Intramural Coronary Delivery of Advanced Antisense Oligonucleotides Reduces Neointimal Formation in the Porcine Stent Restenosis Model

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OBJECTIVES
We evaluated the long-term influence of intramural delivery of advanced c-myc neutrally charged antisense oligonucleotides (Resten-NG) on neointimal hyperplasia after stenting in a pig model.

BACKGROUND
Neointimal hyperplasia after percutaneous coronary interventions is one of the key components of the restenotic process. The c-myc is a critical cell division cycle protein involved in the formation of neointima.

METHODS
In short-term experiments, different doses (from 500 μg to 5 mg) of Resten-NG or saline were delivered to the stent implantation site with an infiltrator delivery system (Interventional Technologies, San Diego, California). Animals were euthanized at 2, 6, and 18 h after interventions, and excised vessels were analyzed for c-myc expression by Western blot. In long-term experiments, either saline or a dose of 1, 5 or 10 mg of Resten-NG was delivered in the same fashion, and animals were euthanized at 28 days after the intervention.

RESULTS
Western blot analysis demonstrated inhibition of c-myc expression and was dose dependent. Morphometry showed that the intimal area was 3.88 ± 0.40 mm² in the control. There was statistically significant reduction of intimal areas in the 5 and 10 mg groups (2.01 ± 0.66 and 1.95 ± 0.91, respectively, p < 0.001) but no significant reduction in the 1 mg group (2.81 ± 0.56, p > 0.5) in comparison with control.

CONCLUSIONS
This study demonstrated that intramural delivery of advanced c-myc neutrally charged antisense morpholino compound completely inhibits c-myc expression and dramatically reduces neointimal formation in a dose dependent fashion in a porcine coronary stent restenosis model, while allowing for complete vascular healing. (J Am Coll Cardiol 2002; 39:1686–91) © 2002 by the American College of Cardiology Foundation

A potential application of molecular therapy with antisense oligonucleotides is the prevention or treatment of restenosis after coronary interventions (1,2). Balloon and/or stent injuries result in neointimal hyperplasia that lead to recurrent narrowing of the lumen within months after intervention (3). Alternatively, direct interference of the critical steps in the smooth muscle cell growth cycle has been attempted, using antisense oligonucleotides in animal models (4–7). Inhibition of several cellular proto-oncogenes have been shown to inhibit smooth muscle cell proliferation in vitro and to reduce neointimal thickening in vivo (7–12).

The clinical use of antisense technology, however, remained limited due to a relative lack of specificity, slow uptake across the cell membrane and rapid intracellular degradation of the oligonucleotide (13). Therefore, one study in humans using local delivery c-myc antisense has yielded a negative result (14).

Recent studies have introduced phosphorodiamidate morpholino oligomers (PMO), which represent an unusual DNA chemistry with a six-membered morpholino ring instead of a deoxyribose sugar. In addition, the charged phosphodiester and uncharged phosphorothioate linkage replaces internucleotide linkage. The lack of internucleoside charge allows the PMO to avoid the tendency of the more common phosphorothioate analogs to produce nonspecific effects through binding of cellular and extracellular proteins (15–17). The PMO are resistant to the nuclease found in serum (18) and exhibit a high degree of specificity and efficacy in both in vitro and cell free translation studies (19–22).

It was also demonstrated that endoluminal delivery of c-myc neutrally charged antisense oligonucleotide with a transport catheter reduces neointimal formation and positively affects the vascular remodeling in the rabbit balloon injury model (23).

The objective of the present study was: 1) to evaluate the effect of local antisense therapy using advanced PMO with c-myc antisense oligonucleotides (Resten-NG) on c-myc...
expression after stent implantation and 2) to determine the long-term influence of local antisense therapy on neointimal hyperplasia after stenting in a pig coronary model.

METHODS

Oligonucleotide synthesis and purification. Phosphorothioate morpholino oligomers were synthesized at AVI BioPharma as described previously (24). The Resten-NG sequence is complementary to the c-myc messenger ribonucleic acid at the translation initiation start site, 5' ACG-TTGAGGGCAGCTTCAGGC-3', and the control sequence employed in cell culture studies contains the sequence 5'-ACTGTGAGGGCGATCGTGC-3'. The PMOs were purified by ion exchange chromatography. Purity was >90% full length 20-mer as determined by reverse phase HPLC (high performance liquid chromatography) MALADITOF mass spectroscopy.

