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PRODUCTION AND APPLICATION OF ANGIOSTATINS FOR THE TREATMENT OF OCULAR NEOVASCULAR DISEASES

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Angiostatins comprise a group of kringle-containing proteolytically-derived fragments of plasminogen/plasmin, which act as potent inhibitory mediators of endothelial cells proliferation and migration. Angiostatins are involved in modulation of vessel growth in healthy tissues and various pathological conditions associated with aberrant neovascularization. The aim of the present paper was to summarize available information, including our own experimental data, on prospects of angiostatin application for treatment of ocular neovascular diseases (OND), focusing on retinal pathologies and corneal injury. In particular, literature data on prospective and retrospective studies, clinical trials and animal models relating to the pathophysiology, investigation and management of OND are described. Special emphasis was made on the laboratory approaches of production of different angiostatin isoforms, as well as comparison of antiangiogenic capacities of native and recombinant angiostatin polypeptides. Several studies reported that angiostatins may completely abolish pathologic angiogenesis in diabetic proliferative retinopathy without affecting normal retinal vessel development and without exhibiting adverse side effects. Angiostatins have been tested as a tool for corneal antiangiogenesis target therapy in order to manage diverse ocular surface pathological conditions induced by traumas, chemical burns, previous surgery, chronic contact lens wear, autoimmune diseases, keratitis and viral infections (herpes, COVID-19), corneal graft rejection, etc. Among all known angiostatin species, isolated K5 plasminogen fragment was shown to display the most potent inhibitory activity against proliferation of endothelial cells via triggering multiple signaling pathways, which lead to cell death and resulting angiogenesis suppression. Application of adenoviral genetic construct encoding angiostatin K5 as a promising tool for OND treatment illustrates a vivid example of upcoming revolution in local gene therapy. Further comprehensive studies are necessary to elucidate the clinical potential and optimal regimes of angiostatin-based intervention modalities for treating ocular neovascularization.

Key words: angiostatins, ocular neovascular diseases, retinopathy, corneal neovascularization, antiangiogenic therapy, local gene delivery.

Ocular neovascular diseases (OND), such as diabetic retinopathy, macular edema and degeneration, neovascular glaucoma, vascularized burn or traumatic corneal pain and others, represent a significant part of the pathologies that lead to vision impairments and loss [1]. A survey from 39 countries estimated that 285 million people suffer from visual impairments. The incidence rate of OND occurrence increases every year, thus these diseases pose a significant global economic and clinical burden because of having a negative impact on patients' quality of life. These diseases are associated with the development of diabetes, domestic and work-related accidences, the development of inflammation due to viral infections, including herpes and COVID-19, uncontrolled wearing of contact lenses, previous surgery, autoimmune diseases, corneal graft rejection, age-linked changes, etc. Despite highly effective treatment procedures are implied to improve

and preserve vision in such categories of patients, innovations are needed to reduce the burden of intravitreal injections and improve outcomes in patients who do not respond adequately to currently available agents. New insights in the pathogenesis of these diseases offer the opportunity to develop targeted therapies that attack the disease process more successfully than ever [2].

Many types of eye diseases, including age-related macular degeneration, diabetic retinopathy and related disorders of the retina, feature abnormal overgrowth of new retinal blood vessel branches, which can lead to progressive loss of vision and total blindness. This phenomenon is called "neovascularization". Retinal neovascularization, abnormal formation of new vessels from pre-existing capillaries, is a common complication of many ocular diseases, such as advanced diabetic retinopathy, neovascular glaucoma, some forms of age-related macular degeneration, and retinopathy of prematurity [1, 3]. Neovascularization leads to fibrosis and eventual damage to retinal tissues. It is a major cause of blindness in the industrialized countries and affects millions of people from infants to the elderly [1, 4, 5]. Angiogenesis is tightly controlled by two counter-balancing systems: angiogenic stimulators such as vascular endothelial growth factor (VEGF) and angiogenic inhibitors such as angiostatin and pigment epithelium-derived factor (PEDF) [6–8]. Endogenous angiogenic inhibitors are essential for keeping the vitreous avascular [9]. In some pathological conditions, such as diabetic retinopathy and retinopathy of prematurity, regions in the retina become hypoxic. Local hypoxia increases the production of angiogenic stimulators and decreases the production of angiogenic inhibitors, breaking the balance between the positive and negative regulators of angiogenesis. As a result, there is an excessive proliferation of capillary endothelial cells, which leads to neovascularization) [1, 7]. As the small vessels supplying the retina or cornea narrow or fail, oxygen levels in the retina decline. This low-oxygen condition, called hypoxia, is sensed by hypoxia-inducible factor-1 (HIF-1), which then triggers a complex hypoxic response. This response includes boosting production of the VEGF protein to bring more blood to areas in need to provide an adaptive beneficial response. However, chronic hypoxia leads to chronic and harmful blindness-causing overgrowth of abnormal, often leaky, new vessels. The development of anti-vascular endothelial growth factor (VEGF) agents has revolutionized the treatment of ocular neovascularization. For example, Ranibizumab (monoclonal inhibitory anti-VEGF antibody) was granted FDA approval in 2006 (Genentech, 2013). Genentech had commercial rights for Ranibizumab in the United States, Canada and Mexico, though now only retains it in the United States. Novel proangiogenic targets, such as angiopoietin and platelet-derived growth factor (PDGF), are under development for patients who respond poorly to anti-VEGF therapy and to reduce adverse effects from long-term VEGF inhibition. A rapidly advancing area is gene therapy, which may provide significant therapeutic benefits. Viral vector-mediated transgene delivery provides the potential for continuous production of antiangiogenic proteins, which would avoid the need for repeated anti-VEGF injections. Gene silencing with RNA interference to target ocular angiogenesis has been investigated in clinical trials (Fig. 1).

Although anti-VEGF drugs stabilize or improve vision quality in most patients, about 40% of patients are not significantly helped by these drugs. Moreover, researchers are concerned that the long-term blocking of VEGF, a growth factor needed for the health of many tissues including the retina, may do harm along with good. Many cases of retinal neovascularization are accompanied by the loss of tiny blood vessels elsewhere in the retina, and blocking VEGF inhibits or prevents the re-growth of these vessels. Therefore, conservative methods of pharmacotherapy of this group of diseases including the use of anti-VEGF drugs (monoclonal antibodies) do not always provide a positive therapeutic effect, which is often associated with disorders of reparative and regenerative processes in the eye and other side effects. Since both vascular endothelial dysfunction and functionally unreasonable activation of angiogenesis play a key role in the pathogenesis of OND, the use of endogenous vascular growth inhibitors for their correction may be of considerable scientific and practical interest. A number of endogenous angiogenic inhibitors have been shown to be the fragments or cryptic domains of parent large protein molecules [10–12]. For example, proteolysis of plasminogen/plasmin by different proteases releases a group of angiogenic inhibitors, referred to as angiostatins. Plasminogen contains 5 kringle domains, with each consisting of 80 amino acids [13]. Angiostatin (kringles 1–4), kringles

