Downregulation of c-myc Expression by Antisense Oligonucleotides Inhibits Proliferation of Human Smooth Muscle Cells

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Background. Proliferation of smooth muscle cells (SMCs) plays an important role in vascular pathobiology, being involved in the development of coronary restenosis and atherosclerosis. The activation of nuclear proto-oncogenes appears to be a final common pathway onto which various mitogenic signals converge. Accordingly, we attempted to determine whether the activation of the c-myc nuclear proto-oncogene is essential for human SMC proliferation and explored the possibility of inhibiting their growth using antisense oligonucleotides directed against c-myc messenger RNA (mRNA).

Methods and Results. Proliferation of human SMCs was associated with an increase in c-myc mRNA expression after growth stimulation. Using 15-mer phosphorothioate oligonucleotides (oligomers), we tested their growth-inhibitory effect in SMCs in vitro. Antisense oligomers directed against the translation initiation region of the human c-myc gene exhibited a significant antiproliferative effect, whereas sense and mismatched oligomers did not inhibit the growth. The growth-inhibitory effect of c-myc antisense oligomers was dose dependent and preventable by an excess of sense oligomers. Furthermore, growth inhibition of SMCs treated with c-myc antisense oligomers was associated with a marked decrease in the c-myc mRNA level. Phosphorothioate oligomers remained stable in medium containing 20% serum and were detectable in SMCs as early as 1 hour after cell exposure. Intact oligomers rapidly accumulated intracellularly and persisted within human SMCs for at least 16 hours.

Conclusions. c-myc antisense oligomers reduced c-myc expression and produced a significant growth inhibition of human SMCs, indicating an important role of c-myc gene activation in the process of SMC proliferation. Furthermore, extracellular stability and rapid cellular uptake provide the basis for future studies assessing the therapeutic role of the c-myc antisense approach in reducing SMC proliferation in the process of vascular restenosis. (Circulation. 1993;88:1190-1195.)

KEY WORDS • oncogenes • muscle, smooth • gene expression

Vascular smooth muscle cells (SMCs) have been identified as playing an important role in the development of atherosclerosis and restenosis after coronary angioplasty. Their presence has been confirmed in both types of lesions 1,2 and results primarily from a change in SMCs from a contractile to a synthetic phenotype. This remarkable characteristic is associated with SMC migration from media to intima, proliferation, and the synthesis of extracellular matrix. 3 In contrast to atherosclerosis, in which this process is extended over several decades, vascular restenosis represents an acute response to balloon injury culminating in a significant narrowing of an initially patent vessel in the course of a few months. 4 Hence, it has become apparent that the inhibition of SMC growth is necessary to control the restenosis process. Recent advances in cellular and molecular biology have provided insight into the molecular mechanisms of SMC proliferation, which is caused by the transduction of signals from the extracellular environment (e.g., growth factors) to the cell nucleus. Several genes become transiently activated during phenotypic modulation of SMCs. 5-8 These findings have stimulated interest in defining the role of abnormal gene expression in SMC growth and in selecting potential therapeutic targets for molecular-based approaches for acquired cardiovascular disorders such as vascular restenosis.

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Nuclear proto-oncogenes are highly conserved phosphoproteins tightly linked to cellular proliferation. The transient increase in nuclear proto-oncogene messenger RNA (mRNA) after mitogenic stimulation has been shown as the cell enters the G1 phase and appears to be necessary for the G1-to-S-phase transition. 9,10 Studies in cultured SMCs have demonstrated that c-fos, c-myc, and c-myb proto-oncogenes are activated shortly after various mitogenic stimuli. 11,12 Proto-oncogene expression has also been induced in the vessel wall after balloon denudation in a pattern similar to in vitro studies. 7 The above observations and the redundancy of signal transduction pathways have raised the possibility that nuclear proto-oncogene activation is a final common pathway onto which many diverse mitogenic signals converge, making it a potential therapeutic target. Antisense RNA technology is emerging as an effective means of lowering the levels of a specific gene product. It is based on the findings that these “antisense”
sequences hybridize to specific RNA transcripts, disrupting normal RNA processing, stability, and translation, thereby preventing the expression of a targeted gene. Administration of antisense oligonucleotides or transfer of expression constructs capable of producing intracellular antisense sequences complementary to the mRNA of interest have been shown to block the translation of specific genes in vitro and in vivo.

