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ЕКСПЕРИМЕНТАЛЬНІ СТАТТІ

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

V. L. BILOUS, L. G. KAPUSTIANENKO, A. A. TYKHOMYROV

Palladin Institute of Biochemistry of the National Academy of Sciences of Ukraine, Kyiv

E-mail: basil.bilous@gmail.com

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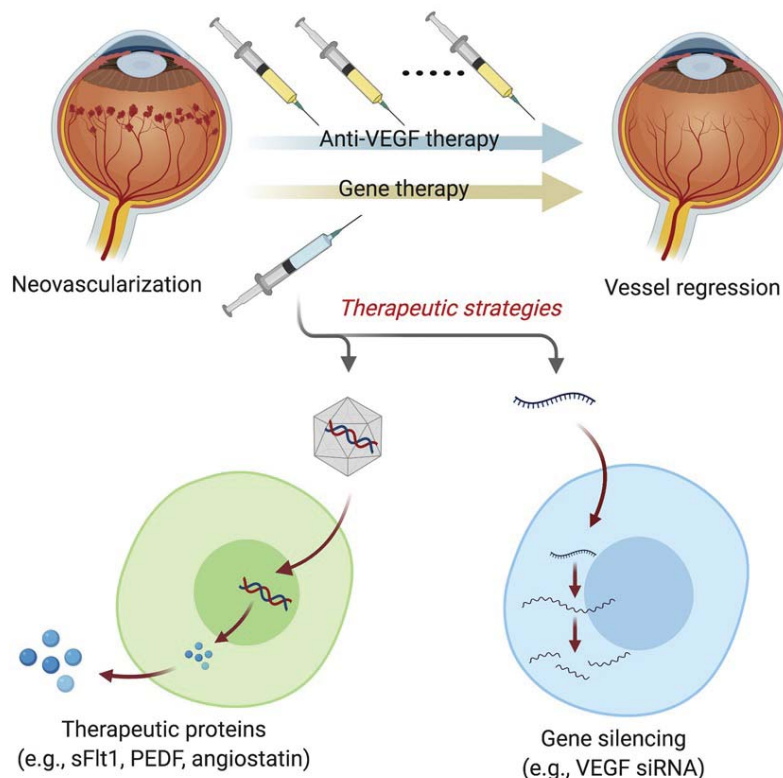
Fig. 1. **Therapeutic strategies of the ocular neovascularization treatment based on anti-VEGF therapy, gene silencing technology, and application of angiogenesis inhibition** (by Lin et al., 2020 [2])

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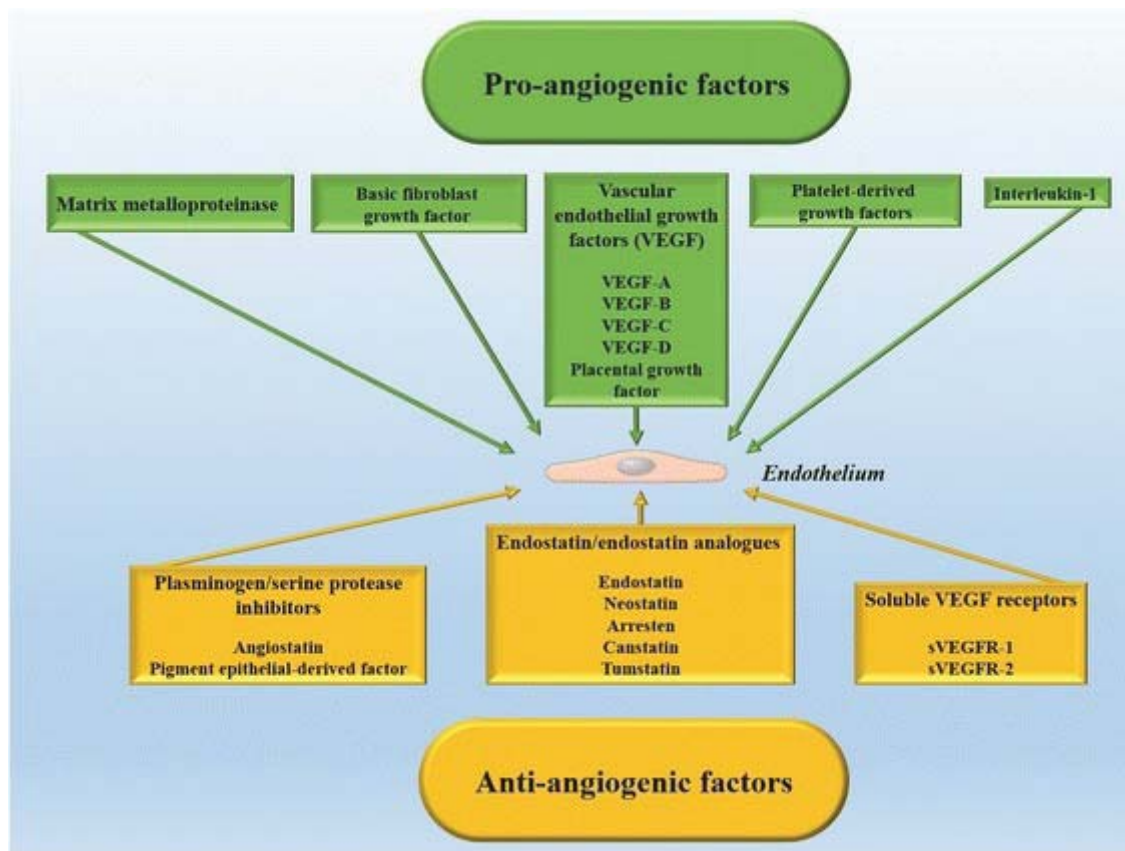
Fig. 2. **Contribution of proangiogenic and antiangiogenic factors to the regulation of neovascularization** (by Feizi et al., 2017 [22])

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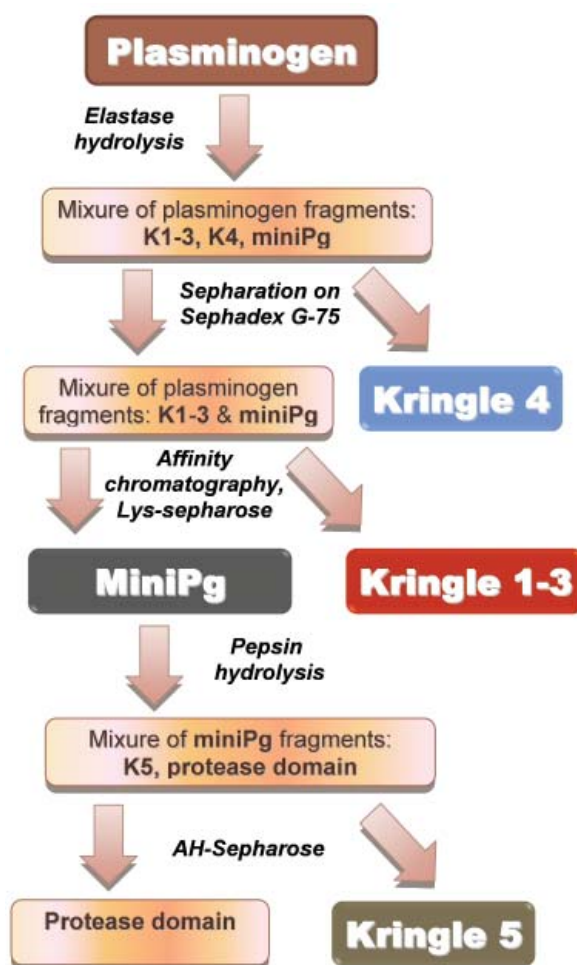
Fig. 3. **The scheme of kringle-containing plasminogen fragments isolation:**
kringle 1-3, kringle 4, kringle 5, and mini-plasminogen

A

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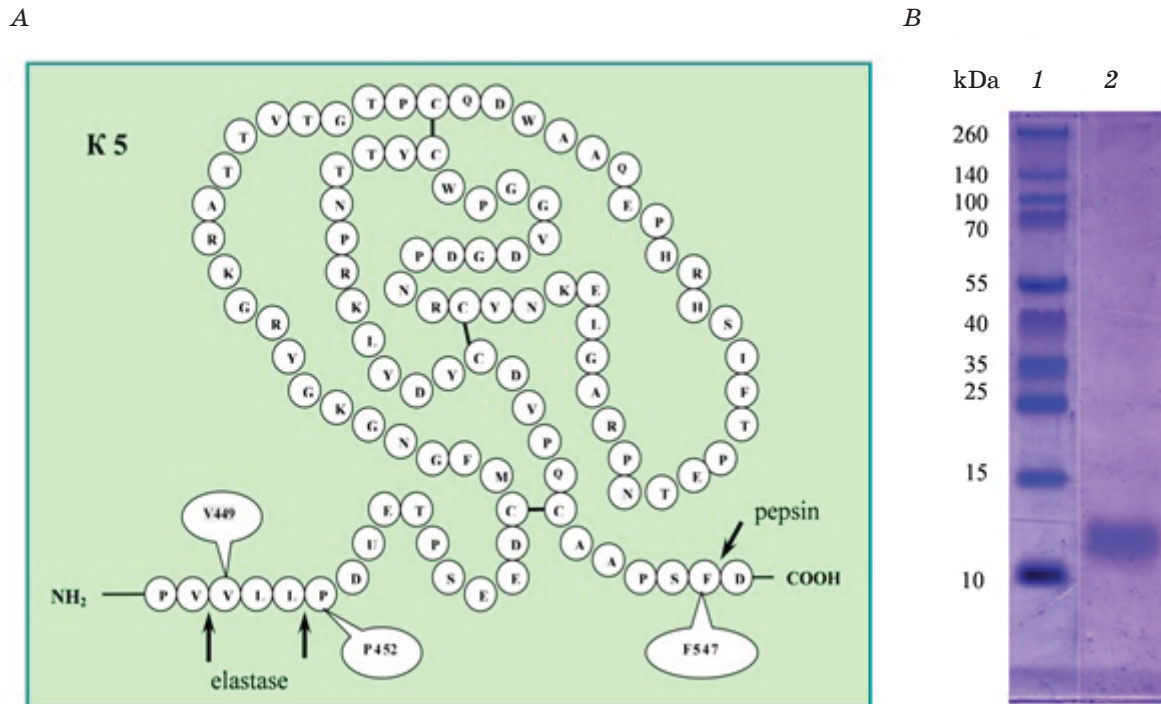
Fig. 4. Structure of the kringle 5 fragment of human plasminogen:
A — structural model and amino acid composition of kringle 5 (arrows indicate sites of specific elastase and pepsin hydrolysis) [17]; *B* — typical electrophoregram of isolated kringle 5
(*1* — molecular weight markers; *2* — kringle 5)

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.

