Potential combination of vector delivered-endogenous anti-VEGF and recombinant tissue plasminogen activator: novel therapy for diabetic retinopathy

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ABSTRACT

Diabetic retinopathy is one of the complications of diabetes mellitus and causes visual disturbances. This increasing number of diabetic retinopathies needs to be addressed because it can affect various aspects of life. The current management of diabetic retinopathy still has limitations in terms of the therapeutic effect and the potential for permanent vision loss. The development of research shows that injection of recombinant adeno-associated virus (rAAV) with sFlt-1 linkage and recombinant plasminogen activator (rTPA) has the potential to be used as an alternative treatment for diabetic retinopathy. This literature review aims to collect and analyze various sources regarding the potential of the combination of rAAV sFLT-1 and rTPA as a treatment for diabetic retinopathy. This literature review was compiled systematically by synthesizing literature from search engines ScienceDirect, PubMed, and Research Gate. This modality was administered by injecting a combination of rAAV sFLT-1 and rTPA subretinally once. The results of the literature analysis show that rAAV sFLT-1 will trigger the body's immune response against adenovirus and recombinant particles carried, while rTPA will inhibit angiogenesis that occurs in diabetic retinopathy. This modality can improve and maintain visual acuity in diabetic retinopathy patients with minimal side effects. The combination injection of rAAV sFLT-1 and rTPA is an appropriate treatment modality for diabetic retinopathy in terms of effects and adherence.

Keywords: Diabetic retinopathy, Recombinant tissue plasminogen activator, Vector delivered-endogenous anti-VEGF.

INTRODUCTION

Diabetes mellitus (DM) is a metabolic disease characterized by hyperglycemia caused by abnormalities in insulin secretion or/and action.[1] DM can cause microvascular complications, namely diabetic retinopathy (DR), which causes disturbances in the visual system. With the increasing number of diabetes in the world, the prevalence of DR is also predicted to increase in the coming years. The literature shows that DR is higher in patients with chronic uncontrolled diabetes, hypertension, and patients from South Asian, African, Latin American, and Pacific ethnicities.[2,3] Although DR itself is not life threatening, it can reduce the patient's quality of life. The literature shows that DR patients have significantly increased socioemotional disturbances.[4-6]

The treatment that has become the gold standard to date, especially in areas with limited resources is panretinal photocoagulation (PRP).[7] PRP is performed by firing a laser at the inner layer of the retina and its photoreceptors with the aim of destroying the ischemic and rapidly metabolizing photoreceptors.[8-11] The loss of parts of the 'metabolic burden' also reduces the oxygenation load so that the hypoxic conditions experienced due to occlusion as a microvascular complication of diabetes are reduced.[8] Reduced hypoxia can suppress the production of vascular endothelial growth factor (VEGF) so that DR does not progress to proliferative diabetic retinopathy (PDR).[8] However, the loss of part of the retina with its photoreceptors certainly results in side effects such as permanent vision loss, choroidal detachment, exudative

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Received: 27.05.2023
Accepted: 25.07.2024

J Ret-Vit 2024; 33: 291-300

DOI:10.37845/ret.vit.2024.33.48

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retinal detachment, choroidal neovascularization, and macular edema.[8] Therefore, the American Academy of Ophthalmology (AAO) began to advocate anti-VEGF intravitreal injection therapies such as ranibizumab and aflibercept which have been proven in the literature as safe and effective alternative methods.[7,12,13] However, the use of this injection has the disadvantage of requiring repeated injections, causing significant discomfort and burden on patients and caregivers, which can reduce medication adherence.[14,15]

Recent science with a focus on gene therapy has recently succeeded in proving in vitro that injection of recombinant adeno-associated virus (rAAV) with sFlt-1 junction which is part of the VEGF-1 receptor (Flt-1) has the potential to be a therapeutic modality to inhibit retinal neovascularization in animals.[16] The literature also shows that administration of recombinant plasminogen activator (rTPA) has the potential to decrease vitreoretinal tractions (VRTs) in PDR.[17] The combination of subretinal injection of rAAV.sFLT-1 with rTPA has the potential as a therapy that is efficacious both in the short term by suppressing coagulopathy and in the long term by naturally forming antibodies against the VEGF-1 receptor. This combination injection has the advantage of having only one administration frequency.[18-20]

This literature review aims to discuss the potential for subretinal injection of a combination of rAAV.sFLT-1 with rTPA as a new therapeutic modality for DR.

