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Corneal Neovascularization as a Target for Nucleotide-Based Therapies

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Abstract

The cornea is a transparent tissue with avascular characteristics (corneal avascularity). It can be compromised by imbalances of angiogenic factors due to chemical injuries, infections, or autoimmune diseases such as Stevens-Johnson's syndrome, which can lead to corneal blindness. Clinically, the etiologies of corneal neovascularization (NV), such as inflammation, immune rejection, limbal stem cell deficiency, or hypoxia, are usually long-lasting; therefore, conventional treatment modalities, including antiangiogenic medications, laser, or surgeries have only a suboptimal effect, and the prognosis is even worse with multiple recurrences. In contrast, novel treatment modalities, such as gene therapy (enhanced intracellular expression of antiangiogenic factors) and nucleotide-based anti-angiogenic therapy (antisense oligonucleotides, silence-RNA, and micro-RNA) have been accomplished in animal models or clinical trials in recent years. Because of its specific and long-lasting effect, as well as the improvement and verification of the safety of expression vectors and carriers, the clinical value of nucleotide-based therapy has been increasingly appreciated. This review summarizes and updates relevant research, and provides a better understanding regarding the mechanism and treatment of corneal NV.

Keywords: Corneal neovascularization; Target gene therapy; Antisense oligonucleotides; Silence-RNA; Micro-RNA

Introduction

The cornea is a convex structure that covers the front surface of the eye ball. It is a specialized transparent collagen tissue, and therefore accounts for the majority of the refractive power of the eye. The corneal epithelium plays a critical role in maintaining corneal transparency and promoting stromal wound healing [1,2]. In contrast, the conjunctival epithelium is rich in goblet cells and microvasculature. The corneal epithelium serves as a physical barrier that prevents invasion of the conjunctival epithelium. Under normal physiological circumstances, corneal avascularity is maintained through a dynamic balance among angiogenic regulatory factors secreted by the corneal epithelium. However, in pathological conditions, either due to invasion of the conjunctival epithelium or an imbalance among angiogenic regulatory factors, corneal neovascularization (CNV) occurs [3,4] and may lead to blindness [5]. Up to 4.1% (1.4 million) patients in the United States presenting at ophthalmological clinics are affected by CNV. Importantly, CNV may cause loss of visual acuity in approximately 12% of these patients [6].

Clinically, factors provoking CNV are usually present and active, while conventional chemically synthesized anti-angiogenic drugs have a biological half-life and cannot work locally in a long-term and continuous fashion. Nucleotide-based drugs bind with expression vectors before they are transported into cells for expression. With the features of specificity in both expression location and target, and continuous expression, nucleotide-based drugs are generally considered to be potential replacements for chemically synthesized and protein-targeting drugs as a third generation drugs.

Development and Molecular Mechanisms of Corneal Neovascularization (CNV)

Vascular growth can be broadly classified into three types: vasculogenesis, angiogenesis, and collateral growth [7-9]. During vasculogenesis, endothelial cells differentiate from endothelial progenitor cells in the embryo or bone marrow and are connected successively to form lumens of blood vessels. Angiogenesis is the formation of sprouting endothelial cells from the pre-existing blood vessel walls, followed by maturation into new vessels and invasion of adjacent tissues. Collateral growth is the development and remodeling of the pre-existing vessels, forming side bridges among vessel webs. Normally, turnover of endothelial cells is rarely observed in well-developed vessels, but vessels may react to environmental stimulations in the form of remodeling. For example, collateral growth is often observed in ischemic hearts and chronically ischemic corneal epithelia. CNV, in the form of angiogenesis, is established from the sprouting of pre-existing vessel webs located in the limbal tissue surrounded by the conjunctiva.