The authors declare no competing interests.

REFERENCES

1. *Flaxman S. R., Bourne R. R. A., Resnikoff S., Ackland P. et al.* Vision Loss Expert Group of the Global Burden of Disease Study. Global causes of blindness and distance vision impairment 1990-2020: a systematic review and meta-analysis. *Lancet Glob. Health.* 2017, 5 (12), e122–e1234. https://doi.org/10.1016/S2214-109X(17)30393-5

2. *Lin F.-L., Wang P.-Y., Chuang Y.-F., Wang J.-H., Wong V. H. Y., Bui B. V., Liu G.-Sh.* Gene therapy intervention in neovascular eye disease: a recent update. *Molecular Therapy.* 2020, 28 (10), 2120–2138. https://doi.org/10.1016/j.ymthe.2020.06.029

3. *Cabral T., Mello L. G. M., Lima L. H., Polido J., Regatieri C. V., Belfort R. Jr., Mahajan V. B.*

Retinal and choroidal angiogenesis: a review of new targets. *Int. J. Retina Vitreous*. 2017, V. 3, P. 31. https://doi.org/10.1186/s40942-017-0084-9.

4. *Friedlander M*. Fibrosis and diseases of the eye. *J. Clin. Invest.* 2007, 117 (3), 576–586. https://doi.org/10.1172/JCI31030

5. *Usui Y., Westenskow P. D., Murinello S., Dorrell M. I., Scheppke L., Bucher F., Sakimoto S., Paris L. P., Aguilar E., Friedlander M*. Angiogenesis and eye disease. *Annu Rev. Vis. Sci.* 2015, V. 1, P. 155–184. https://doi.org/10.1146/annurev-vision-082114-035439

6. *Ma Q., Reiter R. J., Chen Y*. Role of melatonin in controlling angiogenesis under physiological and pathological conditions. *Angiogenesis*. 2020, 23 (2), 91–104. https://doi.org/10.1007/s10456-019-09689-7

7. *Xi L*. Pigment epithelium-derived factor as a possible treatment agent for choroidal neovascularization. *Oxid. Med. Cell. Longev.* 2020, V. 2020, P. 8941057. https://doi.org/10.1155/2020/8941057

8. *Di Somma M., Vliora M., Grillo E., Castro B. Dakou E., Schaafsma W.,·Vanparijs J., CorsiniM.,·Ravelli C.,·Sakellariou E.,·Mitola S*. Role of VEGFs in metabolic disorders. *Angiogenesis*. 2020, 23 (2), 119–130. https://doi.org/10.1007/s10456-019-09700-1

9. *Hu Y., Tang S*. Major challenges in vitreoretinal surgery. *Taiwan J. Ophthalmol.* 2015, 5 (1), 9–14. https://doi.org/10.1016/j.tjo.2014.04.005

10. *Andreoli C. M., Miller J. W*. Anti-vascular endothelial growth factor therapy for ocular neovascular disease. *Curr. Opin. Ophthalmol.* 2007, 18 (6), 502–508. https://doi.org/10.1097/ICU.0b013e3282f0ca54

11. *O'Reilly M. S., Boehm T., Shing Y., Fukai N., Vasios G., Lane W. S., Flynn E., Birkhead J. R., Olsen B. R., Folkman J*. Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. *Cell*. 1997, V. 88, P.277–285. https://doi.org/10.1016/s0092-8674(00)81848-6

12. *Cao Y., Ji R., Davidson D., Schaller J., Marti D., Sohndel S., McCance S., O'Reilly M., Llinas M., Folkman J*. Kringle domains of human angiostatin. Characterization of the anti-proliferative activity on endothelial cells. *J. Biol. Chem*. 1996, V. 271, P. 29461–29467. https://doi.org/10.1074/jbc.271.46.29461

13. *Castellino F. J., McCance S. G*. The kringle domains of human plasminogen. *Ciba Found. Symp.* 1997, V. 212, P. 46–60. https://doi.org/10.1002/9780470515457.ch4

14. *Cao Y., Chen A., An S. S. A., Ji R.-W., DavidsonD., Cao Y., Llinas M*. Kringle 5 of plasminogen is a novel inhibitor of endothelial cell growth. *J. Biol. Chem*. 1997, V. 272, P. 22924–22928. https://doi.org/10.1074/jbc.272.36.22924

15. *Lu H., Dhanabal M., Volk R., Waterman M. J., Ramchandran R., Knebelmann B., Segal M., Sukhatme V. P*. Kringle 5 causes cell cycle arrest and apoptosis of endothelial cells. *Biochem. Biophys. Res. Commun.* 1999, V. 258, P. 668–673. https://doi.org/10.1006/bbrc.1999.0612

16. *Ji W. R., Barrientos L. G., Llinas M., Gray H., Villarreal X., DeFord M. E., Castellino F. J., Kramer R. A., Trail P. A*. Selective inhibition by kringle 5 of human plasminogen on endothelial cell migration, an important process in angiogenesis. *Biochem. Biophys. Res. Commun.* 1998, V. 247, P. 414–419. https://doi.org/10.1006/bbrc.1998.8825

17. *Kapustianenko L. G., Iatsenko T. A., Yusova E. I., Grinenko T. V*. Isolation and purification of a kringle 5 from human plasminogen using AH-Sepharose. *Biotechnol. acta*. 2014, 7 (4), 35–42. https://doi.org/10.15407/biotech7.04.035

18. *Tykhomyrov A. A., Yusova E. I., Diordieva S. I., Corsa V. V., Grinenko T. V*. Production and characteristics of antibodies against K1-3 fragment of human plasminogen. *Biotechnol. acta*. 2013, 6 (1), 86–96. (In Ukrainian). https://doi.org/10.15407/biotech6.01.086

19. *Kapustianenko L. G*. Polyclonal antibodies against human plasminogen kringle 5. *Biotechnol. acta*. 2017, 10 (3), 41–49. https://doi.org/10.15407/biotech10.03.041

20. *Roka-Moya Y. M., Zhernossekov D. D., Yusova E. I., Kapustianenko L. G., Grinenko T. V*. Study of the sites of plasminogen molecule which are responsible for inhibitory effect of Lys-plasminogen on platelet aggregation. *Ukr. Biochem. J*. 2014, 86 (5), 82–88. https://doi.org/10.15407/ubj86.05.082

21. *Niu G., Chen X*. Vascular endothelial growth factor as an anti-angiogenic target for cancer therapy. *Curr. Drug. Targets*. 2010, 11 (8), 1000–1017. https://doi.org/10.2174/138945010791591395

22. *Feizi S., Azari A. A., Safapour S*. Therapeutic approaches for corneal neovascularization. *Eye Vis*. 2017, 4 (28), 1–10. https://doi.org/10.1186/s40662-017-0094-6

23. *Coats D. K*. Retinopathy of prematurity: involution, factors predisposing to retinal detachment, and expected utility of preemptive surgical reintervention. *Trans. Am. Ophthalmol. Soc*. 2005, V. 103, P. 281–312. PMID: 17057808

24. *Campbell M., Humphries P*. The blood-retina barrier: tight junctions and barrier modulation. *Adv. Exp. Med. Biol*. 2012, V. 763, P. 70–84. PMID: 23397619

25. *Cunha-Vaz J., Bernardes R., Lobo C*. Blood-retinal barrier. *Eur. J. Ophthalmol*. 2011,

21 (6), 3–9. https://doi.org/10.5301/EJO.2010.6049

26. *O'Connor A. R., Wilson C. M., Fielder A. R.* Ophthalmological problems associated with preterm birth. *Eye (Lond.).* 2007, 21 (10), 1254–1260. https://doi.org/10.1038/sj.eye.6702838

27. *Sun J. K., Radwan S. H., Soliman A. Z., Lammer J., Lin M. M., Prager S. G., Silva P. S., Aiello L. B., Aiello L. P.* Neural Retinal Disorganization as a Robust Marker of Visual Acuity in Current and Resolved Diabetic Macular Edema. *Diabetes.* 2015, 64 (7), 2560–2570. https://doi.org/10.2337/db14-0782

28. *Campochiaro P.* Ocular Neovascularization. *J. Mol. Med. (Berl.)* 2013, 91 (3), 311–321. https://doi.org/10.1007/s00109-013-0993-52013

29. *GBD 2019 Blindness and Vision Impairment Collaborators on behalf of the Vision Loss; Expert Group of the Global Burden of Disease Study.* Causes of blindness and vision impairment in 2020 and trends over 30 years, and prevalence of avoidable blindness in relation to VISION 2020: the Right to Sight: an analysis for the Global Burden of Disease Study. *Lancet Global Health.* 2020. S2214-109X(20)30489-7. https://doi.org/10.1016/S2214-109X(20)30489-7

30. *Gadzhieva B. K.* Ocular neovascular-related diseases: immunological mechanisms of development and the potential of anti-angiogenic therapy. *Ophthalmol. J.* 2016, 9 (4), 58–67. https://doi.org/10.17826/OV9458-67