MATERIALS AND METHODS

This literature review was compiled using the literature synthesis method. The literature search was carried out on November 8, 2021 to March 17, 2022. The literature was searched using the keywords "diabetic retinopathy", "vector delivered-endogenous anti-VEGF", "recombinant tissue plasminogen activator" on validated search engines, namely ScienceDirect, PubMed, and Research Gate. The appropriate and relevant literature is 58. Information from the literature is reviewed and analyzed, then compiled into scientific literature.

RESULT AND DISCUSSION

Diabetic Retinopathy

DR is a microvascular complication of DM and hyperglycemia plays an important role in the pathogenesis of retinal microvascular damage. Microvasculopathy in DR occurs due to occlusion and leakage of retinal capillaries. Metabolic pathways involved in retinal vascular damage caused by hyperglycemia include the polyol pathway, accumulation of advanced glycation end products (AGEs), the protein kinase C (PKC) pathway and the hexoamine pathway. All of these pathways play a role in increasing oxidative stress, inflammation, vascular occlusion and upregulation of growth factors that contribute to the pathogenesis of diabetic retinopathy.[21,22]

Sustained hyperglycemia causes the blood-retinal barrier to be damaged, resulting in a loose junction of the endothelial cells in the blood-retina barrier. Hyperglycemia triggers the destruction of pericytes, the structural supporting cells of the retinal capillaries, resulting in changes in retinal hemodynamics. The walls of the capillaries where pericytes are lost will dilate and leak, allowing fluid, macromolecules, and even blood to leak out onto the retina. This dilatation of the capillary wall is called a microaneurysm, the earliest lesion of DR that can be identified clinically by ophthalmoscopy as small red dots of bleeding and yellow deposits of hard exudate resulting from an increase in the thickness of the basement membrane. This condition is followed by loss of retinal capillary endothelial cells. These retinal microvascular endothelial cells and pericytes will then undergo apoptosis and an increase in leukostasis occurs. Thickening of the basement membrane, disruption of the blood-retinal barrier and loss of pericytes and endothelial cells lead to ischemic conditions.[21,23,24]

Retinal ischemia or hypoxia leads to upregulation of VEGF, a key factor in the development of PDR and DME through activation of hypoxia-inducible factor 1 (HIF-1) and elevation of phospholipase A2 (PLA2). Other angiogenic factors such as angiopoietin (Ang-1, Ang-2) are also involved in the regulation of vascular permeability by interacting with the endothelial receptor tyrosine kinase Tie2. VEGF and other angiogenic factors cause angiogenesis, damage the blood-retinal barrier, stimulate endothelial cell growth, and increase vascular permeability in the ischemic retina. These newly formed blood vessels emerge through the surface of the retina and proliferate into the vitreous cavity. These vessels are fragile and prone to rupture, so they can cause vitreous haemorrhage and loss of vision, injure the vitreous body, cause retinal detachment and eventually blindness. Neovascularization as a result of this growth factor imbalance then contributes to the development of other retinal diseases.[21,24]

Adenovirus

Adenovirus is an uncoated double-stranded DNA virus with a diameter of 65-80 nm. Adenovirus is composed of two main components, namely an icosohedral outer capsid

and an inner core where the double-stranded DNA genome is sandwiched with histone-like proteins. Adenoviruses can infect a variety of human tissues and are most commonly associated with upper respiratory tract disease in children.[25,26]