Western blot analysis. Western blot analysis involved a 8.7 µg protein sample of HeLA cell lysate that was loaded onto a polyacrylamide gel, a 4% stacking gel and an 8% running gel at 100 V for 2 h. The gel was then electroblotted onto immobilon P membrane from Millipore (Bedford, Massachusetts). Actin served as an internal standard probed with a mouse monoclonal primary antibody, AC40, from Sigma Chemical Co. (St. Louis, Missouri). The c-myc expression was measured with a mouse monoclonal primary antibody, C-33, from Santa Cruz Biotechnology (Santa Cruz, California). Both actin and c-myc antibodies were identified with secondary goat anti-mouse IgG antibodies conjugated with horse radish peroxidase from Santa Cruz Biotechnology. The immunoblot bands were visualized by chemiluminescence with Amersham Pharmacia ECI detection reagents (Piscataway, New Jersey).

Animals and experimental protocol. Animals used in this study received humane care in compliance with the “Principles of Laboratory Animal Care,” formulated by the National Society for Medical Research, and the “Guide for the Care and Use of Laboratory Animals,” prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication #85-23, revised 1985).

Eighteen female or male juvenile pigs fed on a standard natural grain diet (25 to 30 kg) were sedated with a combination of ketamine (20 mg/kg) and xylazine (2 mg/kg) by intramuscular injection. The animals were given pentobarbital (10 to 30 mg/kg IV) and were subsequently intubated and ventilated with oxygen (2 l/min) and isoflu-
euthanized. The arteries were perfusion-fixed, and the injured segments located with the guidance of the coronary angiograms were dissected free from the heart. After artery removal, the hearts were sectioned transaxially at 1-cm intervals, and sections were examined grossly for evidence of myocardial damage. The stented segments were processed for plastic embedding, staining and histomorphometric analysis of arteries (5 to 8 sections per vessel were averaged, expressed as mean value ± SD) by use of published methods (25). The grading scheme was used (25,26) to determine the maturity of vascular repair (endothelialization, intimal fibrin, inflammation, intimal SMC content, adventitial fibrosis).

Transmission electron microscopy. Endothelial structure was studied using transmission electron microscopy. Segments of arteries were fixed in 3% glutaraldehyde for 24 h and then transferred to sodium cacodylate buffer. Dehydrated specimens were embedded in Polybed 812 (Polysciences, Warrington, Pennsylvania), thin-sectioned and stained with uranyl acetate and lead citrate, and examined with a Philips 301 electron microscope (Eindhoven, The Netherlands).

Statistical evaluation. Data (mean ± SD) were analyzed for overall differences between treatment groups, using one-way analysis of variance with the Bonferroni correction. Comparison of the mean values with a p value of <0.05 was considered statistically different. All statistics were performed using SPSS 10.0 for Windows (SPSS, Inc., Chicago, Illinois). The intimal area and injury score were correlated using linear regression analysis.

RESULTS

Eighteen animals underwent intervention on 72 coronary arteries. The antisense drug was delivered to segments of each coronary artery, and coronary stents were implanted successfully at the sites where the antisense drug was delivered. All pigs survived to completion of the four-week study without evidence of myocardial infarction on gross inspection and also after histological evaluation.

C-myc expression after balloon angioplasty and antisense delivery. High performance liquid chromatography analysis of vessel tissue for Resten-NG showed dose dependent delivery of Resten-NG from 0.5 to 5 mg into the injured segment. Local delivery of Resten-NG into coronary arteries by infiltrator catheter blocked expression of c-myc induced by stent implantation by western blot analysis of vessel tissue and was dose-dependent (Fig. 1).

Histological and morphometric analysis. All stents were well developed within the vessel, resulting in thinning of the media adjacent to the stent struts. In the rare vessels with stent protrusion into the adventitia, there was evidence of perivascular hemorrhage (Fig. 2). No cases of thrombosis of the treated segment were observed in any of the treatment groups (Table 1). We observed complete healing with virtually no toxicity in all treatment groups. Re-endothelialization was complete in all treatment groups (Table 1). Transmission electron microscopy of antisense-treated vessels demonstrated normal appearance of endothelial cells with tight junctions between cells. There was no significant intimal fibrin in any groups. Inflammation score as well as adventitial fibrosis was similar in control and antisense treatment arteries (Table 1). The neointima from antisense treated arteries with doses of 5 and 10 mg was smaller in size than the controls. Control arteries exhibited a substantial neointima consisting mostly of stellate and spindle-shaped cells in a loose extracellular matrix. In the antisense treated arteries, the cells of the neointima were morphologically similar to controls.

