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Recent progress in gene therapy for eye diseases

Review Article

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Abbreviations: ARMD, age-related macular degeneration; **C.F.U**., Colony forming unit; **ERG**, retino-electrogram; **PDE**, phosphodiesterase; **RPE**, retinal pigment epithelial; **PDR**, proliferative diabetic retinopathy; **PDGF**, platelet-derived growth factor; **PVR**, proliferative vitreoretinopathy; **RCV**, replication competent virus; **RP**, retinitis pigmentosa; **VEGF**, vascular endothelial growth factor

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Summary

Gene therapy for eye diseases is still developing and requires further effort to bring it to clinical trials. Both a better understanding of the pathological aspect of the diseases and establishment of better viral systems is required. These efforts will facilitate the development of gene therapy-based treatments, which will complement conventional approaches to manage and potentially cure blinding diseases of the eye.

I. Introduction

Despite recent advances in the treatment of ocular diseases, conventional approaches are not yet able to cure many retinal diseases. The retina, being a neuronal tissue, regenerates poorly when damaged, and hence poses additional challenges. The retinal diseases which lack satisfactory treatments or cures include retinitis pigmentosa (RP), proliferative vitreoretinopathy (PVR) and diseases arising from unscheduled neovascularization such as proliferative diabetic retinopathy (PDR), and age-related macular degeneration (ARMD).

The etiology of a disease is usually the foundation for developing cures and treatments. Fortunately, recent advances in the fields of molecular biology and genetics are providing a better understanding of the pathogenesis of these diseases. For example, vascular endothelial growth factor (VEGF), a potent mitogen for endothelial cells, and enhancer of new vessel growth, plays a cardinal role in most neovascular disease of the retina and choroid including PDR and ARMD. Several kinds of mutations have been identified in the proteins for transduction of the light signal. These proteins include rhodopsin, α-, β-phosphodiesterase (α, β PDE), peripherin/rds, and are responsible for some forms of RP. The identification of molecular targets within these diseases provides new opportunity for intervention.

For instance, these findings make it possible to develop a gene therapy-based treatment for the incurable eye diseases mentioned above. Investigators have begun to develop gene therapeutic treatments by employing the appropriate DNA constructs in animal models of the diseases. In this paper, we will review some of the work done so far regarding gene therapies in the eye with a particular focus on retinal diseases and vector systems. Finally we will consider relevant issues for the realization of gene therapy-based treatment of some of the most problematic retinal diseases.

II. Vector systems for expressing proteins in the eye

Because of the unique features of the eye, the following issues should be addressed when considering gene therapy. The relatively small size of the eye limits the injection volume, and hence the gene delivery system must be highly effective. The volume issue becomes especially important when injecting into the subretinal space, which is much smaller than the vitreous cavity and has more

restrictions. For ARMD and RP the subretinal space is the ideal injection site because the target cells are photoreceptor and the RPE cells. Intravitreal injection is technically easier than subretinal injection, but the target cells are limited to those within the inner layer of the retina. In cases where the retina is detached, intravitreal injections will potentially access cells in the outer retina and RPE cells through the retinal break. In summary, two different types of injections are feasible, and the choice depends on the target cells within the eye, as well as the condition of the eye itself.

III. Adenovirus vector

A. Adenovirus has a relatively high titer and infects many cell types, including neuronal cells.

Adenovirus is a member of DNA viruses which can enter both post-mitotic and dividing cells. One of the advantages of this system is the high titer that is readily achieved (greater than 10^{12} C.F.U. (= Colony forming unit) / ml). This increases the likelihood of expressing genes at high levels, and is particularly advantageous for injection into small spaces such as the subretinal compartment mentioned above. The other advantage is that this viral system is suitable for both dividing and postmitotic cells, including neuronal cells. Consequently, the adenoviral approach is appropriate for diseases such as RP, where the target cell type is the postmitotic photoreceptor cells.

B. Adenovirus is easily rejected by the host's immune system

When genes are introduced using adenoviruses, a common consequence is humoral and cellular immunoreaction. A typical observation following an intravitreal adenovirus injection is severe uveitis characterized by infiltration of inflammatory cells into vitreous cavity, retina, and choroid. Retinal thinning is also observed after intravitreal injection of adenovirus, however this change is transient. Retino-electrogram (ERG) examination has shown that the retina functionally recovers within weeks after the injection (Sakamoto et al., 1998). Subretinal injection has been reported to induce a milder immune response than subcutaneous injection. (Bennett et al., 1996) This may be in part due to the immune-privileged status of the subretinal space which would diminish the immune response to the injected virus.