The process of angiogenesis can be divided into three stages: sprouting, stabilization, and maturation [10-12], which are regulated by different growth factors at different stages. Sprouting is the peri-vascular destabilization of mature vessels under environmental stimulation through inhibition of the Tie2 receptor by angiopoietin-2 [13] followed by egress of endothelial cells stimulated by vascular endothelial growth factors (VEGF). Stabilization refers to newly formed vessels that are structurally strengthened by proliferation and formation of the extracellular matrix (ECM) from the peripheral cells. Various signaling pathways are involved in this process, such as the secretion of platelet derived growth factor (PDGF) by vascular endothelial cells, promoting proliferation of peri-vascular cells [14]. On the other hand, binding of angiopoietin-1 (Ang 1) with the Tie2 receptor augments the connection between endothelial cells and peripheral cells, and thereby strengthens surrounding vascular structures while decreasing vascular permeability [15]. Maturation is the process where the transforming growth factor beta-1 (TGF β1) stimulates formation of the ECM and conversion of mesenchymal cells into vascular wall cells for strengthening and maturation of vessels. Neuropilin-1 (NRP1) is expressed on the vasculature, and NRP1-mediated signaling also promotes vascular maturation, a critical step in angiogenesis [16].

Unlike intraocular neovascularization, such as retinal neovascularization, vision is not threatened at the early stage of CNV. Clinically, patients only seek for help at the maturation stage of CNV, which explains the limited effects observed in VEGF inhibitors (bevacizumab) treatment targeting the early stage. Therefore, the pharmacological purpose of CNV treatment is to ameliorate the diseases that incite CNV and to lessen the post-treatment recurrence of CNV.

Pathogenesis of CNV

The development of drugs to inhibit CNV is closely related to the etiology and pathophysiology of CNV, which can be classified as inflammatory reaction, immunologic rejection, limbal stem cell deficiency, and hypoxia.

Inflammatory reaction-induced CNV

Keratitis, by etiology, can be subdivided into exogenous, endogenous, and mixed types. Exogenous keratitis, such as bacterial (*S. aureus, S. pneumonia*), viral (*Herpes Simplex Virus*), fungal, and protozoal (*Acanthamoeba*) is often observed in corneal trauma, dacryoadenitis, dry eye syndrome, or patients wearing long-term contact lens. Additionally, conditions such as neurotrophic keratopathy, corneal dystrophy, diabetes, or immunologic rejection, often result in a corneal epithelial defect combined with secondary exogenous corneal ulcer. Other factors leading to a corneal epithelial defect include autoimmune disorders, such as Stevens-Johnson syndrome, cicatricial pemphigoid, and Mooren's ulcer. Upon initiation of an inflammatory reaction, corneal epithelium, stromal cells, and non-specific immune cells release angiogenic factors such as VEGF. These factors can hasten the production of matrix metalloproteinases (MMPs) from the endothelial cells within the limbal capillary network, facilitate the destabilization of vessels, and simultaneously stimulate neovascularization [17]. Inflammatory reactions also trigger the migration of Langerhans cells to the cornea, releasing more angiogenic cytokines and further recruitment of immune cells [18], which can aggravate CNV.

Immunologic rejection-induced CNV

Apart from autoimmune disorders, rejection in high risk corneal transplantation is also a common immunologic reaction. Immunologic rejection starts with the activation of the complement system through the cellular and humoral immunologic reactions, inducing chemotaxis of neutrophils. Corneal invasion by neutrophils can subsequently lead to activation of fibroblasts and degradation of collagen, and finally ulceration of the cornea [19]. The ulcerated tissue, as an antigen, deteriorates the immunologic reaction, and coupled with the destruction of the corneal epithelium, promotes the occurrence of CNV. Because the attack of the host's immune system is long-term and continuous, traditional drugs, with their confined pharmacokinetics, have only a limited effect. Therefore, it would be beneficial to patients if specific anti-angiogenic factors could be expressed in the cornea.

Limbal stem cell deficiency-induced CNV

The epithelial cells of limbal tissues are densely packed, have special columnar epithelial structures formed in the basal papillary area (called palisades of Vogt) and an abundant internal vascular plexus. It is also well known that the turnover of corneal epithelial cells depends on the stem cells situated in the limbal basal layers to provide new cells [20-22].