The objective of this study was to assess the role of the c-myc nuclear proto-oncogene expression in human SMC proliferation by use of the antisense approach and to explore the therapeutic potential of c-myc antisense oligomers for the inhibition of human SMC proliferation.

Methods

Cell Culture

Human SMCs originated from the saphenous veins of patients undergoing routine bypass surgery. The cells were isolated by an explant method. The explants were placed into tissue culture dishes containing Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 20% heat-inactivated fetal bovine serum (FBS), 100 IU/mL penicillin, 100 µg/mL streptomycin, and 2 mmol/mL glutamine (20% FBS-DMEM). The cultures were maintained at 37°C in a humidified incubator with 5% CO2. The cells exhibited typical morphological characteristics of vascular SMCs, ie, spindle shape and hill-and-valley pattern. The identification of vascular SMCs was further confirmed by in situ smooth muscle α-actin staining.

Synthesis of Oligomers

15-Mer antisense, sense, and mismatched oligonucleotides (oligomers) were synthesized on an Applied Biosystems model 394 high-throughput DNA synthesizer (Applied Biosystems, Foster City, Calif). The oligomers were lyophilized, resuspended in PBS, and quantified by spectrophotometry. Unmodified and modified (phosphorothioate) oligomers from the translation initiation region of the human c-myc gene were used in this study. The sequences were as follows: sense oligomers (5’TGGCCTTCAACGT3’), antisense oligomers (3’TAGGGAGGTTGCA5’), and mismatched oligomers (3’AACGAGTGTGGG5’).

Growth Assay

The early passages (passes 2 and 4) of human SMCs in 20% FBS-DMEM were seeded at a density of 10,000 cells per well in 24-well plates. Twenty-four hours after plating, original medium was replaced with growth-arrest medium (0.5% FBS-DMEM) for the next 48 hours. Then the cell growth was stimulated by the addition of 20% FBS-DMEM. The oligomers were added 24 hours before stimulation, at the time of stimulation, and every 48 hours thereafter unless stated otherwise. At the times indicated, SMCs were trypsinized and counted in a Coulter counter. The degree of inhibition was calculated as follows: % inhibition = 1 – (net growth of antisense-treated cells/net growth of sense-treated cells) x 100. The net growth of human SMCs was obtained by subtracting the starting cell number (at the time the cells are released from the G0 phase) from the cell number at indicated time points of the experiment. Each experiment was carried out in triplicate. Data are expressed as mean±SD. In addition, cell viability after treatment with oligonucleotides was assessed by trypan blue exclusion.

Stability and Cellular Uptake of Oligomers

15-Mer phosphorothioate oligomers were 5’ end labeled with γ-32P-ATP (5 x 106 cpm/µg) using a 5’ DNA terminus labeling protocol (Gibco BRL Life Technologies, Inc, Gaithersburg, Md). To determine the stability of oligomers in serum, 32P-end labeled phosphorothioate oligomers (2 µmol/L) were incubated in culture medium containing 20% FBS. The aliquots of medium were collected at different time points. In experiments assessing intact intracellular oligomers, SMCs were incubated with 2 µmol/L of 32P-labeled phosphorothioate oligomers at the time of stimulation. For the preparation of the cell lysates, SMCs were washed with PBS, trypanized, and centrifuged. The pellet was resuspended in 0.2 mol/L glycine (pH 2.8) to remove membrane-bound oligomers. Cells were lysed in 10 mmol/L Tris-HCl, 200 mmol/L NaCl, 1% sodium dodecyl sulfate, and 200 µg/mL proteinase K (pH 7.4) for 2 hours at 37°C. The oligomers were precipitated from culture medium or cell lysates by the phenol extraction method18 and electrophoresed in a denaturing 20% polyacrylamide gel. The gel was dried and exposed to X-OMAT Kodak film for autoradiography and analyzed by densitometry.

