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Gene Therapy Using SiRNA for Treatment of Ocular Neovascularization

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Recently, gene therapy has become a novel strategy for severe disease treatment, especially using small interfering RNA (siRNA). SiRNA is about 20-25 nucleotide-long, generated from long double-strand RNA (dsRNA) after the cleavage of DICER, to target and degrade complementary mRNA through the RNA interference (RNAi) pathway. The targeted delivery of siRNA has become an important gene silencing strategy because of its highly potent down-regulation of gene expression in targeting cells. Currently, siRNA is being examined as a potential therapeutic treatment to reduce ocular neovascularization. However, successful gene therapy depends on an efficient delivery system. Cationic liposomes are composed of positively charged lipid, these liposomes can condense nucleic acid into small particles and protect loaded genes from nuclease degradation. Therefore, cationic liposome is considered a good candidate for gene delivery. This review will discuss the development of gene therapy for ocular neovascularization. In addition, current delivery systems of cationic liposome with PEGylation on lipid are also introduced.

Key words: siRNA, ocular neovascularization, cationic liposome, nanotechnology

INTRODUCTION

Retinopathy from retinal and choroidal neovascularization is the leading cause of blindness in developed countries. For adult, ocular neovascularization is classified as age related macular degeneration (AMD) and diabetic retinopathy (DR) that can severely damage vision acuity and lead to blindness. In 2002, the WHO estimated there were 161.2 million visually impaired people in the world, among them there were 14 million (8.7%) AMD and 7.7 million (4.8%) DR.[1] In 2000, there were 600 million population over 60, and this will reach 2 billion in 2020.[2] As the aged population increases, neovacularization will become one of the important diseases in the following decade.

AMD is a chronic degenerative disease for the elderly that is further divided into two types, dry form and wet form, according to the disease development. The clinical features of dry AMD include drusen deposits under the retina and geographic atrophy of the retina. The wet AMD has characteristics by growing abnormal choroidal blood vessels to invade the Bruch membranes called choroidal neovascularization or CNV. The clinical features of CNV include subretinal fluid, lipid deposition, hemorrhage and fibrotic scar.[3] However, the dry form AMD is not as serious as the wet form.

DR is a progressive microangiopathy characterized by small vessel damage and occlusion. The prevalence of DR is about 0.75% for people over 40 and 1.15% for those aged 60-74.[2] The early stage of DR is also called non-proliferative diabetic retinopathy (NPDR) or background diabetic retinopathy. As progressive microvascular occlusion, the formation of new vessels in the late stage is termed proliferative diabetic retinopathy or PDR. Type 1 diabetic patients with more than a 20 year history have a rate of proliferative of diabetic retinopathy (PDR) around 50%. Type 2 diabetic patients have a prevalence of about 60% with a history more than 15 years and 20% of the patients will progress to PDR.[4]

Currently, few drugs are available for the treatment of ocular neovacularization. FDA approved AMD treatments include photodynamic therapy with verteporfin, and intravitreal injection with pegatanib and ranibizumab.[2] Gene therapy has great potential for treating neovascularization.[5] Neovascularization is closely related to gene over-expression of vascular endothelial growth factor (VEGF). Thus, gene therapy strategies may be used to treat neovascularization by down regulation of the gene expression of VEGF. Several approaches related to the gene silencing strategy have been used in regulating

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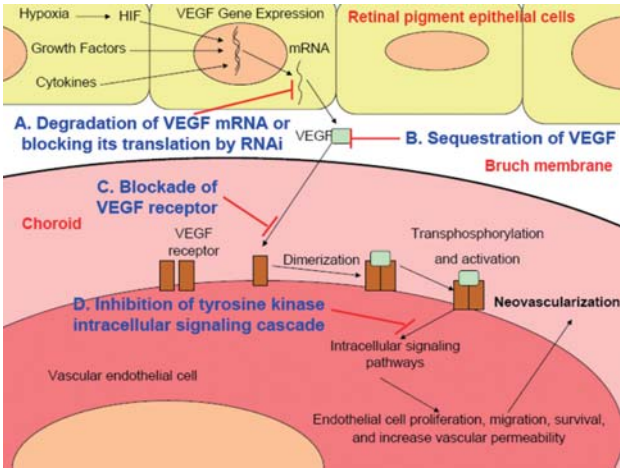