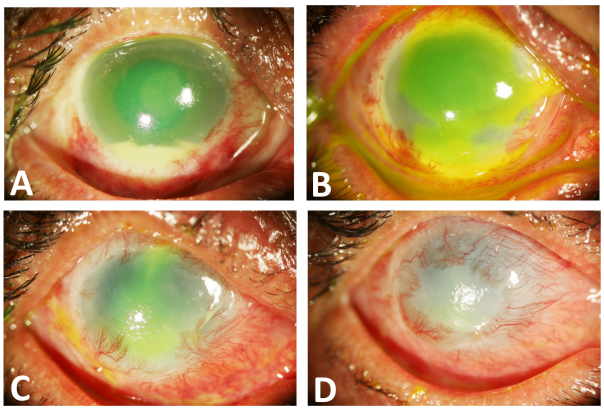

Figure 1: Corneal change after severe alkaline burn. **A.** A 53 years old male suffered from severe alkaline burn, with 360 degrees of limbal ischemia and extensive corneal and conjunctival epithelial defect. Hypopyon and exudative membrane in front of the lens were seen due to severe inflammation. **B.** One and a half month after injury, reepithelialization was seen from 4 and 8 O/C limbal region, accompanied by vascular ingrowth. **C.** Three months after injury, although corneal epithelial defect decreased significantly, corneal neovascularization progressed further. **D.** Four months after injury, neovascularization accelerated that blood vessels now invaded from all directions of the limbus, one of the hall mark of total limbal stem cell deficiency.

To maintain corneal avascularity, corneal epithelium expresses anti-angiogenic factors (i.e., tissue inhibitors of metalloproteinase [23], soluble fms-like tyrosine kinase-1, sflt-1 [24], thrombospondin [25-27], angiostatin [28-30], endostatin [31-34], pigment epithelium-

derived factor, PEDF [35-38]) and anti-inflammatory factors (i.e., interleukin-1 receptor antagonist; IL-1rA [39]). Clinical evidence shows that CNV increases significantly in limbal stem cell deficiency (LSCD) [4]. Therefore, in patients with severe chemical injuries (Figure 1) or Stevens-Johnson syndrome, given the destruction of limbal stem cells, CNV ensues following the invasion of conjunctival epithelial cells [40,41].

Hypoxia-induced CNV

More than 80% of oxygen consumed through respiration by the corneal epithelium is from the air, and thus corneal hypoxia is frequently observed in patients who constantly wear contact lenses. Fortunately, due to the competent anti-angiogenic activity of the corneal epithelium, most of the blood vessels only advances to the margin of the limbal tissue, while only rare, severe cases develop CNV. It has been shown in an animal model that hypoxia inducible factor-1 (HIF-1) is activated with the long-term wearing of contact lenses [42]. Being a transcription activator of VEGF, it is thus proposed that HIF should be critical in the pathway from hypoxia to CNV [43].

Therapies for CNV

Surgery

a. **Argon, dye and Nd:YAG lasers:** Lasers, via coagulation of blood vessels and ablation of tissues, can obliterate sprouting neovascularization, but are not effective for widespread, web-like neovascularization (Figure 2) [44-47].

