Medical Sciences

Gene Therapy for Cardiovascular Disease

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ABSTRACT. PTCA treatment has become more extensive and gained favor as an alternative treatment for coronary artery bypass grafting (CABG). PTCA leading to wound-healing responses that include thrombosis, smooth muscle proliferation and migration, elastic recoil and vascular remodeling that may contribute to recurrent obstruction or vessel narrowing referred to as restenosis. Recent studies have focused on the use of antisense compounds to prevent restenosis following PTCA. Until recently, the clinical applicability of antisense technology to the problem of restenosis has been limited due to a relative lack of target specificity, slow uptake across the cell membranes, and rapid intracellular degradation of the antisense oligonucleotides. The recently introduced AVI-4126 (Resten-NG) belongs to a new family of molecules known as the phosphorodiamidate morpholino oligomers (PMO).
**The most robust of the observations to date include the fact that AVI-4126 is safe and effective in different vascular disorders in multiple species and conducted by multiple investigators. Three different methods for local and systemic delivery have been described, each with advantages and limitations. Efficacy in animal models is encouraging. Further, clinical trials with AVI-4126 indicate that the agent is very safe. The last remaining question if AVI-4126 finds a place in the future therapeutic regimen for the prevention of restenosis and other disorders remains unanswered.** © *2007 Bull. Georg. Natl. Acad. Sci.*

Key words: percutaneous transluminal coronary angioplasty, restenosis, antisense, Resten-NG.

1.0. Introduction

Percutaneous transluminal coronary angioplasty (PTCA) was introduced in 1977 [1] and has become an effective treatment for limited coronary artery disease [2]. Since then, PTCA treatment has become more extensive and gained favor as an alternative treatment for coronary artery bypass grafting (CABG) [3]. Frequently, however, the artery is injured at the site of PTCA leading to wound-healing responses that include thrombosis, smooth muscle proliferation and migration, elastic recoil and vascular remodeling. Each of these responses may contribute to recurrent obstruction or vessel narrowing referred to as restenosis.

Recent studies have focused on the use of antisense compounds to prevent recurrent vessel obstruction or restenosis following PTCA. Antisense refers to a gene inactivating technology which blocks the "sense" of the genetic code (hence antisense) and prevents the normal wound healing responses that can lead to vessel obstruction or restenosis following injury. Some antisense compounds can inhibit the cell cycle response to injury in the G-1 by blocking *c-myc,* a regulatory gene that is the key factor in the cascade of effects that leads to restenosis in many angioplasty patients. Compounds that inhibit the cell cycle in the early phase are often less toxic, a description that fits both rapamycin and AVI - 4126 (Resten-NG).
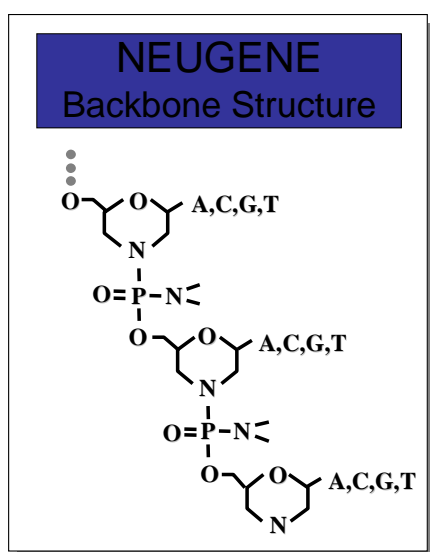
NEUGENE
Backbone Structure

Until recently, the clinical applicability of antisense technology to the problem of restenosis has been limited due to a relative lack of target specificity, slow uptake across the cell membranes, and rapid intracellular degradation of the antisense oligonucleotides [4]. The only randomized study in humans with *c-myc* antisense demonstrated no reduction in restenosis after stent implantation when arteries were pretreated with the drug [5]. However, the recently introduced AVI-4126 (Resten-NG) belongs to a new family of molecules known as the phosphorodiamidate morpholino oligomers (PMO).

These oligomers are comprised of dimethylamino phosphinylideneoxy-linked morpholino subunits. The morpholino subunits contain a heterocyclic base recognition moiety of DNA (A,C,G,T) attached to a substituted morpholine ring system. In general, PMO are capable of binding to ribonucleic acid (RNA) in a sequence-specific fashion with sufficient avidity to be useful for the inhibition of the translation of mRNA into protein *in vivo*, a result commonly referred to as an "antisense" effect.

Although the PMO share many similarities with other substances capable of producing antisense effects, such as deoxyribonucleic acid (DNA), RNA, and their analogous oligonucleotide analogs such as the phosphorothioates (PSO), there are specific differences. Most importantly, the PMO are uncharged and resistant to degradation under biological conditions. The combination of efficacy, potency and lack of nonspecific activities of the PMO chemistry has compelled us to reexamine the approach to antisense *c-myc* for the prevention of restenosis following balloon angioplasty.

AVI-4126 is an antisense phosphorodiamidate morpholino oligomer (PMO) with sequence complementary to the translation initiation start site of the *c-myc* mRNA. The mechanism of action of AVI-4126 involves the interference with ribosomal assembly thus preventing translation of *c-myc* and the interference with intron 1-exon 2 splicing of the *c-myc* pre-mRNA preventing appropriate translation of the *c-myc* mRNA. The IC_{50} for inhibition of *c-myc* by AVI-4126 is 0.3 µM in cell culture [6]. The cellular response to AVI-4126 is diminished cell growth associated with arrest of cells in the G_0/G_1 phase of the cell cycle. Inhibition of *c-myc* would also interfere with expression of downstream genes such as those associated with cellular adhesion, the cell cycle, and connective tissue matrix remodeling.

