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# Nanoparticle formulation enhances the delivery and activity of a vascular endothelial growth factor antisense oligonucleotide in human retinal pigment epithelial cells

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## Abstract

The objective of this study was to investigate the delivery and activity of a vascular endothelial growth factor (VEGF) antisense oligonucleotide in a human retinal pigment epithelial cell line (ARPE-19) using a biodegradable nanoparticulate delivery system. A 19-mer antisense phosphorothioate oligonucleotide (PS-ODN) complementary to bases 6–24 relative to the translational start site of the VEGF mRNA, a sense PS-ODN and a mismatch PS-ODN were examined for the inhibition of secretion and mRNA expression of VEGF using an enzyme-linked immunosorbent assay and reverse transcription–polymerase chain reaction, respectively. Nanoparticles of the antisense oligonucleotides were formulated using a poly(lactide-co-glycolide) (50:50) copolymer using a double emulsion solvent evaporation method. After preparing nanoparticles, drug loading, encapsulation efficiency and particle size were determined. The cells were exposed to either plain solution of oligonucleotide or nanoparticles of oligonucleotide from Day 3 through Day 6. Alternatively, the cells were incubated with PS-ODNs and lipofectin for 4 h on Day 4. In all studies, VEGF secretion and mRNA expression were determined on Day 6. The particle size, drug loading and encapsulation efficiency were 252 nm, 5.5% and 16.5%, respectively. The antisense PS-ODN inhibited VEGF mRNA and protein secretion when delivered using nanoparticles or lipofectin but not in its free form. This was consistent with the ability of nanoparticles and lipofectin to elevate the cellular uptake of the oligonucleotide by 4-fold and 13-fold, respectively. Neither mismatch nor sense oligonucleotides inhibited VEGF secretion. In conclusion, biodegradable nanoparticles enhance cellular delivery of a VEGF antisense oligonucleotide and inhibit VEGF secretion and mRNA expression in a human retinal pigment epithelial cell line.

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## Introduction

Retinal neovascular diseases including diabetic retinopathy, retinopathy of prematurity, and age-related macular degeneration are the leading causes of blindness in the world (Lee et al 1998). These diseases are characterized by extensive proliferation of abnormal blood vessels from the existing retinal blood vessels, vascular leakage, formation of exudative and haemorrhagic lesions, contraction of the retina and retinal detachment, all of which cause vision disturbance or loss. It is hypothesized that an increase in oxidative stress and hypoxia increase the retinal expression of growth factors, including vascular endothelial growth factor (VEGF), a potent endothelial cell mitogen implicated in the neovascularization and vascular hyperpermeability associated with these diseases. Thus, inhibition of VEGF in the retinal cells is currently under investigation for the treatment of these retinal vascular diseases.

Retinal pigment epithelium is a non-renewable single cell layer of the retina situated in the posterior segment of the eye between the neural retina and the choroid. Its functions include maintenance of blood–retinal barrier properties, phagocytosis and continuous digestion of growing photoreceptor outer segments, and maintenance of the volume and composition of retinal fluids (Steinberg 1985;

Schraermeyer & Heimann 1999). Elevation of VEGF in retinal pigment epithelium has been observed in retinal neovascular diseases, suggesting a role for retinal pigment epithelium in VEGF-induced neovascularization. Thus, retinal pigment epithelium can be a very important target for VEGF suppression to treat these diseases. This study was aimed at the inhibition of VEGF in retinal pigment epithelial cells using an antisense oligonucleotide.

Antisense oligonucleotides are single-stranded pieces of nucleic acids ranging from 15 to 25 bases with a sequence specifically designed to bind a target sequence of intracellular RNA, thereby blocking the expression of target protein by altering the splicing, stability and/or translation of the mRNA (Baker & Monia 1999; Crooke 1999). The cellular delivery of antisense oligonucleotides is challenging due to their length, charge and degradation by intracellular and extracellular nucleases (Berton et al 2001). Phosphorothioate modification of antisense oligonucleotides by substituting one oxygen atom in the phosphodiester bond with a sulfur atom improves the stability of the oligonucleotide (Stein & Cohen 1988). However, this modification does not completely prevent the enzymatic degradation of the antisense oligonucleotide. Entrapment of oligonucleotide in delivery systems such as microparticles, nanoparticles, liposomes and micellar systems can protect an oligonucleotide from nuclease hydrolysis, localize it at the site of the cells, reduce toxicity to other tissues, and increase its intracellular levels (Zelphati et al 1994; Bennett et al 1998; Pichon et al 2000). Furthermore, delivery of macromolecules into phagocytic cells can be enhanced using particulate delivery systems because these cells have the inherent ability to take up particles.