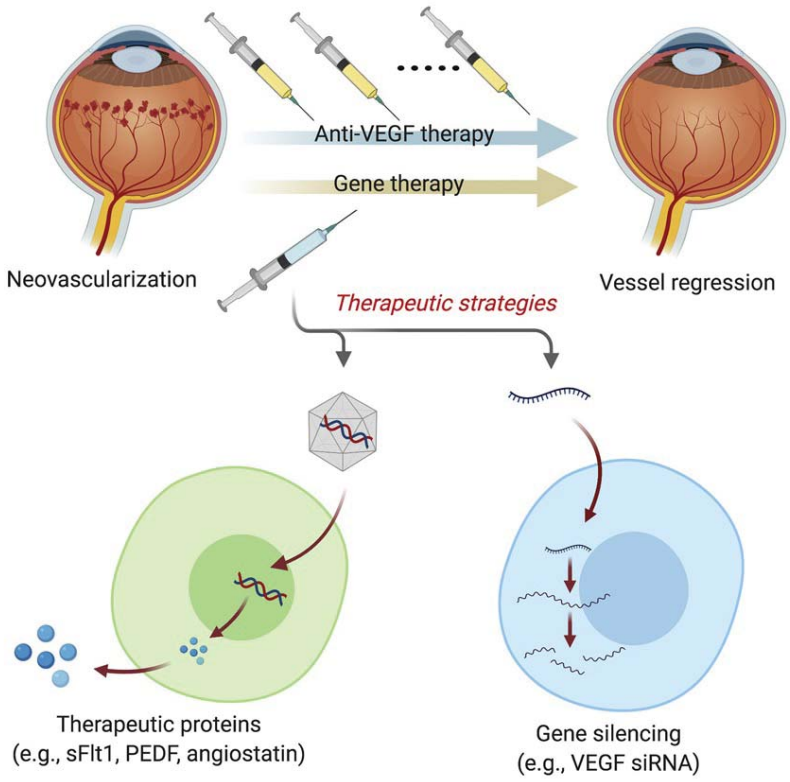

Fig. 1. **Therapeutic strategies of the ocular neovascularization treatment based on anti-VEGF therapy, gene silencing technology, and application of angiogenesis inhibition** (by Lin et al., 2020 [2])

1–5, kringles 1–3, and kringle 5 (K5) are all angiogenic inhibitors [10, 12]. Angiostatins effectively inhibit angiogenesis by specifically inducing apoptosis in endothelial cells and inhibiting their proliferative and migratory activity. Among them, isolated K5 displays the most potent inhibitory activity to endothelial cell proliferation [14]. K5 induces apoptosis and causes cell cycle arrest in proliferating endothelial cells [15]. K5 also inhibits endothelial cell migration [15, 16]. Thus, angiostatins can be used as a tool to study the molecular mechanisms of diseases associated with pathological neovascularization, as well as to serve as prototypes for the development of new effective and safe antiangiogenic drugs. Using the technology of limited proteolysis of plasminogen and purification of its products by affinity chromatography, a scheme for producing different angiostatin species (K1-3, K1-4, K4, K5) has been elaborated and successfully developed in the Department of Enzyme Chemistry & Biochemistry of IBC NASU [17–20].

Our own strong experience in the field of anti-angiogenic materials and accumulated results of current literature are believed to provide both practical and fundamental basis for further development of the highly effective ophthalmic drugs for the prevention and treatment of eye diseases associated with pathological neovascularization. In this review, we highlight the recent attempts of angiostatin application for the treatment of OND, such as corneal injuries and retina diseases. Although additional work remains, the progress described herein may pave the way to new, highly effective and important ocular medicines.

Ocular neovascular diseases: occurrence, risks, molecular basis of pathogenesis, and current treatment approaches

Ocular neovascular diseases (OND), which affect the cornea and retina, are a significant part of the pathology of the organ of vision, while studying of their mechanisms are of great medical and social importance. Today, more than 300 million people worldwide suffer from OND. The need to study the role of angiogenesis in ophthalmology is associated with a variety of conditions that are the main causes of blindness and low

vision in people of working age and are accompanied by the emergence of newly formed vessels. For example, both diabetic retinopathy and macular degeneration are associated with abnormal growth of blood vessels (neovascularization) in the retina. The macula is a specialized area of the retina that can be significantly affected by pathological processes, including age-related macular degeneration and diabetic retinopathy. The main molecular cause of disease is an imbalance between pro-angiogenic and anti-angiogenic factors (Fig. 2). The success in OND treatment achieved in the recent years was accompanied by the development of anti-neovascularization strategies primarily associated with the use of VEGF-inhibiting drugs [21].

Retinal neovascular diseases

Retinopathy with the subsequent macular degeneration in patients with diabetes develops in 50–98% of cases within 15 years after diagnosis and is the most common diabetic complication. It is initiated by microaneurysms, which are accompanied by increased permeability of the blood-retinal barrier (BRB). Subsequently, the pathological process is aggravated by macular edema, ischemic changes (focal capillary blockage), dilatation of venules, thickening of the basement membrane, sericite degeneration and the background of abnormally high levels of proliferation of fibroblasts and endothelial cells. It should be noted that the clinical manifestations of DR in insulin-dependent and non-insulin-dependent diabetes different from each other: in the first case, proliferative angioretinopathy is more often noted, and in the second — macular degeneration (maculopathy). The main problems in the treatment of this complication are retinal detachment and intraocular bleeding, so the prevention of retinal neovascularization is an important area of modern biomedicine [23].

Human retina is the deepest, light-sensitive layer of the eye tissue. Retinal blood vessels are similar to the cerebral blood vessels by their function, actually, retina is a part of the central nervous system [24]. Pericytes, glial and endothelial cells form BRB [25]. The retina is a structure with relatively high metabolic activity, cellular