1.1. Designing Treatment Regimens for Preventing Restenosis

The interpretation of antisense data tends to be more complex than that of small molecule inhibitors. This is due to the fact that the antisense mechanism of action involves inhibition of protein synthesis but the rate of protein turnover is generally not influenced. The equation for inhibition of *c-myc* expression can be viewed as (1) below:

$$[MYC]^t = [MYC]_{ss} +$$

$$+ \text{induced } [c\text{-}myc \text{ mRNA}] - MYC_{turnover} \quad (1)$$

note: *MYC* all caps refers the protein, *c-myc* mRNA refers to the transcript and *c-myc* refers to the gene.

The magnitude of injury will determine the magnitude of new transcription of *c-myc* mRNA and the amount of *c-myc* mRNA is also a balance between the rate of synthesis and rate of decay.

The rate of *MYC* synthesis depends upon the concentration of *c-myc* mRNA. Equation (1) can be simplified to (2) as follows:

$$[MYC]^t = [MYC]_{ss} +$$

$$+ \text{injury induced } MYC \text{ synthesis} - MYC_{turnover} \quad (2)$$

Critical questions in the development of a delivery tool for the AVI-4126 involve: (a) What is the time course for *MYC* synthesis? (b) Can AVI-4126 inhibit MYC expression in the appropriate cells in the vessel wall following appropriate magnitude and duration of injury and (c) Is the amount and duration of AVI-4126 delivered relative to injury to the vessel wall sufficient? The expected result of a study in which the initial synthesis of *MYC* is not inhibited is shown in Figure 1 Part B.

1.2. MYC Expression Timecourse

Background. Considerations important to the evaluation of *MYC* expression and antisense inhibition include the stability of the transcript and the translated protein. The half-life of the transcribed mRNA is between 30 minutes and one hour [7]. The half-life of the

Table 1

Comparison of No injury with seven days post injury

No injury	Seven days post	*P* value	Interpretation
0.50 ± 0.11 (3)	0.42 ± 0.06 (12)	0.53; 13 df	No Difference

The numbers in parenthesis indicate the number of vessels evaluated.

MYC protein is 20 to 50 minutes [8,9]. *MYC* expression is induced within 2 to 6 hours of injury [10].

Interpretation. The injury caused by stent placement produces a rapid increase in *MYC* detected by western blot within 3 hours. The peak *MYC* expression is followed by reduced but ongoing transcription and translation of *c-myc* mRNA which occurs simultaneously with the normal degradation of *MYC* protein. The expected result of a polymer-coated stent delivering AVI-4126 into the vascular wall will be to reduce the translation of *c-myc* mRNA. The rate of *MYC* degradation is not influenced so the result will be to observe an enhanced rate of loss of *MYC*.

Concern for lingering *MYC* expression influences duration for antisense present in the injured vessels. This combined with a report of *MYC* expression at seven days post injury prompted further evaluation at seven days. Swine coronary vessels were injured by balloon overstretch and then vessels were recovered 2 hours later. The expression of *MYC* was determined by western blot and a ratio of blot intensity for *MYC* divided by intensity for β-actin, an internal control, to compensate for sample preparation variability (Table 1).

Conclusion. These data indicate that elevated *MYC* expression is shorter than seven days after vessel injury. This is true for bare stents and polymer-coated stents. Therefore delivery of AVI-4126 does not require prolonged release (greater than 4 days).

2.0. Catheter Delivery

Local drug delivery was designed to bring the antisense agent to the coronary artery during the period of time corresponding to peak injury response. The earliest attempts to deliver antisense agents for prevention of restenosis involved a rat carotid artery model using adventitial [11] or surgical application [12]. The initial clinically applicable devices were catheter-based providing local delivery as a bolus injection, followed by the subsequent withdrawal of the catheter. The combination of antisense targeting to *c-myc* with a catheter-based delivery to coronary arteries of pigs for prevention of restenosis began with the phosphorothioate oligonucleotides [13]. The bolus injection of phosphorothioate oligomers produced reduced heart rate, blood pressure and cardiac output in primate models which in some cases were lethal [14-18]. The phosphorodiamidate morpholino oligomers (PMO) have been evaluated for similar effects after intravenous bolus injections in both primates (Good Laboratory Practice [GLP] studies by Sierra Biomedical) and man (Good Clinical Practice [GCP] studies at MDS Harris). No alterations in heart rate, blood pressure or cardiac output have been observed. In summary, bolus injections of PMO by local catheter-based delivery devices are feasible.

2.1. Transport Catheter Studies in Rabbit Iliac Vessels

Twenty-five, male, New Zealand, white, atherosclerotic rabbits maintained on a diet of 0.25 percent cholesterol were anesthetized, a Transport Catheter[TM] inserted into the iliac artery and PTCA performed (8 atm for 30 seconds, 3 times). The endoluminal delivery of saline or 0.5 mg of AVI-4126 to the PTCA site was at 2 atm via the outer balloon for two minutes [19]. The area of the intima and media were determined by planimetry (Table 2). Quantitative angiography from these animals shows the maximum lumen diameter (MLD) at the time of harvest (60 days after PTCA) was significantly greater in the antisense-treated group than in the control animals. The morphometric analysis confirms the angiography in demonstrating significantly greater lumen area than in the control. The intimal area was also significantly smaller in the AVI-4126-treated animals. We also observed positive remodeling of the vessel. Vessel area was significantly greater ($P < 0.05$) in the treated animals.