Although it is well known that retinal pigment epithelial cells take up particles, the factors affecting the uptake is not well characterized. Understanding the factors influencing particle uptake would be critical in developing a suitable particulate system for drug delivery to the retinal pigment epithelium. In a previous investigation, we used a retinal pigment epithelial cell line, ARPE-19, to determine the factors affecting the particle uptake (Aukunuru & Kompella 2002). ARPE-19 cells, developed from retinal pigment epithelial cells obtained from a male donor, have structural and functional characteristics similar to retinal pigment epithelial cells in-vivo (Dunn et al 1998), including polarized distribution of cell surface markers (Holtkamp et al 1998). The results of our investigation demonstrated that ARPE-19 cells take up particles and that the particle uptake increases with decreasing particle size over the tested range of 2000 to 20 nm. The objective of the current study was to develop a nanoparticle delivery system for a VEGF antisense oligonucleotide and to determine whether it enhances the delivery and hence the ability of the oligonucleotide to reduce VEGF secretion and mRNA expression in ARPE-19 cells. Our previous investigations demonstrated that ARPE-19 cells secrete VEGF (Aukunuru et al 2002), which can be up-regulated by oxidative stress (Ayalasomayajula & Kompella 2002).

## Materials and Methods

### Synthesis of oligonucleotides and selection of sequence targets

A 19-mer antisense phosphorothioate oligonucleotide (PS-ODN) complementary to bases 6–24 relative to the translational start site of the VEGF mRNA, the control sense and mismatch PS-ODNs, previously reported to inhibit VEGF in epidermal keratinocytes (Smyth et al 1997), were synthesized by the phosphoramidite method (Itakura et al 1984) using a DNA/RNA synthesizer (Applied Biosystems 394; Applied Biosystems, Foster City, CA, USA) at the University of Nebraska Medical Center DNA core facility. The reagents used in the synthesis were obtained from Glen Research, Sterling, VA, USA. The sequences are CACCCAAGACAGCAGAAAAG, GTGGGTTCTGTCGTCTTTC and CTCCAAAGACAGCAGAAAG for the antisense, sense and mismatch oligonucleotides, respectively. Fluorescent labelled PS-ODNs were synthesized by labelling the 5′ end of the antisense PS-ODN with 5′-fluorescein phosphoramidite (6-FAM).

### Fabrication of nanoparticles

Poly(D,L-lactide-co-glycolic acid) (PLGA 50:50) of inherent viscosity 0.65 dL $g^{-1}$ (in methylene chloride at 30 °C) (Birmingham Polymers Inc., Birmingham, AL, USA) was used in the fabrication of nanoparticles. Particles were prepared using a water-in-oil-in-water emulsion solvent evaporation technique. Initially, 4 mg of PLGA was dissolved in 800 $\mu$L of methylene chloride and a solution of 5% polyvinyl alcohol (PVA) was prepared in cold distilled water. PVA solution was filtered through a 0.22-$\mu$m hydrophilic polysulfonic membrane syringe filter (25 mm Nalgene filter unit; Nalgene Co., Rochester, NY, USA) to remove any undissolved PVA. Antisense oligonucleotide (150 $\mu$g) was reconstituted in 86 $\mu$L sterile distilled water and this solution was added to methylene chloride containing PLGA. The mixture was then placed on an ice bath for 5 min and emulsified using a microtip probe sonicator set at 50 W of energy output (XL 2015 Sonicator ultrasonic processor; Misonix Inc., Farmingdale, NY, USA) for 30 s to obtain a primary water-in-oil emulsion. A double emulsion (water-in-oil-in-water) was obtained by adding the above water-in-oil emulsion to 1.5 mL of 5% PVA and ultrasonicating the mixture at 50 W of energy output for 10 s. The organic phase in the water-in-oil-in-water emulsion was evaporated by vortexing for 3 h at room temperature to obtain a suspension of nanoparticles. The suspension was transferred into Ultra-Clear centrifuge tubes (25 × 89 mm) (Beckman Coulter, Palo Alto, CA, USA) and ultracentrifuged at 110 000 *g* for 20 min to obtain a pellet. The pellet was reconstituted with distilled water and subsequently washed twice with distilled water to remove any adsorbed drug. Finally, the particles were resuspended in water, frozen at −70 °C for 45 min and subsequently freeze-dried (VirTis Company, Inc., Gardiner, NY, USA).

