggest that previous AS data obtained using these and similar, contiguous guanosine-containing AS sequences be reevaluated and that there may be an additional class of nucleic acid compounds that have potential as antirestenosis therapeutics.**

A great deal of interest has been focused on the potential for developing human therapeutics based on antisense (AS) phosphorothioate oligonucleotide (oligo) strategies. The elegant specificity of Watson–Crick base pairing between the AS oligo and the target mRNA or gene could form the basis for a highly specific and effective drug. AS oligo drugs could be designed to eliminate the expression of, in principle, any cellular protein (1). Recently, several groups have identified restenosis as a candidate for this type of therapeutic intervention (2–7).

Restenosis, the reclosure of coronary arteries after angioplasty, limits the long-term benefits of this nonsurgical intervention. It is estimated that of the ≈400,000 procedures performed in the United States this year to open atherosclerotic arteries, 30–50% will restenose (reclose) within 6 months (8). Excessive proliferation of smooth muscle cells (SMCs) is thought to be a major contributing factor to restenosis and one viable strategy for intervention is the inhibition of this proliferative response (9).

Using oligos complementary to c-myb and c-myc, a number of investigators have reported AS inhibition of SMC proliferation *in vitro* (5, 7, 10–14) and inhibition of restenosis in two different animal models (2, 5, 7). However, our results, presented here, suggest that the antiproliferative activity of these specific oligos on SMCs is not due to a hybridization-dependent AS mechanism. Rather, a stretch of four contiguous guanosine (4G) residues, which is present in both the AS c-myb and AS c-myc oligos used in the above studies, is responsible for the sequence-specific but non-AS antiproliferative effects of these oligos. Based on these findings, 4G-containing oligos may be developed into therapeutically viable drugs for the treatment of restenosis.

MATERIALS AND METHODS

Primary SMC Isolation, Characterization, and Growth Assays. New Zealand White rabbit (rb) aortic SMCs were isolated by an explant procedure (15). SMCs from tissue outgrowths were passaged twice at a 1:5 split and were frozen. Culture medium consisted of DME (GIBCO) supplemented with glutamine, penicillin, and streptomycin; growth and starvation medium contained 10% and 0.1% fetal bovine serum, respectively. The purity of the SMC cultures was determined by immunostaining with monoclonal antibodies to smooth muscle actin (1A4; Sigma) and smooth muscle myosin (hSM-V; Sigma). SMCs were seeded directly into 12-well dishes at 20,000 cells per well; after 1 day, the cells were placed in starvation medium for 3–4 days to induce entry into G_0. These growth-arrested cells were stimulated to proliferate by the addition of growth medium containing the indicated oligos. After 3 days, cells were treated with trypsin and assayed with a Coulter Counter. The percentage suppression of proliferation with oligos compared to parallel cultures without oligos was calculated according to the formula

$$\% \text{ suppression} = \frac{(Fc - Zc)_{\text{control}} - (Fc - Zc)_{+\text{oligo}}}{(Fc - Zc)_{\text{control}}},$$

where Zc (zero cell count) is the number of cells per well immediately after the starvation period and Fc (final cell count) is the number of cells per well after the 3-day proliferation period.

BrdUrd Incorporation into Primary SMCs. SMCs plated onto Nunc chamber slides at 6000 cells per cm^2 were growth arrested and then incubated with serum with or without oligos and BrdUrd labeling reagent (Amersham) for 24 hr. The BrdUrd-labeled nuclei were detected with a kit from Amersham and the AEC substrate from Vector Laboratories. Total

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Abbreviations: SMC, smooth muscle cell; oligo, phosphorothioate oligonucleotide; rb, rabbit(s); hu, human; AS, antisense; SCR, scrambled.
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nuclei were detected with propidium iodide, and the percentage of BrdUrd-labeled nuclei was determined by image analysis.