Table 2

Rapid bolus local delivery in rabbit iliac vessels

	Control	Treated
Maximum lumen diameter (MLD)	0.8 ± 0.2	1.6 ± 0.3*
Late loss	1.8 ± 0.3	0.9 ± 0.2*
Lumen (mm^2)	0.62 ± 0.73	1.89 ± 0.35*
Intima (mm^2)	1.67 ± 0.44	0.82 ± 0.32*

* *P* value less than 0.05

2.2. Infiltrator Catheter Studies in Swine

We evaluated the long-term influence of intramural delivery of advanced *c-myc* antisense on neointimal hyperplasia following stenting in a pig model [20]. In acute experiments different doses (from 500μg to 5mg) Resten-NG (n=11) or saline (n=14) were delivered prior to the stent implantation site with the Infiltrator™ delivery system. Animals were sacrificed at 2, 6 and 18 hours after intervention, and excised vessels were analyzed for *c-myc* expression by western blot. In chronic experiments (n = 20) saline or 1, 5 and 10 mg of AVI-4126 were delivered in the same fashion and the animals were sacrificed at 28 days following intervention.

Western blot analysis demonstrated inhibition of *c-myc* expression and was dose dependent. Morphometry showed that the intimal area was significantly reduced relative to the control (Table 3). There was a statistically significant reduction of intimal areas in 5 mg 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 the control. 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.

3.0. Coated Stents

3.1. Phosphorylcholine (PC) Matrix for Drug Delivery

PC stents were loaded with AVI-4126 using soak-trap (ST) and dry-trap (DT) methods. Twelve pigs un-derwent AVI-4126 PC coronary stent implantation (3 stents/animal). Two to 6 hours post- procedure, 3 pigs were sacrificed and stented segments were analyzed by western blot for *c-myc* expression. In chronic experiments, 9 pigs (27 stent sites) were sacrificed at 28 days following intervention and vessels were perfusion-fixed. High performance liquid chromatography (HPLC) analysis of plasma showed minimal presence of the antisense oligomer. Western blot analysis involved determination of both *c-myc* and *β-actin* (an internal control protein) band intensities. The ratio of MYC to *β-actin* is 48 percent lower in the AVI-4126-treated vessels than in the untreated control vessels with stent implantation. The concentration of AVI-4126 in those vessels was 52 nM as determined by high performance liquid chromatography. Quantitative histologic morphometry (Table 4) showed that the neointimal area was significantly reduced (by 40%) in the ST group compared with control (2.2 ± 0.7 vs. 3.7 ± 0.7, mm^2 respectively, $P = 0.0077$). Immunostaining and electron microscopy demonstrated complete endothelialization, without fibrin deposition, thrombosis, or necrosis in all groups.

Control arteries exhibited a substantial neointima consisting mostly of stellate and spindle-shaped cells, in a loose extracellular matrix. The neointima from treated arteries with antisense-loaded stent implantation was significantly smaller in size. Most importantly, there was no difference in the appearance of re-endothelialization. The transmission electron microscope (TEM) revealed a virtually normal appearance of the endothelium. A semi- quantitative histological grading system demonstrated similar smooth muscle cell (SMC) colonization in all groups and minimal residual fibrin deposition for the ST-eluting stents. However dry trap (DT) and control polymer-coated (PC) stents had higher intimal fibrin scores.

We also observed less inflammation after implantation of the antisense-loaded stent. In general, the

Table 3

Rapid bolus local delivery in swine coronary vessels

	Control	Treated
Lumen area (mm^2)	3.26 ± 1.57	5.62 ± 1.40*
Intimal area (mm^2)	3.88 ± 1.04	1.95 ± 0.91*
IA/IS	4.08 ± 0.76	2.13 ± 0.55*

* *P* value is less than 0.05

Table 4

Drug-eluting stent in swine coronary vessels

	Control	Treated
Maximum lumen diameter (MLD)	2.3 ± 0.6	3.3 ± 0.6*
Late loss	1.1 ± 0.2	0.4 ± 0.5*
Lumen area (mm^2)	3.4 ± 0.9	5.2 ± 0.9*
Intima area (mm^2)	3.9 ± 0.8	2.3 ± 1.1*

Rate of *MYC* loss in injured vessels

	MYC: β-actin and GAPDH ratios		
Group	Three hour vessels	24 hour vessels	dx/dt
Control	1.51 ± 0.31 (12)	1.24 ± 0.79 (12)	0.27
Low dose stent	1.68 ± 0.59 (6)	0.82 ± 0.45 (6)	0.86
High dose stent	2.61 ± 1.77 (5)	1.15 ± 0.47 (6)	1.46

neointima of the ST- and DT-coated stents consisted of smooth muscle cells, matrix proteoglycans, and minimal focal regions of residual fibrin adjacent to the stent struts. Focal medial necrosis or intimal hemorrhage was an infrequent observation within any of the control or drug-coated stents. The antisense-loaded stent had a favorable influence on hyperplasia (reduction of intima by 40%) in the absence of endothelial toxicity and may represent an advantage over more destructive methods such as brachytherapy [21] or cytotoxic inhibitors [22]. Recently, local antiproliferative strategies including pharmacological stent coatings (paclitaxel, rapamycin, etc.) have demonstrated inhibition of smooth muscle cell proliferation *in vitro*, reduced neointimal thickening in animal models of restenosis and produced promising results in the pilot human studies [23] (Sousa et al. 2001). However, questions remain about the reendothelialization process after stent implantation with certain cytotoxic compounds which could put patients at risk for late stent thrombosis and cause late complications [23]. In contrast with other chemotherapeutic agents (paclitaxel, actinomycin D), Resten-NG (AVI-4126) is an antisense compound that inhibits the cell cycle in the G-1 by blocking *c-myc*, a regulatory gene that is the key factor in the cascade of effects that lead to restenosis in many angioplasty patients.