Research has shown that viruses can be modified to send important instructions to the body's cells. These modified viruses are referred to as viral vectors. Viral vector vaccines have been used to treat the threat of infectious diseases, such as Zika, flu and SARS-CoV2. These viral vectors are not only used in vaccines, but are also used in treating cancer and gene therapy.[27,28]

Recombinant adeno-associated virus sFLT-1 (rAAV sFLT-1)

rAAV sFLT-1 is a combination gene therapy consisting of rAAV as a vector of the virus and sFLT-1 is a protein tyrosine kinase which is a VEGF-1 receptor that has been modified by the Polymerase Chain Reaction (PCR) technique.[29,30] rAAV was chosen as a vector because it is a viral vector that is more efficient in cell transduction when compared to non-viral vectors and has gene expression that can survive for a long time. In addition, rAAV also has a single stranded DNA genome of about 4.6 kilobases (kb), capsid organization, and small size that can facilitate the desired genetic modification. In terms of toxicity, rAAV is a safe viral vector because it cannot replicate without the help of helper viruses such as Adenovirus (Ad) and Herpes Simplex Virus.[31,32] sFLT-1 was chosen because it has antiangiogenic properties that function to inhibit the formation of new blood vessels in the retina due to the activity of VEGF.[30,33]

Recombinant Tissue Plasminogen Activator (rTPA)

Tissue Plasminogen Activator (TPA) is a serine protease that can convert plasminogen into plasmin which plays a role in breaking blood clots. In addition, TPA also functions to inhibit angiogenesis by reducing fibrin expression.[34,35] TPA is composed of five structural domains consisting of fibronectin (F), epidermal growth factor (E), two disulfide-bound triple-loop structures known as kringles (K1 and K2), and, serine proteases (S). Structural domains that can be used as rTPA consist of K2 which functions to bind to fibrin in TPA and S which functions to convert plasminogen to fibrin for the breakdown of blood clots.[36] rTPA is a landfill made with recombinant biotechnology. This modality is used to treat ischemic stroke, myocardial infarction, pulmonary embolism, and thrombolysis.[37,38] rTPA can also be an anti-angiogenic agent in treating choroidal neovascularization (CNV).[34] rTPA is also a complementary drug in vitrectomy to treat submacular hemorrhage due to Age-macular degeneration (AMD).[18] With potential as an anti-angiogenic, rTPA has the potential to treat DR.

Construction Mechanism

Production of rAAV.sFlt-1

rAAV.sFlt-1 was produced in HEK-293 cells using the triple plasmid transfection method and purified by heparin affinity chromatography. The viral genome titer was 8 × 10^{12} particles/mL.[29] HEK-293 cells were thawed and cultured on expression media. Cultures were carried out at 37°C with stirring at 135 rpm in an incubator humidified with 5% CO2. Cells were cultured at a volume of 30 mL in a 125 mL Erlenmeyer flask (on a flat bottom with a ventilated lid), and subcultures were performed between 0.3 and 3 million cells/mL. At all times, cell viability was maintained above 90%. Samples of 50 mL of cell culture samples were taken daily from an Erlenmeyer flask, mixed with 50 mL of trypan blue dye. Then the number of cells and their viability were analyzed using Automated Cell Counter.[32]

The triple plasmid transfection method used a plasmid with an AAV serotype-specific replication gene (rep) and a capsid (cap) gene as vector DNA plasmids, and an essential Ad gene on the third plasmid (pXX6) to activate rAAV. This aims to eliminate Ad production in transfected cells so as to produce rAAV vectors so that they do not cause infection for users.[31] Prior to transfection, an aliquot of cells in the exponential growth phase were transferred to a new Erlenmeyer flask and diluted to a cell density of 1 million cells/mL to a total volume of 24 mL by adding preheated media. Each plasmid solution was measured using a spectrophotometer. The three plasmids were mixed at a 1:1:1 molar ratio with a total plasmid dose of 2 mg plasmid per milliliter of culture. Then, 1 mL of the transfection mixture was prepared by sequential addition of the plasmid mixture, culture medium, and maximal polyethylenimine (PEI) mixture. The amount of PEI Max added was calculated so that the mass ratio of PEI:DNA out was 2:1. The transfection mixture was stirred vigorously for 10 seconds then, incubated at room temperature for 10 minutes, and then added to the cell culture.[32]

The final volume of culture in each Erlenmeyer flask was 25 mL. For control cultures, 25 mL of culture was prepared at 1 million cells/mL without adding the transfection mixture. All Erlenmeyer flasks were cultured as described above.