Proliferation of SMCs in Arteries. The model used to assay proliferation of SMCs in the media of the artery wall is a modification of the rb ear crush protocol of Banai *et al.* (16). New Zealand White rb were anesthetized and the hair on the dorsal surface of the ear was shaved. A pair of acrylic discs (12 × 2 mm) was positioned over the central vascular bundle of the ear and clamped firmly in place with Kelly clamps for 30 min, after which they were removed, and the rb was terminated. The crushed vascular bundle segments were removed to culture medium containing 5% fetal bovine serum and the appropriate treatment. After 54 hr of incubation, BrdUrd (Aldrich) was added to a final concentration of 10 μg/ml and incubated an additional 18 hr. The segments were fixed and processed into paraffin blocks. Serial sections were stained with antibodies to BrdUrd (17) and image analysis was performed on a Quantimet 520 (Leica) to determine the density of BrdUrd-positive SMCs in the media.

Oligo Synthesis. All of the oligos were synthesized on an Applied Biosystems DNA synthesizer (model 394). Phosphorothioate linkages were introduced by oxidizing the phosphite linkages with 3*H*-1,2-benzodithiol-3-one 1,1-dioxide instead of the standard iodine reagent (18). Oligonucleotides were purified by gel-exclusion chromatography, lyophilized to dryness, and resuspended as stock solutions in sterile water.

Oligo Sequences. The sequences for the c-myb oligos were from the published human (hu) and mouse sequences (19, 20). The rb and rat sequences were determined by reverse transcriptase PCR of total RNA isolated from SMCs by the acid phenol/chloroform method (21). The hu c-myc oligo sequences and the 4-bp mismatch were from Shi *et al.* (7); other oligo sequences were designed by the authors.

RESULTS

The Antiproliferative Activity of c-myb Oligos in Primary SMCs Is Not Due to an AS Mechanism. A standardized *in vitro* cell proliferation assay was developed whereby the increase in cell number of primary rb SMCs was determined after serum stimulation of growth-arrested cells. We found, as have others (10, 11), that the normal proliferative response of SMCs could be significantly inhibited by the addition of rb AS c-myb oligos to the culture medium (Table 1, sequences 1, 2, and 8; Fig. 1 *A* and *C*). Based on the observation that there is little inhibition when the corresponding sense oligo is used, this antiproliferative effect has been interpreted to be due to a sequence-specific AS mechanism. Consistent with this interpretation, we found that both the rb c-myb sense (sequence 3) and a randomized or scrambled (SCR) sequence (sequence 4) inhibit SMC proliferation much less than the AS sequence (Fig. 1*A*).

Surprisingly, in rb SMCs we found that the exact AS oligo to rb c-myb was consistently less inhibitory than the corresponding murine or hu c-myb AS oligos (sequences 2 and 8), which differ by 1 nucleotide from the rb sequence (Fig. 1 *A* and *C*). Because a single mismatch may cause only a small decrease in the melting temperature of an oligo–mRNA hybrid, we designed several 2-base mismatches to explore whether the inhibition of proliferation was dependent on hybridization. For example, we found that disrupting potential base pairing at positions 4 and 12 (sequence 6) or 2 and 18 (data not shown) of the rb c-myb AS sequence did not reduce the antiproliferative activity (Fig. 1*A*). One further 2-base mismatch at positions 6 and 12 (sequence 7) was, however, found to be significantly less inhibitory than the other mismatch sequences (Fig. 1*A*). It seemed unlikely that this was a fortuitous combination of mismatched bases that disrupted hybridization, but rather that some other feature of the oligos actually led to the inhibition of SMC proliferation.