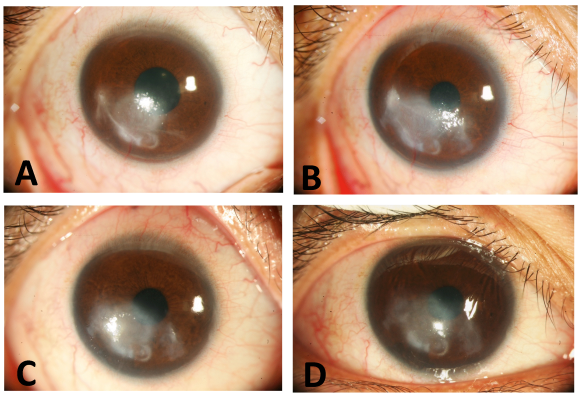

Figure 2: Successful obliteration of corneal NV by laser photocoagulation. **A.** A 34 years old male was a victim of herpes stromal keratitis. Since 2012, deep corneal stromal NV at 7 O/C was noted, which exended and spreaded out into the opacified scar area. **B.** In November 2014, stromal infiltration and opacity extended and corneal NV became more engorged. His best corrected vision reduced to 20/200. **C.** Corneal NV was ablated by yellow dye laser. Photo taken 10 days after treatment. **D.** Three months after treatment, corneal opacity reduced without recurrence of NV. Best corrected vision increased to 20/30.

b. **Photodynamic therapy (Visudyne; verteporfin):** After administration of verteporfin, targeted laser therapy can damage and embolize vascular endothelial cells [48].

c. **Diathermy (electrical burn):** After diathermy treatment in patients with lipid keratopathy complicated by CNV, vessels remain obliterated for eight months postoperatively [49]. In addition, with fine-needle diathermy, 50% to 100% of CNV can be obliterated with mild blanching of the corneal stroma [50].

d. **Ocular surface reconstruction:** The limbal epithelium is a barrier to invasion of neovascularization to the cornea and is the location of corneal epithelial stem cells. Depending on the extent of injury to the cornea and limbal stem cells, different options for ocular surface reconstruction can be adopted, such as corneal or limbal transplantation. While limbal autografts can be successfully applied for treatment of limbal deficiency [51], limbal allografts are not satisfactory for patients with bilateral limbal deficiency in terms of long-term preservation [52]. The amniotic membrane, due to its efficacy in inhibiting inflammation and promoting wound healing, has been applied clinically for a long time [53-55]. To increase anti-angiogenic effect, limbal stem cells can be cultivated on amniotic membranes for transplantation [34,56], however, which is also limited to autologous transplantation.

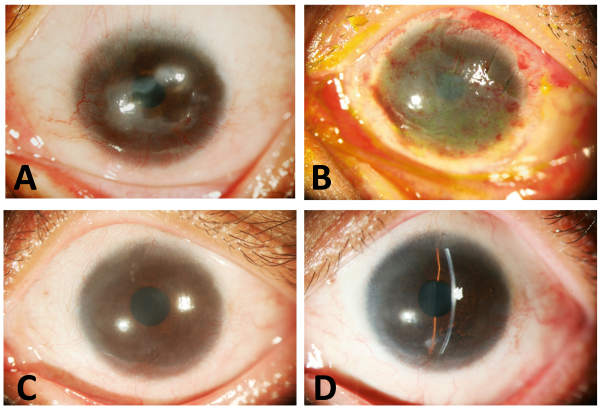

Figure 3: Restoration of corneal clarity by cultivated oral mucosal epithelial transplantation (COMET). **A.** A 27 years old male suffered from acid burn OS during childhood, and pre-op best corrected vision was 20/300. **B.** COMET was performed in August 2009. Photo taken one week later. **C.** Photo taken one year after operation. **D.** Three years after operation, the cornea remained clear, and best corrected vision imporved to 20/30. However, peripheral corneal NV ingrowth from 3 to 6 O/C was noted.

e. **Cultivated oral mucosal transplantation:** For patients with bilateral ocular surface diseases, where autologous ocular epithelial stem cells are not available, cultivated oral mucosal epithelial transplantation (COMET) is an alternative for reconstruction [57]. Moreover, in patients with severe chemical injury or Stevens-Johnson syndrome, when limbal stem cell transplantation has a poor prognosis, COMET can be viewed as a bridge therapy to promote wound healing in the acute stage of disease [58]. It has been demonstrated that long-term survival of transplanted OMEC can be detected in the ocular

surface [59]. However because of the limited antiangiogenic activity of OMEC, CNV can still occur (Figure 3). Accordingly, the technology of COMET may be refined if engrafted OMEC can express antiangiogenic factors by means of gene therapy.