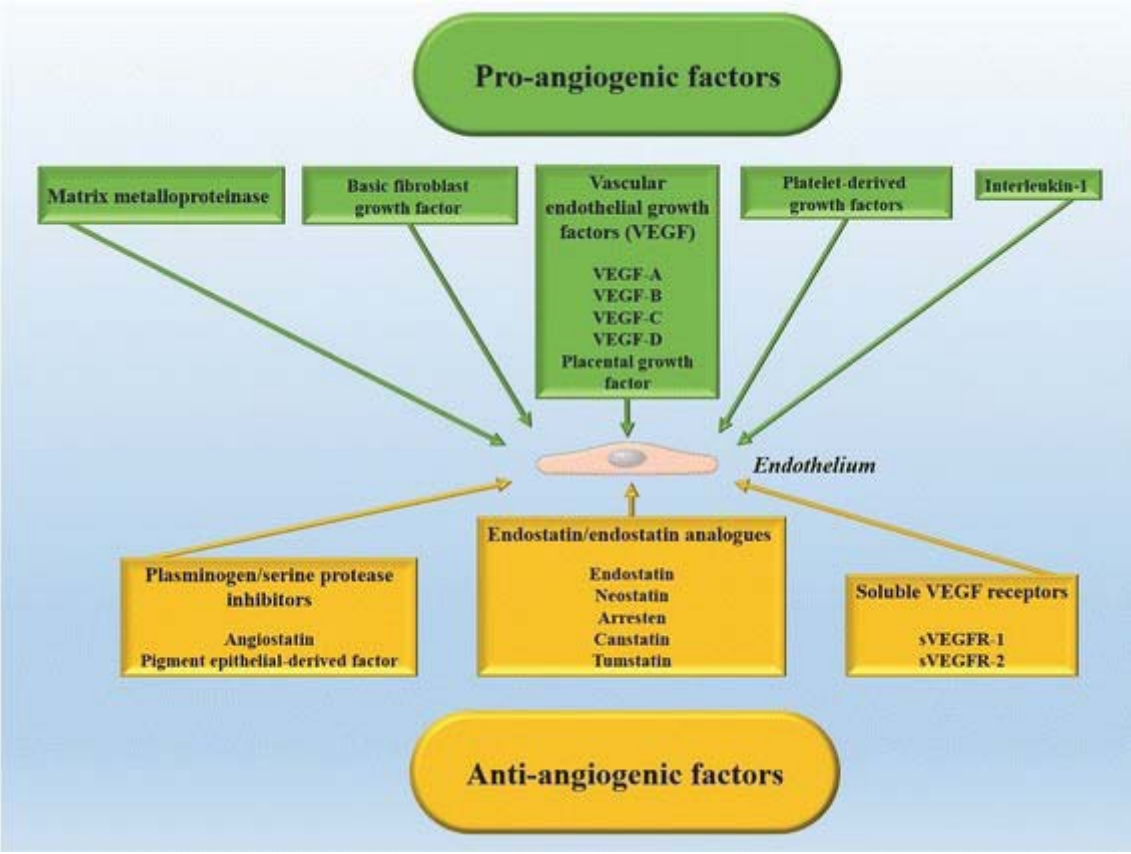

Fig. 2. **Contribution of proangiogenic and antiangiogenic factors to the regulation of neovascularization** (by Feizi et al., 2017 [22])

respiration and oxygen demand, so diabetes-induced tissue ischemia can lead to irreversible consequences. Retinal blood vessels are the main suppliers of metabolites and oxygen for neuronal and glial cells, while in turn, cells of neural origin provide retinal blood vessels with growth factors. Thus, there is a constant communication between neurons and vessels in the retina. In addition to this, the BRB plays quite a great role in maintaining functioning of retina. There is plenty of evidence indicating that retinal neovascularization is often caused by neuroinflammation [26, 27], but the ways, in which neuroinflammation regulates retinal neovascularization, remains to be discovered.

New blood microvessels proliferate during neovascularization. The new blood vessels lack tight junction proteins and consequently they differ from normal blood vessels. Blood plasma leaks from the aberrantly structured capillary into the surrounding tissue and causes the degeneration of the vitreous, resulting in vitreous hemorrhage. Severe vision loss can be caused by retinal detachment accompanied by the subsequent pull on the retina by degraded vitreous, which involves the macula [28]. Neovascularization is involved in the development of a plenty of ocular diseases, such as age-related macular degeneration (AMD), retinopathy of prematurity (ROP) and diabetic retinopathy (DR). According to The World Health Organization (WHO) data, AMD is the second the most important disease in the world, leading to visual impairment and blindness (8.7%), and the leading cause of reduced vision in economically developed countries [29]. This pathology has great socio-medical impact because of general disability due to the loss of central vision [30].

Corneal injury

Healthy cornea is an optically transparent, avascular tissue located anterior to the iris and the pupil. The transparency and avascularity is very important to protect the eye from infection and injury. New abnormal vessels tend to penetrate into the corneal stroma as a result of imbalance between angiogenic and antiangiogenic factors. This balance ensures the transparency of the cornea, and its disturbance may lead to neovascularization [31]. This can be caused by a wide range of factors such as infection, ischemia, degeneration, trauma, and loss of the limbal stem cell barrier. Lipid keratopathy, infectious keratitis, ulcers, corneal scars, eye sand, chemical burns, transplant rejection, hypoxic strokes as a result of wearing contact lenses are among the main pathologies of the cornea that can lead to neovascularization [32].

Corneal neovascularization is of great interest and concern. The efforts of scientists and physicians are focused on identifying the molecular mechanisms of diseases caused by pathological neovascularization. The main task is to find new and safe treatments for this group of diseases. According to the prognostic data, about 1.4 million patients with corneal abnormalities are predicted per year, 12% of which would lose their sight [33]. In corneal transplantation, 20% of the samples confirmed pathological neovascularization [34].

An important issue in the treatment of OND is the maturation state of blood vessels. Mature vessels do not require angiogenic growth factors, unlike immature counterparts. The latter depends on the growth factors that are required for proliferation. Therefore, the current approach in treatment is to remove the established vascular system or prevent angiogenesis [35].

Antiangiogenic drugs act through, at least, three main mechanisms: direct binding and inhibition of VEGF, suppression synthesis of VEGF, or suppression of VEGF-mediated signaling pathways [36]. The most current therapies are based on the inhibition of VEGF and its receptors. However, the use of such drugs often does not provide a full-fledged positive effect. The drugs currently used in medical practice, in addition to having a positive effect, cause a number of side effects. The proposed drugs-inhibitory antibodies to VEGF or its receptors increase intraocular pressure, exert allergic and cytotoxic effects, and may induce endophthalmitis [37].

Proteolytically-derived plasminogen fragments (angiostatins): structure, biological activity, production of the native and recombinant forms

Angiogenesis is a process of generation of new blood vessels from the pre-existing ones. Angiogenesis is a fundamental and complex process, which is mostly restricted in adults. Normally, it is involved in reproduction and wound healing. Several pathological processes, such as inflammation, cancer, endometriosis, autoimmunity, and adiposity are linked with abnormally activated angiogenesis [38]. In addition, an aberrant vessel growth plays an important role in some eye diseases leading to loss of vision. The discovery of factors that mediate this process has significantly expanded our understanding

of many normal and pathological states. Angiogenesis is strictly controlled by a wide number of pro-angiogenic (VEGF, PDGF, bFGF, EGF, MMP, fibrinogen, fibronectin, etc.) and anti-angiogenic (AS, ES, TSP-1, PF4, PEDF, TGF-b1, PAI-1, α_2-AP, TIMP, etc.) factors [39]. Imbalance of these factors may occur after eye injuries and promotes the development of various pathologies, such as neovascular glaucoma, diabetic retinopathy, chemical burns, and viral infections of the cornea [40].

Angiogenesis is a multi-stage process that includes endothelial cell proliferation, migration, basement membrane degradation, and the organization of a new lumen. Angiostatins are one of the most potent specific inhibitors of angiogenesis that specifically affect proliferating vascular endothelial cells [41]. In 1994, it was first discovered that a fragment of the heavy chain plasmin(ogen), containing the first four (of five in total) kringle domains (K1–4) and called angiostatin, suppresses angiogenesis and tumor growth [41]. Traditionally, angiostatin is considered as a structure corresponding to the kringle domain 1–3 fragment (K1–3) or kringle domain 1–4 (K1–4) fragment of plasminogen/plasmin molecule. Each kringle consists of 80 amino acids held together by three disulfide bonds and formed in loops. Later, by proteolysis of plasminogen or autolysis of plasmin, angiostatin K1–3, containing the first three kringles, and angiostatin K1–4.5, containing kringles 1–4 and 85% kringle 5 of plasminogen, were obtained. It has been shown that angiostatin K1–3 is a weaker inhibitor of endothelial cell proliferation than angiostatin K1–4 [42]. Angiostatin K1–4.5 inhibited angiogenesis and tumor growth at a dose 50 times less than K1–4 [43]. Comparative studies of plasminogen fragments (angiostatin, K1, K3, K2–3, etc.) have shown that kringle 5 (K5) exerts the most profound inhibitory activity [44] (Table).