### Particle size and charge measurements

A suspension of particles was prepared in distilled water and sonicated for 15 s. The sample was filled in a cuvette and the charge and the size of the particles were determined using a particle size analyser. The size of the nanoparticles was determined using photon correlation spectroscopy with quasi elastic light scattering equipment (ZetaPlus zeta potential analyser; Brookhaven Instruments Corp., Holtsville, NY, USA) and ZetaPlus particle sizing software (version 2.07). This instrument gives the hydrodynamic diameter, which is the particle diameter plus the thickness of the surrounding solvent double layer. This instrument also gives the zeta potential of the particle.

### Determination of antisense oligonucleotide loading and encapsulation efficiency in the nanoparticles

Fluorescent-labelled PS-ODNs were used to determine the drug loading and encapsulation efficiency in the nanoparticles. For these determinations, particles were prepared as described above and 200 $\mu$g of particles was added to 250 $\mu$L of dimethylsulfoxide in a 2-mL centrifuge tube to allow polymer dissolution. Subsequently, 500 $\mu$L distilled water was added to the centrifuge tube and mixed thoroughly. This mixture was allowed to stand for 15 min at room temperature followed by vigorous shaking for 30 min to facilitate the extraction of the PS-ODNs into water. The fluorescent PS-ODN in this aqueous phase was quantified using a fluorescent spectrofluorometer. Loading and encapsulation efficiency were calculated using the formulae:

Loading = Amount (mg) of antisense oligonucleotide in 1 mg of particles $\times$ 100

Encapsulation efficiency = (amount of antisense oligonucleotide in the particles/amount of antisense oligonucleotide used for the fabrication) $\times$ 100

### In-vitro drug release

To determine the release of the antisense oligonucleotide from the nanoparticles, 1 mg of nanoparticles containing unlabelled antisense oligonucleotide was suspended in 250 $\mu$L of distilled water in a 1-mL centrifuge tube and shaken on a Dubnoff metabolic shaker incubator (Precision Scientific, Chicago, IL, USA) at 37 °C. At pre-determined time intervals, the particles were pelleted by centrifuging the suspension at 100 000 $g$ for 10 min. Supernatant was collected and analysed for the antisense oligonucleotide using a reverse-phase high-performance liquid chromatography (HPLC) method. The release study was continued after redispersing the pellet in 250 $\mu$L distilled water.

### HPLC analysis

Antisense oligonucleotide in the release study samples was determined using a reverse-phase HPLC assay. Aliquots (50 $\mu$L) of the samples were injected onto an OligoDNA RP (15 cm $\times$ 4.6 mm i.d., 5 $\mu$m) HPLC column. The mobile phase consisted of 80% aqueous buffer (ammonium acetate buffer, pH 6.8) and 20% acetonitrile. The antisense oligonucleotide was monitored at 275 nm using a photodiode array detector. The retention time of the oligonucleotide was 5.5 min.

### Cell culture

ARPE-19 cells obtained from ATCC (Rockville, MD, USA) were used between passages 19–29. ARPE-19 cells were cultured in 1:1 DMEM/F12 medium (Gibco, Grand Island, NY, USA) with 56 mM of sodium bicarbonate, 2 mM L-glutamine, 15 mM HEPES buffer, 10% fetal bovine serum (Gibco) and 1% penicillin G (100 IU mL$^{-1}$)/ streptomycin (100 $\mu$g mL$^{-1}$) solution (Gibco). Cells were grown in a 37 °C incubator supplied with 5% $CO_2$ and 95% air. ARPE-19 cells grown to 60% confluency in 48-well tissue culture plates (Becton-Dickinson, Franklin Lakes, NJ, USA) were used for the uptake studies. Confluent ARPE-19 cells grown in 96-well plates and t-25 flasks were used in the VEGF secretion and mRNA expression studies, respectively.