3.2 Experimental Matrices in Coated Stents

Some polymer coatings induce *MYC* to greater levels perhaps as a result of greater vessel wall injury. The bare stent vessel represents the best measure of the rate of the concentrations of *MYC* returning to steady state, which is approximately 4 days.

Table 5 below shows tabular data describing the rate of *MYC* loss in the injured vessels. The column referred to as dx/dt represents the difference in *MYC*: internal control ratio (both β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were utilized as internal controls) from the three-hour point to the 24-hour point. This rate of MYC loss appears to be dose dependent.

If we assume the injury does not alter steady state *MYC* expression, then the time to recovery in the control group can be determined by $(1.51-0.5)/(0.27/21) = 78.3$ hours. If synthesis of *MYC* were inhibited by 100

percent, then the *MYC* half-life would bring *MYC* to steady state in 5 half-lives or approximately 2.5 hours. In the case of the low-dose stent we observe $(1.68-0.5)/(0.86/21) = 28.8$ hours which represents a 63 percent reduction in the time to *MYC* steady state (IC_{63}). The high-dose stent would be $(2.61-0.5)/(1.46/21) = 30.4$ hours or a 61.2 percent inhibition (IC_{61}). In these studies, the amount of AVI-4126 in the vessel wall was determined and the dose dependence of the rate of loss of *MYC* versus the vessel concentrations were measured at 24 hours. These data are presented in Table 6. The studies demonstrate a remarkable agreement of inhibition of *MYC* with a resident amount of AVI-4126 in the vessel wall. Table 6 below shows the concentrations of AVI-4126 in the vessels at the indicated times.

The observations in Table 6 reveal an excellent agreement between the published IC_{50} of 300 nM for Resten-NG in inhibiting *MYC* [6] and the observed (IC_{61}) at the 415 nM in the high-dose group. The inhibition by the low-dose stent was essentially equal to that of the high-dose stent. The concentration at 24 hours was somewhat less but was still in reasonable agreement given the differences between cell culture and *in vivo* blood vessels.

Concentration of Resten-NG in vessel walls (nM)

Group	24 hour vessels
Control	63.4 ± 92.8 (12)
Low dose	233 ± 55.1 (6)
High dose	415 ± 204 (6)

GAPDH = glyceraldehyde-3-phosphate dehydrogenase

Limitations. The studies did not include a polymer-coated stent or scrambled PMO polymer-coated stent as controls. The polymer coating the stent tends to induce greater *MYC* expression observed at the three-hour time point over the bare stent. The analysis of the rate of loss of *MYC* tends to minimize the shortfall in direct comparisons of observed *MYC* level at a given time point.

Conclusion. AVI-4126 inhibits *MYC* expression in the current polymer-coated stent format in proportion to the amount of AVI-4126 that remains resident in the vessel wall. The degree of inhibition is quantitatively in

good agreement with earlier studies investigating *MYC* inhibition. The polymer-coated stent delivers micromolar concentrations into the vessel wall within 3 hours of placement. The delivery would need to be faster in order to prevent initial expression of MYC demonstrated by the substantial *MYC* detected at three hours. The polymer tends to add to vessel injury as measured by elevated *MYC* three hours after placement. Ultimate success will require polymers capable of rapid elution of AVI-4126 with minimal capacity to inflame or otherwise additionally injure the vessel wall.

4. Perflourocarbon Gas Microbubble Carriers (PGMC) for Site-Specific Drug Delivery

Perfluorobutane gas microbubbles with a coating of dextrose and albumin efficiently bind antisense oligomers [24]. These 0.3 to 10 µm particles bind to sites of vascular injury. Further, the perfluorobutane gas is an effective cell membrane fluidizer. The potential advantages of micro bubble carrier delivery include minimal addition to vessel injury from delivery, no resident polymer to degrade leading to eventual inflammation, rapid bolus delivery, and repeated delivery is highly feasible. Further, the potential for PGMC to deliver to vessel regions both proximal and distal to stents in vessels suggests this mode of delivery will be excellent as an adjuvant to a variety of catheter and coated-stent delivery techniques.

4.1. PGMC Delivery Assisted by Diagnostic Ultrasound

Based on these advantages, 21 pigs received AVI-4217 (a pig version of AVI-4126) bound to PGMC, AVI-4217 alone or no antisense treatment after carotid bal-loon injury [25]. The vessels were evaluated 30-days post injury. The results are expressed in Table 7.

4.2. PGMC for Site-Specific Delivery of AVI-4217 in Porcine Coronary Vessels

These results are impressive and additional studies have been conducted in coronary vessels with stents and did not involve ultrasound for site-specific delivery of the AVI-4217. Seven pigs underwent stent implantation (3 stents/animal). Four pigs received intravenous (IV) injection of PGMC and 1 mg of AVI-4217 and two served as controls. Four hours post-procedure, 3 pigs were sacrificed and stented segments analyzed by HPLC and western blot. In chronic experiments, 4 pigs were sacrificed at 28 days.