Table 1. Oligonucleotide design

Sequence no. and abbreviated name	Sequence
1. rb c-myb AS (G4)	GTG CCG GGG TCT CCG GGC
2. mu c-myb AS (G4)	GTG **T**CG GGG TCT CCG GGC
3. rb c-myb sense	GCC CGG AGA CCC CGG CAC
4. rb c-myb SCR (random)	CGC CGT CGC GGC GGT TGG
5. rb c-myb SCR (G4)	GCT GCG GGG CGG CTC CTG
6. rb c-myb AS 4/12 (G4)	GTG tCG GGG TCc CCG GGC
7. rb c-myb AS 6/12 (G3)	GTG CCt GGG TCg CCG GGC
8. hu c-myb AS (G4)	GTG CCG GGG TCT **T**CG GGC
9. hu c-myb SCR (G4)	GCT GTG GGG CGG CTC CTG
10. hu c-myb SCR 10/17 (G4)	GCT GTG GGG tGG CTC CcG
11. hu c-myb SCR 6/13 (2 × G3)	GCT GTc GGG CGG gTC CTG
12. hu c-myb SCR 8/15 (2 × G2)	GCT GTG GcG CGG CTg CTG
13. hu c-myb SCR (random)	TGC CTG CGC GGC GGT TGG
14. hu c-myc AS (G4)	AAC GTT GAG GGG CAT
15. hu c-myc SCR (G4)	GTA CAC ATG GGG AGT
16. hu c-myc sense	ATG CCC CTC AAC GTT
17. hu c-myc AS (4-bp mm)	AAC GTg GAt tGG CAg
18. hu c-myc SCR (random)	GAA CGG AGA CGG TTT

Oligo sequences are based on codons 2–7 of c-myb or codons 1–5 of c-myc and are shown in the 5′ to 3′ direction. Nucleotides shown in boldface type are naturally occurring sequence differences between species. Nucleotides shown as lowercase letters have been altered to generate either mismatch (mm) controls for the rb c-myb or hu c-myc AS sequences or are various permutations of the hu c-myb SCR (G4) control. Contiguous guanosine residues discussed in the text have been underlined and are indicated parenthetically in the oligo name. mu, Murine.

Contiguous Guanosine Residues Are Responsible for the Antiproliferative Activity of c-myb and c-myc AS Oligos. Comparison of the sequences tested above revealed that all of the antiproliferative oligos shared a set of four contiguous guanosine residues (G4) between positions 6 and 9 of the oligos (sequences 1, 2, 6, and 8), while all of the noninhibitory oligos lacked this G4 sequence (Table 1, sequences 3, 4, and 7). It is especially relevant to note the different effects of the rb AS 4/12 and the rb AS 6/12 oligos (Fig. 1*A*, sequences 6 and 7): these two very similar sequences inhibit SMC proliferation to very different degrees, likely due to reducing the G4 motif in the 4/12 oligo to G3 in the 6/12 oligo. To confirm the role of this G4 sequence motif, we designed a G4 oligo in which the position and length of the guanosine residues were maintained, but the bases both 5′ and 3′ of this region were SCR (sequence 5). This control G4 oligo inhibits SMC proliferation at least as well as the other G4-containing oligos—clearly this oligo could not be eliciting its antiproliferative effect via a hybridization-dependent AS mechanism on c-myb mRNA as it is an 11-bp mismatch relative to its target (Fig. 1*A*). Virtually identical results were obtained with these same oligos in primary pig SMCs (data not shown).

Interestingly, we noticed that the c-myc AS oligo that had been used to inhibit SMC proliferation was also a G4 oligo (5, 7, 12, 13) and decided to test a parallel series of oligos to test the G4 vs. AS hypothesis. We compared the level of suppression of SMC proliferation by using five different c-myc oligos (Table 1, sequences 14–18). The effects of the c-myc AS, a G4 SCR control, the c-myc sense, a 4-bp mismatch (from ref. 7, which disrupts the G4 motif), and a SCR oligo on SMC proliferation are shown in Fig. 1*B*. Both of the G4-containing oligos (AS and G4 SCR control; sequences 14 and 15) suppressed SMC proliferation, while all of the non-G4-containing oligos (sense, 4-bp mismatch, and SCR; sequences 16–18) were significantly less inhibitory. These results are consistent with a