### Uptake of VEGF antisense oligonucleotide into ARPE-19 cells

For uptake, 1.2 mL of either plain solution or nanoparticle suspension containing fluorescent-labelled antisense oligonucleotide at an effective concentration of 1 $\mu$M prepared in 10% serum containing DMEM/F12 was exposed to ARPE-19 cells from Day 3 through Day 6 after seeding. On Day 6, the cells were washed once with DMEM and twice with ice-cold phosphate-buffered saline (PBS; 137 mM NaCl, 3 mM KCl, 8 mM $Na_2HPO_4$, 1 mM $KH_2PO_4$, pH 7.4) and lysed with 0.5% Triton-X. The amounts of antisense oligonucleotide in the lysates were determined using a spectrofluorometer. Lipofectin (BRL Laboratories, Carlsbad, CA, USA), a commercial transfection agent composed of cationic liposomes containing dioleoyl phosphatidyl ethanolamine and the cationic amphiphile DOTMA (Felgner & Ringold 1989) was used as a positive control. For this purpose, Day-4 ARPE-19 cells were incubated with 1.2 mL of lipofectin (10 $\mu$g mL$^{-1}$) and fluorescent-labelled antisense oligonucleotide (1 $\mu$M) (ODN:lipid ratio = 0.576 ng $\mu$g$^{-1}$) in serum-free DMEM/F12 for 4 h on Day 4. At the end of incubation, the cell monolayer was washed with ice-cold PBS and lysed with 0.5% Triton-X and the lysates estimated for antisense oligonucleotide using a spectrofluorometer.

### Effect of VEGF antisense, sense and mismatch oligonucleotides

Lipofectin (BRL Laboratories) was used as a positive control to facilitate the cellular entry of the oligonucleotide and to determine the activity and selectivity of the

antisense approach. The cells were incubated with antisense, sense and mismatch oligonucleotides in serum-free DMEM/F12 medium in the presence of lipofectin for 4 h on Day 4, followed by replacement with normal media. VEGF secretion and expression were determined as mentioned above. All oligonucleotides in this study were used at a concentration of 1 $\mu$M in 1.2 mL of medium containing lipofectin at a concentration of 10 $\mu g\,mL^{-1}$.

### Effect of VEGF antisense oligonucleotide encapsulated in nanoparticles on VEGF mRNA expression

To determine the effectiveness of nanoparticle formulation of antisense oligonucleotide on VEGF expression and secretion, ARPE-19 cells were exposed to 1.2 mL of antisense oligonucleotide solution, a suspension of PLGA nanoparticles containing the antisense oligonucleotide, or a suspension of blank PLGA nanoparticles in 10% serum containing DMEM/F12 from Day 4 through Day 6 after seeding. The concentration of the oligonucleotide and nanoparticles in the above preparations was 1 $\mu$M and $\sim$105 $ng\,mL^{-1}$, respectively. The sense and mismatch oligonucleotides were not assessed using nanoparticle formulations.

For determining VEGF secretion, on Day 6, the media was replaced with serum-free medium. After 12 h of incubation, VEGF secretion over an additional 12 h into serum-free medium was determined using a previously described enzyme-linked immunosorbent assay (Research Diagnositics Inc., Flanders, NJ, USA) capable of detecting $VEGF_{165}$ and $VEGF_{121}$ (Aukunuru et al 2002; Ayalasomayajula & Kompella 2002). For determining VEGF mRNA levels, on Day 6, total RNA was extracted and reverse transcription–polymerase chain reaction (RT-PCR) for VEGF and GAPDH was performed using the Access RT-PCR System (Promega Corporation, Madison, WI, USA) as described elsewhere (Bandi & Kompella 2001; Ayalasomayajula & Kompella 2002). The amplified products were separated on a 2% agarose gel and VEGF and GAPDH mRNA were quantified using densitometry analysis. This method is capable of detecting mRNAs of all five human VEGF isoforms.

### Statistical analysis

Data are expressed as mean $\pm$ s.d. Comparison of mean values between the treatments was done using two-way analysis of variance followed by Tukey's post-hoc analysis using SPSS (version 8.0) software. Differences were considered statistically significant at $P < 0.05$.