K5-induced antiproliferative effect is several times higher than that of angiostatin, as well as that of any single kringle domain. Observed anti-endothelial activity of K5 as well as that of other kringle domains is mediated by different mechanisms. For example, electro-dependent anion channel (VDAC1) may play a role of receptor for K5 on the surface of endothelial cells. K5 binding to endothelial cells reduces intracellular pH and mitochondrial membrane hyperpolarization [45]. Both ATP synthase, associated with the cytoplasmic membrane of endothelial cells, and integrin $\alpha_v\beta_3$ have been reported to be angiostatin receptors [46].

It is concluded from these *in vitro* studies [47] that the ranking order of endothelial cell inhibition is K5 > K1, K2, K3 > K1, K2, K4 > K1 > K3 > K2 > K4. However, these *in vitro* data have not been directly translated into antiangiogenic activity *in vivo*. For example, K5 has been found to be less active than angiostatin in suppression of angiogenesis in the chick chorioallantoic membrane assay and the mouse corneal angiogenesis model [12, 48]. Insufficient suppression of *in vivo* angiogenesis by K5 is mainly due to its relatively short half-life *in vivo*. Thus, the antiangiogenic effect of a given compound should be tested in *in vivo* angiogenesis models and not only in *in vitro* endothelial cell cultures [49].

Binding of angiostatin to tissue plasminogen activator causes a decrease in migration and invasion of endotheliocytes. Angiostatins are involved in many cellular processes, including binding to ATP synthase located on the cell surface, participation in

The effects of various plasminogen fragments (angiostatins) on proliferation and migration of endothelial cells [12]

Plasminogen fragments	Inhibition of endotheliocyte proliferation		Inhibition of endotheliocyte migration	
	Effect	IC_{50}, nM	Effect	IC_{50}, nM
K1	+	320	+/–	> 1 000
K2	+	< K1, K3	+	> 100
K3	+	460	+	> 100
K4	–	–	+	500
K5	+++	50	+++	50
K1-3	+++	70	+/–	> 1 000
K2-3	+	≈ K2	++	100
K1-4	++	135	+++	50
K1-4.85	+++	10	+++	0.05
K1-5	+++	0.05	+	600

the Krebs cycle and bind to integrin, which is involved in the processes of angiogenesis. Moreover, angiostatins suppress the ability to stimulate endothelial cells and smooth muscle cells for hepatocyte growth factor, interfering with the transition from G2 phase to mitosis in the cell cycle and significantly blocking neovascularization and the growth of tumor metastases [50].

Recombinant kringle 5 of human plasminogen inhibits the migration of endothelial cells with an IC50 of approximately 50 nM. LBS kringle 5 is not involved in its antimigration activity. The antimigration activity of kringle 5 is similar to that of angiostatin. Kringle 5 shows selective inhibition of endothelial cells. Compared with its native form, the reduced kringle 5 shows a significant increase in antimigration activity, i. e. kringle conformation can prevent its effective interaction with cells. Thus, kringle 5 plasminogen is a well-established selective inhibitor of endothelial cell migration [51].

To have an experimental tool for our research, earlier we developed a method for producing functionally active fragment of human plasminogen kringle 5 with the use of chromatography on AH-Sepharose (Fig. 3). Proposed method includes the following stages: hydrolysis of plasminogen by pancreatic elastase, separation of mini-plasminogen from kringle fragments 1–3 and 4 on the Lys-Sepharose, pepsin hydrolysis of mini-plasminogen, affinity chromatography on AH-Sepharose, analytical electrophoresis in polyacrylamide gel [17]. Electrophoretically pure fragment of human plasminogen kringle 5 was isolated, while the ability of kringle 5 to bind specifically with the AH-Sepharose demonstrates its functional activity with respect to the ligands of high and low molecular weight (Fig. 4).

Studies of the therapeutic effects of angiostatins are divided into two groups depending on the nature of the tested proteins, either native or recombinant.

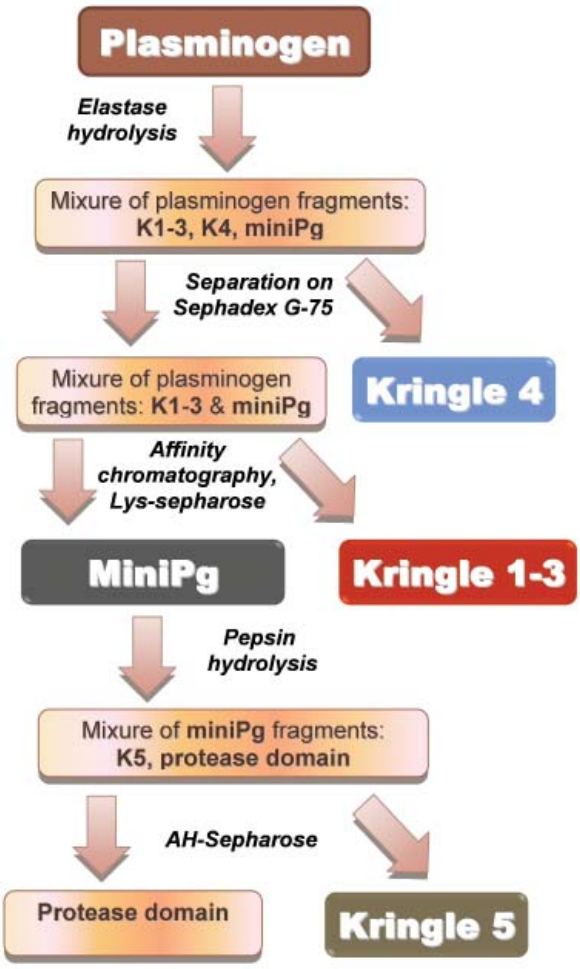

Fig. 3. **The scheme of kringle-containing plasminogen fragments isolation:** kringle 1-3, kringle 4, kringle 5, and mini-plasminogen