As mentioned above, the introduced adenovirus triggers both cellular and humoral responses. The cellular response leads to the appearance of cytotoxic T cells which kill the virally infected cells. Whereas the humoral response is characterized by the production of antibodies against the adenoviral proteins. These antibodies persist even after the expression of the introduced proteins has ceased, and hence decreases the success of repeat infections. An additional drawback of this system for gene transfer is that most of the human population probably have antibodies to these adenovirus from the previous infection with adenovirus which are the cause of the common cold.

Despite the immunological reaction following gene therapy using adenovirus, it is possible to achieve high expression of the introduced protein. Expression tends to be transient, usually restricted to a month especially the retinal tissue (Bennett et al., 1994; Li et al., 1994), and the shutdown in expression is most probably due to the immune response. This hypothesis is supported by the observation that adenovirus-dependent transgene expression lasts much longer in nude mice or immunosuppressive-reagent treated mice (Dai et al., 1995).

C. Adenovirus is not integrated into the host genomic DNA

In addition to transient expression of proteins, a second feature of adenovirus-mediated gene transfer is that the introduced DNA is not integrated into the host's genomic DNA. The introduced DNA is replicated extrachromosomally in the nucleus. Replication-deficient adenovirus can be generated by replacing the E1 region which is a critical part for virus replication with the gene of interest. In this case, recombinant vectors are replicated only in the cells expressing the E1 gene. In summary, the adenoviral gene delivery system enables high level transient expression. This approach may not be appropriate for treating chronic diseases such as RP, PDR or ARMD. Even in PVR, which has a much quicker onset, it is not clear therapeutic genes would be expressed long enough when introduced with an adenovirus.

IV. Adenovirus associated vector (AAV)

A. AAV has a high titer and can be integrated into genomic DNA

AAV is a simple and non-pathogenic single-stranded DNA virus, and is newer than either adenovirus or retrovirus in the gene therapy area. The advantage of the vector is that it integrates into genomic DNA of the infected cells, which enables permanent expression in the tissue. The other advantage is that AAV has relatively higher titer (greater than 10^{12} C.F.U./ml), and that both growing and resting cells can be infected. This viral approach has been used to stably express lacZ or green fluorescent protein (GFP) in photoreceptors of mice and rabbits (Ali et al., 1996; Flannery et al., 1997).

B. Production of AAV is technically involved, and accommodates only relatively small DNA inserts

The disadvantage of AAV is that preparation of virus is challenging, and requires that the investigator be vigilant to avoid contamination with adenoviruses. The other disadvantage is that AAV cannot accommodate DNA inserts bigger than 4.0 kbp. This characteristic limits the size of the gene which can be expressed by AAV. The advantages include high titer, wide target range, and prolonged expression. As with the other gene transfer systems, the characteristics of the disease and genes that will be expressed should be matched to the features of the approach for gene expression.

V. Retroviral vector

A. Retroviral vector is the most commonly used

The retrovirus belongs to the family of RNA viruses, which convert genomic RNA into DNA with RNA transcriptase. Of the viral vehicles, the retrovirus vector is the most widely used both in basic research and clinical trials. Retroviruses have been studied extensively, and consequently more is known about this viral system for gene therapy as compared with the other available approaches. Retroviruses are easier to generate and purify than adenovirus or AAV. The retroviral gene transfer system is the most commonly used approach in gene therapy, and many of the past and current clinical trial protocols employ this vector system.

B. Retroviral vector can be integrated into genomic DNA of the target cells

An important feature of retroviruses is that they integrate into the genomic DNA of the target cells by passing through the cellular and nuclear membrane. Despite the integration of the introduced gene into the cell's genome, expression of protein is not as long as with the AAV system. This reason is still unclear (Verma and Somia, 1997). A caveat of integrating the gene into the host genome is the potential for insertional mutagenesis.

C. Retroviruses preferentially infect proliferating cells

A feature of this system is that retrovirus poorly infect post-mitotic cells such as neuronal cells. This is a potential problem to treat the diseases in which neuronal cells are the target cell type. In fact, some phenotypes of the RP result from point or null mutation of rhodopsin, or peripherin / RDS gene (Dryja et al., 1990; Kajiwara et al., 1994), both of which are specifically expressed in the photoreceptor cells. So while photoreceptor cells are likely a target for gene therapy to treat RP, and a retroviral vector delivery system is probably not a good choice. Also the viral titer possible with retroviruses is much lower (greater than 10^8 C.F.U./ml) as compared with adenoviruses or AAV.