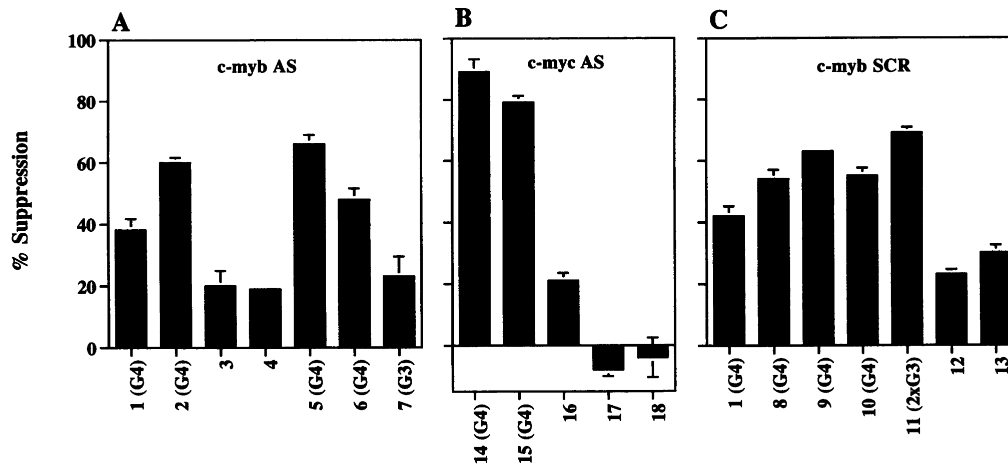

FIG. 1. Percentage suppression of rb SMC number *in vitro* by AS and G4 oligos at 30 μM. (*A*) c-myb AS oligos vs. SCR, sense, and other control oligos (see Table 1). (*B*) c-myc AS vs. control oligos. (*C*) G4 control oligos. Note that in all cases the G4 (and 2 $\times$ G3)-containing oligos are significantly more inhibitory than those lacking such contiguous G sequences. Data are presented as means $\pm$ SD of triplicate samples from a representative experiment. Other experiments performed with 10 or 60 μM oligos showed lower or higher levels of suppression, respectively, but similar relative differences were clearly observed.

G4 rather than an AS mechanism for suppression of SMC proliferation by the AS c-myc oligo.

A series of non-AS oligos was designed to further define the guanosine sequences that lead to significant inhibition of SMC proliferation (Fig. 1*C*). This set of oligos was based on a G4-containing SCR control sequence with the same base composition as the hu c-myb AS [hu c-myb SCR (G4), sequence 9]. Two-base swaps in this sequence were used to maintain the base composition while specifically altering the G4 sequence or not. As shown in Fig. 1*C*, we found that all of the oligos containing a G4 motif significantly inhibit SMC proliferation (sequences 1 and 8–10). Interestingly, an oligo containing two G3 sequences is also antiproliferative in SMCs (hu c-myb SCR 6/13; sequence 11), demonstrating that other multi-G sequences can be antiproliferative in SMCs. In contrast, a very similar oligo, hu c-myb SCR 8/15 (sequence 12, which lacks a G4 sequence), and the SCR sequence (sequence 13) had dramatically reduced antiproliferative activity (Fig. 1*C*).

The specific role of the requirement for guanosine residues in the c-myb inhibitory oligos was probed by substituting inosine, which lacks the 2-amino group found in guanosine but is otherwise identical, for two or four of the guanosine residues in hu c-myb SCR (G4) (sequence 9). Replacing the guanosine residues of the G4 sequence with inosine led to a significant reduction in the level of inhibition of SMC proliferation at 30 μM (42% vs. 74% suppression). There was, however, still considerably more inhibition with the inosine oligos than is seen with the hu SCR (random) sequence (sequence 13; 19% suppression), suggesting that the structurally related base inosine can at least partially substitute for guanosine in the (unknown) mechanism of antiproliferation caused by these oligos; however, some unique feature of guanosine is also involved.