## Results

### Fabrication and characterization of nanoparticles containing VEGF antisense oligonucleotide

Using a double emulsion solvent evaporation technique, nanoparticles encapsulating antisense oligonucleotide were fabricated. The diameter and zeta potential of the particles were $252 \pm 3.4$ nm and $-12.98 \pm 1.80$ mV, respectively. The oligonucleotide loading and the encapsulation efficiency were 5.5% and 16.5%, respectively.

### In-vitro release of oligonucleotide from nanoparticles

The in-vitro release of the antisense oligonucleotide into PBS was measured at 37 °C. Following an initial burst, the drug release occurred at a slower rate over the 10-day study period. The cumulative oligonucleotide release from the particles at the end of 10 days was 39% (Figure 1).

### Nanoparticle encapsulation increases the uptake of VEGF antisense oligonucleotide

Compared with controls, both lipofectin and nanoparticles resulted in higher levels of fluorescence in the cells, suggesting that both approaches are effective in enhancing the oligonucleotide uptake compared with a plain solution. The uptake of VEGF antisense oligonucleotide increased 13-fold with lipofectin ($0.6 \pm 0.08\ ng\,mg^{-1}$ of protein or $9.0 \pm 1.1\%$, $P = 0.0004$) and 4.3-fold with nanoparticles ($0.2 \pm 0.03\ ng\,mg^{-1}$ of protein or $3.1 \pm 0.6\%$, $P = 0.014$) compared with controls ($0.046 \pm 0.03\ ng\,mg^{-1}$ of protein or $0.55 \pm 0.25\%$) (Figure 2).

### Effect of VEGF antisense oligonucleotide on VEGF mRNA expression

The VEGF antisense oligonucleotide solution did not significantly inhibit the VEGF mRNA expression compared with controls. However, co-incubation with lipofectin significantly reduced the mRNA expression of both $VEGF_{165}$ ($P = 0.032$) and $VEGF_{121}$ ($P = 0.006$). Also, nanoparticles containing VEGF antisense oligonucleotide

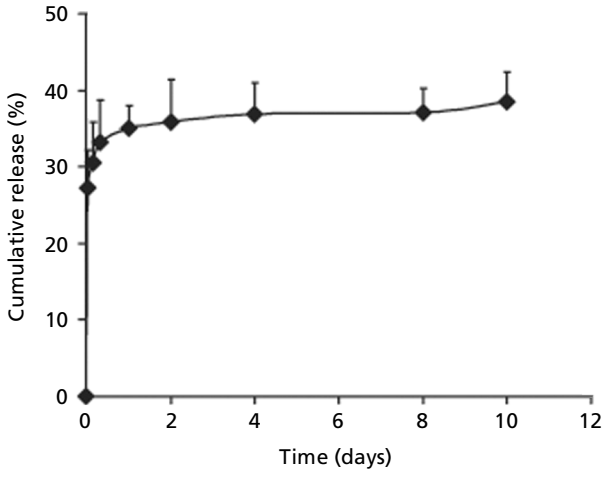


**Figure 1** In-vitro release of vascular endothelial growth factor antisense oligonucleotide from PLGA nanoparticles. Data are expressed as mean $\pm$ s.d., n = 4.

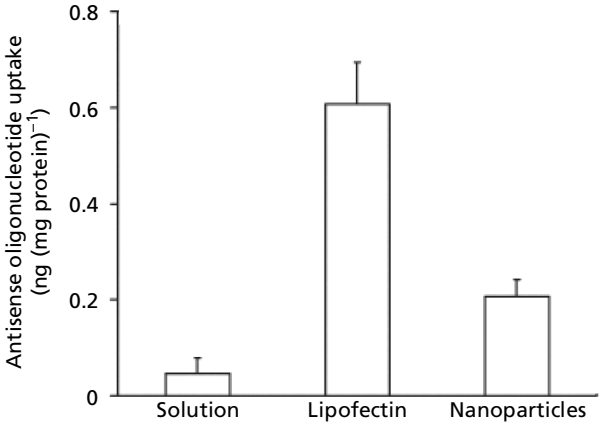


**Figure 2** In-vitro uptake of antisense oligonucleotide from solution, lipofectin and PLGA nanoparticle formulation in ARPE-19 cells. Data are expressed as mean ± s.d., n = 4. *$P < 0.05$ compared with solution group.

significantly reduced the mRNA expression of both $VEGF_{165}$ ($P = 0.044$) and $VEGF_{121}$ ($P = 0.023$) (Figure 3). Based on replicates in conjunction with the RT-PCR gel shown in Figure 3, the increase in VEGF mRNA expression with VEGF sense oligonucleotide and decrease with mismatch oligonucleotide are not statistically significant.