31. *Beebe D. C.* Maintaining transparency: a review of the developmental physiology and pathophysiology of two avascular tissues. *Semin. Cell. Dev. Biol.* 2008, 19 (2), 125–133. https://doi.org/10.1016/j.semcdb.2007.08.014

32. *Chang J. H., Gabison E. E., Kato T., Azar D. T.* Corneal neovascularization. *Curr. Opin. Ophthalmol.* 2001, 12 (4), 242–249. https://doi.org/ 10.1097/00055735-200108000-00002

33. *Lee P., Wang C. C., Adamis A. P.* Ocular neovascularization: an epidemiologic review. *Surv. Ophthalmol.* 1998, 43 (3), 245–269. https://doi.org/10.1016/s0039-6257(98)00035-6

34. *Abdelfattah N. S., Amgad M., Zayed A. A., Salem H., Elkhanany A. E., Hussein H., Abd El-Baky N.* Clinical correlates of common corneal neovascular diseases: A literature review. *Int. J. Ophthalmol.* 2015, 8 (1), 182–193. https://doi.org/10.3980/j.issn.2222-3959.2015.01.32

35. *Sharif Z., Sharif W.* Corneal neovascularization: updates on pathophysio-logy, investigations & management. *Rom. J. Ophthalmol.* 2019, 63 (1), 15–22. https://doi.org/10.22336/rjo.2019.4

36. *Pons-Cursach R., Casanovas* O. Mechanisms of Anti-Angiogenic Therapy. *Tumor Angiogenesis.* 2017, P. 1–25. https://doi.org/10.1007/978-3-319-31215-6_2-2

37. *Roodhart J. M., Langenberg M. H., Witteveen E., Voest E. E.* The molecular basis of class side effects due to treatment with inhibitors of the VEGF/VEGFR pathway. *Curr. Clin. Pharmacol.* 2008, 3 (2), 132–143. https://doi.org/10.2174/157488408784293705

38. *Griffioen A. W., Molema G.* Angiogenesis: potentials for pharmacologic intervention in the treatment of cancer, cardiovascular diseases, and chronic inflammation. *Pharmacol. Rev.* 2000, 52 (2), 237–268. PMID: 10835101

39. *Polverini P. J.* Angiogenesis in health and disease: insights into basic mechanisms and therapeutic opportunities. *J. Dent. Educ.* 2002, 66 (8), 962–975. PMID: 12214844

40. *Yang H., Yu X., Sun X.* Neovascular glaucoma: handling in the future. *Taiwan J. Ophthalmol.* 2018, 8 (2), 60–66. https://doi.org/10.4103/tjo.tjo_39_18

41. *O'Reilly M. S., Holmgren L., Shing Y., Chen C., Rosenthal R. A., Moses M., Lane W. S., Cao Y., Sage E. H., Folkman J.* Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. *Cell.* 1994, V. 79, P. 315–328. https://doi.org/10.1016/0092-8674(94)90200-3

42. *Lee T. Y., Muschal S., Pravda E. A., Folkman J., Abdollahi A., Javaherian K.* Angiostatin regulates the expression of antiangiogenic and proapoptotic pathways via targeted inhibition of mitochondrial proteins. *Blood.* 2009, 114 (9), 1987–1998. https://doi.org/10.1182/blood-2008-12-197236

43. *Cao R., Wu H. L., Veitonmäki N., Linden P., Farnebo J., Shi G. Y., Cao Y.* Suppression of angiogenesis and tumor growth by the inhibitor K1-5 generated by plasmin-mediated proteolysis. *Proc. Natl. Acad. Sci. USA.* 1999, 96 (10), 5728–5733. https://doi.org/10.1073/pnas.96.10.5728

44. *Cao Y., Chen A., An S. S., Ji R. W., Davidson D., Llinás M.* Kringle 5 of plasminogen is a novel inhibitor of endothelial cell growth. *J. Biol. Chem.* 1997, 272 (36), 22924–22928. https://doi.org/10.1074/jbc.272.36.22924

45. *Gonzalez-Gronow M., Kalfa T., Johnson C. E., Gawdi G., Pizzo S. V.* The voltage-dependent anion channel is a receptor for plasminogen kringle 5 on human endothelial cells. *J. Biol. Chem.* 2003, 278 (29), 27312–27318. https://doi.org/10.1074/jbc.M303172200

46. *Tarui T., Mazar A. P., Cines D. B., Takada Y.* Urokinase-type plasminogen activator

receptor (CD87) is a ligand for integrins and mediates cell-cell interaction. *J. Biol. Chem.* 2001, V. 276, P. 3983–3990. https://doi.org/10.1074/jbc.M008220200

47. *Bohnsack R. N., Patel M., Olson L. J., Twining S. S., Dahms N. M.* Residues essential for plasminogen binding by the cation-independent mannose 6-phosphate receptor. *Biochem.* 2010, 49 (3), 635–644. https://doi.org/10.1021/bi901779p

48. *Llombart-Bosch A., López-Guerrero J. A., Felipo V.* New trends in cancer for the 21st century. *Springer Netherlands.* 2006, P. 251–275. https://doi.org/10.1007/978-1-4020-5133-3

49. *Cao Y., Xue L.* Angiostatin. *Semin. Thromb. Hemost.* 2004, 30 (1), 83–93. https://doi.org/10.1055/s-2004-822973

50. *Böhm M. R., Hodes F., Brockhaus K., Hummel S., Schlatt S., Melkonyan H., Thanos S.* Is angiostatin involved in physiological foveal avascularity? *Invest. Ophthalmol. Vis. Sci.* 2016, 57 (11), 4536–4552. https://doi.org/10.1167/iovs.16-19286

51. *Tykhomyrov A. A., Shram S. I., Grinenko T. V.* The Role of angiostatins in diabetic complications. *Biochemistry (Moscow). Supplement Series B. Biomedical Chemistry.* 2014, 8 (2), 94–107 https://doi.org/10.1134/S1990750814020140

52. *Spranger J., Bühnen J., Jansen V.* Systemic levels contribute significantly to increased intraocular IGF-I, IGF-II and IGF-BP3 [correction of IFG-BP3] in proliferative diabetic retinopathy. *Horm. Metab. Res.* 2000, 32 (5), 196–200. https://doi.org/10.1055/s-2007-978621

53. *Guzyk M. M., Tykhomyrov A. A., Nedzvetsky V. S., Prischepa I. V., Grinenko T. V., Yanitska L. V., Kuchmerovska T. M.* Poly(ADP-Ribose) polymerase-1 (PARP-1) inhibitors reduce reactive gliosis and improve angiostatin levels in retina of diabetic rats. *Neurochem. Res.* 2016, 41 (10), 2526–2537. https://doi.org/10.1007/s11064-016-1964-3

54. *Lai C. C., Wu W. C., Chen S. L.* Suppression of choroidal neovascularization by adeno-associated virus vector expressing angiostatin. *Vis. Sci.* 2001, 42 (10), 2401–2407.

55. *Liguori I., Russo G., Curcio F., Bulli G., Aran L., Della-Morte D., Gargiulo G., Testa G., Cacciatore F., Bonaduce D., Abete P.* Oxidative stress, aging, and diseases. *Clin. Interv. Aging.* 2018, V. 13, P. 757–772. https://doi.org/10.2147/CIA.S158513

56. *Ali M. M., Janic B., Babajani-Feremi A., Varma N. R., Iskander A. S., Anagli J., Arbab A. S.* Changes in vascular permeability and expression of different angiogenic factors following anti-angiogenic treatment

in rat glioma. *PLoS One.* 2010, 5 (1), e8727. https://doi.org/10.1371/journal.pone.0008727

57. *Penn J. S., Madan A., Caldwell R. B., Bartoli M., Caldwell R. W., Hartnett M. E.* Vascular endothelial growth factor in eye disease. *Prog. Retin. Eye Res.* 2008, 27 (4), 331–371. https://doi.org/10.1016/j.preteyeres.2008.05.001

58. *Fitzgerald K. A., O'Neill L. A. J., Gearing A. J. H., Callard R. E.* Angiostatin. *The Cytokine Facts Book and Webfacts (Second Edition), Academic Press.* 2001, P. 139–141. https://doi.org/10.1016/B978-012155142-1/50026-9

59. *Sima J., Zhang S. X., Shao C.* The effect of angiostatin on vascular leakage and VEGF expression in rat retina. *FEBS Letters.* 2004, 564 (1–2), 19–23. https://doi.org/10.1016/S0014-5793(04)00297-2

60. *Macsai M., Mojica G.* Ocular surface disease: cornea, conjunctiva and tear film. *Saunders.* 2013, P. 293–308. https://doi.org/10.1016/C2010-0-68489-6

61. *Klopfer J., Tielsch J. M., Vitale S., See L. C., Canner J. K.* Ocular trauma in the United States. Eye injuries resulting in hospitalization, 1984 through 1987. *Arch. Ophthalmol.* 1992, 110 (6), 838–842. https://doi.org/10.1001/archopht.1992.01080180110037

62. *Thylefors B.* Epidemiological patterns of ocular trauma. *Aust N. Z. J. Ophthalmol.* 1992, 20 (2), 95–98. https://doi.org/10.1111/j.1442-9071.1992.tb00718.x

63. *Lim C. H., Turner A., Lim B. X.* Patching for corneal abrasion. *Cochrane Database Syst. Rev.* 2016, 7 (7), CD004764. https://doi.org/10.1002/14651858.CD004764.pub3

64. *Hossain R. R., Papamichael E., Coombes A.* East London deliberate corrosive fluid injuries. *Eye (Lond).* 2020, 34 (4), 733–739. https://doi.org/10.1038/s41433-019-0593-x

65. *Dave V. P., Pathengay A., Braimah I. Z., Panchal B., Sharma S., Pappuru R. R., Mathai A., Tyagi M., Narayanan R., Jalali S., Das T.* Enterococcus endophthalmitis: clinical settings, antimicrobial susceptibility, and management outcomes. *Retina.* 2020, 40 (5), 898–902. https://doi.org/10.1097/IAE.0000000000002462