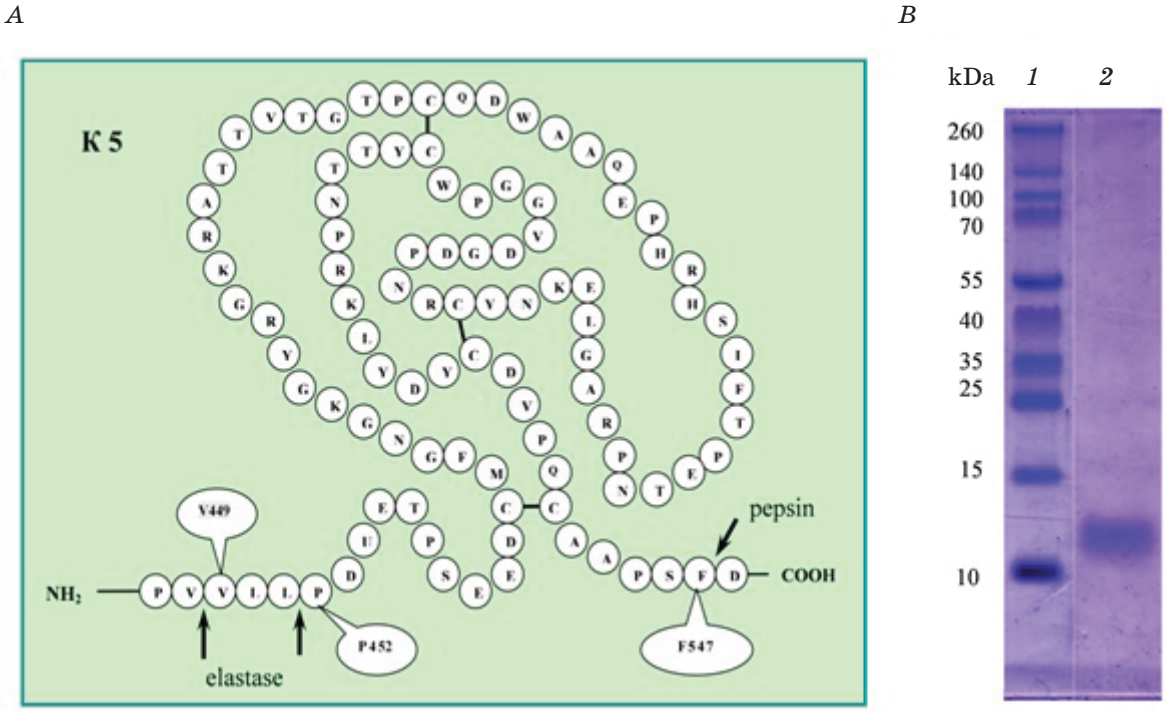

Fig. 4. **Structure of the kringle 5 fragment of human plasminogen:**
A — structural model and amino acid composition of kringle 5 (arrows indicate sites of specific elastase and pepsin hydrolysis) [17]; *B* — typical electrophoregram of isolated kringle 5 (*1* — molecular weight markers; *2* — kringle 5)

It has been suggested that one of the mechanisms of therapeutic action of laser retinal photocoagulation to prevent vision loss in retinopathy is the induction of the formation of endogenous pool of angiostatins [52]. Results of another study have indicated possibility of pharmacocorrection of diabetes-induced retinopathy by modulating angiostatin levels in the injured retina. It has been shown that inhibitors of proapoptotic enzyme PARP-1 are able to restore production of angiostatins in retinas of diabetic rats near to control levels [53]. The prospect of delivery of a genetically engineered construct containing an angiostatin-coding sequence (rAAV-AS K1-4) to retinal tissue in diabetic retinopathy has been declared [54].

It is known that under conditions of prolonged hyperglycemia and hypoxia, the formation and accumulation of advanced glycation end-products (AGEs) in the injured tissues occur. AGEs are known to be powerful inducers of oxidative stress [55]. AGEs trigger irreversible biochemical changes in protein structure, activate endothelial cells and provoke diabetic tissue fibrosis, and cause excessive production of free radicals, including reactive forms of oxygen (ROS), which in turn activate major pathways of cell death. It is known that ROS in synergism with HIF-1α increase the expression of both VEGF and its receptor. VEGF is a potent angiogenic factor, also known as vascular permeability factor (VPF), which is 50,000 times more angiogenic than histamine [56]. VEGF is able to increase the degree of permeability of retinal microvessels even at very low concentrations. Therefore, increased expression of VEGF in ischemic retinal tissue leads to accelerated proliferation of endothelial cells and, as a consequence, to the formation of microvessels with impaired structure [57]. In addition, VEGF is thought to induce retinal microangiopathy by affecting the protein metabolism of close occlusal contacts. Phosphorylation, abnormal occlusal redistribution, ubiquitination, and endocytosis of this protein caused by VEGF induce disruption of the structure of tight contacts and, subsequently, increased vascular permeability. Angiostatin is a VEGF antagonist, as indicated by preclinical studies in rats, for example with experimental diabetes mellitus [58]. It was found that the introduction of recombinant DNA encoding the sequence of angiostatin reduced VEGF levels in the retina of animals with hyperglycemia, displayed a protective effect on the components

of tight contacts and, ultimately, led to the normalization of capillary structure. It is possible that the shift in angiogenic balance in the retina in diabetes occurs because angiostatins are formed in amounts that are insufficient to balance the proangiogenic effects of VEGF. At present, the mechanisms of angiostatin formation in the retina and their contribution to the development of retinopathy still need to be established. In particular, the question of which cells are responsible for generation of angiostatins in retinal tissue remains completely unexplored. The involvement of these molecules in the pathophysiological mechanisms of diabetic retinopathy development is evidenced by the results of other preclinical trials of genetic constructs that encode a sequence corresponding to the structure of angiostatins. Their involvement in the regulation of retinal neovascularization is indicated by observations made during laser photocoagulation, which has been successfully used in clinical management of diabetic retinopathy and prevention of vision loss [59].

Modern drugs "artificial tear", in addition to the traditional polymer base, also contains ingredients that stimulate regeneration, provide a specific layer of the tear film and osmoprotection for the corneal epithelium, and enhance production of endogenous interferon [60].

Angiostatin application for corneal injury treatment

Eye trauma accounts for about 3% of all emergency department visits, with approximately 80% of these visits for corneal abrasions or foreign bodies [61, 62]. The incidence of corneal abrasion is higher among people of working age, with automotive workers between the ages of 20 and 29 years having the highest incidence of eye injuries [63]. Corneal abrasions can be caused by any type of objects including fingernails, contact lens wear, plant branches, and foreign objects blown or thrown into eyes. Lack of eye protection can result in high-speed projectile objects penetrating the cornea resulting in more serious damage. Corneal laceration and perforation can be accidental, however more often involve activities that cause high-speed projectiles such as saws, angle grinders, and pounding metal objects, with or without eye protection. It is important to elicit from the history the type of object i.e. wood or metal and estimated projectile speed.

Exposure-related burns of the eye can be categorized into chemical (acid and alkali burns), radiation burns from ultraviolet (UV) sources, and thermal burns. Alkali corneal injuries are more common than acid due to the prevalence of household cleaning agents containing ammonia and lye. Acidic burns are typically work-related injuries involving industrial processes, but can also be intentional assault [64]. Radiation burns result in ultraviolet keratitis from tanning beds, high-altitude environments, welding arcs, and the occasional solar eclipse. Thermal burns are distinctly uncommon but can occur with objects such as curling irons and with fire-related injuries. However, the prognosis after ocular burns and corneal perforations is guarded. Many of these patients may require prolonged care and some of them even have a visual loss despite of adequate treatment [65, 66].

The cornea is normally avascular and transparent structure. Control of neovascularization in both normal and pathological conditions is necessary to maintain the transparency of the cornea. Corneal neovascularization plays an important role in the pathogenesis of a number of corneal disorders. The specific angiogenic factors leading to corneal neovascularization are likely to be multiple and diverse (Fig.2). Some factors, such as FGF and TGF-α, appear to have a direct effect in inducing endothelial cell proliferation. Various approaches of the inhibition of corneal neovascularization have been investigated. Steroids, heparin [67], amiloride [68, 69], and inhibitors of arachidonic acid metabolism [70–72] have all been shown to inhibit corneal neovascularization.