The antiproliferative activity of these multi-G-containing oligos is fully reversible, indicating that the suppression of growth is not due to cellular toxicity. Replacement of the oligo-containing growth medium with fresh medium (after a typical 3-day incubation) leads to a rapid and complete reentry of the cells into the cell cycle. The cells begin to proliferate after a brief lag, and their doubling time is indistinguishable from cells being released from serum deprivation (data not shown).

The Antiproliferative Activity of c-myb Oligos on SMCs in Arteries Is Not Due to an AS Mechanism. BrdUrd incorporation into proliferating cells in the artery wall is illustrated in Fig. 2. The results of image analysis of the labeling density in the media of these arteries are summarized in the graphs in Fig. 3. Incubation of the crush-damaged artery segments with the hu c-myb SCR (G4) and hu c-myb SCR 6/13 (2 $\times$ G3) oligos (sequences 9 and 11) resulted in substantial reduction in the number of BrdUrd-labeled SMC nuclei in the media compared to the control untreated arteries or the arteries treated with the other oligos. The greater potency of the hu c-myb SCR (G4) (sequence 9) oligo compared to the perfect AS match rb c-myb AS (G4) (sequence 1) has also been observed *in vitro* (Fig. 1*C*) and in several experiments on crushed rb arteries by using thymidine incorporation as an endpoint assay. A statistically significant reduction of 90% or greater is consistently observed with the hu c-myb SCR (G4) (sequence 9) oligo, whereas the rb c-myb AS (G4) sequence (sequence 1) causes about a 30% reduction in thymidine incorporation (data not shown). These observations *in vitro* and *ex vivo* suggests that the context of the G4 sequence affects the potency of the antiproliferative activity in SMCs and that the antiproliferative activities of the oligos *ex vivo* parallel the *in vitro* results (Figs. 1–3).

Oligos Containing Contiguous Guanosine Residues Prevent Growth-Arrested SMCs from Reentering the Cell Cycle. To explore the mechanism by which the G4-containing oligos exert their antiproliferative activity, we asked whether the oligos prevent growth-arrested SMCs from reentering the cell cycle. BrdUrd incorporation into DNA was used to measure the percentage of cells that have reentered S phase of the cell cycle after treatment with many of the G4 and control oligos

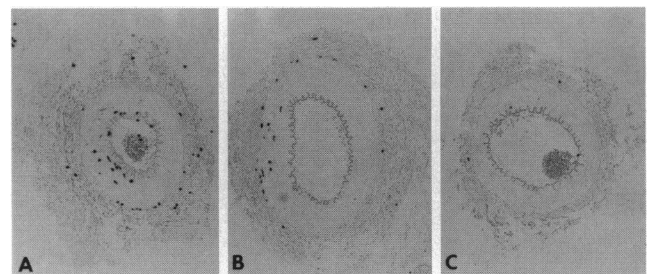

FIG. 2. Photomicrographs of cross-sections of arteries that were incubated with vehicle (*A*), rb c-myb AS (G4) (sequence 1) (*B*), and hu c-myb SCR (G4) (sequence 9) (*C*) at 100 μM and immunostained with antibodies to BrdUrd. The medial SMC nuclear labeling density calculated from these particular sections is representative of the median data presented in the bar graphs of Fig. 3.