Fig. 1 Inhibition of VEGF signaling pathway in neovascularization

VEGF, including aptamers, antisense oligonucleotides and small interfering RNA (siRNA).[6] Presently, RNAi through siRNA has become an important research topic in targeting and controlling over-expressed genes in the angiogenesis of cancer and neovcascularization in retinopathy. However, the application of siRNA as a therapeutic agent has met many challenges; an efficient delivery system plays an important role in achieving targeted delivery.[7] Recently, some studies have indicated an effective vector for siRNA delivery depends on nano-scale particle size with a stealth delivery feature to extend the retention time of the vector in the blood circulation and target specific cells in the tissues.[8] This report reviews recent development of gene therapy through siRNA using a nanotechnology delivery system for treating retinopathy associated with neovascularization.

RETINOPATHY AND NEOVASCULARIZATION

Ocular neovascularization is associated with retinopathies including AMD, PDR, and retinopathy of prematurity (ROP). These ocular diseases cause visual impairment or even acute vision loss in infants (ROP), working age adults (PDR), and in the elderly (AMD).[9] In the vertebrate eye, the retinal pigment epithelium (RPE) cells play a critical role in the development and maintenance of adjacent photoreceptors in the retina. When in the hypoxia or ischemia condition, hypoxia inducible factor (HIF) will increase and make the RPE cell transcribe VEGF mRNA.[10] Thus, VEGF mRNA translates into VEGF which accumulates at the Bruch membrane. VEGF is the ligand for 2 membrane-bound tyrosine kinase receptors, VEGFR-1 and VEGFR-2. Most of the proangiogenic functions of VEGF are mediated by VEGFR-2. Ligand binding triggers VEGFR-2 dimerization and transphosphorylation with subsequent activation of an intracellular tyrosine kinase domain. The ensuing intracellular signaling causes vascular endothelial cell proliferation, migration, survival, and leads to neovascularization. These new growing blood vessels are incomplete and fragile. Blood easily leaks from abnormal new vessels into the macula to damage visual acuity, ultimately leading to blindness. The current therapeutic strategy of neovascularization is to develop drugs for inhibiting the activities of VEGF (Fig. 1). Some approaches have been extensively examined, including the sequestration of VEGF, the blockade of the VEGF receptor, or the inhibition of the tyrosine kinase signaling cascade.

MECHANISM OF siRNA

RNAi was proposed by Andrew Z. Fire and Craig C. Mello in 1998.[11] They found a scenario when the sense strand or antisense strand of gene unc-22 was injected into the embryo of *Caenorhabditis elegans*. The regulated phenotype of unc-22 cannot be observed in elegans. However, a complete double-stranded RNA would efficiently knockdown the expression of unc-22 protein. The phenomenon of gene silence was termed RNAi. At the same year, Fire's group reported a more detailed mechanism of RNAi to inhibit gene expression.[12] Presently, the technology of RNAi is applied to the gene knockdown study to explore the role of an investigated gene, well known as microRNA (miRNA), short hairpin RNA (shRNA) and siRNA.[13] Fire and Mello were announced as 2006 Nobel laureates in medicine physiology to honor their contribution in the field of RNAi.

The mechanism of RNAi operates through a process of mRNA regulation by siRNA. The exogenous long double-stranded RNA (dsRNA) might be from microbials or other sources through cell uptake. Consequently, dsRNA can be cleavaged by the dsRNA specific RNase III enzyme or DICER into small fragments. These small fragments made of 19-23 base pair duplexes are termed siRNAs. The siRNA molecules are then assembled into a multiprotein complex, termed RNA-induced silencing complex (RISC). The RISC contains helicase that unwinds the two strands of RNA molecules. Then, endonuclease hydrolyzes the target mRNA homologous at the site where the antisense strand is bound. The mRNA is defective and will be degraded and, consequently, the expression of protein from the translation of the mRNA is significantly inhibited.[14]