The quantitative histomorphometry results of the control and antisense treated arteries are shown in Table 2. No significant difference was seen in vessel size between control and antisense treated groups. These results demonstrate a statistically significant reduction of intimal area (IA) and IA normalized to injury score (IS) after treatment with antisense of 5 and 10 mg. There was a strong trend favoring the...
Endothelialization

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attention of antisense technology has focused on the short-

restenotic process, it is not surprising that most of the

lar therapy.

DISCUSSION

1-mg group versus control, and a significantly larger lumen
area with use of antisense treatment with 5 and 10 mg versus
control. The regression analysis between IS and IA demon-

strated that the signi

cant decrease in slope between
treated and control groups indicates that local delivery of
antisense results in significantly less intimal growth despite
similar degrees of arterial injury (Fig. 3).

Table 1. Antisense Histopathologic Findings

<table>
<thead>
<tr>
<th>Tissue Event</th>
<th>Antisense Dose</th>
<th>0 µg</th>
<th>1 µg</th>
<th>5 µg</th>
<th>10 µg</th>
</tr>
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<tbody>
<tr>
<td>Endothelialization</td>
<td>score</td>
<td>3+</td>
<td>3+</td>
<td>3+</td>
<td>3+</td>
</tr>
<tr>
<td>Inflammation score</td>
<td></td>
<td>0.47 ± 0.49</td>
<td>0.29 ± 0.25</td>
<td>0.39 ± 0.47</td>
<td>0.25 ± 0.17</td>
</tr>
<tr>
<td>Intimal vascularity</td>
<td></td>
<td>0.47 ± 0.45</td>
<td>0.08 ± 0.13</td>
<td>0.33 ± 0.60</td>
<td>0.13 ± 0.32</td>
</tr>
<tr>
<td>Medial wall tissue nerosis</td>
<td></td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Intimal fibrin</td>
<td></td>
<td>0.53 ± 0.39</td>
<td>0.58 ± 0.30</td>
<td>0.33 ± 0.25</td>
<td>0.50 ± 0.35</td>
</tr>
<tr>
<td>Neointimal SMC</td>
<td></td>
<td>2.44 ± 0.37</td>
<td>2.33 ± 0.21</td>
<td>2.81 ± 0.33</td>
<td>2.78 ± 0.30</td>
</tr>
<tr>
<td>Adventitial fibrosis</td>
<td></td>
<td>0.40 ± 0.36</td>
<td>0.48 ± 0.2</td>
<td>0.58 ± 0.64</td>
<td>0.40 ± 0.8</td>
</tr>
<tr>
<td>Thrombosis</td>
<td></td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Data are presented as the mean value ± SD. (Grading scheme, see Methods).

ND = not detected; SMC = smooth muscle cells.

1-mg group versus control, and a significantly larger lumen area with use of antisense treatment with 5 and 10 mg versus control. The regression analysis between IS and IA demonstrated that the significant decrease in slope between treated and control groups indicates that local delivery of antisense results in significantly less intimal growth despite similar degrees of arterial injury (Fig. 3).

Rationale for vascular applications of antisense molecular therapy. Because of the proliferative component of the restenotic process, it is not surprising that most of the attention of antisense technology has focused on the short-lived regulators of the final common pathway of mitogenic stimuli, the cell cycle. Inhibition of the production of several of the mediators of the cell cycle with antisense oligonucleotides has been shown to be effective for the prevention of restenosis in several different animal models of vascular injury (5–11).

However, the results of a single-center clinical trial performed in Rotterdam that examined the effectiveness of an antisense compound directed against c-myc were recently reported with disappointing results (14). The authors used a self-expanding stent, which can cause chronic injury to stented arteries. In these circumstances a single injection of antisense may not be adequate to reduce a neointimal response to ongoing injury (vessel stretch). Additionally, the RNAse H competent nucleotide chemistries, such as the phosphorothioate used in the Investigation by the Thoracentre on Antisense DNA Given by Local Delivery and Assessed by IVUS after Coronary Stenting (ITALICS) clinical trial, lacks specificity, in part because duplexes as short as five base pairs in length can be cleaved by RNAse H (26). Cleavage of the RNA in such short duplexes might occur at a rate of 1 in every 1,000 bases of RNA sequence or nearly once in every transcript from every gene. Hence, the advantages of the PMO chemistry represent fundamental improvements in the selective inhibition of target gene mRNA over earlier antisense approaches and, therefore, worth consideration in the prevention of restenosis.

We choose early onco-gene c-myc as a target because of its multiple role in development of neointimal hyperplasia. It was previously demonstrated that, along with the profound antiproliferative and antimigrative effects, c-myc antisense has significant anti-inflammatory properties, and most importantly, it also inhibits matrix production (23).