Reverse Transcription and Polymerase Chain Reaction

To determine c-myc mRNA levels in human SMCs, a modified reverse transcription and polymerase chain reaction (RT-PCR) technique was used.19 Total RNA was isolated with a single-step procedure using the acid guanidinium thiocyanate–phenol–chloroform extraction method. The quality of RNA samples was confirmed by denaturing gel electrophoresis. To distinguish between amplification of genomic DNA and complementary DNA (cDNA), the primer pairs were designed to enclose at least one intron on the genomic sequence of c-myc and β-actin. The primers were synthesized as described above, and the primer sequences were as follows: c-myc primer A, 5’TGGTGGCTCATGGAGAGACA 3’ (1271 to 1290); primer B, 5’GGTGTTCCTGAACGTG3’ (1845 to 1826); β-actin primer A, 5’AAGGATTCTATGTTTGC3’ (1438 to 1455); primer B, 5’CATCTCTGTCAGAGT3’ (2412 to 2395). The primers were 5’ end labeled with 50 µCi of [γ-32P]ATP according to 5’ DNA terminus labeling protocol (Gibco BRL Life Technologies, Inc). The PCR amplification efficiency was determined by amplifying a series of dilutions of RNA sample to obtain the PCR products during the exponential phase of amplification. One and two micrograms of total RNA were reverse transcribed into cDNA by 200 units of SuperScript reverse transcriptase for β-actin and c-myc, respectively. The PCR amplification of cDNA was carried out by use of the GeneAmp RNA PCR protocol (Perkin-Elmer Corp, Hayward, Calif). Briefly, an aliquot of cDNA was added to a reaction mixture containing 20 µmol/L of primers and 5 units of Taq polymerase. Amplification was performed with a DNA thermal cycler (Perkin-Elmer Cetus) for 15 (β-actin) and 30 (c-myc) cycles. A cycle profile consisted of 1 minute at 94°C for denaturation, 2 minutes at 60°C (c-myc) or 55°C (β-actin) for annealing, and 2 minutes at 72°C for primer extension. The RT-PCR products were electrophoresed in a 6% polyacrylamide gel and exposed to Kodak film. The restriction enzyme digestion of PCR products was carried out to confirm PCR products.
Results

Proto-oncogene c-myc Expression in Human SMCs

To assess the level of expression of the c-myc proto-
oncogene in human SMCs, c-myc mRNA was deter-
mined in quiescent (arrested for 48 hours) and pro-
liferating cells (2, 4, and 24 hours after serum
stimulation). RT-PCR was performed with specific
c-myc primers. Quiescent human SMCs expressed a low
level of c-myc mRNA. In proliferating SMCs, in con-
trast, c-myc mRNA levels increased at 2 and 4 hours
after cell growth stimulation. The c-myc mRNA de-
clined at 24 hours, although its level remained higher
than in quiescent cells (Fig 1).

Inhibition of SMC Proliferation and c-myc Expression

The incubation of human SMCs with c-myc antisense
phosphorothioate oligomers resulted in a significant
growth-inhibitory effect, whereas sense or mismatched
phosphorothioate oligomers exerted no effect on cell
growth (Fig 2). A significant inhibition was maintained
for at least 4 days with a continuous exposure to
oligomers (P<.001). In contrast to the growth-inhibitory
effect of phosphorothioate oligomers, unmodified an-
tisense oligomers had no effect on SMC growth in doses
up to 40 μmol/L. As expected, the antiproliferative effect
of c-myc antisense phosphorothioate oligomers was
dose-dependent within a range of 1 to 10 μmol/L
(Fig 3). In the experiments with oligomers added at the
time of cell stimulation, the incubation of human SMCs
with c-myc antisense oligomers (10 μmol/L) for 8 and 24
hours produced comparable growth inhibition of
58±13% and 70±14%, respectively. Similar studies
were carried out to determine the growth-inhibitory
effect of c-myc antisense oligomers in porcine SMCs.
Growth inhibition >90% was observed in porcine SMCs
after c-myc antisense treatment compared with control,
sense-treated, or two base pair mismatched antisense
oligomer-treated cells (data not shown). In all growth
experiments, the treated SMCs demonstrated normal
morphology, and no cell death was noted by trypan blue
exclusion at the dose range tested.