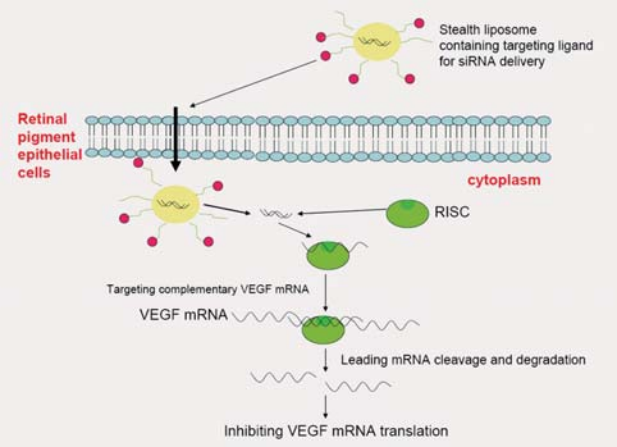

Fig. 2 The pathway of siRNA-loaded liposome vector in the gene therapy of ocular neovascularization

BIOLOGICAL BARRIER FOR siRNA DELIVERY

Despite the excitement of RNAi through siRNA for sequence specific gene regulation, many hurdles need be overcome for developing siRNA as a targeted therapeutic agent. Three aspects should be considered to design an ocular gene delivery system, including the siRNA nature property, delivery efficiency and ocular physiological process. The half-life of naked siRNA in the bloodstream is very short, being about 6 minutes.[15] SiRNA cannot penetrate the cellular lipid membrane into the cytoplasm due to siRNA having a negative charge and the hydrophilic macromolecule. In addition, biological barriers are critical issues to deliver siRNA to target sites such as interaction with blood components as well as off-target tissue uptake. Direct intravitreal injection of siRNA into the vitreous provides straightforward access to the posterior segment of the eye, such as vitreous and retina, the administration route bypassed the systemic pathway of blood circulation to prevent the interaction of blood components and the elimination of system circulation. In addition, to achieve a long duration, the delivery system with controlled-release in the retina is also an important consideration for siRNA delivery.

DRUG DELIVERY SYSTEM

SiRNA vector for gene delivery is mainly classified into viral and non-viral vector systems. The design of the viral vector depends on the essential gene sequences. The non-essential genes of the viral vector, such as the retrovirus, lentiviral vector, adenovirus, or adeno-associated virus, were replaced with essential genes to exert the desired effects.[16] Although viral vectors usually obtain high potency and tranfection efficiency, but in regard to the safety issues including mutation, immune-responsiveness, oncogene and recombinant problems, viral vectors are not widely applied in gene delivery.

Compared with the viral vector, the safety issue of non-viral vector is not of much concern. However, the low transfection efficiency of the non-viral vector becomes a critical problem to be overcome. Non-viral delivery can be divided into physical and non-physical methods. Physical methods include muscle injection[17], hydrodynamic methods[18], gene gun[19] and electroporation.[20] Non-physical methods mainly involve cationic liposome and polymer nanoparticle.

SiRNA delivery has been achieved using polymer nanoparticles. These particles are made from various polymers including polyethyleneimine (PEI), chitosan, polylysine (PLL) and polyethylene glycol (PEG) based polymers.[21-23] They are polycations or polycation-containing block copolymers. Strong electrostatic interactions between oppositely charged polyelectrolytes can significantly prevent enzymatic degradation of the incorporated gene in the bloodstream. The complex of gene-carried polycation generally possesses a globally positive surface charge. It easily attaches itself to the negatively charged cell surface for subsequent endocytosis.[24]

The non-physical method for siRNA delivery has been extensively studied using novel cationic lipids. Many cationic lipids have been prepared since the first cationic lipid of DOTMA (*N*-[1-(2, 3,-dioleyloxy) propyl] -*N*, *N*, *N* trimethylammonium chloride) was introduced in 1987.[25] The cationic charge lipid can form a lipoplex with the gene to avoid enzymatic degradation and increase the interaction with the cell membrane carrying the negative charge. Cationic liposome-mediated transfer of gene is a promising approach associated with low immunogenicity and toxicity. The cationic liposome could be prepared without difficulty, which could be connected with a suitable ligand to achieve active targeting in siRNA delivery. Fig. 2 shows the liposome vector of the siRNA is used to deliver the siRNA into the cell for treating ocular neovascularization.