These unique features of the retrovirus make this delivery system particularly well suited to certain diseases. For instance, since retroviruses selectively infect proliferating cells, administration of virus to an anatomical site that has both resting and dividing cell will result in infection of only the cells that are proliferating. In an individual with a brain tumor, one approach would be to use retroviruses containing a conditional suicide gene such as thymidine kinase. Subsequent administration of gancyclovir will kill only the infected tumor cells. The application of this approach to ocular diseases is discussed below.

D. An ex vivo approach can increase the efficacy of infection

To circumvent the relatively low viral titer, an *ex vivo* method is sometimes employed instead of *in vivo* infection. This method involves following four steps: (**i**) remove some of the target cells from the body; (**ii**) culture them under condition where they proliferate; (**iii**) infect the target cells *in vitro* with the retrovirus harboring the gene of interest, expand them; (**iv**) reintroduce the modified cells into the body. Although a high transfer efficacy can be achieved with this *ex vivo* method, it can only be applied when the target cells are readily accessible and proliferate in tissue culture. Another problem is that expression of the introduced gene sometimes shuts off after transplantation. While the mechanism involved has not been clearly resolved, one solution for this problem is to use receptors with different / stronger promoters (Verma and Somia, 1997).

VI. Lentivirus

A. Lentivirus is the newest approach for gene transfer

Lentivirus belongs to the retroviral family, and human immunodeficiency virus (HIV) is the best-known member of this group. The advantages of this vector include: (**i**) integration into the host genomic DNA; (**ii**) both dividing and non-dividing cells can be effectively infected; (**iii**) like AAV, expression of target protein is sustained. Green fluorescent protein (GFP) expression in the retina was still stable even 12 weeks after gene transfer with this vector. (Miyoshi et al., 1997).

A relatively minor drawback is that titers of only 10^8 can be readily achieved for lentivirus. A much more serious disadvantage of this vector is the possibility of generating replication competent virus (RCV). Since this vector system

is based on the HIV, contamination of these unwanted RCV would lead the host infection of acquired immunodificiency syndrome (AIDS), a deadly disease. The usage of this vector endangers not only the recipient but the medical staff and researchers. Efforts have been made to develop safe lentivirus based on the HIV virus. For instance, the required viral genes have been divided into three independent plasmid in order to minimize recombinations leading to RCV (Naldini et al., 1996). While the possibility of generating RCV has been severely reduced, it is prudent to exercise caution when using this viral system. Furthermore, it is likely that a variety of safety issue will need to be addressed before the clinical use of lentiviruses becomes widespread.

VII. Specific strategies for each ocular diseases

A.Retinitis pigmentosa

Retinitis pigmentosa (RP) is often an inherited disease characterized by progressive degeneration of photoreceptor cells in the retina. The degeneration leads to apoptotic cell death of the photoreceptor cells (Portera-Cailliau et al., 1994). The degenerated retina is recognized as a pigmented area of the retina, which results from accumulation of RPE cells. Typically, it initially involves the mid-peripheral retina, and then progresses toward the central part of retina, called macula. Since the macula is responsible for central vision, loss of this portion of the retina compromises visual acuity. Even if macular function is preserved, the visual field is sometimes severely impaired.

The severity and progression of the disease generally depends on whether both of the patient's alleles are mutated. Homozygous patients usually have an early onset, and have worse prognosis than heterozygous patients. Heterozygous patients have a relatively late onset, and sometimes retain good vision throughout their entire life. These facts suggest that RP includes multiple genotypes. Genetic studies have revealed mis- or null mutations in multiple genes including rhodopsin, peripherin / RDS, or RPE 65 (Dryja, 1997; Morimura et al., 1998).

The existence of an animal model for the disease has enabled investigators to test gene therapy as a means of treatment. The rd (rd/rd) mouse harbors a mutation in the beta subunit of cGMP phosphodiesterase gene (βPDE), and this results in progressive retinal degeneration similar to RP. Adenoviruses have been used to successfully transfer normal βPDE into the photoreceptors of these mice, and retinal degeneration is delayed in such animals (Bennett et al., 1996). Thus, this seems to be an appropriate approach to transduce a gene encoding a functional protein into autosomal recessive patients that suffer from a disease caused by a dysfunctional protein.