To assess potential long-term effects of c-myc an-
tisense treatment on cell growth, the oligomers were
withdrawn after 8 hours of incubation, and human
SMCs were subcultured 7 days later. The cell counts
were obtained at 1, 2, 4, and 6 days thereafter. The
growth rates of antisense-treated and control SMCs
were identical. This demonstrates normal SMC viability
after antisense oligomer treatment.

We expected that the antiproliferative effect of c-myc
antisense oligomers would be abrogated by the addition
of excess sense oligomers to the SMC culture. As shown
in Fig 4, increasing the ratio of sense to antisense
oligomers completely abolished the growth inhibition
(sense, 0 to 20 μmol/L and antisense, 2 μmol/L). This
was probably because of the formation of heteroduplexes

FIG 1. Blot showing expression of c-myc mRNA in human
smooth muscle cells. Total RNA was isolated; samples were
reverse transcribed to cDNA and then amplified by poly-
merase chain reaction with c-myc and β-actin sequence specific
32P-5'-end labeled primers. O, growth-arrested cells (0.5% fetal
bovine serum/Dulbecco's modified Eagle's medium [FBS-
DMEM]); 2, 4, 24, proliferating cells (20% FBS-DMEM) at
2, 4, and 24 hours after stimulation, respectively. The experi-
ment was repeated twice with similar results.

FIG 2. Growth curves of human smooth muscle cells
(SMCs) incubated with 10 μmol/L of c-myc antisense, sense,
and mismatched oligomers. Control cells were incubated
without addition of oligomers. SMCs were growth-arrested
with 0.5% fetal bovine serum/Dulbecco's modified Eagle's
medium (FBS-DMEM) for 48 hours and then stimulated
(Stim) with 20% FBS-DMEM. Oligomers were added 24
hours before stimulation and at the time of stimulation and
then replenished every 2 days. Results are presented as
mean±SD (n=3). The experiments were repeated three times
on different occasions, yielding similar results. Antisense-
treated cells demonstrated significantly slower growth com-
pared with any remaining group (P<.001).

FIG 3. Curve showing dose-dependent growth inhibition of
human smooth muscle cells after c-myc antisense oligomers.
Percent inhibition was calculated at day 1 by comparison of cell
count in antisense-treated cells with the number of cells treated
with c-myc sense oligomers. Results are expressed as mean±SD
(n=3). The experiment was repeated three times on different
occasions with similar results. Conc, concentration.
between the two oligomers, which indicates a hybridization-based mechanism of the growth-inhibitory effect of antisense oligomers.

To determine whether the antiproliferative effect of antisense oligomers was caused by a reduction in c-myc expression, c-myc mRNA was determined in antisense- and sense-treated cells. C-myc antisense phosphorothioate oligomers (10 μmol/L) reduced the target mRNA in proliferating human SMCs, whereas sense-treated cells demonstrated unchanged levels of c-myc mRNA compared with those in cells without oligomer treatment (Fig 5). Simultaneous determination of β-actin mRNA in the control and sense- and antisense-treated cells demonstrated no major differences.

Stability and Cellular Uptake of Oligomers

Gel electrophoresis coupled with autoradiography was used to determine the intact oligomers. 15-Mer phosphorothioate oligomers demonstrated a slow degradation in medium containing 20% FBS with about 71% and 49% of oligomers remaining intact (undegraded) at 24 and 36 hours, respectively. The kinetics of cellular uptake of oligomers in human SMCs are shown in Fig 6. Cells were incubated with 32P-end labeled phosphorothioate oligomers. The intact oligomers were detectable as early as 1 hour after incubation and rapidly continued to increase for 16 hours. Similar results were obtained by use of fluorescent activated cell sorter with FITC-labeled oligomers (data not shown). There was no difference in cellular uptake of oligomers between quiescent and proliferating cells. Hence, phosphorothioate oligomer stability in serum and a rapid cellular uptake allowed for their sustained levels within human SMCs.