Media exchange was performed 6 h after transfection: cells were transferred to 50 mL centrifuge tubes and centrifuged at 300 g for 5 min to remove the supernatant; then resuspended in 25 mL of warmed fresh medium; and transferred back to the original Erlenmeyer flask and cultured as described above. 2 mL culture samples were taken at time points of 10, 24, 34, 46, and 55 hours. A volume of 50 mL of culture broth from each sample was used to measure cell counts and immediate viability. Another cell culture sample was put into 500 mL in each Eppendorf tube and centrifuged at 1,000 rpm for 5 min. The supernatant and cells were separated into different tubes and stored at 800C.[32]

Twenty-four hours after transfection, Dulbecco's Modified Eagle's Medium (DMEM) culture medium with 10% Fetal bovine serum (FBS) was replaced with the same medium without serum. After 48 h, culture media and cells were harvested separately for vector purification. Vector particles from the culture medium were precipitated with PolyEthylene Glycol (PEG-400). Then, the cells were lysed with three freeze/thaw cycles and the vector particles were precipitated by ammonium sulfate. Both precipitates were treated with benzonase and then purified by two centrifugation cycles using cesium chloride. For the first cycle, both precipitates were dissolved in 1.37g/ml CsCl solution. Twenty milliliters of virus sample in 1.37g/ml CsCl was propagated at 0.5ml to 1.5g/ml CsCl in a 12.5ml centrifuge tube. Samples were centrifuged for 36 to 48 h at 288,000 × g, 15°C at 41,000 rpm and 10 drop fractions were collected from the bottom of the gradient. Repartition of viral particles through gradients was followed by Dot/Blot analysis with a biotinylated DNA probe specific to the transgene. The fraction containing rAAV was collected, volume adjusted to 12ml with 1.37g/ml CsCl solution for the second round of CsCl purification under the same conditions. The collected fraction containing rAAV after the second gradient was dialyzed in a MWCO 10,000 Slide-A-Lyzer dialysis cassette against three changes of 500ml sterile 1× PBS for at least three hours at 4 °C each and stored at -80°C. Viral genome count (vg) was obtained by PCR chain reaction of extracted vector DNA and vector physical particle count (pp) by ELISA-based method. The ratio of the viral genome to physical particles in the prepared vector varies from 1/3 to 1/10.[39]

The sFlt-1 cDNA coding was performed by PCR technique using a forward primer (TCGGGATCC TCGCCACCATGGTCAGCTACTGGGACACC) combining the BamHI site and a reverse primer (ATAGCGGCCGCTTAATGTTTTACATTACTTTGTG TGGT) incorporating the NotI site. PCR was performed using the proof-reading enzyme Pfu polymerase. The 2.1 kb product digested with BamHI and NotI was then subcloned onto the pEGFP-N1 mammalian vector expression cassette by replacing the BamHI-NotI EGFP cDNA fragment with a PCR-modified sFlt-1 cDNA fragment. sFlt-1 cDNA was verified by sequencing. The resulting expression cassette was then subcloned to derivatives of the deleted AAV plasmid for all viral genes to generate the plasmid AAV. CMV.sFlt-1 also known as rAAV sFLT-1. Purification of rAAV sFLT-1 was carried out by purification of the CsCl