However, this approach is not suitable for patients with autosomal dominant RP, because the disease arises from an accumulation of mutated protein. The build up of such proteins is believed to be cytotoxic to the photoreceptors, and consequently induces cell death. In this case, therapy requires the elimination of the mutant gene/protein. One way is to use a ribozyme to cleave the mutant mRNA while leaving the wild type RNA. This approach has proven to be effective in slowing retinal degeneration in autosomal dominant retinal degeneration mice (Lewin et al., 1998).

A second approach to treat autosomal dominant RP is by expression of a neurotrophic factor which has a protective effect on the photoreceptor cells. So far, ciliary neurotrophic factor (CNTF), and basic fibroblast growth factor (bFGF) have shown to rescue the photoreceptor cell death. (Akimoto et al., 1999; Cayouette and Gravel, 1997) Similar to these, some investigators have shown that brain-derived neurotrophic factor (BDNF) prevented ganglion cell death from the optic nerve axotomy (Di Polo et al., 1998). Both the ribozyme and neurotrophic factor approaches hold promise for treating patients with autosomal dominant RP.

B. Proliferative vitreoretinopathy

Proliferative vitreoretinopahty (PVR) is the most common reason for failure of retinal reattachment surgery in patients with retinal detachment (RD). It is characterized by pre- and/or subretinal membrane formation and contraction resulting in tractional retinal detachment with or without rhegmatogenous component. Membrane formation is believed to be caused by unscheduled proliferation and collagen synthesis of the RPE cells migrating through the retinal break (Pastor, 1998). Once this occurs, surgery is the only treatment, and only 50% of such patients are cured. In the other patients PVR reoccurs, and often lead to at least a partial loss of vision (Pastor, 1998; Yoshida et al., 1984).

The pathogenesis of PVR is still unproven, but growth factors are commonly believed to make an important contribution to disease progression. While many growth factors have been implicated, platelet-derived growth factor (PDGF) is thought to be the strongest candidate for this process. The data to support this idea include: (**i**) PDGF is a strong mitogen and chemoattractant for RPE cells and the retinal glial cells both of which are present in the membrane (Bressler et al., 1985; Campochiaro and Glaser, 1985), (**ii**) cultured RPE cell secrete PDGF and express PDGF receptors, thereby establishing a functional autocrine loop (Campochiaro et al., 1994), (**iii**) the concentration of PDGF is elevated in the vitreous of PVR patients and PDGF can be found in the epiretinal membrane (Cassidy et al., 1998; Robbins et al., 1994) and (**iv**) in an animal model of PVR,

the ability to respond to PDGF greatly enhances the PVR potential of cells (Andrews et al., 1999).

Transforming growth factor-beta (TGF-β) and hepatocyte growth factor (HGF) are thought to be other potential inducer of PVR, because both TGF-β and HGF are a mitogen for RPE cells and both are up-regulated in PVR. (Connor et al., 1989; Lashkari et al., 1999) These findings suggest that growth factors contribute to the development of PVR, and identify growth factors and their receptors as a targets for gene therapy-based strategies to prevent PVR.

Some investigators have shown that gene therapy can be successfully applied to an animal model of PVR (Sakamoto et al., 1995). They injected fibroblasts with or without a retrovirus harboring herpes simplex virus thymidine kinase (HSVtk) gene which makes the infected cells sensitive for gancyclovir. Gancyclovir was given after the injection, and they found that PVR progression was inhibited by the retrovirally transferred HSVtk gene using this *ex vivo* approach. Subsequent studies showed that an *in vivo* approach was also effective for preventing PVR (Kimura et al., 1996).

C. Other disease

The lack of effective treatment of neovascular diseases of the choroid and retina requires the development of new approaches, one of which is likely to be gene therapy. Retinal neovascularization occurs in PDR, and in retinopathy of prematurity (ROP), whereas new vessel growth in the choroid is a hallmark of ARMD. In all of these diseases, it is likely that VEGF plays an important role in the pathological angiogenesis. Up-regulation of VEGF due to hypoxia in the retinal glial cells and/or the RPE cells is thought to be the strongest inducer for these new vessels. (Frank, 1997; Kliffen et al., 1997; Lopez et al., 1996; Pe'er et al., 1996). Hence, VEGF, and its receptors are excellent targets for gene therapy-based treatments for these diseases.

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