Discussion

The major findings of this study are that (1) proliferation of human SMCs is associated with increased expression of the c-myc nuclear proto-oncogene, (2) c-myc antisense phosphorothioate oligomers decrease c-myc expression and produce a significant growth-inhibitory effect in human SMCs, and (3) the rapid cellular uptake and relative stability of phosphorothioate oligomers suggest potential applicability of the antisense approach as a therapeutic modality in the cardiovascular system.

Nuclear Proto-oncogene c-myc in SMC Proliferation

The c-myc proto-oncogene, one of the first described nuclear proto-oncogenes, is an evolutionarily conserved gene expressed in numerous cell types. It has been postulated that c-myc has a role as a direct transcriptional activator,20 stimulates DNA replication,21 or regulates gene expression at the posttranscriptional level.22 C-myc protein demonstrates extensive homology with known transcriptional regulators. In addition, helix-loop-helix and leucine zipper domains in c-myc protein are functionally relevant for protein-protein interaction.23

The results of this study have demonstrated that the c-myc transcripts were elevated in proliferating but not quiescent human SMCs. The c-myc transcripts increased early after cell stimulation and then declined, although their level remained elevated at 24 hours after stimulation. These findings are consistent with observations that c-myc mRNA and protein levels are highest before DNA synthesis (S phase) and then remain above the basal level throughout the cell cycle in the presence of growth factors.11 Studies using other cell systems have shown that downregulation of c-myc expression was invariably associated with the inhibition of cell growth and differentiation.24 This was further supported by the evidence that known inhibitors of cell proliferation reduced c-myc mRNA levels, although conflicting data regarding the effect of heparin on c-myc mRNA in SMCs have been reported.25,26

Decrease in c-myc Expression Inhibits Human SMC Proliferation

Despite the evidence showing that c-myc activation is associated with mitogenic stimulation, the cause-and-effect relation between enhanced c-myc expression and
human SMC proliferation has not been addressed previously. Since antisense oligonucleotides inhibit expression of specific genes, this approach provides the opportunity to examine the role of the c-myc proto-oncogene in SMC proliferation. This study has shown that c-myc antisense oligomers targeting the c-myc translation initiation region markedly reduced c-myc mRNA levels in human SMCs, which was paralleled by a significant growth inhibition. Although we have not shown reduction in c-myc protein, several lines of evidence suggest a sequence-specific effect of antisense oligomers used in the present study. Using the same antisense sequence, Holt et al. demonstrated the formation of an intracellular duplex with the target mRNA and a selective decrease of c-myc protein in human promyeloctytic leukemia HL-60 cells. The growth-inhibitory effect of c-myc antisense oligomers reported in the present study was observed in a low dose range and was eliminated by the addition of sense oligonucleotides. The antiproliferative effect was elicited even without pretreatment; a significant growth inhibition was observed after an incubation time as short as 8 hours after SMC stimulation. In contrast, sense or mismatched oligonucleotides produced no effect on SMC growth. The above observations and a marked reduction in c-myc mRNA after antisense treatment suggested that the inhibition of proliferation was probably caused by a sequence-specific effect of antisense oligonucleotides downregulating c-myc expression in human SMCs. An alternative mechanism of action for antisense oligomers may include binding of oligomer molecules to protein(s) involved in cell proliferation rather than specific mRNA. Recently, the ability of single-stranded DNA molecules (aptamers) to bind and inhibit thrombin has been described. This mechanism, however, appears unlikely, since the growth-inhibitory effect of oligomers described in this study was sequence-specific and was associated with decreased c-myc expression. RT-PCR was used to measure c-myc transcripts in the present study. Although quantitative PCR can be difficult to achieve, a high reproducibility of c-myc amplification in the sense- and antisense-treated samples (Fig 5) enabled us to use this methodology.