Conventional drugs

a. **Antibiotics:** For CNV of infectious origin, antibacterial or antifungal drugs can be used to eliminate factors causing inflammation. In addition, tetracycline can inhibit CNV of non-infectious origin through inhibition of MMPs [60].

b. **Corticosteroids:** As a mainstream drug to inhibit antigen-presenting cells, a corticosteroid prohibits synthesis of proinflammatory cytokines, and suppresses proliferation and migration of vascular endothelial cells. Corticosteroids can inhibit the inflammatory reaction and decrease the destructive effect to the tissue. However, corticosteroids are not effective for the pathogens per se and thus are contraindicated in acute bacterial and fungal keratitis.

c. **Non-steroidal anti-inflammatory drugs (NSAIDs):** COX-2 inhibitors, for example, can alleviate CNV through decreased production of prostaglandins [61,62].

d. **Cyclosporin A and FK506:** Both have been shown to inhibit T lymphocytes and to significantly suppress post-transplantation rejection and neovascularization after chemical burns, and thereby to prolong the survival time of implants [63,64].

Molecular-targeted therapy

With improved understanding of regulatory mechanisms of angiogenesis, a series of targeted drugs have been developed, aiming at factors related to regulation of angiogenesis and signal pathways.

Anti-VEGF agents: SU5416 was the first reported agent to be effective in the inhibition of tumor angiogenesis via inhibition of the VEGF receptor, Flk-1 [65]. Successive studies also showed SU5416 inhibited rat corneal angiogenesis [66].

The monoclonal antibody to VEGF, or bevacizumab (Avastin), acting via inhibition of endothelial cell mitosis to suppress CNV, is currently a prevalent anti-cancer drug. Ranibizumab (Lucentis), a Fab fragment of bevacizumab used to neutralize VEGF, has a molecular weight of only one third that of bevacizumab, and a better pharmakinetic penetration effect. Ranibizumab was therefore developed and approved for intravitreal injection to treat age-related macular degeneration (AMD). Clinically, due to price concerns, bevacizumab is often chosen to treat intraocular NV [67], leaving AMD still as an off-label indication.

Amano et al. showed that VEGF is the main angiogenic factor of inflammatory CNV in rats [68]. Manzano et al. reported the use of bevacizumab in the form of eye drops to inhibit rats' CNV [69]. Subconjunctival injection or topical use of ranibizumab has been reported to treat CNV induced by recurrent pterygium [70] and corneal graft rejection [71,72]. However, corneal epithelialization is impaired, and wound healing is thus compromised, most likely because anti-VEGF agents can impede the normal repair effect of VEGF on the corneal nerve, and adversely affect the nerve's growth and regeneration [73,74].

VEGF-related agents: Sorafenib (Nexavar) can inhibit cytokines related to angiogenesis, such as the C-Raf, B-Raf, and mutant B-Raf [75], and cell surface kinases such as Kit, Flt-3, VEGFR-2, VEGFR-3, and PDGFR-β can thereby block pathological angiogenesis [76,77]. Not yet applied in clinical CNV, Sorafenib can effectively inhibit silver nitrate applicator-induced CNV in rats [78].

Sunitinib (Sutent) can inhibit the activity of VEGFR (1, 2, and 3), PDGFR (α and β), CSF-1R, Ret, Kit, and Flt-3, and therefore block the formation of pathological angiogenesis [79]. Topical administration of Sunitinib can effectively inhibit suture-induced CNV in rabbits [80].

Regorafenib can inhibit angiogenic factors (VEGF receptor, Tie2, PDGFR-β, mutant oncogenic kinases, and the fibroblast growth factor receptor), which are involved in neovascularization [81]. Regorafenib in the form of topical drops can effectively inhibit CNV after chemical injuries in rats [82].