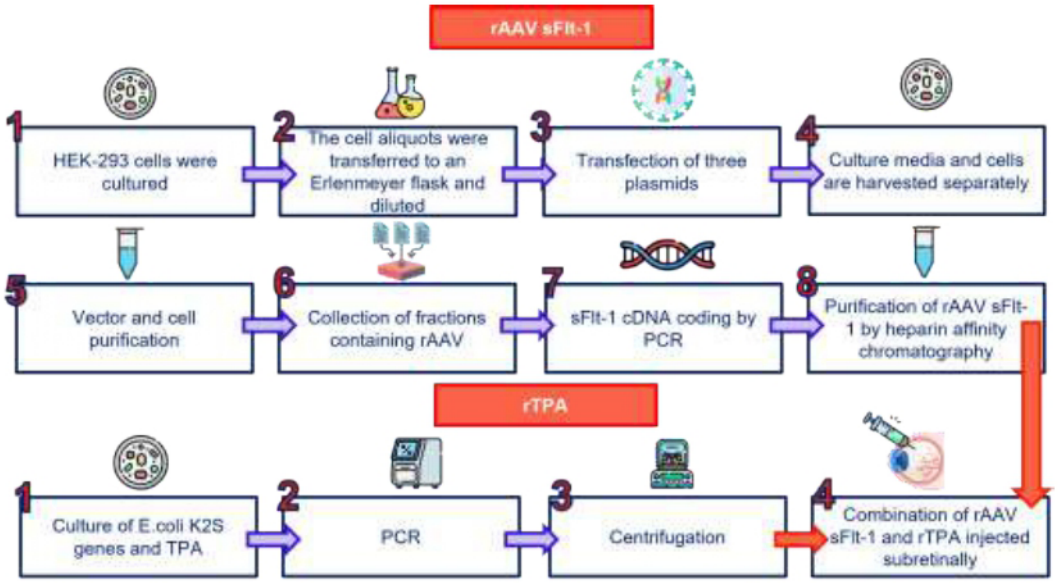

Figure 1: *The construction mechanism of the combination modality of rAAV sFLT-1 and rTPA*