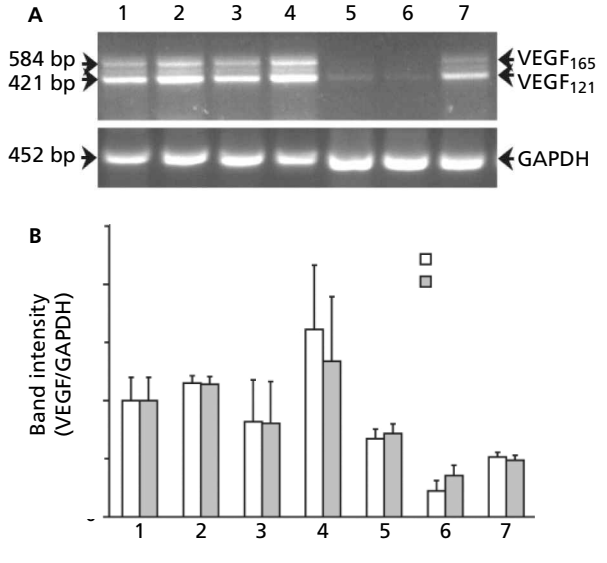


**Figure 3** Effect of vascular endothelial growth factor (VEGF) antisense oligonucleotide nanoparticles on VEGF mRNA expression (one representative gel out of three replicates is shown) (A) and VEGF band intensity in ARPE-19 cells (B). Lanes: 1, control media; 2, antisense oligonucleotide; 3, lipofectin; 4, lipofectin + sense oligonucleotide; 5, lipofectin + mismatch oligonucleotide; 6, lipofectin + antisense oligonucleotide; 7, PLGA nanoparticles containing antisense oligonucleotide. Data are expressed as mean ± s.d., n = 3. *$P < 0.05$ compared with control media.

### Effect of VEGF antisense oligonucleotide on VEGF secretion

Treatment of ARPE-19 cells with VEGF antisense oligonucleotide solution decreased the VEGF protein secretion from $605.35 \pm 115$ to $520.7 \pm 102.2$ pg mg$^{-1}$ of protein, which was not statistically significant. However, co-treatment with lipofectin significantly decreased VEGF secretion from ARPE-19 cells to $348.5 \pm 27.7$ pg (mg protein)$^{-1}$ ($P = 0.004$) (Figure 4). VEGF secretion from ARPE-19 cells treated with sense and mismatch oligonucleotides was $536.1 \pm 74$ and $579.5 \pm 128.9$ pg mg$^{-1}$, respectively, which was not significantly different from controls. The blank PLGA particles did not affect the VEGF secretion ($583 \pm 52.9$ pg (mg protein)$^{-1}$). However, the nanoparticles containing VEGF antisense oligonucleotide significantly reduced VEGF secretion to $371.8 \pm 49.5$ pg (mg protein)$^{-1}$ ($P = 0.011$) (Figure 4).

## Discussion

Nanoparticles are colloidal polymeric particles over the range of 1 to 1000 nm in diameter, with a therapeutic agent distributed in their matrix, encapsulated in their core or simply bound to their surface (Desai et al 1997; Aukunuru & Kompella 2002). Several biodegradable polymers, both synthetic and natural, have been investigated for the formulation of nanoparticles. In the present work, PLGA (50:50) was chosen for the formulation of nanoparticles containing a VEGF antisense oligonucleotide using a water-in-oil-in-water emulsion solvent evaporation technique. PLGA is a biodegradable and biocompatible copolymer that has been approved by the USA Food and Drug Administration for application in drug delivery (Jain et al 1998). The purpose of this study was to develop a formulation that can inhibit VEGF