66. *Ross M., Deschênes J.* Practice patterns in the interdisciplinary management of corneal abrasions. *Can. J. Ophthalmol.* 2017, 52 (6), 548–551. https://doi.org/10.1016/j.jcjo.2017.03.016

67. *Folkman J., Weisz P. B., Joullie M. M.* Control of angiogenesis with synthetic heparin substitutes. *Science.* 1989, V. 243, P. 1490–1495. https://doi.org/10.1126/science.2467380

68. *Avery R. L., Connor T. B., Farazdaghi M.* Systemic amiloride inhibits experimentally

induced neovascularization. *Arch. Ophthalmol.* 1990, V. 108, P. 1474–1478. https://doi.org/10.1001/archopht.1990.01070120122041

69. *Romani P., Valcarcel-Jimenez L., Frezza C., Dupont S.* Crosstalk between mechanotransduction and metabolism. *Nat. Rev. Mol. Cell Biol.* 2021, 22 (1), 22–38. https://doi.org/10.1038/s41580-020-00306-w

70. *Deutsch T. A., Hughes W. F.* Suppressive effects of indomethacin on thermally induced neovascularization of rabbit corneas. *Am. J. Ophthalmol.* 1979, V. 87, P. 536–540. https://doi.org/10.1016/0002-9394(79)90245-9

71. *Duffin R. M., Weissman B. A., Gtasser D. B.* Flurbiprofen in the treatment of corneal neovascularization induced by contact lenses. *Am. J. Ophthalmol.* 1982, V. 93, P. 607–611. https://doi.org/10.1016/s0002-9394(14)77376-3

72. *Verbey N. L. J., Van Haeringen N. J., De Jong P. R. V. M.* Modulation of immunogenic keratitis in rabbits by topical administration of inhibitors of lipoxygenase and cyclooxygenase. *Curr. Eye Res.* 1988, V. 7, P. 361–365. https://doi.org/10.3109/02713688809031785

73. *Hanahan D., Folkman J.* Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *J. Cell.* 1996, V. 86, P. 353–364. https://doi.org/10.1016/s0092-8674(00)80108-7

74. *Plouët J., Moro F., Bertagnolli S., Coldeboeuf N., Mazarguil H., Clamens S., Bayard F.* Extracellular cleavage of the vascular endothelial growth factor 189-amino acid form by urokinase is required for its mitogenic effect. *J. Biol. Chem.* 1997, V. 272, P. 13390–13396. https://doi.org/10.1074/jbc.272.20.13390

75. *Kim J. H., Kim J. C., Shin S. H., Chang S. I., Lee H. S., Chung S. I.* The inhibitory effects of recombinant plasminogen kringle 1-3 on the neovascularization of rabbit cornea induced by angiogenin, bFGF, and VEGF. *Exp. Mol. Med.* 1999, V. 31, P. 203–209. https://doi.org/10.1038/emm.1999.33

76. *Sack R. A., Beaton A. R., Sathe S.* Diurnal variations in angiostatin in human tear fluid: a possible role in prevention of corneal neovascularization. *Curr. Eye Res.* 1999, 18 (3), 186–193. https://doi.org/10.1076/ceyr.18.3.186.5367

77. *Wang H., Berman M., Law M.* Latent and active plasminogen activator in corneal ulceration. *Invest. Ophthalmol. Vis. Sci.* 1985, V. 26, P. 511–524. PMID: 8578451

78. *Chesnokova N. B., Aisina R. B., Mukhametova L. I., Pavlenko T. A.,* Gulin D. A., Beznos O. V. Fibrinolysis components and angiogenesis regulation by example of burn-induced corneal neovascularization in rabbits. *Vestn. Oftalmol.* 2012, 128 (4), 62–65. PMID: 22994111

79. *Ahmed A., Berati H., Nalan A., Aylin S.* Effect of bevacizumab on corneal neovascularization in experimental rabbit model. *Clin. Experiment. Ophthalmol.* 2009, 37 (7), 370–376. https://doi.org/10.1111/j.1442-9071.2009.02112.x

80. *Zhang Z., Ma J. X., Gao G., Li C., Luo L., Zhang M., Yang W., Jiang A., Kuang W., Xu L., Chen J., Liu Z.* Plasminogen kringle 5 inhibits alkali-burn-induced corneal neovascularization. *Invest. Ophthalmol. Vis. Sci.* 2005, 46 (11), 4062–4071. https://doi.org/10.1167/iovs.04-1330

81. *Ambati B. K., Joussen A. M., Ambati J., Moromizato Y., Guha C., Javaherian K., Gillies S., O'Reilly M. S., Adamis A. P.* Angiostatin inhibits and regresses corneal neovascularization. *Arch. Ophthalmol.* 2002, 120 (8), 1063–1068. https://doi.org/10.1001/archopht.120.8.1063

82. *Albini A., Brigati C., Ventura A., Lorusso G., Pinter M., Morini M., Mancino A., Sica A., Noonan D. M.* Angiostatin anti-angiogenesis requires IL-12: the innate immune system as a key target. *J. Transl. Med.* 2009, V. 14, P. 5–7. https://doi.org/10.1186/1479-5876-7-5

83. *Murata M., Nakagawa M., Takahashi S.* Inhibitory effects of plasminogen fragment on experimentally induced neovascularization of rat corneas. *Graefe's Arch. Clin. Exp. Ophthalmol.* 1997, V. 235, P. 584–586. https://doi.org/10.1007/BF00947088

84. *Vassalli J. D., Sappino A. P., Belin D.* The plasminogen activator/plasmin system. *J. Clin. Invest.* 1991, V. 88, P. 1067–1072. https://doi.org/10.1172/JCI115405

85. *Vogten J. M., Reijerkerk A., Meijers J. C., Voest E. E., Borel Rinkes I. H., Gebbink M. F.* The role of the fibrinolytic system in corneal angiogenesis. *Angiogenesis.* 2003, 6 (4), 311–316. https://doi.org/10.1023/B:AGEN.0000029414.24060.fe

86. *Mignatti P., Rifkin D. B.* Plasminogen activators and angiogenesis. *Curr. Top. Microbiol. Immun.* 1996, V. 213, P. 33–50. https://doi.org/10.1007/978-3-642-61107-0_3

87. *O'Reilly M. S., Holmgren L., Chert C., Folkman J.* Angiostatin induces and sustains dormancy of human primary tumors in mice. *Nat. Med.* 1996, V. 2, P. 689–692. https://doi.org/10.1038/nm0696-689

88. *Gao G., Li Y., Gee S., Dudley A., Fant J., Crosson C., Ma J. X.* Down-regulation of

vascular endothelial growth factor and up-regulation of pigment epithelium-derived factor: a possible mechanism for the anti-angiogenic activity of plasminogen kringle 5. *J. Biol. Chem.* 2002, V. 277, P. 9492–9497. https://doi.org/10.1074/jbc.M108004200

89. *Ng E. W. M., Adamis A. P.* Targeting angiogenesis, the underlying disorder in neovascular age-related macular degeneration. *Can. J. Ophthalmol.* 2005, V. 40, P. 352–368. https://doi.org/10.1016/S0008-4182(05)80078-X

90. *Miller J. W., Adamis A. P., Aiello L. P.* Vascular endothelial growth factor in ocular neovascularization and proliferative diabetic retinopathy. *Diabetes Metab. Rev.* 1997, 13 (1), 37–50. https://doi.org/10.1002/(sici)1099-0895(199703)13:1<37::aid-dmr174>3.0.co;2-k

91. *Buch P. K., Bainbridge J. W., Ali R. R.* AAV-mediated gene therapy for retinal disorders. *Gene Therapy.* 2008, V. 15, P. 849–857. https://doi.org/10.1038/gt.2008.66

92. *Zhang D., Kaufman P. L., Gao G., Saunders R. A., Ma J. X.* Intravitreal injection of plasminogen kringle 5, an endogenous angiogenic inhibitor, arrests retinal neovascularization in rats. *Diabetologia.* 2001, 44 (6), 757–765. https://doi.org/10.1007/s001250051685

93. *Ma J., Li Ch., Shao Ch., Gao G., Yang X.* Decreased K5 receptor expression in the retina, a potential pathogenic mechanism for diabetic retinopathy. *Mol. Vis.* 2012, V. 18, P. 330–336. PMID: 22355244

94. *Ferrara N., Gerber H. P., LeCouter J.* The biology of VEGF and its receptors. *Nat Med.* 2003, 9 (6), 669–676. https://doi.org/10.1038/nm0603-669

95. *Gao G. Q., Li Y., Zhang D. C., Gee S., Crosson C., Ma J. X.* Unbalanced expression of VEGF and PEDF in ischemia-induced retinal neovascularization. *FEBS Lett.* 2001, V. 489, P. 270–276. https://doi.org/10.1016/s0014-5793(01)02110-x

96. *Aiello L. P., Avery R. L., Arrigg P. G., Keyt B. A., Jampel H. D., Shah S. T., Pasquale L. R., Thieme H., Iwamoto M. A., Park J. E.* Vascular endothelial growth factor in ocular fluid of patients with diabetic retinopathy and other retinal disorders. *N. Engl. J. Med.* 1994, V. 331, P. 1480–1487. https://doi.org/10.1056/NEJM199412013312203

97. *Pe'er J., Folberg R., Itin A., Gnessin H., Hemo I., Keshet E.* Vascular endothelial growth factor upregulation in human central retinal vein occlusion. *Ophthalmology.* 1998, 105 (3), 412–416. https://doi.org/10.1016/S0161-6420(98)93020-2

98. *Pierce E. A., Avery R. L., Foley E. D., Aiello L. P., Smith L. E.* Vascular endothelial growth factor/vascular permeability factor expression in a mouse model of retinal neovascularization. *Proc. Natl. Acad. Sci. U. S. A.* 1995, V. 92, P. 905–909. https://doi.org/10.1073/pnas.92.3.905