Immunomodulatory agents: Thalidomide can assert anti-inflammatory action by inhibiting chemotaxis of neutrophil/monocytes and suppressing phagocytosis of polymorphocytes [83]. Thalidomide is involved in regulating activities of cytokines such as TNF-α, interferon, IL-2, IL-4, and IL-5. Moreover, through suppression of VEGF and basic fibroblast growth factor (bFGF), thalidomide can block the proliferation of vascular endothelial cells. However, indications for thalidomide should be cautiously reviewed due to its risk of causing neonatal malformation [84].

Other agents targeting CNV: As a mammalian target of the rapamycin kinase (mTOR) inhibitor, rapamycin (Sirolimus) can modulate the hypoxia-inducible factor (HIF), and then regulate the expression of related genes, including VEGF and PDGF, and also inhibit CNV [85-87]. Sirolimus can also block the activation of T-cells and B-cells by cytokines that exhibit immunosuppression [88]. Doxycycline (a MMP inhibitor) can strengthen corneal structure to block CNV [89]. The combination of doxycycline and bevacizumab can synergize the inhibitory effect of CNV [90].

Nucleotide-Based Therapies (Antisense Oligonucleotides, Silence-RNA and Micro-RNA)

Recently, in addition to the well-known central dogma or the two-step process of DNA-coding mRNA-protein, the role of non-coding RNA (including micro-RNA or miRNA) has been appreciated and discussed in terms of regulatory functions of cellular proteins. Moreover, in earlier times, biochemists would use antisense oligonucleotides to study inhibition of gene expression, which were later developed into short RNA fragments (silence-RNA or siRNA). Importantly, it has also been confirmed that miRNA regulatory mechanisms (the Dicer-RISC pathway) have already been present in cells; the *ex-vivo* synthesized short RNA fragments are able to inhibit specific translation functions [91]. With the specificity in inhibiting the translation of certain proteins, the siRNA can work as a target therapy. Recent evidence shows that a single miRNA, through modulating various proteins in the same pathway, is adequate for regulation of specific biological processes such as hypoxia. In hypoxia, miR-429 was induced in HUVECs. Additionally, a number of angiogenesis-related genes have been shown to be regulated by miR-429 (e.g., hypoxia-inducible factor 1A and sirtuin 1) [92]. Therefore, compared with the influence of siRNA on a single protein, the effect of miRNA is more significant on specific biological processes.

To be effective, oligonucleotides should be delivered into cells. Delivery methods, such as the gene gun and electroporation, have been reported to transfer naked nucleotide (e.g., GAPDH or opioid growth

factor receptor) into corneal cells [93,94], but they are still in the preclinical stage. It has also been reported that antisense oligonucleotides (targeting insulin receptor substrate 1), in the form of subconjunctival injection, are competent inhibitors for gene function [95]. However, recombination of genes into expression vectors and delivery into cells by carriers may soon become more clinically applicable options, given that linear nucleotides have open ends and are susceptible to exonucleases, leading to uncertainty in efficacy and half-lives. Depending on different forms of carriers, expression vectors are expected to be active from a couple of days to an extended period after incorporation into the genome.

Expression vectors and carriers

Expression vectors are utilized in gene therapy for producing specific proteins, or for interfering with specific pathways by the generation of siRNA or miRNA in cells. To guarantee the effectiveness, safety, and oncogenicity-free features in incorporated exogenous genes, the expression of a gene product should be controllable. Expression vectors with site-specific promoters, as a controlling method in gene expression, are supposed to provide better safety and site specificity in the expression of exogenous genes.

Carriers of oligonucleotides can be viral: adenovirus (AV), adeno-associated virus (AAV), and lentivirus, or non-viral such as liposomes and cationic polymers. The most common non-viral carriers are liposomes, which are aggregated by the hydrophobic ends of bipolar phospholipids at high concentrations. If mixed with DNA, the hydrophobic ends would wrap up the DNA, while exposing the hydrophilic ends to the outer surface. Phospholipids, the main components of cell membranes, would transfer DNA between cells by fusion. In general, this method has been widely used to transfect DNA into cells, but with insufficient transfer efficiency. In contrast, viral carriers have better transfection efficiency but carry the weakness of potential biohazards.