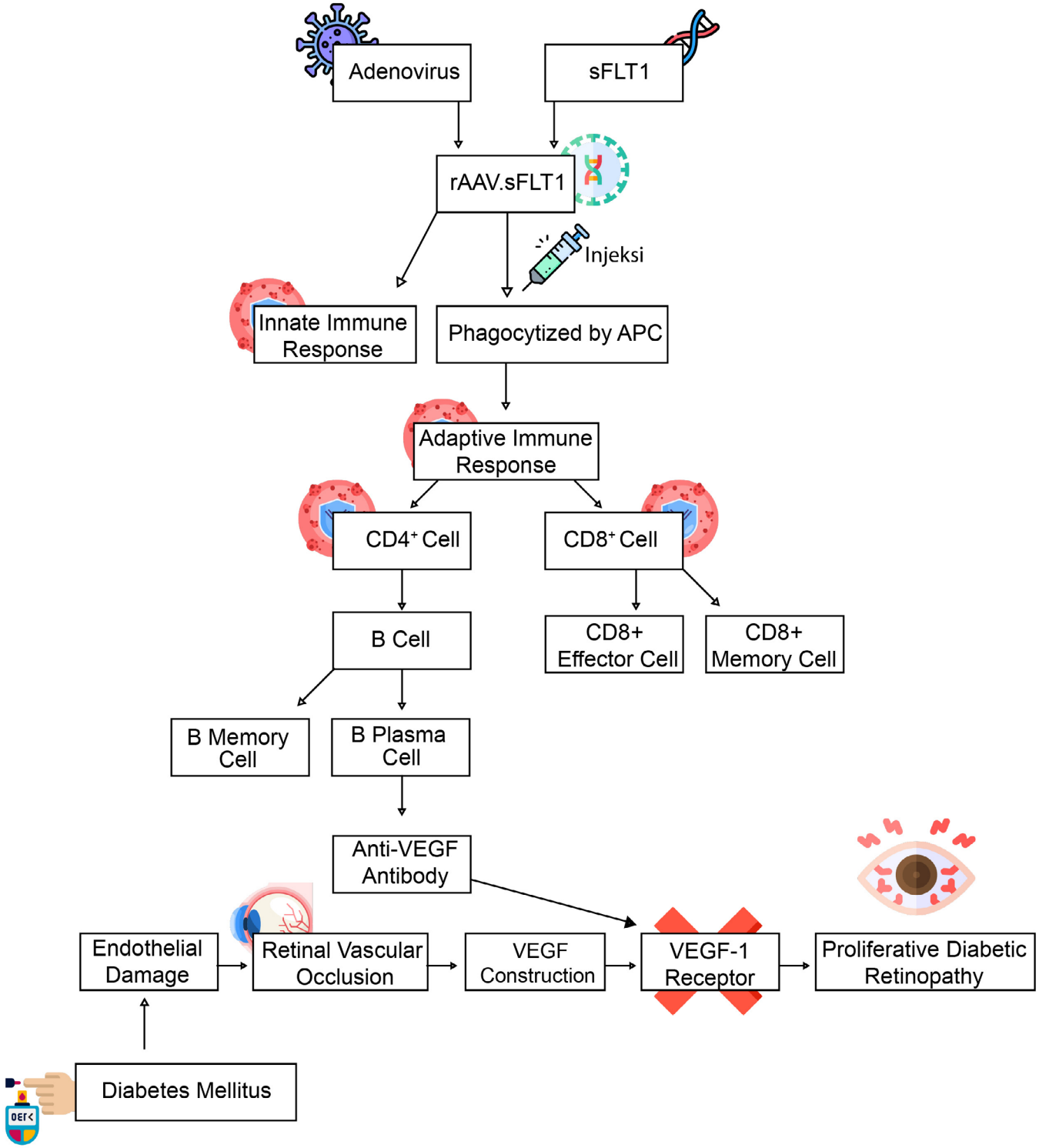

Figure 2: *Mechanism of action of rAAV.sFLT-1*

gradient converted to an iodoxianol gradient followed by a one-step affinity purification procedure on a Heparin column.[40]

Production of rTPA

rTPA can be produced in cultures of Escherichia coli (E.coli) bacteria with the K2S gene from human TPA. E. coli is a suitable host for expressing stably folded globular proteins of prokaryotes and eukaryotes.[41] The advantage of using E.coli as a host is that it can be modified with simple laboratory equipment; many vectors and host strains have been developed to maximize expression; rapid expression begins with eukaryotic cDNA clones; can produce a potentially unlimited, and cost-effective supply of recombinant protein.[42] The E.coli strain used was E. coli DH5α with pET40b (+) plasmid. The growing