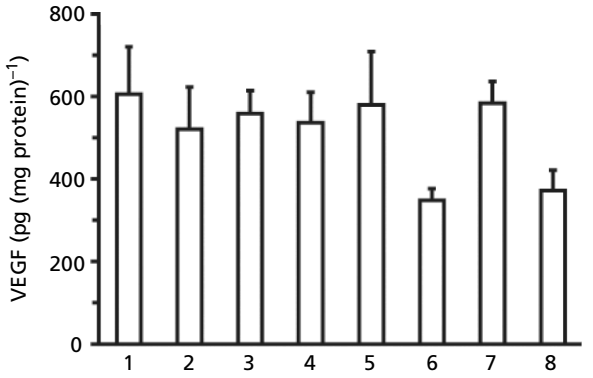


**Figure 4** Effect of antisense oligonucleotide on vascular endothelial growth factor (VEGF) secretion from ARPE-19 cells. 1, control media; 2, antisense oligonucleotide; 3, lipofectin; 4, lipofectin + sense oligonucleotide; 5, lipofectin + mismatch oligonucleotide; 6, lipofectin + antisense oligonucleotide; 7, blank PLGA nanoparticles; 8, PLGA nanoparticles containing antisense oligonucleotides. Data are expressed as mean ± s.d., n = 4. *$P < 0.05$ compared with control medium.

expression in ARPE-19 cells. Since VEGF is the main growth factor implicated in the retinal neovascular diseases, inhibition of VEGF would lead to the inhibition of neovascularization in these diseases. Currently, laser photocoagulation therapy is commonly used to treat retinal neovascular diseases (Stefansson et al 1981; Sullivan et al 1990; Spranger et al 2001). In photocoagulation therapy, several tiny burns are made on the retina using laser beams to form tiny spots of scar tissue, thereby sealing the leaks in the retina and the associated neovascularization (Petrovic & Bhisitkul 1999). However, laser therapy will not prevent vision loss due to obstructing vitreous haemorrhage or severe neovascularization in some patients. There are no drugs that are specifically approved for the treatment of these neovascular diseases. More effective prevention or treatment strategies are therefore desirable. Inhibition of VEGF would be an interesting approach in the early stages of neovascularization. In addition, reducing the neovascularization with pharmacotherapy prior to laser photocoagulation therapy would likely improve the success of the latter.

Evidence suggests an important role for VEGF in retinal neovascular diseases. VEGF levels in the vitreous of diabetic retinopathy are several fold higher compared with the vitreous of the normal population, suggesting a role for VEGF in diabetic retinopathy (Adamis et al 1994; Ambati et al 1997; Zhou & Zhang 1997; Endo et al 2000). Zhou & Zhang (1997) reported an increase in vitreal VEGF levels from 0.35 ng mL$^{-1}$ (range: 0.18–0.60 ng mL$^{-1}$) in healthy humans to 5.66 ng mL$^{-1}$ in proliferative diabetic retinopathy patients (range: 0.84–1.64 ng mL$^{-1}$). Similarly, Ambati et al (1997) reported significantly higher vitreal VEGF levels in patients with proliferative diabetic retinopathy ($1759 \pm 1721$ pg mL$^{-1}$) compared with controls ($27 \pm 65$ pg mL$^{-1}$). Increased VEGF is observed in several cells of retina, including retinal pigment epithelial cells in age-related macular degeneration (Kliffen et al 1997). In addition, in advanced retinopathy of prematurity, elevated VEGF levels are found in subretinal fluid (Lashkari et al 2000). Interestingly, specific inhibition of VEGF prevents ocular neovascularization in animal models (Aiello et al 1995; Sone et al 1999). Upon injecting VEGF-neutralizing chimeric proteins into the vitreous of a murine model of ischaemic retinopathy, retinal neovascularization was reduced in 100% of the animals (Aiello et al 1995). Intravitreal injection of VEGF in cynomologus monkeys resulted in dilated and tortuous vessels, venous bleeding, oedema, microaneurysms, intraretinal haemorrhages and capillary closure with ischaemia (Tolentino et al 1996). All the above results are consistent with a role for VEGF in retinal neovascular diseases. Thus, inhibition of VEGF is being actively investigated by our group to treat retinal neovascular diseases (Ayalasomayajula & Kompella 2003; Kompella et al 2003).