**c-myc Antisense Approach as a Therapeutic Modality**

The use of antisense oligonucleotides offers the opportunity to shed additional light on the molecular basis of SMC proliferation and vascular restenosis as well as to provide a new therapeutic modality. The advantages of this approach may include selective inhibition of proliferating cells, suppression of specific gene products in target SMCs, and bypassing of cell surface events such as the activation of membrane receptors. However, a slow cellular uptake and the instability of oligomers in a biological environment raised questions about their applicability in the cardiovascular system, since antisense oligomers must reach sufficient intracellular concentration to exert a therapeutic effect. Phosphorothioate oligomers used in this study remained relatively stable in serum, with about 50% oligomers intact after 36 hours of incubation. These findings are consistent with previous reports indicating that phosphorothioate (ie, sulfur-protected) oligomers are more resistant to enzymatic degradation by deoxyribonucleases present both in serum and within the cells. Our observations that oligonucleotides are actively transported across cell membranes via receptor-mediated endocytosis suggest that these molecules may be effective in vivo. Since cellular uptake may differ considerably between different cell lines, it was important to determine oligonucleotide transport into human SMCs. The results of this study demonstrate rapid accumulation of intact oligomers within human SMCs starting as early as 1 hour after exposure. A significant amount of intact oligomers persisted within human SMCs for at least 16 hours. Hence, the presence of intact oligonucleotides in the extravascular environment allowed maintenance of their sustained intracellular concentration. Recently, Simons and Rosenberg have shown the growth-inhibitory effect of c-myb antisense oligomers in rat SMCs. Our own observations indicate that c-myb antisense oligomers (10 μmol/L) inhibit human SMC growth as well, with antiproliferative activity comparable to that of c-myc antisense oligomers (unpublished data). Thus, inhibition of different nuclear proto-oncogenes appears to be effective, confirming their essential role in signal transduction in human SMCs. In contrast to a low dose requirement for c-myc and c-myb antisense oligomers (10 μmol/L) in human SMCs, the growth inhibition of rat SMCs has been previously shown with much lower concentrations of phosphorothioate antisense oligonucleotides complementary to proliferating cell nuclear antigen and c-myc mRNA. These data suggest that the type of oligomers used, species differ-

**FIG 6.** Graph and blot showing intracellular accumulation of intact oligomers in human smooth muscle cells. Cells were incubated with 32P-end labeled oligomers (2 μmol/L) beginning at the time of cell stimulation. At indicated time points, intracellular oligomers were extracted from cell lysates and analyzed on a 20% polyacrylamide denaturing gel. Bands indicate intact intracellular oligomers (insert). The curve represents densitometric measurements.
ences, and selection of the target mRNA play an important role in biological response to antisense molecules. Further studies are necessary to evaluate the c-myc antisense approach in vivo. Several issues should be taken into consideration, including the mode of local delivery of oligomers to the site of vascular injury and the selection of relevant animal models. Recently, Simons et al. reported encouraging results regarding adventitial delivery of c-myb antisense oligomers, which reduced formation of neointima after balloon injury in rat carotid arteries. However, the efficacy of the antisense approach with more practical (eg, endoluminal) means of oligomer delivery remains to be evaluated.

Conclusions

In conclusion, c-myc antisense oligomers reduced c-myc expression, which was associated with a significant growth inhibition of human SMCs. This demonstrates that c-myc gene activation plays an important role in the process of human SMC proliferation. A potent growth-inhibitory effect and pharmacokinetic profile of c-myc antisense oligomers provide the basis for studies assessing the therapeutic role of this approach in vascular restenosis. Note added in proof. Since the submission of this manuscript, we have observed a significant inhibition of vascular SMC proliferation in vivo. Proto-oncogene c-myc antisense oligomers delivered locally via a porous balloon reduced neointima formation in denuded porcine coronary arteries.

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