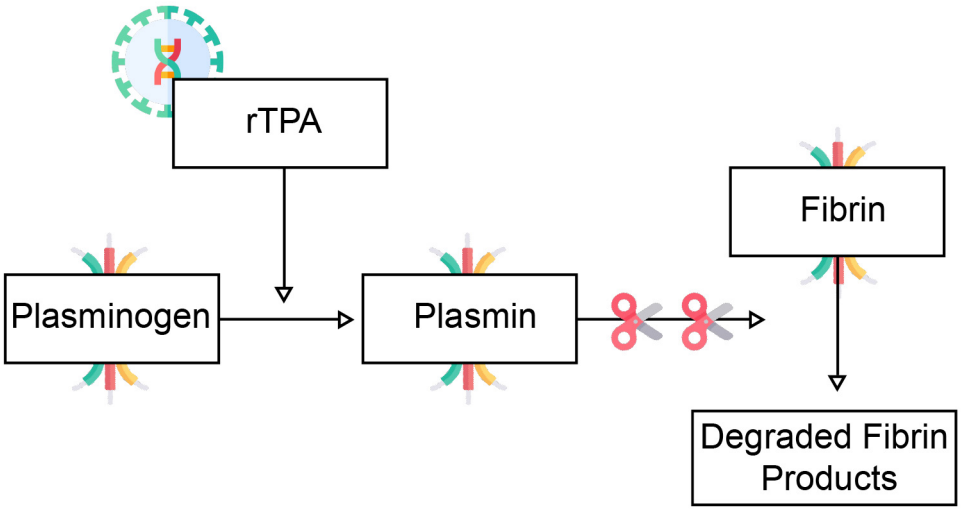

Figure 3: *Mechanism of Action of rTPA*

medium used was L-broth (per liter, 5 g NaCl, 5 g yeast extract, and 10 g triptone; Oxoid). Kanamycin (50 g/ml) was used to select recombinant bacteria. K2S gene amplification was carried out by synthesizing a pair of primers, K2S-1 (5'CGGGGATCCGATCGAAGGTCGT-TCTTACAAGGAAA CAGTG-3') and K2S-2 (5'-GGGCTCGAGATTACGGTCGCATGTTGTCACGA ATCCAG-3'). The K2S-1 primer combines the BamHI site and the Factor Xa protease cleavage sequence, and the K2S-2 primer combines the XhoI site and a translation termination codon at the 5'-terminus. The pMD18T-tPA recombinant plasmid was used as a template. PCR was carried out at 94°C for 5 minutes, then 28 cycles at 94°C for 45 seconds, at 64°C for 45 seconds, and at 72°C for 1.5 minutes; Extension was carried out at 72°C for 10 min. The 1.1-kb PCR product digested with BamHI and XhoI was subcloned onto the pET40b(+) plasmid to generate a fusion protein with DsbC. The recombinant constructs were analyzed by restriction enzyme digestion and sequencing to determine the orientation of the reading frame and confirm sequence accuracy, and the positive recombinant plasmid was named pET40b(+)-K2S. In addition, between the coding sequences of DsbC and K2S there is a His-Tag that is useful for the separation of fusion proteins by Ni^{2+} chelating affinity chromatography.[36]

The recombinant plasmid pET40b(+)-K2S was converted to E. coli strain BL21 for expression. In summary, the transformants were inoculated in 100 ml of Luria-Bertani medium at pH 7.0 in the presence of kanamycin (50 mg/ml) at 37°C. When the uptake of OD600 reached 0.6-1.0, 0.6 mM, IPTG was then added to induce protein synthesis. The bacteria were then cultured by centrifugation (200 rpm) for 12 hours at 30°C. Bacterial cells were harvested by centrifugation at 13,000g for 20 min, resuspended in 25 ml of 20 mM Tris-HCl (pH 7.9), and then lysed with Ultrasonic Cell Crusher at 400W for 30 min in ice water. Protein content was determined by the bicinchoninic acid test method. The centrifuged cell lysate supernatant was applied to a column containing 10 ml of Ni2+ chelating resin which had previously been equilibrated with NTA-0 buffer (0.5 M NaCl, 20 mM Tris-HCl, 10% glycerol, pH 7.9). After washing to initial absorption with NTA-0 buffer, the column was washed with elution buffer containing 10, 20, 50, 100, 200, 300, and 400 mM imidazole at a flow rate of 2 ml/min. Each peak was collected and subjected to SDS-PAGE, and the relative amount of fusion product was measured by Quantity One software. For the K2S cleavage of the DsbC tag, the purified fusion protein was cleaved by the factor Xa protease until mature K2S was obtained by centrifugation.[36]

Administration

The combination of rAAV sFLT-1 and rTPA was administered using the subretinal injection method. This method has a better direct effect on target cells in the subretinal space, compared to intravitreal injection, so it can provide a therapeutic effect in vitreoretinal disease.[43] The dose of rAAV sFLT-1 that can be given to DR patients is $1x10^{10}$ vg. The dose of rTPA that can be given is 0.1 ml of fluid (20 g/0.1 ml of rtPA). rAAV sFlt-1 and rTPA will be injected subretinal simultaneously with the help of a 41G (23G/0.6 mm) cannula with a volume of 100 µL.[44,45]