HPLC analysis of plasma samples of the treated animals showed minimal detected concentration of AVI-4217 but analysis of treated vessels demonstrated easily detected AVI-4217. Western blot analysis of the stented vessels demonstrated modest inhibition of *c-myc* with no alteration in expression of p21 or p27. Morphometry showed that the neointimal area was significantly reduced (Table 8). These data represent a limited number of vessels and studies that are underway to expand the use of PGMC for site-specific delivery of antisense agents.

5.0. Clinical Studies with AVI-4126

5.1. Phase I Clinical Studies

A Phase I study was conducted to evaluate the safety and pharmacokinetic properties of AVI-4126 at five dose levels (1 mg, 3 mg, 10 mg, 30 mg, and 90 mg) administered intravenously. Six subjects were tested at each dose level. Safety laboratory assessments (chemistry, hematology and urinalysis) were performed at baseline,

Table 7

Perflourocarbon gas microbubble carrier delivery in swine carotid vessels

	Control	Treated
Maximal intimal thickness (mm^2)	0.34 ± 0.06	0.20 ± 0.06*
Lumen area (mm^2)	12.4 ± 6.6	21.2 ± 2.9*
Percent area stenosis	19 ± 8	8 ± 2*

Table 8

The results of morphometry regarding to neointimal area carotid vessels

Lumen area (mm^2)	3.3 ± 0.7	6.1 ± 3.2
Percent area stenosis	57.8 ± 13.2	33.3 ± 24.6
Inflammatory score	0.7 ± 0.5	0.2 ± 0.3
Intimal vascularity	0.4 ± 0.5	0.2 ± 0.3
Intimal smooth muscle	3.0 ± 0.0	3.0 ± 0.0
Adventitial fibrosis	1.2 ± 0.8	0.7 ± 0.6

and 24 hour, 48 hour, 72 hour, 1 week and 2 weeks post dose. Adverse experiences were collected on an ongoing basis from time of dosing to discharge from the study at follow-up week 2.

The most frequent adverse events reported included lower extremity aches and headache. The majority of adverse events were graded as mild in intensity and were generally self-limiting. Serum complement C3a was measured. Four subjects had elevated C3a greater than 2x the upper limit of normal (ULN) (normal = 0-400 ng/mL), including two subjects at the 3 mg dose level, one subject in the 10 mg cohort and one subject in the 90 mg dose group. Three of the four elevations occurred at 24 hours post dose and one occurred at 0.5 hours post dose. In light of pharmacokinetic studies of the investigational compound, elevations of C3a at 24 hours post dose are unlikely to be related to administration of AVI-4126 but rather to spurious assay results. Further, there was no concurrent clinical symptomatology accompanying the elevated C3a, which is expected with elevated split complement levels.

5.2. Phase II Clinical Studies, AVAIL

The purpose of the AVAIL study was to investigate both safety and efficacy of AVI-4126 delivered locally via Infiltrator catheter after PCI in humans.

The AVAIL trial is prospective, evaluator blinded, randomized study including clinical follow-up at 30 days and 6 months after intervention and 6 month Angiographic and IVUS follow-up. Infiltrator catheter was advanced to target lesion and either drug was delivered (group A and B) or catheter was inflated (group C) after stent implantation in de novo lesions or PTCA in restenotic lesions. Primary endpoints include MACE, TVR, angiographic restenosis and IVUS at 6 months.

Forty four patients either with de novo lesions or restenosis were randomized into three groups: A (low dose)-3mg (19pts); B (high dose) - 10mg (15Pts) and C-control (10 pts). Baseline angiographic characteristics did not differ between the groups (reference vessel diameter: 2.5-4 mm, lesion length< 16mm). Procedural success was 81, 82 % (unable to advance Infiltrator catheter to target lesion in 8pts (5 pt group B and 3 pts from group C). There was no in-hospital or 30 days MACE recorded in any group. Clinical follow-up was available in 25 pts. At six months 4 pts (50%) from control group (n=8) and 7(100%) pts from low dose A group (n=7) required TVR.

In contrast in high dose group B (n=10) only 1 patient (10%) needed TVR. Angiographic follow-up in 25 pts (8 pts-group A, 7 pts-group B and 10 patients – group C) demonstrated late loss of 1.4+-0.54, 0.8+-0.55 and 1.5+-0.65 respectively (p=0.025). Binary restenosis

was 38% in group C (control), 29 in group A (low dose) and 0% in group B (high dose).

We conclude that Local delivery of antisense is safe and feasible. These preliminary findings from the small cohort of patients require confirmation in a larger trial utilizing more sophisticated drug eluting technologies.

6.0. CABG Applications

Saphenous vein and internal mammary artery became essential tools in aorto-coronary bypass surgery. When blood flow to the heart is limited because of arteriosclerosis of coronary arteries saphenous vein graft is commonly used to bypass the diseased area to bring the necessary blood to the surface of heart [1]. More than 50% of aorto-coronary saphenous vein grafts are occluded 10 years after surgery [2,3]. Intimal hyperplasia is an initial, critical step in the progression toward occlusion [4-6]. Late occlusion of saphenous vein grafts is due to medial and neointimal thickening secondary to migration and proliferation of smooth muscle cells (SMCs) and the subsequent formation of atherosclerotic plaques [7]. Scar tissue is formed at the point where the graft attaches to the blood vessel and as this scar tissue builds up, significant blockage of the blood vessel can occur.