The adenovirus is the earliest viral carrier to be developed and has currently been applied in approximately 60 clinical trials involving gene therapy (clinicaltrials.gov). Severe side effects have been found in past clinical studies using adenovirus, which were attributed to the intense immune response elicited by the genes of the adenovirus in cells. Therefore, to alleviate the immune rejection, some genes (e.g., E2a) of the adenovirus have been eliminated [96]. In rat animal models, CNV can be effectively inhibited by adenovirus carriers expressing VEGF antisense RNA [97]. Antiangiogenic factors, such as the soluble variant of the VEGF receptor (sFlt-1) [98,99], endostatin [100], TIMP-3 [101], and vasohibin-1 [102], have also been demonstrated to inhibit rats' CNV through adenovirus carriers.

Adeno-associated virus (AAV) carriers have been used in approximately 30 clinical trials involving gene therapy. With the characteristics of prominent stability, low pathogenicity, low immunogenicity, a broad range of hosts, and long-term expressivity, the AAV gene drugs are increasingly appreciated. In preclinical animal studies, AAV carrying antiangiogenic factors, such as sFlt-1, endostatin, and angiostatin have been shown to suppress murine CNV [103-105]. However, because of random incarceration of AAV into the genome of host cells, the study designs should be carefully reviewed to prevent the risks of cellular abnormalities originating from destruction of functional genes in humans. Lentivirus carriers have been applied in approximately 20 clinical trials involving gene therapy, in which antiangiogenic factors such as endostatin, angiostatin, and endostatin: kringle-5 fusion protein have been demonstrated to inhibit CNV [106,107]. Similar to AAV, the lentivirus would integrate exogenous sequences into host cells' genomes. As a result, targeted integration of exogenous sequences into the safety zone of chromosomes should be considered as a safety factor before clinical applications [108,109]. In addition to targeted integration, the induced immune response modulated by the toll-like receptors (TLR), which are provoked by viral carriers, is also an important safety concern [110] (Table 1).

Expression vector	Reference
Soluble variant of the VEGF receptor (sFlt-1)	[98,99]
Tissue inhibitor of metalloproteinase-3 (TIMP-3)	[101]
Endostatin	[100,104]
Angiostatin	[103]
Vasohibin-1	[102]
Antisense oligonucleotides	
Vascular endothelial grwoth factors (VEGF)	[97]
Insulin receptor substrate 1 (IRS-1)	[95]
Silence-RNA	
VEGF-A	[113-115]
VEGF Receptor-1	
VEGF Receptor-2	
Hypoxia inducible factor-1 (HIF-1α)	[42]
Micro-RNA	
miR-31, miR -150, miR -184	[122]
miR-132	[125]
miR-204	[124]
miR-210	[122]

Table 1: Pre-clinical trials of new nucleotide-base drugs for anti-angiogenesis.

Antisense oligonucleotides for the treatment of CNV

The insulin receptor substrate 1 (IRS-1) is the downstream factor of the insulin receptor-tyrosine kinase, and suppression of IRS-1 leads to reduced expression of angiogenic factor IL-1β to inhibit CNV. It has been reported that antisense oligonucleotides of IRS-1 (GS-101) significantly suppress the expression of IRS-1 and IL-1β, and the inhibition of CNV [95]. Clinical trials of antisense oligonucleotides of GS-101 have also been completed [111,112].