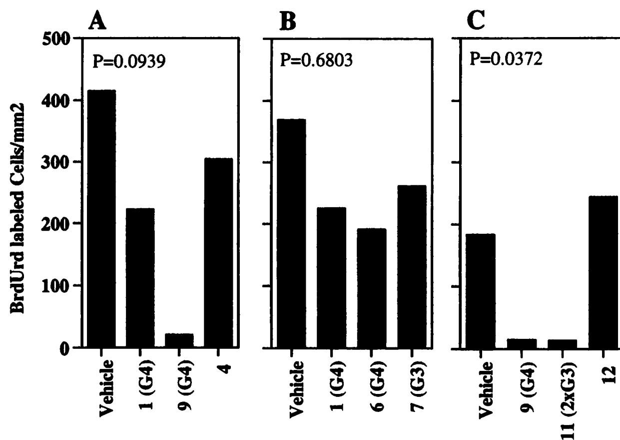

FIG. 3. Twelve rb produced 24 ear artery segments that were randomized into one of four treatments (control or one of three oligos at 100 μM) for a value of $n = 6$ per treatment in each of three experiments (*A*, *B*, and *C*). Image analysis was used to measure medial area and BrdUrd-labeled nuclei in 20 sections from each segment. These were totaled and used to calculate a single medial SMC nuclear labeling density measurement for each segment. These individual measures for each artery were ranked and the median value of four to six segments is represented by the bars in the graphs. A Kruskall–Wallis test was used to test nonparametrically for intergroup differences. The oligos used here are based on the c-myb sequences (as in Fig. 1 *A* and *C* and Table 1).

described above. Data from a representative experiment of this type are shown in Fig. 4. The trends of the results are in very good agreement with our cell count data presented above (Fig. 1) and indicate that SMC proliferation (actual increases in cell number) is inhibited by oligos with G4 sequences because the cells are unable to progress into S phase.

DISCUSSION

In this report, we provide evidence that AS oligos to c-myb and c-myc inhibit the proliferation of SMCs *in vitro* and *ex vivo* by a non-AS mechanism. We have identified a contiguous stretch of 4 guanosine residues that was shown to be responsible for the antiproliferative effects of these specific oligos on SMCs *in vitro* and in arteries *ex vivo*. Our results suggest that the conclusions drawn previously using these c-myb and c-myc AS oligos in SMCs and efforts to develop these AS oligos as restenosis therapeutics need to be reevaluated. However,

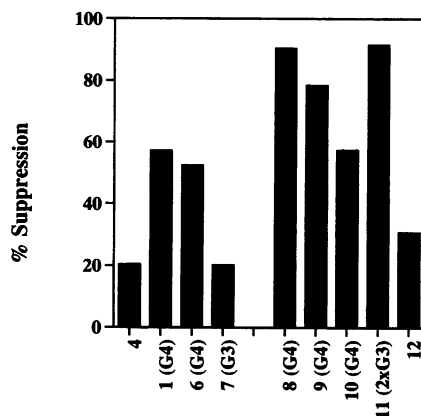

FIG. 4. Percentage suppression of BrdUrd incorporation into the nuclei of rb SMCs treated with the indicated oligos at 30 μM (see Table 1). Data from a representative experiment (performed in duplicate) are presented. Note that in all cases the G4 (and 2 × G3)-containing oligos are significantly more inhibitory than those lacking such contiguous G sequences.

exploring non-AS, contiguous guanosine-containing oligos as potential antirestenosis drugs is attractive. Our data do not directly address the many other systems in which AS c-myb and c-myc oligos have been used or studies that used other AS sequences containing contiguous guanosine residues. However, our results strongly suggest the necessity of using additional control sequences and cautious interpretation of the data from such experiments.

We have developed the *ex vivo* assay system used in these studies to facilitate screening of oligos for their antiproliferative effects on SMCs in arteries without the complications of working *in vivo*. The injury response of arteries is well documented and it has been established that the peak proliferation of medial SMCs occurs within a few days after injury in a variety of *in vivo* models (22, 23). This is also the case for crush-injured ear arteries *in vivo* (16) and in the *ex vivo* experiments presented here. Time course studies of medial proliferation in these arteries *ex vivo* demonstrated that there was very little proliferation 1 or 2 days after injury, that it peaked at 3 days, and that it subsequently subsided almost to baseline levels 6–8 days later (data not shown). Delivery in the *ex vivo* system was achieved by incubation of the segments in culture medium containing the oligo, allowing the response to be evaluated simply and with confidence. Uptake studies with fluorescently labeled oligos demonstrated rapid penetration into the crush-injured artery wall and binding to the SMC nuclei (C.L.F., unpublished data). Thus, the inhibition of SMC proliferation in the *ex vivo* model provides a rapid and effective system in which to further screen the efficacy of various nucleic acids for their potential use as antirestenosis therapeutics.