It has been shown that proteolytic enzymes, including components of the fibrinolytic system, are involved in the regulation of angiogenesis. Plasmin plays a dual role in controlling the process of endothelial proliferation, depending on the phase of angiogenesis. It is known that plasmin itself, together with plasmin-activated metalloproteinases at the initial stage of vascular growth, destroys the extracellular matrix, preparing conditions for the migration of endothelial cells necessary for the formation of new vessels [73]. In addition, plasmin can stimulate the entry of cell growth factors such as VEGF and bFGF into tissues [74]. The proliferation of blood vessels is prevented by endogenous inhibitors of angiogenesis, including angiostatins, which are formed through plasminogen proteolysis by various

proteases or plasmin autolysis [12]. It was found that angiostatin K1-3 has an inhibitory effect on endothelial proliferation during neovascularization of the cornea in rabbits caused by angiogenin, bFGF, and VEGF [75]. Thus, plasmin is involved in the development of angiogenesis, while *vice versa* the plasmin(ogen) degradation product is involved in inhibition of angiogenesis.

It is well-documented that local angiostatin formation may play a crucial role in supporting angiogenic balance by counteracting pro-angiogenic VEGF signaling in cornea. It was found that neovascularization is suppressed in the human cornea as eye is closed. This might be explained by angiostatin conversion from plasminogen in the tear fluid when the eye is closed. In the tear fluid collected after a night sleep, the investigated level of plasminogen, as well as its fragments, such as K1-3, K1-4 and K5, appeared to be increased [76]. As a result, it can be concluded that plasminogen fragments perform the protective function in the cornea during compelled physiological hypoxia and prevent neovascularization and inflammation.

It has been shown that in humans, even a minor corneal trauma, observed, for example, in the Schirmer test, leads to the activation of plasminogen and an increase in the content of plasmin in the tear fluid [77]. An increase in the activity of plasmin in tears was found in patients with chemical burns, after mechanical trauma, with bacterial and difficult-to-heal corneal ulcers [78]. Thus, the corneal burn model vividly reflects all stages of angiogenesis [79]. In case of corneal burns in rabbits, plasmin activity, plasminogen levels in the lacrimal fluid, moisture of the anterior chamber, conjunctiva and cornea were studied, and the effect of instillations of the plasminogen kringle fragment (K1-4.5) on alveolar corneal neovascularization caused by alkaline burns and other clinical manifestations of eye burn disease was evaluated [78]. The revealed increase of plasmin and plasminogen levels in tears after a corneal burn in rabbits indicates the active involvement of this proteolytic system in the reparative processes in burn-wounded tissue.

Angiostatin K1-4.5 administration in the case of eye burns resulted in a powerful inhibition of corneal neovascularization. Within two weeks, there was a much slower growth of blood vessels, and they were single, in contrast to the control group. However, the subsequent administration of angiostatin caused a sharp branching of the vessels in the cornea. Therefore, it can be concluded that angiostatin to suppress corneal neovascularization should be used for no more than 2 weeks. According to the literature, suppression of endothelial proliferation, regression of newly formed corneal vessels and an anti-inflammatory effect was observed after instillations of plasminogen kringle fragment K5 in rabbits with corneal burns [80]. After alkaline eye burn, a decrease in corneal neovascularization was also found in mice after administration of K5 through an osmotic pump [81]. The decrease in the intensity of the development of corneal ulcers during local treatment with angiostatin is apparently explained not only by the suppression of the production of metalloproteinases, but also by the effect of angiostatins on the immune processes. It has been shown that endogenous angiostatins are immunomodulators, since they enhance the production of interleukin-12 by macrophages [82]. The data obtained indicate that the development of drugs based on angiostatin K1-4.5 is promising for suppressing neovascularization of the cornea, as well as for the treatment of diseases accompanied by corneal ulceration.

Angiostatins have been shown to inhibit neovascularization induced in rabbit corneal burns *in vivo*. Inhibition of plasminogen generation by angiostatins is one of the mechanisms of their complex antiangiogenic action. Thus, corneal angiogenesis inhibition was revealed with the use of plasminogen fragment [83]. Plasminogen is converted by plasminogen activator to plasmin [84]. Thus, plasminogen activators play an important role in the angiogenic process, especially degradation of the basement membrane [85, 86]. Plasminogen fragment may also inhibit corneal neovascularization by reducing activation of plasminogen activator and therefore it may be useful for the treatment of corneal angiogenic disorders. The adequate concentration and the histopathology of plasminogen fragment are still being investigated. Recently, plasminogen fragment has been reported to inhibit the growth of primary carcinoma in mice without detectable toxicity [87]. Therefore, such non-toxic angiostatic polypeptides as plasminogen fragments, can find wide clinical application.

Angiostatin application for retinopathy treatment

Diabetic retinopathy is the leading cause of visual loss in the working age group in all developed countries. Visual loss associated with diabetic retinopathy is primarily

caused by complications arising from neovascularization in proliferative retinopathy or exudation and retinal thickening associated with the development of diabetic macular edema [88]. The pathogenesis of neovascular age-related macular degeneration (AMD) is complex, the underlying cause of vision loss being choroidal neovascularization (CNV). CNV can be initiated by a number of events, such as reduction in choriocapillaris blood flow, accumulation of lipid metabolic byproducts, oxidative stress, and alterations in Bruch's membrane. In response to metabolic distress, the retinal pigment epithelium and the retina produce soluble factors that act through a variety of mechanisms, leading to CNV. Hypoxic conditions in the eye tissues induce over-expression of the signaling protein VEGF, a potent angiogenic stimulator. VEGF serves as a 'master switch' for many ocular neovascular conditions through promotion of endothelial cell proliferation and survival, vascular permeability, and ocular in ammation [89]. VEGF and other related signaling molecules increase expression of the Ras gene that encodes proteins involved in maintaining vascular growth [90].

Sima et al. [59] described effects of K1-4 on VEGF expression and other physiological parameters in the retina of diabetic animals [59]. As a result of the single injection of K1-4 7.5 mcg into the vitreous body, the degree of vascular permeability of the retina of rats with oxygen-induced and diabetic retinopathy was significantly reduced. The observed effects of angiostatin at the cellular and tissue levels correlated with a decrease of abnormally enhanced content of VEGF in the retinal tissue of diabetic animals. At the same time, angiostatin had no effect on the normal VEGF expression and the structure of retinal vessels in healthy rats. Based on the obtained data, it was suggested that angiostatin is able to suppress the development of proliferating retinopathy not by directly inhibiting vascular endotheliocytes, but rather by suppressing VEGF synthesis in the retina under hypoxic conditions caused by chronic hyperglycemia.

A wide range of retinal disorders can potentially be treated using viral vector-mediated gene therapy. The most widely used vectors for ocular gene delivery are based on adeno-associated virus (AAV), because they elicit minimal immune responses and mediate long-term transgene expression in a variety of retinal cell types. Proof-of-concept experiments have demonstrated the efficacy of AAV-mediated transgene delivery in a number of animal models of inherited and acquired retinal disorders [91].

Currently, a comprehensive approach to diabetic retinopathy therapy is being developed, which combines the use of traditional laser photocoagulation and targeted delivery of a gene construct with a vector that provides long-term expression of the angiostatic transgene. The rAAV-based vector (rAAV-AS) was used to express DNA encoding AS (K1-4) [54]. It was shown that subretinal administration of rAAV-AS to rats with streptozocin (STZ)-induced diabetes significantly reduced the degree of capillary permeability and the development of choroidal neovascularization induced by laser photocoagulation. The use of this gene delivery system opens up broad prospects for the treatment of eye diseases, since rAAV-AS is highly stable and capable of long-term expression, which allows achieving a significant therapeutic effect even after a single injection.