Immunogenicity Mechanism of Recombinant AAV

Gene therapy modalities in the form of recombinant vaccines with adenovirus vectors work by inducing

immunogenicity of the body's immune system against both the adenovirus and the recombinant particles carried by the vector.[46,47] As a vector, adenovirus can be modified into replication-defective (DR) which closes the possibility of the virus replicating and causing prolonged infection or sepsis, so it is relatively safer.[46,48–50]

Research shows that adenovirus elicits an innate immune response very strongly in mouse in vivo experiments.[51] Adenovirus vector type 5 (Ad-5) binds to coagulation factor X and is then recognized by macrophage toll-like receptor 4 (TLR4), resulting in NF-k activation and the adaptive immune system.[52]

After anti-adenovirus antibodies are formed, the opsonization process can phagocytize viral particles more easily.[53] The uptake process of replication-defective will run until the point where the adenovirus vector and its recombinant part are exhausted in the body.[53] In the end, the body will be free of recombinant adenovirus but will still store the information in memory cells so that the process of re-responding to the same particles can occur more quickly.[19]

Mechanism of Action of rTPA

rTPA as a modality to treat DR has the same mechanism as TPA, namely inhibiting angiogenesis by converting plasminogen to plasmin to reduce fibrin expression.[35] Fibrin is one of the extracellular matrix (ECM) which has a role in angiogenesis to carry out endothelial cell migration, morphogenesis, and maintain the stability of new blood vessels.[54] The TPA molecule binds to fibrin via the F, K2, and E domains. The activation of plasminogen by TPA increases markedly when it binds to fibrin. TPA cleaves the peptide bond Arg561-Val562 which is the site of the formation of two-chain plasmin. Five kringles present a heavy chain, the light chain contains a catalytic triad with His603, Asp646 and Ser741. Plasmin degrades fibrin, fibrinogen and plasma proteins such as factors V, VIII, IX, XI and XII, insulin and growth hormone.

Clinical Effect

Research conducted by Rakoczy et al showed that there was no visual loss, paramacular atrophy, and retinal thinning at the end of the 52nd week of observation in a group of patients who received subretinal injection of 100 L AAV2 at a dose of 1×10^{10} vg or 1×10^{11} vg. Furthermore, best corrected visual acuity increased from a median of 40 letters of the Early Treatment Diabetic Retinopathy Study (EDTRS) at baseline to 50 letters of EDTRS.[44] This visual improvement can also be maintained significantly in some patients within 3 years. The administration of rAAV sFLT-1 was well tolerated, and there were no ocular, cardiovascular and systemic adverse events associated with this modality.[20] Constanble et al also demonstrated that visual acuity improved after rAAV sFLT-1 administration and that ocular side effects were usually procedure-related and self-limiting. Based on the description above, rAAV sFLT-1 is a safe modality and has the potential to be used as a long-term treatment.[56]

Research conducted by Mayer et al showed that treatment with rTPA and gas in patients with subretinal hemorrhage gave improved functional outcome after 1 year. Patients who received rTPA also did not experience side effects. Therefore, rTPA can be considered as a treatment to maintain visual acuity in neovascular macular degeneration patients with a pathogenesis similar to that of DR.[57]

CONCLUSION

The current limitations of therapeutic modalities to treat diabetic retinopathy have prompted various innovations. The combination of rAAV sFLT-1 and rTPA administered subretinally has the potential to be used as a treatment modality for diabetic retinopathy with minimal side effects. This modality works by modulating the immune system and inhibiting angiogenesis. The combination of rAAV sFLT-1 and rTPA can provide benefits for diabetic retinopathy patients and is effective in terms of adherence.

RECOMMENDATION

Clinical trials related to this modality are still very limited and use a small sample, so further tests regarding the effectiveness, side effects and potential of this modality need to be conducted.

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