Indeed, we recently evaluated the long-term impact of local delivery of c-myc neutrally charged antisense oligonucleotides on intimal hyperplasia after percutaneous transluminal coronary angioplasty in a rabbit model (23). Morphometry at eight weeks showed significant inhibition of intimal hyperplasia.

Comparison with other antirestenotic strategies. Because of its low toxicity and local application, intramural delivery of antisense may be theoretically more attractive than other methods of suppressing excessive intimal response after mechanical injury (radiation, etc.).

A large number of experimental and clinical studies have demonstrated that endoluminal-ionizing radiation reduces cellular proliferation and restenosis (27). However, edge effect, late thrombosis, and increased risk of myocardial infarction represent significant limitations of this therapeutic modality (27,28).

Most recently, local antiproliferative strategies, including pharmacological stent coatings (actinomycin-D, paclitaxel, rapamycin, etc.), have demonstrated inhibition of smooth muscle cell proliferation in vitro, have reduced neointimal thickening in animal models of restenosis (26,29,30) and have produced promising results in a pilot human study.

Table 2. Histomorphometric Results in Porcine Coronary Arteries 28 Days After Local Delivery of Antisense and Stent Implantation

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Control (n = 6)</th>
<th>1 mg (n = 8)</th>
<th>5 mg (n = 9)</th>
<th>10 mg (n = 7)</th>
<th>p Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vessel area (mm²)</td>
<td>9.29 ± 1.24</td>
<td>9.54 ± 1.79</td>
<td>9.02 ± 1.54</td>
<td>9.72 ± 1.98</td>
<td>0.262</td>
</tr>
<tr>
<td>Stent strut area (mm²)</td>
<td>7.14 ± 1.04</td>
<td>7.56 ± 1.48</td>
<td>6.86 ± 1.11</td>
<td>7.63 ± 1.59</td>
<td>0.585</td>
</tr>
<tr>
<td>Luminal area (mm²)</td>
<td>3.26 ± 1.57</td>
<td>4.76 ± 1.85</td>
<td>4.91 ± 1.36</td>
<td>5.62 ± 1.40†</td>
<td>0.062</td>
</tr>
<tr>
<td>IA (mm²)</td>
<td>3.88 ± 1.04</td>
<td>2.81 ± 0.56</td>
<td>2.01 ± 0.66†</td>
<td>1.95 ± 0.91†</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Medial area (mm²)</td>
<td>2.05 ± 0.31</td>
<td>1.97 ± 0.42</td>
<td>2.04 ± 0.42</td>
<td>2.16 ± 0.49</td>
<td>0.459</td>
</tr>
<tr>
<td>IS</td>
<td>0.95 ± 0.46</td>
<td>0.91 ± 0.46</td>
<td>0.90 ± 0.34</td>
<td>0.94 ± 0.44</td>
<td>0.546</td>
</tr>
<tr>
<td>IA/IS</td>
<td>4.08 ± 0.76</td>
<td>3.09 ± 0.50</td>
<td>2.17 ± 0.67†</td>
<td>2.13 ± 0.55†</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

*By analysis of variance (ANOVA). †p < 0.05 vs. control (ANOVA post-test with Bonferroni correction). Data are presented as the mean value ± SD.

IA = intima area; IS = injury score.
However high local toxicity may alter the endothelialization process after stent implantation and may cause late complications.

By contrast with other chemotherapeutics (paclitaxel, actinomycin-D), Resten-NG inhibits cell cycle in the G-1. Compounds that inhibit the cell cycle in the early phase are often less toxic. Therefore, Resten-NG as well as Rapamycin fit this description. Additionally, antisense can inhibit the cell cycle at a number of points, so it may be effective regardless of the stage of cell growth at which the drug is applied.

This study also showed that re-endothelialization was completed at 4 weeks in all treatment groups. Vascular healing, inflammation, fibrin deposition and adventitial fibrosis was similar to control arteries.

**Study limitations.** The major limitation of this study is that relevance of different animal models of neointimal hyperplasia to human restenotic process is not yet well established. The long-term effect of local delivery of antisense is unknown. However, observed complete vascular healing at 28 days after antisense delivery and stent implantation may suggest that possible late complications, including late thrombosis, are not likely to take place at three or six months in this animal model or in clinical circumstances.

**CONCLUSIONS**

This study demonstrated that intramural delivery of advanced c-myc neutrally charged antisense morpholino compound inhibits c-myc expression and dramatically reduces neointimal formation in a dose dependent fashion in a porcine coronary stent restenosis model while allowing for complete vascular healing. Clinical trials (AVI-4126) are underway to further establish the efficacy of this intervention in man.

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REFERENCES