The proteolytic fragment of plasminogen kringle 5 (K5) competes with VEGF for binding to VEGFR [44]. Intravitreal administration of K5 inhibits retinal neovascularization and reduces vascular permeability in models of diabetic retinopathy [92].

Muller cells are the main glial cells of the retina, which are present both in the area of the spot and on the peripheral part of the retina. They play important roles in the functioning of nerve cells, metabolism and activation of light receptors in the eye. The importance of Muller cells for normal retinal function suggests that their dysfunction leads to many eye diseases, including diabetic retinopathy and macular telangiectasia. Decreased K5 receptor (K5R) expression was observed in both Muller cell culture during hypoxia or hyperglycemia (conditions that simulate some stages of proliferative diabetic retinopathy) and in the retina of rats in experimental models of oxygen– and STZ-induced retina. K5 inhibits hypoxia-induced overexpression of VEGF in cultured Muller cells. [93].

K5 is believed to have therapeutic potential in the treatment of neovascular diseases as a potent angiogenic inhibitor [14]. Recently, it was shown that intravitreal injection of recombinant K5 prevents the development and arrests the progression of ischemia-induced retinal neovascularization in a rat model [93]. In contrast to its potential therapeutic significance, little is known about the mechanism underlying the anti-angiogenic activity of K5 and other fragments

of plasminogen. It is evident that there is a delicate balance between angiogenic stimulators and angiogenic inhibitors, and this balance plays a key role in maintaining the angiogenesis rate [6, 94, 95]. Under hypoxic conditions in the retina during proliferative diabetic retinopathy and retinopathy of prematurity, the angiogenic stimulators are overproduced while the angiogenic inhibitors are down-regulated [94, 95]. The consequent disruption in the balance between these factors results in retinal neovascularization. VEGF is a major angiogenic stimulator in the retina, and increased VEGF levels have been shown to be a common pathologic factor in OND of humans, as well as in the animal model of ischemia-induced retinopathy [96–99]. PEDF has been identified as a major angiogenic inhibitor in the vitreous [6]. Reduced PEDF levels have been associated with ischemia-induced retinal neovascularization and proliferative diabetic retinopathy in patients [95, 100]. Recently, it was shown that the ratio between angiogenic stimulators and inhibitors is crucial for the control of angiogenesis in the retina. Elevated retinal angiogenic stimulators such as VEGF and decreased angiogenic inhibitors such as PEDF, resulting in an increased ratio of angiogenic stimulators to angiogenic inhibitors, contribute to retinal neovascularization in the ischemia-induced retinopathy rat model [95]. The recent study reports that K5 down-regulates an endogenous angiogenic stimulator, vascular endothelial growth factor (VEGF) and up-regulates an angiogenic inhibitor, pigment epithelium-derived factor (PEDF), in a dose-dependent manner in vascular cells and in the retina. The regulation of VEGF and PEDF by K5 in the retina correlates with its anti-angiogenic effect in a rat model of ischemia-induced retinopathy. Since PEDF has been shown to induce apoptosis [101], the up-regulation of PEDF expression by K5 may be responsible for K5 effect on the induction of apoptosis in endothelial cells.

Retinal RNA levels of both VEGF and PEDF are also changed by K5. The plasminogen kringle 5 inhibits the p42/p44 MAP kinase activation and nuclear translocation of HIF-1α, resulting in the down-regulation of VEGF. Decreased levels of endogenous angiogenic stimulators and up-regulation of endogenous angiogenic inhibitors, thus leading toward restoration of the balance in angiogenic control, may represent a mechanism for the anti-angiogenic activity of K5. The results herein support an idea that the regulation of endogenous angiogenic factors may contribute to the anti-angiogenic activity of K5 [88]. Interestingly, angiostatin has been recently shown to reduce the activation of MAP kinase ERK-1/ERK-2 (p42/p44) in human dermal microvascular endothelial cells [102]. Therefore, angiostatin and K5 may have similar anti-angiogenic mechanisms.

Multiple angiogenic stimulators and inhibitors are expressed in the retina and vascular cells [103, 104]. Insulin-like growth factor-1 has been shown to regulate the expression of VEGF in RPE cells [105], suggesting that regulatory interactions exist among angiogenic stimulators. The regulatory interactions between two counter-balancing systems of angiogenic stimulators and inhibitors have been reported [88]. This study reported that an angiogenic inhibitor can suppress the expression of angiogenic stimulators while enhancing the expression of other endogenous angiogenic inhibitors. These regulatory interactions accelerate the restoration of the balance between angiogenic stimulators and inhibitors and thus, may represent a mechanism of angiogenic control. HIF-1α is a major positive regulator of VEGF expression under hypoxia [106, 107]. Nuclear translocation of HIF-1α is a critical step in the induction of VEGF expression. The study described the nuclear HIF-1α level to be significantly elevated in the retina with neovascularization, correlating with increased VEGF expression [95]. K5 injection significantly reduced the nuclear HIF-1α levels in the retina of the retinopathy model, suggesting a decreased HIF-1α nuclear translocation. These results suggest that inhibiting HIF-1 activation is responsible, at least partially, for the decreased VEGF expression by K5 [88].

The finding that K5 specifically inhibits the activation of p42/p44 raised the question of how K5 interacts with this intracellular pathway. It was performed the study of receptor-binding assay using ^{125}I-labeled K5 and cultured endothelial cells [88]. No specific binding of K5 with endothelial cells was detected, suggesting that K5 does not have a specific receptor on endothelial cells. As VEGF can also activate the MAP kinase pathway through its receptor [108], blocking the VEGF receptor may also result in the inhibition of MAP kinase pathway. Therefore, it was also measured the effect of K5 on VEGF binding with VEGF receptor, and the results showed that K5 does not interfere with VEGF binding to its receptor. These results indicate that the inhibitory effect of K5 on MAP kinase is

neither through binding to a specific receptor on the endothelial cells nor through blocking the VEGF binding. It is possible that K5 may block the binding of other factors to their receptors and subsequently inhibit certain signal transduction pathways. It is also possible that the K5 effect is mediated by molecules in the extracellular matrix such as integrin that is essential for the sustained activation of MAP kinase by angiogenic stimulators [109, 110].

It was demonstrated that K5 affects VEGF and PEDF expression more significantly under hypoxia than under normoxia. This phenomenon may be explained by the fact that the basal level of VEGF is elevated, while that of PEDF is decreased by hypoxia in the absence of K5. The retinal hypoxia elevates VEGF, but reduces PEDF levels [95]. It can be concluded that K5 has anti-angiogenic activity only in the retina with neovascularization, but not in the normal retina [92].

Recent breakthroughs in our understanding of the molecular pathophysiology of OND have allowed specifically targeting pathological angiogenesis. Different anti-VEGF agents and other molecules affecting diverse proangiogenic secreted factors have shown potential benefit in the treatment of ocular neovascularity-based diseases, but the use of these preparations is often associated with various adverse effects. Continuous innovations in pharmacotherapy and progress in understanding of pathophysiology of eye diseases make us believe that improvements in their treatment using anti-angiogenic therapy will continue to provide minimizing side effects of therapy. From these circumstances, the therapeutic potential of angiostatins as effective and safe antiangiogenic agents for the management of a variety of retinal diseases, corneal injuries and other neovascular complications holds great promise in the near future.