The saphenous vein graft disease with its increasing clinical significance represents an unresolved problem. Because of the lack of effective pharmacological interventions for treatment, antisense therapy for vein graft protection offers a promising alternative for the treatment of the disease [8]. Saphenous vein grafts prepared for grafting with c-myc antisense oligomers demonstrate reduced medial cellular proliferation and macrophage infiltration, and preserved medial smooth muscle. These findings suggest that early inhibition of cellular proliferation and inflammatory infiltration results in a sustained reduction in neointimal formation and favorable graft remodeling [9].

Resten-CP5 (AVI-5126, peptide conjugated morpholino oligomer of c-myc) was developed by AVI BioPharma to improve cellular uptake, retention (reduced efflux) and inhibition of SMC proliferation. These indicate c-myc is an appropriate target and the peptide enhanced delivery tool makes this a feasible clinical approach to protect vein grafts. Studies with Resten-CP5 are highly favorable with no significant toxicity, thus setting the stage for further testing in an animal. We hypothesized that by enhancing retention of antisense the efficacy of pretreatment of vascular conduit may be increased. Resten-CP5 (peptide conjugated morpholino oligomer of c-myc) was developed to improve cellular uptake, retention (reduced efflux) and inhibition of SMC proliferation. The objective of the study was to determine the efficacy and potential toxic limitations of Resten-CP5 on late intragraft events *in vivo* in a porcine vein graft interpositioned in a carotid artery.

In 34 animals internal jugular veins were harvested and kept in a bath of Resten-CP5 at various concentrations (0, 1, 3, 10 and 30 µM in 10 ml) for 30 minutes followed by interposition of the veins into the carotid artery. The animals were allowed to recover and sacrificed 8 weeks later. Control angiography was performed after surgery and at the time of sacrifice. The vein and artery were fixed and submitted for histopathology.

The mortality was similar in all groups and in the vast majority of cases was attributed to vein perforation. Pathology demonstrated excellent dose–response. At 30 µM dose level, the samples show prominent mural inflammation with apoptosis and an undeveloped intima. Intimal maturation was improved in the 3 and 10 µM samples with evidence of endothelial cell covering. The degree of mural inflammation and apoptosis was also significantly less although the impact on restenosis at 3µM dosage was not apparent. In contrast in the 10 µM group the neointimal hyperplasia was negligible. The control group with zero dose level demonstrated some inherent injury and inflammation that can be attributed to the procedure and significant neointimal hyperplasia.

These data indicate c-myc is an appropriate target for vein graft remodeling and the peptide enhanced delivery of Resten-CP5 is safe and may become feasible clinical approach to protect vein grafts.

6.1. Regional Treatment of Vulnerable Plaque: Targeting Inflammatory Component of Unstable Atherosclerosis

Coronary artery disease (CAD) remains the leading cause of death in US despite significant advances in pharmacological and invasive treatment modalities. The key reason for this is our inability to identify and treat patients at high risk for plaque rupture prior to the actual clinical event (myocardial infarction). Over the last decade, several groups have shown that inflammatory processes play a key role in plaque rupture [1,2]. In Particular, macrophages have taken a central stage in this process [3].

Several groups have used various catheter-based technologies in an attempt to image the vulnerable plaque such as intravascular ultrasound elastography [4], thermography [5], optical coherence tomography [6], Raman spectroscopy [7], and near infra-red spectroscopy [8]. Each one of these technologies has its advantages and limitations, but a unifying feature is that it is based on an invasive platform. It is clear that for a screening test to gain wide spread use it has to be non-invasive.

Electron Beam Computed Tomography (EBCT) and Magnetic Resonance Imaging (MRI) are both well established non-invasive imaging modalities. However, EBCT can only identify calcium in the athersclerotic plaque, while MRI can image the atherosclerotic plaque but cannot ascertain whether the plaque is vulnerable or not [9]. Since plaque vulnerability is a biologic process, the application of biologic imaging for its diagnosis is intuitive. An ideal scenario would be to use a compound that has quantitative selectivity for activated macrophages, has properties for noninvasive imaging, and functions as an intermediary to destroy the targeted macrophages (a Trojan horse).

Indeed, inflammation is one of the key components of the process of restenosis. We evaluated the influence of adventitial delivery of a charge-neutral c-myc antisense oligonucleotide (AVI-4126) on inflammation and neointimal hyperplasia following stenting in a pig model of Arterial over-stretch injury was performed using V-Flex stent implantation in the coronary arteries of 7 pigs (four stents per animal: two in LAD and two in CX). Mixture of contrast and 2mg of AVI-4126 (n=5) or saline (n=2) were delivered to the vessel at sites between stents with the EndoBionics MicroSyryinge delivery system that allows for longitudinal drug delivery. Animals were sacrificed at 28 days following intervention and vessels were fixed in formalin, processed and stained with Hematoxylin and Eosin and elastic Van Gieson stains. All sections were examined by light microscopy for the presence of inflammation, thrombus, neointimal formation, and localized toxic effects.

Histological analysis by planimetry showed that the intimal area was 4.00 ± 1.32 mm^2 in the control group. Comparison of intimal areas in the c-myc group, intimal area 3.2 ± 1.40, with controls reveals a statistically significant reduction. There also was significant reduction in the incidence of granulomas in treatment group in comparison with control (30.7 ± 39.9 versus $56.3 \pm 42.6.9$ $p < 0.01$).