99. *Adamis A. P., Miller J. W., Bernal M. T., D'Amico D. J., Folkman J., Yeo T. K., Yeo K. T.* Increased vascular endothelial growth factor levels in the vitreous of eyes with proliferative diabetic retinopathy. *Am. J. Ophthalmol.* 1994, V. 118, P. 445–450. https://doi.org/10.1016/s0002-9394(14)75794-0

100. *Umeda N., Ozaki H., Hayashi H., Miyajima-Uchida H., Oshima K.* Colocalization of Tie2, angiopoietin 2 and vascular endothelial growth factor in fibrovascular membrane from patients with retinopathy of prematurity. *Ophthalmic Res.* 2003, 35 (4), 217–223. https://doi.org/10.1159/000071173

101. *Stellmach V., Crawford S. E., Zhou W., Bouck N.* Prevention of ischemia-induced retinopathy by the natural ocular antiangiogenic agent pigment epithelium-derived factor. *Proc. Natl. Acad. Sci. U. S. A.* 2001, V. 98, P. 2593–2597. https://doi.org/10.1073/pnas.031252398

102. *Redlitz A., Daum G., Sage E. H.* Angiostatin diminishes activation of the mitogen-activated protein kinases ERK-1 and ERK-2 in human dermal microvascular endothelial cells. *J. Vasc. Res.* 1999, V. 36, P, 28–34. https://doi.org/10.1159/000025623

103. *Jacobson B., Basu P. K., Hasany S. M.* Vascular endothelial cell growth inhibitor of normal and pathologic human vitreous. *Arch. Ophthalmol.* 1984, V. 102, P. 1543–1545. https://doi.org/10.1001/archopht.1984.01040031259031

104. *Glaser B. M., Campochiaro P. A., Davis J. L., Sato M.* Retinal pigment epithelial cells release an inhibitor of neovascularization. *Arch. Ophthalmol.* 1985, V. 103, P. 1870–1875. https://doi.org/10.1001/archopht.1985.01050120104029

105. *Punglia R. S., Lu M., Hsu J., Kuroki M., Tolentino M. J., Keough K., Levy A. P., Levy N. S., Goldberg M. A., D'Amato R. J., Adamis A. P.* Regulation of vascular endothelial growth factor expression by insulin-like growth factor I. *Diabetes.* 1997, V. 46, P. 1619–1626. https://doi.org/10.2337/diacare.46.10.1619

106. *Morwenna S., Ratcliffe W. P.* Mammalian oxygen sensing and hypoxia inducible factor-1. *Int. J. Biochem. Cell Biol.* 1997, V. 29, P. 1419–1432. https://doi.org/10.1016/s1357-2725(97)00129-5

107. *Ferrara N.* Molecular and biological properties of vascular endothelial growth

factor. *J. Mol. Med.* 1999, V. 77, P. 527–543. https://doi.org/10.1007/s001099900019

108. *Seko Y., Takahashi N., Tobe K., Ueki K., Kadowaki T., Yazaki Y.* Vascular endothelial growth factor (VEGF) activates Raf-1, mitogen-activated protein (MAP) kinases, and S6 kinase (p90rsk) in cultured rat cardiac myocytes. *J. Cell. Physiol.* 1998, V. 175, P. 239–246. https://doi.org/10.1002/(SICI)1097-4652(199806)175:3<239::AID-JCP1>3.0.CO;2-P

109. *Eliceiri B. P., Klemke R., Stromblad S., Cheresh D. A.* Integrin alphavbeta3 requirement for sustained mitogen-activated protein kinase activity during angiogenesis. *J. Cell Biol.* 1998, V. 140, P. 1255–1263. https://doi.org/10.1083/jcb.140.5.1255

110. *Tarui T., Miles L. A., Takada Y.* Specific interaction of angiostatin with integrin alpha(v)beta(3) in endothelial cells. *J. Biol. Chem.* 2001, V. 276, P. 39562–39568. https://doi.org/ 10.1074/jbc.M101815200

ОДЕРЖАННЯ ТА ЗАСТОСУВАННЯ АНГІОСТАТИНІВ ДЛЯ ЛІКУВАННЯ НЕОВАСКУЛЯРНИХ ЗАХВОРЮВАНЬ ОКА

В. Л. Білоус
Л. Г. Капустяненко
А. О. Тихомиров

Інститут біохімії ім. О. В. Палладіна НАН України, Київ

E-mail: basil.bilous@gmail.com

Ангіостатини становлять групу крингл(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

В. Л. Белоус
Л. Г. Капустяненко
А. А. Тихомиров

Институт биохимии им. А.В. Палладина НАН Украины, Киев

E-mail: basil.bilous@gmail.com

Ангиостатины составляют группу крингл(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.

CURRENT CONCEPT OF THE STRUCTURAL AND FUNCTIONAL PROPERTIES OF ALFA-FETOPROTEIN AND THE POSSIBILITIES OF ITS CLINICAL APPLICATION

O. K. GULEVSKYY, YU. S. AKHATOVA

Institute for Problems of Cryobiology and Cryomedicine
of the National Academy of Sciences of Ukraine, Kharkiv

E-mail: Julija_Veselovskaja@meta.ua

This paper was aimed to review the literature data from native and foreign sources accumulated for 40-years period of research of the features of the molecular structure, functions, production and application of human alpha-fetoprotein (AFP), which is known as one of the most studied and increasingly demanded proteins. Results of fundamental studies performed with the use of modern methods, including various types of electrophoresis, chromatography, electron microscopy and immunoassay, in order to characterize the principal physicochemical capacities and localization of free and bound forms of AFP, as well as polypeptide structure, heterogeneity and topography of AFP receptors are highlighted here. The data on the mechanisms of AFP synthesis, its conformational features, binding sites and intracellular metabolism are also presented. The concepts of physiological functions and mechanisms of AFP transport in an organism are presented. Data on AFP isolation from the natural primary products and its production by means of recombinant and synthetic methods are shown. This review also summarizes information on the current possibilities of clinical application of AFP and the prospects for its usage in anticancer therapy for targeted delivery of chemotherapy drugs, with emphasis on the description of the recent progress in this field.

Key words: alpha-fetoprotein, sources of alpha-fetoprotein production, application of alpha-fetoprotein, targeted delivery of chemotherapy drugs.

General characteristics

Alpha-fetoprotein (AFP) is a single-chain oncofetal glycoprotein with a molecular weight of about 70 kDa, including something like 600 amino acids and 4% carbohydrates. In terms of its structure and physicochemical properties, AFP is homologous to the main transport protein of blood serum of adults — albumin. AFP is synthesized by the cells of the visceral endoderm of the yolk sac, the embryonic liver and in a small amount in the gastrointestinal tract. Then it enters the blood of the fetus, amniotic fluid, and from them partially into the mother's blood. Normally, AFP can be detected in fetal serum starting from the 4th week of pregnancy [1, 2]. Its concentration attains peaks between 12 and 16 weeks and then decreases gradually until nascency. Since AFP crosses the placenta, it can be found at a fairly high concentration in the maternal serum, reaching a maximum between 32 and 36 weeks of gestation, with AFP levels in the maternal serum differing between primiparous and multiparous (Fig. 1) . Immediately after nascence, the serum AFP level decreases by several orders of magnitude. In the blood of healthy people, the normal concentration of AFP is 0–12 IU/mL [3].
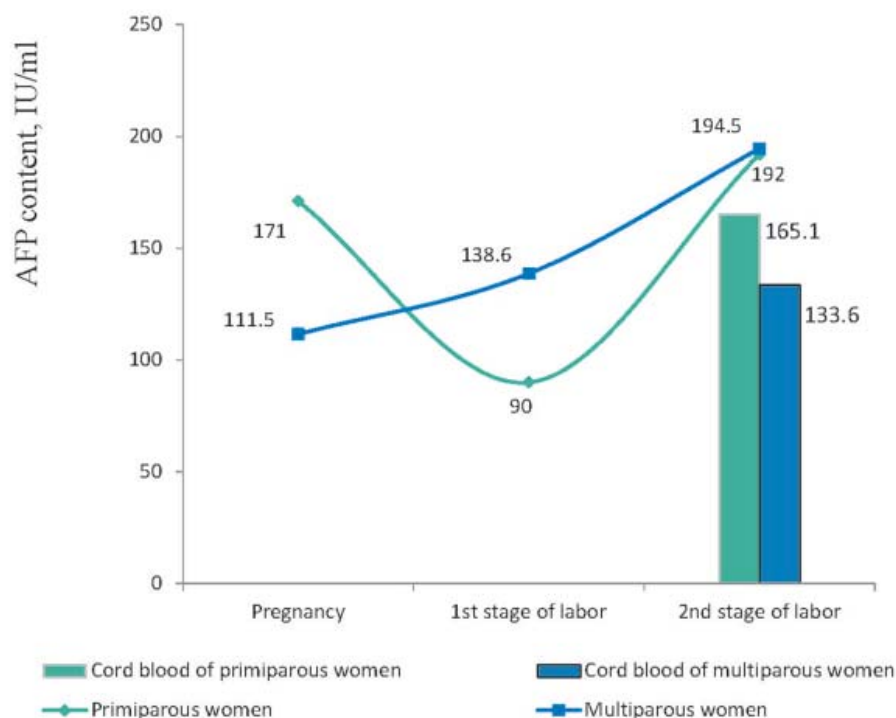
Fig. 1. **The content of AFP in the cord and peripheral blood
of pregnant women at the end of pregnancy [4]**

When studying the levels and sites of AFP localization, it was found that it is present in all organs and tissues of the human fetus, in the blood serum of pregnant women and newborns, as well as in the tissues of the placenta, amniotic fluid and cord blood [5, 6].