Angiostatins comprise a group of kringle-containing proteolytically-derived fragments of plasminogen/plasmin, which exert potent endothelial cell inhibitory activity, including the induction of apoptosis and inhibition of migration. The intact kringle structures are believed to be necessary for the antiangiogenic activity. Angiostatins are involved in modulation of vessel growth in healthy tissues and contribute to the development of various pathological conditions associated with aberrant neovascularization, including ocular neovascular diseases, or OND. Several studies reported that angiostatins may completely abolish pathologic angiogenesis in diabetic proliferative retinopathy without affecting normal retinal vessel development and without exhibiting adverse side effects. Angiostatins are being tested as a promising tool for corneal anti-angiogenesis target therapy in order to manage diverse ocular surface pathological conditions induced by diabetic complications, chemical injury, trauma, previous surgery, chronic contact lens wear, autoimmune diseases, keratitis and viral infections (herpes, COVID-19), corneal graft rejection, etc. Among all known angiostatin species, isolated K5 plasminogen fragment was shown to display the most potent inhibitory activity against proliferation of endothelial cells via triggering multiple signaling pathways, which lead to endotheliocyte cell death and resulting angiogenesis suppression. Application of adenoviral genetic construct encoding angiostatin K5 as a promising tool for OND treatment illustrates a vivid example of upcoming revolution in local gene therapy. Efforts are now underway to translate the understanding of the biology of angiostatins to clinical practice to provide an important new tool for the treatment of OND by inhibition of angiogenesis.

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ОДЕРЖАННЯ ТА ЗАСТОСУВАННЯ АНГІОСТАТИНІВ ДЛЯ ЛІКУВАННЯ НЕОВАСКУЛЯРНИХ ЗАХВОРЮВАНЬ ОКА

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Ангіостатини становлять групу кринг(К)-вмісних протеолітичних фрагментів плазміноген/плазміну, які функціонують як потужні інгібувальні медіатори проліферації та міграції ендотелійних клітин. Вони беруть участь у модулюванні росту судин у тканинах за норми та різних патологічних станів, асоційованих з аберантною неоваскуляризацією. Метою роботи було узагальнення наявної інформації, включаючи власні експериментальні дані авторів, щодо перспектив застосування ангіостатину для лікування неоваскулярних захворювань ока (НЗО). Головну увагу зосереджено на патологіях сітківки та ушкодженні рогівки. Зокрема, описано дані літератури стосовно перспективних та ретроспективних досліджень, клінічних випробувань і патофізіологічних тваринних моделей, створених для дослідження та лікування НЗО. Особливий акцент було зроблено на лабораторних підходах до отримання різних ізоформ ангіостатину, а також на порівнянні антиангіогенних властивостей нативних та рекомбінантних поліпептидів-ангіостатиків. Результати серії досліджень свідчать, що ангіостатини можуть повністю пригнічувати патологічний ангіогенез за діабетичної проліферативної ретинопатії, не впливаючи на нормальний розвиток судин сітківки та не виявляючи несприятливих побічних ефектів. Ангіостатини випробовують як інструмент для таргетної антиангіогенної терапії рогівки з

ПОЛУЧЕНИЕ И ИСПОЛЬЗОВАНИЕ АНГИОСТАТИНОВ ДЛЯ ЛЕЧЕНИЯ НЕОВАСКУЛЯРНЫХ ЗАБОЛЕВАНИЙ ГЛАЗА

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Ангиостатины составляют группу кринг(К)-содержащих протеолитических фрагментов плазминоген/плазмина, которые функционируют как мощные ингибирующие медиаторы пролиферации и миграции эндотелиальных клеток. Ангиостатины участвуют в модулировании роста сосудов в тканях в норме и при различных патологических состояниях, ассоциированных с аберрантной неоваскуляризацией. Целью работы было обобщение имеющейся информации, включая собственные экспериментальные данные авторов, о перспективах применения ангиостатинов для лечения неоваскулярных заболеваний глаза (НЗГ). Основное внимание сосредоточено на патологиях сетчатки и повреждениях роговицы. В частности, описываются данные литературы о перспективных и ретроспективных исследованиях, клинических испытаниях и патофизиологических животных моделях, созданных для исследования и лечения НЗГ. Особый акцент был сделан на лабораторных подходах к получению различных изоформ ангиостатина, а также на сравнении антиангиогенных возможностей нативных и рекомбинантных полипептидов– ангиостатиков. Результаты серии исследований свидетельствуют, что ангиостатины могут полностью подавлять патологический ангиогенез при диабетической пролиферативной ретинопатии, не влияя на нормальное развитие сосудов сетчатки и не вызывая неблагоприятных

метою лікування різноманітних патологічних станів очної поверхні, спричинених травмами, хімічними опіками, попередніми оперативними втручаннями, постійним носінням контактних лінз, автоімунними захворюваннями, кератитами та вірусними інфекціями (герпес, COVID-19), відторгненням трансплантата рогівки тощо. Серед усіх відомих ізоформ ангіостатинів ізольований фрагмент плазміногену K5 виявляє найпотужнішу інгібувальну активність стосовно проліферації ендотеліальних клітин, пригнічуючи її через активацію множинних сигнальних шляхів, що призводять до загибелі клітин та супресії ангіогенезу. Застосування аденовірусної генетичної конструкції, що кодує ангіостатин K5, як перспективного засобу корекції НЗО є яскравим прикладом революційного підходу в таргетній генній терапії. Вважаємо за доцільне проведення подальших комплексних досліджень для з'ясування клінічного потенціалу та оптимальних режимів використання засобів на основі ангіостатину для лікування неоваскулярних патологій ока.

Ключові слова: ангіостатини, неоваскулярні захворювання ока, ретинопатія, неоваскуляризація рогівки, антиангіогенна терапія, локальне доставлення генів.

побочных эффектов. Ангиостатины испытывают как инструмент для таргетной антиангиогенной терапии роговицы с целью лечения различных патологических состояний глазной поверхности, вызванных травмами, химическими ожогами, предыдущими оперативными вмешательствами, постоянным ношением контактных линз, аутоиммунными заболеваниями, кератитами и вирусными инфекциями (герпес, COVID-19), отторжениями трансплантата роговицы и т. д. Среди всех известных изоформ ангиостатинов изолированный фрагмент плазминогена K5 обнаруживает самую мощную ингибирующую активность по отношению к пролиферации эндотелиальных клеток, подавляя ее в результате активации множества сигнальных путей, приводящих к гибели клеток и супрессии ангиогенеза. Применение аденовирусной генетической конструкции, кодирующей ангиостатин K5, как перспективного средства коррекции НЗГ представляет яркий пример революционного подхода в таргетной генной терапии. Целесообразным является проведение дальнейших комплексных исследований для выяснения клинического потенциала и оптимальных режимов использования средств на основе ангиостатинов для лечения неоваскулярных патологий глаза.

Ключевые слова: ангиостатины, неоваскулярные заболевания глаза, ретинопатия, неоваскуляризация роговицы, антиангиогенная терапия, локальная доставка генов.