This study clearly demonstared that regional adventitial delivery of AVI-4126 by EndoBionics MicroSyryinge Catheter reduced inflammation and neointimal hyperplasia following stent implantation in swines for up to 28 days along the entire length of artery.

6.2 Future Directions of Antisense Molecules: Antihyperlipidemic Properties of Antisense

Statins are an important class of therapeutic for the treatment of heart disease by lowering serum cholesterol. The use of statins may become limited due to toxicities including life threatening rhabdomyolysis, teratogenicity and immune modulatory effects. We hypothesized that inhibiting HMG CoA Reductase gene expression with Phosphorodiamidate Morpholino Oligomers (PMO) will result in potential therapeutic lipid lowering with fewer limiting toxicities.

A. Rapid Bolus Drug Delivery

Injury

[MYC]

Drug

2   6   10   14

Time (hrs)

B. Prolonged Elution Drug Delivery
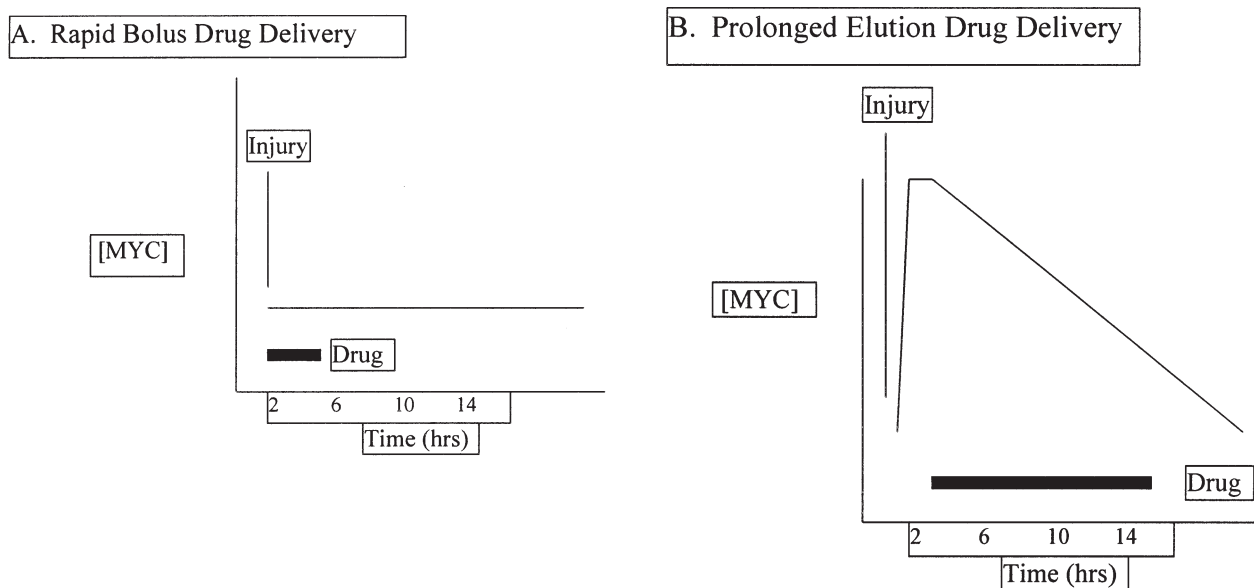
Injury

[MYC]

Drug

2   6   10   14

Time (hrs)

Fig. 1. Graphic cartoon of *MYC* expression as the dependent variable on the ordinate and time in hours as the independent variable on the abscissa. Part A in the upper portion represents the situation when AVI-4126 is delivered to the vessel wall at or near the time of vascular injury. The *MYC* concentration is efficiently prevented by inhibiting expression of new protein. Part B in the lower portion represents the situation when AVI-4126 is delivered to the vessel wall shortly after induced *MYC* protein synthesis has begun. The *MYC* protein level is elevated prior to AVI-4126 blockade. In this situation the antisense inhibition is reflected only in creating a steeper slope in the decline in *MYC* concentrations in the vessel wall due to inhibition of lingering translation which occurs approximately 3 hours after balloon injury.

The objective of the study was to determine the efficacy and potential toxic limitations of PMO targeting HMG CoA Reductase in rodent models.

C57/BL mice were fed a diet composed of 45 percent caloric content from fat for 4 weeks. Beginning at the end of week 2 the mice were treated with daily intraperitoneal injections for two weeks with saline, or 2.5 mg/kg atorvistatin, lovastatin, PMO directed at HMG CoA Reductase, PMO directed at DGAT2, or PMO directed at cytochrome P450 3A2 (control sequence). Blood samples were recovered from the ophthalmic plexus weekly to monitor total serum cholesterol.

The high fat diet induced elevated serum cholesterol in the mice over the entire four week study period. Both Atorvistatin and Lovistatin significantly reduced total serum cholesterol to less than 50 percent of the saline treated mice (p < 0.05). Both PMOs directed at HMG CoA Reductase and DGAT2 significantly reduced total serum cholesterol relative to saline treated mice (p < 0.05) but these levels were not significantly different from Atorvistatin and Lovistatin. The PMO directed at cytochrome P450 3A2 did not significantly reduce total serum cholesterol with levels not significantly different from saline treated mice. No toxicity was observed in the PMO treated mice and a review of literature where PMOs have been utilized zebrafish embryos no evidence of teratogenicity was obvserved.

The preclinical data indicate antisense PMOs to HMG CoA Reductase and DGAT2 are effective in lower-

ing serum cholesterol in a selective, sequence- specific manner with no evidence of toxicity.