Intensive studies of the conformational states of AFP carried out in the early 2000s showed that, despite its stability in solution, AFP has sufficient conformational mobility and can form a molten globule [7]. It was shown that in the native AFP molecule, part of its biologically active sites are hidden inside the globule and do not participate in complexation with other molecules. They become available only upon conformational modification of AFP as a result of the action of various physicochemical factors, such as temperature, pH, and salt composition of the medium [7]. The study of conformational changes in AFP under the influence of such factors enables to understand the molecular mechanisms of its functioning, the peculiarities of interaction with ligands in the circulatory channel and intracellular metabolism.

Reception and intracellular AFP pathway

For the first time, the assumption of the existence of specific receptors to AFP (receptor for alpha-fetoprotein, ReCAF) was made by R. Moro in 1981 to explain the absorption of AFP by growing cells under the mechanism of receptor-mediated endocytosis [8]. ReCAFs are membrane-embedded AFP-binding transmembrane glycoproteins. However, such receptors are still poorly known. The problem of AFP receptors seems to be key for understanding not only the transport, but also other possible functions of AFP. Currently, this is one of the topical points in the study of the AFP role in the development process. There are 4 types of membrane receptors for AFP with molecular weights of 18, 31, 60, and 62–67 kDa [9, 10]. The first two are "scavenger receptors" and are located mainly on the cells of the vascular endothelium. Their role is to remove denatured and modified AFP molecules and albumin from the bloodstream. The third is also found on the surface of the endothelium. It is a sialoglycoprotein and its properties are close to the fourth, classical ReCAF, which is found on monocytes, lymphocytes, some cells of the reproductive system and a number of tumors [9].

Due to the fact that AFP belongs to the class of transport proteins, a significant proportion of it, both in serum and in tissues and cells, is in a bound state, i.e. in the form of complexes with transported ligands and/ or with receptor molecules. There are data in the literature on the intracellular distribution and state of AFP in different types of cells, in

particular, in hematopoietic stem cells (HSC) [11]. It is reported that labeled AFP is detected in the cytoplasm, Golgi complex, endoplasmic reticulum and mitochondria, and exists in the cytoplasm in two forms — free and bound. During native electrophoresis of cytoplasmic protein fractions, labeled AFP was detected in the form of several protein bands with different molecular weights: 65–68 kDa (free AFP), 125–130, 210–220, and 255–260 kDa (bound AFP) (Fig. 2) [11].

This indicates that upon leaving the endosome into the cytoplasm, AFP interacts with cytoplasmic distributor proteins (p52, p62, p67, p182, p55, and p147, depending on the cell type) (Fig. 2, *a*, *b*). The authors emphasize the fact that this interaction is reversible, since during SDS electrophoresis of the same protein fractions of the HSC cytoplasm, only one fraction of labeled AFP was detected — free (Fig.*2, c*).

It has also been shown by electron microscopy that covalently bound AFP conjugates with horseradish peroxidase in the process of endocytosis are first found in clathrin vesicles, then in endosomes and folded membranes of the central region of the Golgi apparatus, while egg albumin after internalization (the degree of its internalization is very low) was found in lysosomes. After internalization, the main

part of AFP is not degraded and is re-released into the extracellular environment, i.e, AFP is recirculated [3]. In addition, it is known that AFP is able to bind not only to cells, but also to structures of the extracellular matrix [12].

AFP functions

At the level of the organism, AFP is a multifunctional protein with selective cellular stimulating and inhibitory activity. Its main function is transport namely transfer of low molecular weight substances to tissues and cells of the embryo. AFP binds and transfers such ligands as bilirubin, fatty acids, steroids, retinoids, flavonoids, phytoestrogens, dyes, heavy metals, dioxin, as well as various drugs [12] At the same time, some hydrophobic ligands, such as fatty acids or estrogens, cause conformational changes in the protein tertiary structure.

AFP has an exceptionally high affinity for polyunsaturated fatty acids (PUFA), which is necessary for building cell membranes and a special class of biologically active substances — eicosanoids. Due to the fact that PUFAs are not synthesized in the body, but are part of the cell membranes, they are used in the synthesis of prostaglandins and are especially important for the formation of nervous tissue, selective binding of PUFAs in the placenta and their transfer from the mother's blood to the blood and embryonic cells is one of the key AFP
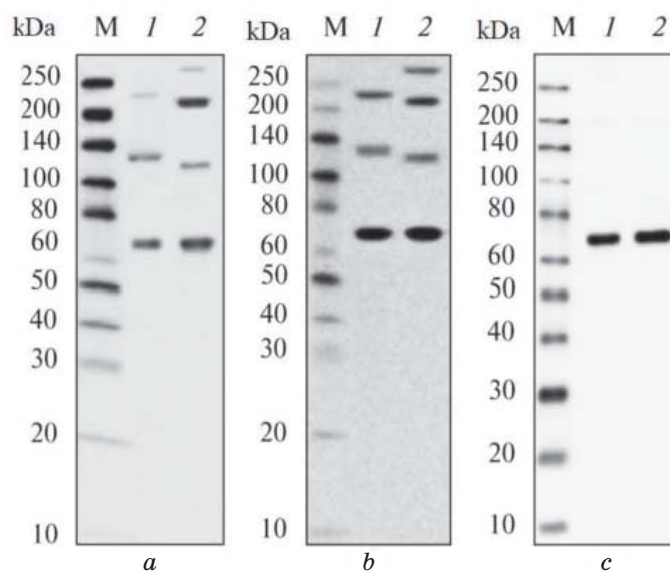
Fig. 2. **Interaction of 125I-labeled AFP with AFP-binding proteins/receptors in the cytoplasm of bone marrow HSCs [11]:**
a — detection of 125I-labeled AFP in the cytoplasm of bone marrow HSCs during native electrophoresis of the protein fraction of the cytoplasm; *b* — detection of the interaction of 125I-labeled AFP with AFP-binding proteins/cytoplasmic receptors in vitro during native electrophoresis; *c* — detection of 125I-labeled AFP in the cytoplasm during SDS-electrophoresis of the cytoplasmic protein fraction;
M — protein markers; *1* — CD34+ 133-117-135+ HSC; *2* — CD34+ 133-117+ 135-HSC

functions. The affinity of AFP for PUFA is 105 times higher than that of albumin [2].

In the embryonic period, AFP plays obviously an important role in the regulation of growth and differentiation of fetal tissues, in protecting the fetus and mother from the oncoming attack of the immune systems, in limiting the effect of steroid hormones, in particular maternal estrogens, on the fetus [12]. Among the probable functions, AFP is immunosuppressive, i.e., suppression of immune responses to antigens in a developing fetus. Since new proteins (antigens) appear in the development process, antibodies to them will also arise, which will lead to a number of complications. Therefore, the embryo's own immune system is suppressed, and AFP is possibly involved in providing this suppression [12]. This is supported by numerous studies confirming that AFP suppresses the expression of the molecules of the major histocompatibility complex and thereby prevents the presentation of antigens by macrophages. This is one of the main mechanisms responsible for the immunological incompetence of the developing fetus.

The effect of AFP on specific immunity has also been elucidated [2]:

• AFP inhibits the production of antibodies and the maturation of cytotoxic T-lymphocytes to T-dependent antigens without affecting the activity of mature T− and B-lymphocytes. The main target of the fetal protein is proliferating T-helpers corresponding to cytotoxic T-lymphocytes in the main histocompatibility complex.

• AFP suppresses the proliferative response of lymphocytes to mitogen.

• AFP increases the activity of specific T-suppressors.

• AFP reduces the phagocytic ability of macrophages.

• Depending on the experimental conditions, AFP can increase or decrease the production of prostaglandin E2 in macrophages, while indomethacin (an inhibitor of cyclooxygenase), together with AFP, loses the ability to suppress the synthesis of prostaglandin E2.

• A consequence of the stimulating effect of AFP on the production of prostaglandin E2 by monocytes is that AFP significantly suppresses the synthesis of TNF-α by activated monocytes (by 58%) and IL-1β (by 67%).

• AFP reduces the activity of natural killer cells.

Thus, AFP has definitely multidirectional immunomodulatory effects. In high doses, comparable to the concentrations in fetal blood, it inhibits lymphocyte proliferation, as demonstrated in cell cultures [2]. At the same time, no interactions of AFP with IL-2 supporting the proliferation of T cells were revealed, which made it possible to draw a conclusion about the direct effect of AFP on T cells.

On the one hand these data is indicative of the mother's immune system participation in pregnancy control even in the case of complete genetic compatibility of the mother and fetus, and on the other hand of AFP important role in this process [12]. When considering AFP from the standpoint of its immunosuppressive properties Clark et al. put forward the fetoembryonic hypothesis of the body's defense against autoimmune influences [13]. The gist of the hypothesis is that developing humans and gametes are protected by soluble immunosuppressive glycoproteins found in amniotic fluid and ergastoplasm known as glycodelin-A (GdA) and glycode lin-S (GdS), respectively. Structural analysis of their nucleoligosaccharide sequences suggests that GdA and GdS have very unusual carbohydrate functional group sequences that allow them to exhibit immunosuppressive properties. AFP has similar GdA and GdS sequences in its structure.

A study was carried out aimed at elucidating the role of AFP in the activation of cytokine synthesis *in vitro*. The production of IL-1β, IL-6 and TNF-β in whole blood cell cultures of 10 healthy subjects was studied after polyclonal activation by lipopolysaccharide in the presence of AFP. As a result of the experiments, it turned out that AFP is intact in relation to the studied cytokines [14].

Much attention is paid to the effect of AFP on the mitotic cycle of cells. The antiproliferative effect of this protein was studied in the works of the 70s−80s. The authors note the dependence of the effect on the AFP dose, purification degree, type of the cells and medium composition [15]. In the works of the 90s. much attention is paid to the synergistic (amplifying) properties of AFP. It has been shown that, without having a mitotic effect, AFP increases significantly (by 2−3 times) the effect of epidermal growth factor [16], insulin-like growth factor, and TGF-β [17].