Inhibition of VEGF using antisense oligonucleotides has been reported in human endothelial cell lines (Hallscheidt et al 2001), human glioma cells (Zheng et al 2000) and retinopathy animal models (Robinson et al 1995; Garrett et al 2001). Robinson (1996) demonstrated that antisense oligonucleotides against VEGF inhibited retinal neovascularization in a murine model of proliferative retinopathy, suggesting their potential use in the treatment of retinal neovascular diseases. An antisense PS-ODN complementary to bases 6–24 relative to the translational start site of VEGF mRNA, previously reported to inhibit $VEGF_{121}$ and $VEGF_{165}$ in normal human epidermal keratinocytes (Smyth et al 1997), were used in this study.

When used in a lipofectin formulation, a widely used approach for enhancing cellular uptake of macromolecules, the antisense oligonucleotide formulation reduced VEGF secretion by 46% and the mRNA expression was reduced by 33% (Figures 3 and 4). Lipofectin formulations of both sense and misense oligonucleotides did not inhibit VEGF secretion and mRNA expression, suggesting that the inhibition is specific for the antisense sequence. Because lipofectin is an experimental approach that may prove toxic for chronic retinal use, we fabricated a PLGA nanoparticle formulation encapsulating a VEGF antisense oligonucleotide to improve the cellular uptake. We observed that the cellular uptake of oligonucleotide was significantly increased when oligonucleotide was entrapped in nanoparticles (Figure 2). These studies demonstrate that the nanoparticle approach could be used for improving cellular delivery of oligonucleotide. These nanoparticles containing antisense oligonucleotide significantly reduced both $VEGF_{165}$ and $VEGF_{121}$ mRNA levels and VEGF protein secretion from ARPE-19 cells (Figures 3 and 4), suggesting that the antisense oligonucleotide is able to reach intracellular compartments at VEGF inhibitory concentrations. The $VEGF_{165}$ and $VEGF_{121}$ are the secretory isoforms of VEGF and are responsible for the neovascularization at distant locations following secretion (Ferrara 1999). On the other hand, placebo nanoparticles and antisense oligonucleotide solution did not reduce either the secretion or expression of VEGF, suggesting that enhanced cellular uptake with nanoparticles improves the effectiveness of the antisense oligonucleotide.

The effectiveness of nanoparticles in the cellular delivery of antisense oligonucleotides can be explained based on previous literature. Berton et al (1999) demonstrated that nanoparticles deliver oligonucleotides into a non-sequestering intracellular compartment, whereas the free oligonucleotide solution delivers them to the acidic intracellular environment, resulting in their enhanced degradation when administered as solutions. Recent evidence suggests that PLGA nanoparticles escape the endolysosomes and enter the cytosol (Panyam et al 2002). It is also likely that nanoparticles protect the antisense oligonucleotides from extracellular and intracellular hydrolysis by nucleases. The above possibilities, as well as the possible diffusion of the PS-ODNs from lysosomes to cytoplasm before they are degraded by endonucleases, can explain the observed effects of the VEGF antisense oligonucleotide encapsulated in nanoparticles.

## Conclusions

This is the first study to demonstrate the effectiveness of nanoparticles encapsulating VEGF antisense oligonucleotides

in suppressing VEGF secretion and expression. This work demonstrates that nanoparticles are useful in enhancing the cellular activity of a VEGF antisense oligonucleotide, possibly due to increased cellular uptake. This is an in-vitro study and further investigations with in-vivo models would lead to a better understanding of a nanoparticle approach in the delivery of VEGF-containing antisense oligonucleotides. The possible site of administration of this formulation to inhibit VEGF is the subretinal space. The subretinal space is the space between the choroid and retinal pigment epithelial cells. Previously, microspheres containing VEGF injected into subretinal space resulted in excessive neovascularization at the site of injection. In addition, several genes were administered into the subretinal space using drug delivery systems such as adenoviral vectors and liposomes, suggesting that this route is a possible route for the administration of nanoparticles. Alternatively, these particles can be administered subconjunctivally, since subconjunctivally administered nanoparticles deliver drug to the retina (Kompella et al 2003).

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