Targeting Viral Disease: Antisense Inhibition of Coxsackie B1 and B3 Significantly Ameliorated Morphological Changes in the Myocardium of Mice Infected with Coxsackie B1 and B3 Viruses

We examined the possibility of antisense inhibition of Coxsackie B1 and B3 viruses to protect myocardium of mice in different periods of viral infection and myocarditis.

One hundred clean-linear mice were subdivided into 5 equal groups: 1 – treated with antisense phosphorodiamidate Morpholino oligomer (PMO) Coxsackie B1 and subsequently infected with Coxsackie B1 virus; 2 – treated with antisense PMO to Coxsackie B3 and subsequently infected with Coxsackie B3 virus; 3 and 4 - infected with Coxsackie B1 and Coxsackie B3 virus, accordingly; 5 – control: buffer solution was used instead of the vaccine. Antisense treatment was performed by intraperitoneal injection (PMO CVB-1 and CVB-3, respectively) in a dose of 120μg 14 times throughout first 28 days of the experiment. All mice from the Groups 1 to 4 were infected with appropriate viruses (0.2 ml of axenic virus culture with 100 LD50) at 38th day of the experiment and killed at days 3,5,7,9,15 and 30 post viral infection to obtain materials for light-

(haemotoxillin-eosin) and electron-microscopic investigations. Compliment-fixation reaction was used to determine the antiserum titer.

Accumulation of the antibodies in Groups 1 and 2 reached a titer of 1:512 and 1:256, respectively, 3 days after injection of infection. In non-treated mice (Groups 3 and 4) the antiserum titer never rose above 1:16 for up to 15 days post infection. Morphologic changes in myocardium were consistent with viral myocarditis (focal fiber necrosis with mononuclear infiltration and interstitial edema) in all 4 experimental groups, although they were more prominent and widespread in groups 3 and 4. Subsequent morphological changes in the course of infection became more pronounced in non-treated animals. By the 30th day post infection mice in groups 3 and 4 show diffuse spreading of fibrosis throughout the walls of the left ventricle, vasodilatation and retained mononuclear infiltration. In contrast, the antisense PMO treated animals had only focal changes and diffuse cell reaction in the interstitium against the background of diffuse myocardial dystrophy. No morphologic changes were observed in the control group.

Pretreatment with antisense phosphorodiamidate Morpholino oligomers designed to inhibit Coxsackie B1 and B3 viruses did not protect from acute viral myocarditis, but significantly ameliorated course of disease and morphological changes associated with pathology in myocardium.

7.0. Conclusions

The most robust of the observations to date include the fact that AVI-4126 is safe and effective in different vascular disorders in multiple species and conducted by multiple investigators. Three different methods for local and systemic delivery have been described, each with advantages and limitations. Efficacy in animal models is encouraging. Further, clinical trials with AVI-4126 indicate that the agent is very safe. The last remaining question: Will AVI-4126 find a place in the future therapeutic regimen for the prevention of restenosis and other disorders remains unanswered.

სამედიცინო მეცნიერებანი

გულ-სისხლძარღვთა დაავადებების გენური თერაპია

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პერკუტანული ტრანსლუმინური კორონარული ანგიოპლასტიკა მკურნალობის ფართოდ გავრცელებულ და წარმატებულ მეთოდს წარმოადგენს კარდიოლოგიაში, თუმცა აღნიშნული მკურნალობის ფონზე სისხლძარღვის კედელში განვითარებული ლოკალური ცვლილებები (თრომბოზი, სისხლძარღვის კედლის გლუვი კუნთების პროლიფერაცია და სისხლძარღვის კედლის რემოდელირება), მნიშვნელოვნად განაპირობებს მკურნალობის შედეგად განვითარებულ სისხლძარღვთან შევიწროებას, რაც რესტენოზის სახელწოდებით არის ცნობილი. ამჟამად, კვლევების უმრავლესობა ანტისენს-შემცველი სტენტების გამოყენებაზეა

ფოკუსირებული, რაც მნიშვნელოვნად ამცირებს რესტენოზის აღმოცენების რისკს კორონარული ანგიოპლასტიკის ფონზე. რესტენოზის პრევენციის მიზნით, ანტისენსების კლინიკურ გამოყენებას გარკვეული ლიმიტირება ახასიათებს, რაც, უპირველეს ყოვლისა, უჯრედის მემბრანაში მათი ნელი შეღწევადობით და უჯრედის შიგნით სწრაფი დაშლით ხასიათდება. ახლად წარმოებული AVI-4126 (Resten-NG) ანტისენს-მოლეკულების ახალ ოჯახს მიეკუთვნება. მრავალრიცხოვანი კვლევების მონაცემებით აღნიშნული ანტისენსი საკმაოდ უსაფრთხო და ეფექტური საშუალებაა სისხლძარღვთა სხვადასხვა დაავადებების მკურნალობის თვალსაზრისით. ამჟამად, პრეპარატის ლოკალური და სისტემური გამოყენების სამი განსხვავებული მეთოდია მოწოდებული, თითოეული მათგანისთვის დამახასიათებელი უპირატესობითა და შეზღუდვით. ექსპერიმენტული კვლევის მონაცემები საკმაოდ წარმატებულია, თუმცა არსებობს კითხვები, რომლებიც AVI-4126-ის მომავალ გამოყენებას შეეხება რესტენოზისა და სისხლძარღვთა სხვა დაავადებების პრევენციის მიზნით.

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