Silencing-RNA for the treatment of CNV

Kin et al. reported that a mixture of siRNA with VEGF-A, VEGFR-1, and VEGFR-2 is more potent than a single siRNA to inhibit the mouse CNV [113]. Using a mouse model of chemically induced CNV, Zuo et al. demonstrated that VEGF-A siRNA can inhibit the protein expression of VEGF-A, vascularized areas, and the number of new vessels [114]. Singh et al. also demonstrated that VEGF siRNA can also suppress the hypoxia-induced VEGF in human corneal cells

[115]. Using a mouse model, Chen et al. showed that inhibition of HIF-1α by siRNA could ameliorate CNV induced by wearing contact lens [42]. Clinically, siRNA still has not been applied for the treatment of CNV. On the contrary, for intraocular neovascularization, for example, AMD, VEGF siRNA (Bevasiranib and C and 5), and VEGF receptor siRNA (siRNA-027) are now used in clinical trials [116-118]. Based on similar mechanisms of neovascularization, it is feasible that such reseach could be applied to CNV in the future.

Regulation of NV by the micro-RNA

The clinical applications of miRNA are based on the fact that several miRNA have been linked to the pro-angiogenic or anti-angiogenic biological processes [119,120]. In an animal model of myocardial infarction, Hu et al. demonstrated that miRNA-210 is able to promote proliferation and inhibit apoptosis of vascular cells [121]. Shen et al. observed reduced miR-31, miR-150, miR-184, and formation of choroidal NV in the ischemic retina, and also reduced areas of choroidal NV after intraocular injection of pre-miR-31, -150, or -184 [122]. Chen et al. showed that hypoxia promotes the expression of miRNA-130a while it inhibits the expression of GAX and HoxA5 (anti-angiogenic factor) by HUVEC and therefore enhances angiogenesis [123]. In a KLEIP$^{-/-}$ mouse model of a CNV-related study, Kather et al. demonstrated that miR-204 could inhibit the expression of angiopoietin-1 (proangiogenic factor) [124]. Moreover, given an enhanced expression of miR-132 in infectious CNV caused by herpes simplex virus (HSV), Mulik et al. designed a single-stranded small RNA antagomir-132 according to the sequences of the miRNA, and found that *in-vivo* silencing of miR-132 can effectively suppress HSV-induced CNV [125] (Table 2).

Registry No.	Drug	Targeted Gene	Indications	Phase of Trial
Gene expression				
NCT01024998	AAV2-sFLT01	*sFLT*	AMD	Phase I
NCT01494805	rAAV.sFlt-1	*sFLT*	AMD	Phase I
NCT00109499	AdGVPEDF.11D	*PEDF*	Macular degeneration	Phase I
NCT01301443	RetinoStat	*Endostatin Angiostatin*	AMD	Phase I
Antisense oligonucleotides				
GS101-P3-CG (EudraCT)	GS-101	*IRS-1*	CNV	phase III
siRNA				
NCT00722384 NCT00259753 NCT00499590	Bevasiranib	*VEGF*	Macular degeneration	Phase I Phase II Phase III
NCT00557791	Bevasiranib	*VEGF*	AMD	Phase III
NCT00363714	AGN211745 (siRNA-027)	*VEGF* receptor	Choroidal NV AMD	Phase II Phase I
NCT00395057	AGN211745 (siRNA-027)	*VEGF* receptor	Choroidal NV AMD	Phase II
NCT01445899	PF-04523655	*RTP801*	Choroidal NV	Phase II
NCT00725686	PF-04523655	*RTP801*	AMD	Phase I
miRNA				
(none)				

Table 2: Clinical trials of new nucleotide-base drugs for anti-angiogenesis.

Conclusion

CNV is the result of interactions among various pathological factors. With advanced research in the modulation of CNV biological processes, and better understanding of the interaction of multiple factors for angiogenesis, more effective and specific treatment modalities for CNV have been designed. Because the factors inciting CNV are usually continuous and long-term, in contrast to anti-VEGF antibodies which have to be applied periodically, the nucleotide-based therapy, with the feature of persistent action, is supposed to provide better therapeutic outcome than conventional drugs. With the maturation of transgenic techniques and safety validation, nucleotide-based therapy, after confirmed by relevant clinical trials, is likely to provide an alternative treatment for CNV.

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