Among other AFP properties, its regulatory role in the metabolism of steroid hormones and the ability to block the binding of antibodies to the acetylcholine receptor are noted [2, 16]. Thus, it was found that AFP negatively affects the metabolism and properties of estrogens. AFP isolated from umbilical cord blood or amniotic fluid inhibits

dose-dependently estradiol production *in vitro*. In this case, progesterone production was insensitive to the action of AFP [2, 16].

It is also known that AFP is capable to have an impact on cell proliferation and differentiation. In particular, its effect on the intracellular signaling pathway with the participation of phosphatidylinositol 3-kinase (PI3K)/AKT was shown, and a direct effect on the production of insulin-like growth factor was established [18, 19]. Since AFP acts as a transport protein, it can deliver regulatory molecules to the cells having receptors for AFP, or act as one of the independent regulators of signal transduction pathways. Since receptors for AFP are found in dividing cells (including tumor cells, hematopoietic cells, immunity, etc.), so in fact, it is able to exercise informational control of proliferating cells and significantly affect the level of their functional activity.

Isolation and purification of AFP

Currently, there are several ways to isolate and purify AFP from various biological raw materials. Most often, material with a high AFP content is used for these purposes: serum of cord or abortive blood of the second trimester of pregnancy and amniotic fluid [2]. AFP is also obtained from fetal and tumor tissues [2]. The main problem that arises during the isolation of a highly homogeneous preparation of AFP is purification from serum albumin, since it is present in serum in high concentrations and has a number of properties similar to AFP (molecular weight, close isotopes, etc.). In addition, human AFP isolated by different methods and from various sources has significant microheterogeneity that revealed either by native electrophoresis or isofocusing, while one or two bands are present in electrophoresis under denaturing conditions [20]. Thus, isofocusing the protein isolated from cord blood serum at pH values from 4.5 to 5.2 using polyclonal and monoclonal antibodies, 9 AFP isoforms were found (Sittenfeld and Moreno, 1988). When isolating protein from hepatomas, the authors found even greater heterogeneity than when isolating AFP from normal tissues and fluids [20]. It is believed that the heterogeneity of this protein is associated with varying degrees of glycosylation and with the presence or absence of ligands, primarily fatty acids. Back in the 70s and 80s. it was shown (Parmelee et al., 1978; Kerckaert et al., 1975, 1979b; Nunez et al., 1976; Bayard and Kerckaert, 1977; McMahon et al., 1977; Nagai et al., 1982) that AFP delipidisalia leads to a change in its isotope, and unsaturation with fatty acids promotes the

transition of the protein to another isoform. The possibility of linking the heterogeneity of AFP with the primary structure was not confirmed when constructing peptide maps of AFP from a normal source and hepatoma: electrophoresis of hydrolysis was identical for both proteins (Ruoslahti and Seppala, 1971). These data were also confirmed by the analysis of the primary structure of cDNA, mRNA and the amino acid sequence of AFP derived from them from carcinoma (Morinaga et al., 1983) and normal liver (Gibbs et al., 1987), as well as by comparing the N-terminal sequence of the protein from various sources. Thus, the problem of AFP heterogeneity during its production remains open.

The technology for isolation and purification of AFP is a complex, laborious and multistage process with a large amount of losses of the target material. The bulk of research in this direction was carried out in 1980–2000. At present, either recombinant technologies [21] or fragmentary synthesis of its segments with an average of 15–40 amino acids [22] are used to obtain AFP.

In general, the method for AFP obtaining from natural sources can be divided into several main stages [2, 20]:

• fractionation of source material — separation of ballast proteins and blood cells;

• inactivation of viruses (solvent/detergent type TRITON-X100 or TWEEN-80);

• stepwise isolation of AFP (on average 2–4 stages) using affinity/immune-affinity/reverse-phase chromatography on columns with a sorbent (Sepharose, Sephadex, agarose, metal chelates). In the case of immuno-affinity chromatography, antibodies to human AFP covalently immobilized in the matrix are used. The elution solutions vary depending on the type of antibodies;

• ultrafiltration of the eluate;

• checking the purity of the protein by electrophoresis or immunochemical method;

• stabilization of the resulting AFP solution by freeze drying with stabilizing additives.

Depending on the method of obtaining, the AFP yield from different authors averages 60–85%, but there are also indicators with more significant losses of AFP. For example, in [2] a practical example is described, in which 2.5 liters of serum containing 250 mg of AFP was obtained from 3 liters of abortive blood. After 12 stages of the production cycle, the dry matter yield was 86.4 mg AFP, i. e. about 35% of the initial protein content in abortive blood.

One of the most highly efficient schemes for the isolation and purification of AFP from

human cord blood was proposed by the authors of [20]. The scheme includes sequential chromatography on four columns (blue sepharose, two metal chelates, and one reverse phase), which contributes to the efficient isolation of high-purity protein and the yield of the target product is about 85% [20].

Most of the AFP isolation and purification schemes are described in detail in [2]. Thus, one of the most common is the immune-affinity method for AFP isolation using specific high-affinity polyclonal antibodies. It is optimal for isolation from a source with a high AFP content. In this case, the antiserum is pretreated with serum obtained from an adult organism (to get out it from antibodies to impurity proteins), after which it is mixed with the starting material to form a precipitate. The precipitate is dissolved in a low pH buffer. AFP and free antibodies are separated by gel filtration. Antibodies conjugated to various matrices (agarose, Sephadex or Sepharose) are also used. In this case, AFP is eluted with a solution with a low pH value or high concentrations of chaotropic agents (8 M urea).

A less common immuno-affinity method is an isolation scheme using monoclonal antibodies with low affinity and antibodies to impurity proteins [2, 20]. It is obvious that the use of low pH values and high concentrations of chaotropic agents for elution of the target product, in the case of using positive antibodies, can affect the nativeness of the protein. The disadvantage of this scheme is the strong interaction between the antibody and the target protein, which leads to a significant loss of the target product. When using low affinity monoclonal antibodies and negative antisera, the problems arise associated with high cost (monoclonal antibodies) and low sorbent capacity in both cases.

Other schemes for AFP purification have been developed, for example, using immobilized concanavalin A, with which AFP bound to the estradiol matrix binds specifically, separation using a two-phase system, various isolation schemes using high pressure chromatography or metal chelate sorbents [2].

All these methods make it possible to obtain a substance for the manufacture of an injectable form of the AFP drug. But at the same time, the general and main disadvantage of all the above schemes for obtaining AFP is the heterogeneity of the isolated protein, which implies additional purification steps, the removal of hydrophobic ligands, and regular immunochemical or electrophoretic control. In addition, there are problems associated with low capacity, low degree of purification and large losses of the target product up to 40%.

It is interesting that for all described schemes of AFP isolation from cord or abortive blood, it is fractionated with the removal of corpuscles and ballast proteins. However, as it was discussed above, a significant amount of AFP is bound to receptor complexes on cells of leukocyte origin, localized in the cytoplasm, or forms complexes with other proteins and ligands [3, 11]. From this point of view, it would be appropriate to introduce the stage of decellularization of the starting material, which, possibly, will significantly increase the final yield of the target product.

The most common methods of decellularization are destruction by hypotonic lysis, heating to high temperatures ($+70-100\ ^{\circ}$C), destruction by ultrasound, as well as by exposure to low temperatures. It should be noted that when extracted at room or high temperature, the structure of proteins is disrupted and, as a consequence, a significant decrease or complete loss of their biological activity occurs. The use of low temperatures will completely avoid heating. Therefore, cryotechnologies are the most promising and prevailing in terms of a number of qualitative characteristics for these purposes [23−25]. The use of low temperatures in the preprocessing of biological raw materials contributes to a more complete destruction of cellular structures and the release of proteins into the extraction solution. At the same time, the degree of decellularization and destruction of cellular structures can be varied using a combination of fast or slow freezing and thawing modes, which is important due to the localization of AFP not only in the cytoplasm, but also in the Golgi complex, endoplasmic reticulum, and mitochondria [11]. It is known that the greatest destruction of cellular components occurs during slow cooling of biological material in the temperature range of water crystallization (especially at the eutectic point) and recrystallization [23]. Based on this, the study of the effectiveness of various modes of low-temperature destruction of cord or abortive blood for the isolation of AFP will be of high scientific and practical importance.

The purified AFP preparation, obtained from aborted human tissues, was registered (Russia, USA) and was used in clinics in the 80−90s mainly for the treatment of autoimmune diseases (Alfetin® 75 µg/ampoule; Profetal® 75 µg/ampoule), but was withdrawn in 2008 for ethical reasons. Advances in biotechnology over the past decade make it possible not only to

improve the methods of isolation and large-scale purification of natural AFP, but also to produce a recombinant protein [26]. Preconceptual study is underway as well to create artificially synthesized active peptide fragments of AFP, consisting of an average of 30–60 amino acid residues, and to study their properties and therapeutic efficacy [22, 27, 28].

At the same time, the clinical application of AFP, both natural and synthetic, is controversial [26]. On the one hand, natural AFP is glycosylated (3–5%), while synthetic AFP is not, which can affect the binding affinity/specificity of the drug or its reception. On the other hand, in the case of using synthetic AFP, medicinal substances can be preliminarily bound to stabilize the tertiary structure of the synthetic protein [29] and/or additional stability can be achieved due to the binding of metal ions at the stage of production [30, 31], which will eliminate the loss of pharmaceutical ligand, for example, by competition for binding to albumin. In addition to all that has been said, the potential risks of administering therapeutic doses of this oncofetal protein to adult patients are still actively discussed in the scientific literature [26, 31].