THE DEVELOPMENT FOR LIPOSOME

The disadvantages of conventional cationic liposome are it is unstable, and it easily interacts with bio-components leading to decomposition. Therefore, gene delivery failed in the in vivo study. The evolution for liposome

is added with a lipid linker polyethylene glycol (PEG) called PEGylated or stealth liposome. PEG is a biocompatible uncharged hydrophilic polymer, which is soluble in water as well as in many organic solvents. The long chain structure of PEG has good conformational flexibility and high chain mobility, causing a steric exclusion effect that prevents the adsorption of proteins. PEG couples covalently to another substance called PEGylation. The molecular weight and density of PEG in liposome can substantially affect the circulation time of liposome in the body. In preclinical studies, the PEG-liposome with a PEG chain of about 2000 Daltons (PEG2000) conjugating a grafting density of 5-7.5 mol% is often used.[25-28] The PEG-liposome of 100-200 nm can accumulate in the tumor or angiogensis sites because nano-size particles can avoid rapid uptake by the RES and remain in blood circulation. These particles have more opportunities to extravasate through discontinuous capillaries in the newly growing vessels.[29] It can change its pharmacokinetic characteristics, reduce toxicity, and increase bioavailability compared with conventional liposome. However, PEG-lipid substantially decreases the in vitro uptake of gene loaded particles into the cells by reducing the interaction with cell membranes and reduces gene transfer by decreasing the escape of genes from the endososome. For increasing the amount of the drug entering the tumor cell and overall therapeutic efficacy, the liposome could be delivered through both active and passive targeting. Some tumor sites will over-express unique receptors such as sigma, HER2 and transferin receptor in the pathogenesis condition. By conjugating with special ligands or antibodies, the drug-loaded liposome would significantly increase drug uptake into the targeted cells.[30]

THE DEVELOPMENT OF siRNA FOR NEOVASCULARIZATION TREATMENT

Recently, the regulation of VEGF mRNA by siRNA has been reported.[31-35] The VEGF-knockdown strategy has shown a significant effect on the inhibition of vascular growth and permeability in animal models of laser-induced choroidal neovascularization or oxygen induced ischemia retinopathy. Likewise, the knockdown VEGFR-1 also demonstrated anti-angiogenic effects in a mouse ocular model. Some studies report upstream regulation gene HIF-1a also plays an important role in neovascularization.

Currently, there are seven siRNA drugs undergoing clinical trial from 2004 to 2008.[36] Bevasiranib or Cand5 is the first siRNA drug targeting VEGF mRNA for treating AMD. The clinical trials of bevasiranib have been conducted since 2004 for AMD, and were recently in Phase III. Bevasiranib trials are also being conducted for treating diabetic macular edema at the Phase II clinical stage. The second siRNA drug for AMD is AGN211745 or Sirna-027, which targets the VEGF receptor and is currently in the Phase II of clinical trials. The third siRNA drug for AMD is RTP801i, which is examined for targeting a hypoxia-inducible gene in Phase I clinical trials. As well as VEGF dependent neovascularization, Ambati et al. found a chemokine receptor CCR3 might be an important receptor in angiogensis for CNV patients.[37] Chemokine receptor is well known for its expression on eosinophils. Eosinophils would be expressed by eotaxins and attracted to the site of inflammation. The chemokine receptor is related to eye allergic conjunctivitis.[38-39] Besides inflammation, chemokine also plays an important role in angiogensis. The mechanism of CCR3 expressed on CNV is still unclear, but it is an important finding for the development of ocular neovascularization to provide a new target for drug discovery in treating retinopathy.[40]

CONCLUSION

Ocular neovascularization is the leading cause of blindness in adults. Presently, many drugs undergoing clinical trial have great potential to be developed in the treatment of ocular neovascularization. SiRNA is the newest potential therapy for ocular neovascularization. The eye is a potential organ for siRNA delivery due to its closed system and small anatomic structure. Retinal neovascularization and CNV are supposed to suit a specific environment to be easily accessed by intravitreal injection for drug targeting. Several delivery systems are developed to successfully deliver to the target site. Liposome is a good vector for gene delivery because of its easy preparation, low toxicity and high biocompatibility. For accurate targeting, lower toxicity and long duration release, the vector still has many issues to be improved. Liposome combined with PEGylation technology and special targeting ligand provides the newest delivery system for siRNA therapy. PEGylation and ligand help gene delivery efficiently target the cells requiring treatment. Using this system, the drug can enter the right place and have the right effect at the right time. It also provides more opportunity for anti-angiogensis in treating other diseases such as cancer therapy.

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