A variety of different biological responses have been ascribed to oligos containing G4 sequences including antiproliferative responses, antiviral responses, and inhibition of specific enzymatic activities (24–27). Yaswen *et al.* (24) found that G4-containing oligos were antiproliferative in some cells but not in others. Their results, along with ours, suggest that antiproliferative activities ascribed to G4-containing AS oligos should be reevaluated on a case by case basis. Wyatt *et al.* (26) provided evidence that a heat-sensitive "guanosine-quartet" structure was responsible for the observed antiviral activities. And recently, the crystal structure of such a parallel-stranded "guanine tetraplex" was described (28). However, we were unable to alter the antiproliferative effects of the G4 oligos described here after heat denaturation (data not shown), suggesting that the mechanism of antiproliferation may be fundamentally different from the antiviral activities of similar G4 oligos. Recently, Bennett *et al.* (27) showed that phospholipase A_2 activity was inhibited by oligos containing at least two sets of three or more consecutive guanosine residues. They speculate that the guanosine-quartet structure may be responsible. Interestingly, Smith *et al.* (29) demonstrated that $G_3T_4G_3$ oligos form an asymmetric, diagonally looped dimeric quadruplex structure. Whether such structural motifs are responsible for some or all of these biological effects remains to be documented. However, preliminary experiments suggest that G4 oligos bind to several proteins present in serum and in cell extracts (Gregory S. Brown and Cy Stein, personal communication). We suggest that protein–oligo interactions, perhaps mediated by specific structural conformations, may underlie the antiproliferative activities of G4 oligos.

Inhibition of SMC proliferation and restenosis using c-myb and c-myc AS oligos has been documented in rat, pig, and hu models (2, 5, 7, 10–14). The present data would suggest that in at least some of these experiments the mechanism of action of the oligo may not be due to the complementarity of the oligos with the intended target sequence. The present study shows that even the use of several control sequences (e.g., sense, random, and mismatches) may not reveal non-AS effects that are due to unusual structural features of the oligo sequence.

A recent editorial by Stein and Krieg (30) provides an excellent framework from which to conduct AS experiments. They suggest that investigators use a direct measurement of the target protein or mRNA in comparison to a control protein or mRNA with a similar half-life, since a decrease in target gene expression (c-myb or c-myc), although necessary, is not sufficient to prove that the oligo is acting via an AS mechanism; one must also show that there is no decrease of the control protein. The choice of the appropriate control for c-myb or c-myc is further complicated because blocking an upstream cell cycle target will likely prevent expression of downstream proteins.

Another issue of fundamental importance in AS research is the uptake and intracellular trafficking of oligos. It is generally accepted that most oligos enter cells via the endocytic pathway but apparently it is still not appreciated that in the absence of a delivery aid (such as cationic lipids or direct microinjection), oligos do not readily cross the permeability barrier of the endosomal or plasma membrane (1, 30, 31). Interestingly, as mentioned above, balloon injury of cells in arteries seems to remove this permeability barrier, perhaps by disrupting the cell membrane (C.L.F., unpublished data). However, in the course of our experiments on SMCs *in vitro*, we have been unable to find any evidence that naked oligos can escape from SMC endosomes (T.L.B., unpublished data). Thus, naked oligos added to the medium of cultured cells may never (or rarely) come into contact with their intended mRNA/gene targets in the cytoplasmic/nuclear compartment. Our current working hypothesis is that the G4 oligos elicit their antiproliferative effects on SMCs *in vitro* and *ex vivo* (and possibly *in vivo*) by an extracytoplasmic mechanism. Regardless of the mechanism, we believe that the potential therapeutic benefits of G4 oligos in restenosis should be explored.

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