Clinical application

To date, there have been many studies of the therapeutic efficacy of an AFP-based drug in laboratory and clinical practice [7, 32–35]. However, as it was discussed above, there is a latent danger of using therapeutic doses of AFP in adults, which consists in the abundance of its reversible and transitional conformational states depending on environmental conditions (pH, temperature, osmolality, excessive ligand concentrations, oxidation and heat/glucose shock) [7]. Contrary to the earlier reports that AFP induces cancer cell apoptosis [36], it has also been shown that it can inhibit this process in several types of cancer cells, thereby promoting their proliferation, growth and progression [37–40]. In addition, AFP is able to dimerize with other proteins, such as nuclear receptors (eg, retinoic receptor), transcription factors and caspases, which, as a result, can also promote the growth of tumor cells [34, 37].

Nevertheless, the ability of AFP to bind and target drug delivery to target cells with receptors for it remains an attractive object for further research. The capture of the drug by AFP leads to a change in its conformation, which provides a high binding affinity, thus, it acts as a natural nanocontainer for the selected hydrophobic ligands [31]. AFP has 5 or more possible binding sites. Of these, the major hydrophobic binding pocket is of particular interest, since other sites cannot compete with albumin for ligand binding. Since albumin is present in the bloodstream in a huge excess, all non-covalently bound ligands on the surface of the AFP molecule will be lost [41].

Inside the cells with a low pH, AFP changes its conformation again and releases the transported substance. In a ligand-free conformation, AFP returns to the bloodstream in an intact form (AFP recirculation takes about 1 hour) and continues to transfer dozens of ligands in a shuttle manner [41].It was shown that AFP can bind and release the ligand several times with a change in pH [30, 42].

In an adult organism, AFP receptors are present extensively, primarily in AFP-positive myeloid suppressor cells and cancer cells. Other cell populations are practically not affected by the AFP-ligand complex, since they do not have receptors or there are too few of them, or the cells themselves are in an inactive state (for example, stem cells have a set of receptors for AFP, but are in an inactive state in the bone marrow) [43]. Thus, with the help of AFP, it is possible to exert a targeting effect on myeloid suppressors and cancer cells. In this regard, there are 3 main directions in the use of drugs based on AFP:
- immunotherapy (the first registered AFP drugs belong to the class of immuno-modulators);
- therapy of autoimmune diseases;
- anticancer therapy.

Immunotherapy and therapy of autoimmune diseases. The ability of AFP with the help of transported ligands to activate or inhibit suppressor cells of myeloid origin makes it possible to control the intensity of immune responses in a targeted manner, which is used in the complex treatment of autoimmune diseases [31, 44, 45] and other immunopathological conditions caused by impaired synthesis of cytokines that regulate T-cell immunity (ulcerative colitis, autoimmune thyroiditis, etc.). In addition, as it was already discussed, AFP is capable of enhancing the action of alpha-interferon, a tumor necrosis factor, and modulating the activity of prostaglandins and leukotrienes, which are important in the development of the inflammation process [46, 47]. It has been shown that due to these properties, AFP is a participant in the acute phase of inflammation [48].

The use of synthetic AFP (MM-093) as monotherapy has been investigated in autoimmune diseases such as rheumatoid

arthritis, psoriasis and uveitis [49] and has been successful in phase I and II of clinical trials: the established safe doses can be used for several indications. In rheumatoid arthritis, however, MM-093 has not been shown to be effective in phase II of clinical trials. Perhaps this is due to the fact that AFP is not a drug in itself, and it should be used as a part of complex therapy, since the successful treatment of autoimmune diseases using AFP from natural sources was carried out in combination with other drugs (for example, in a complex with reduced doses of glucocorticoids) [2].

At the same time, the study of experimental autoimmune myasthenia in animal models provided clear evidence of the effectiveness of MM-093 monotherapy. It has been found that the use of MM-093 leads to a decrease in the complement cascade, which plays an important role in the onset of clinical symptoms of autoimmune myasthenia [50]. It is possible that AFP delivers local natural ligands to myeloid suppressors, which is sufficient for the positive results obtaining, or other mechanisms of action are involved. There were no safety concerns in this study.

Anticancer therapy. Another promising area of drugs application based on AFP is the delivery of cytostatics to tumor cells. Chemotherapy is one of the most commonly used methods of fighting cancer. The effectiveness of chemotherapy drugs is ensured by the fact that they are all especially effective in killing actively dividing and metabolizing cells. Unfortunately, in addition to tumor cells, the cells of the bone marrow, gastrointestinal tract, etc. normally have the same characteristics. As a result, the entire spectrum of side effects of chemotherapy is associated with the effect of the drug on normal actively dividing cells in the body. In view of this, one of the promising ways to increase the effectiveness of chemotherapy drugs is their selective delivery to tumor cells. The essence of the method is that the chemotherapy is attached to a vector molecule, which delivers it to a certain type of cells. Targeting of delivery is provided due to specific receptors characteristic only of target cells. AFP is one of the most promising proteins that can be used as vector molecules capable of targeting the delivery of chemotherapy drugs. It was shown that conjugates of natural AFP with cytostatics inhibited the growth of tumor cells *in vitro* and *in vivo* [51–54]. However, the use of natural AFP is limited by the abundance of its conformational states depending on the conditions of the microenvironment (which makes it difficult to predict the therapeutic effect), as well as

technical and ethical aspects, since its only source is material of fetal-placental origin. For this reason, numerous studies are being carried out on the development and use of functionally complete recombinant AFP fragments. It is known that the receptor binding site is located in the C-terminal domain of the AFP molecule. Therefore, for the purpose of targeted delivery, it is optimal to use recombinant protein fragments based on the C-terminal domain [7, 55–57]. One of the main problems when using recombinant proteins is their production in a functional form. In most cases, when proteins are produced in large quantities in the bacterial system, they form in cells the so-called inclusion bodies, which consist of protein in an aggregated state [55, 58–60]. To obtain a functionally active protein, it is necessary to carry out the procedure of its renaturation. For hydrophobic proteins with a large number of disulfide bonds, such as the C-terminal domain of AFP, this problem is especially difficult. The development of an effective renaturation technique is the main difficulty in obtaining a functional form of a recombinant AFP fragment. This problem solution will make it possible to obtain a functional fragment of AFP in sufficient quantities to create its conjugates with cytostatics.

The creation of conjugates of AFP with cytostatics is a method aimed at the cancer cells themselves. But another approach to the use of AFP in the framework of anticancer therapy is also possible — depressing effect on myeloid suppressors. The point is that the accumulation of these cells is a key process of immunosuppression in various types of cancer [61]. Myeloid suppressors and T-regulatory cells are the main components of the immunosuppressive tumor microenvironment [62], providing tumor-mediated evasion from immune control [63]. Being normal cells in the body, myeloid suppressors enhance cancer growth and inhibit the activity of natural killer cells and specialized T cells that can destroy cancer cells. Thus, in order to overcome the tolerance to tumor cells on the part of the immune system and activate adaptive and innate immunity to cancer, myeloid suppressors must be depleted [31, 64].

It has been shown that such common chemotherapy drugs as doxorubicin, paclitaxel, gemcitabine, and 5-FU can deplete myeloid suppressors [65]. These drugs can be used not only as direct toxins against cancer cells, but also as immunomodulators that can selectively reduce myeloid suppressor-mediated immunosuppression and increase

the effectiveness of other therapies. Myeloid suppressors are more important target in anticancer therapy than cancer cells themselves, since killing one suppressor has more potent anticancer effect than killing a single cancer cell. For example, the effect of 5-FU immunotherapy within the framework of a complex treatment regimen prevailed over the effect of classical chemotherapy, as it is seen when comparing tumor growth suppression in mice [66]. Low-dose paclitaxel blocks myeloid suppressor-mediated immunosuppression *in vivo*, suppresses chronic inflammation in the tumor microenvironment, and can be used to increase the effectiveness of concomitant antitumor therapy [67]. Moreover, unlike 5-FU or doxorubicin, paclitaxel strongly binds to AFP, which ensures its targeted delivery to myeloid suppressors and enhances the antitumor effect of AFP [31]. Similar regimens for the combination of chemotherapy and immunotherapy are already used in the treatment of cancer [68, 69]. Chemotherapy leads to self-destruction of cancer cells (apoptosis, autophagy, etc.), while immunotherapy alters the tolerance of the patient's immune system to cancer cells. The combination of these two mechanisms in many cases can provide victory over cancer [70]. The use of non-covalent AFP-chemotherapy complexes combines immunotherapy and targeted chemotherapy. To date, a large number of drugs for the treatment of oncological diseases containing AFP or its fragments are at the stage of development, studies of the efficacy and safety of use [71–78].

Thus, summarizing the data presented, we can conclude that the study of the molecular mechanisms of AFP functioning and the therapeutic potential of drugs based on it is a promising area of modern fundamental science, pharmacy and medicine. It should be noted that despite the huge array of works devoted to the study of this protein, there are a number of unresolved issues that require further research. From a fundamental point of view, it is important to clarify the number of active AFP binding sites for interaction with various ligands, to assess the strength of this interaction and the stability of the complexes formed. It is extremely important to establish the biological activity of not only the native protein, but also its fragments, which may reveal new possibilities for its therapeutic use. Biotechnological solutions also require a solution to issues related to the production of AFP from natural raw materials, namely, the search for the most effective and gentle ways of its isolation, including the use of cryotechnologies, which together will avoid large losses of the target product during the production cycle and provide a high degree of its purification. Questions about the mechanisms of AFP reception also require further in-depth study. It is assumed that solving these problems will allow the creation of a registered AFP drug in the consecutive 5–10 years.

REFERENCES

1. *Moldogazieva N. T., Terentiev A. A., Shait K. V.* Relationship between the structure and functions of alpha-fetoprotein: its conformational changes and biological activity. *Biomed. chem*. 2005, 51 (2), 127–151. (In Russian).

2. *Chereshnev V. A., Rodionov S. Yu., Cherkasov V. A., Malyutina N. N., Orlov O. A.* Alfa-fetoprotein. *Yekaterinburg: Uralskoe otdelenie RAN*. 2004, 376 p. (In Russian).

3. *Treshalina H. M., Smirnova G. B., Tsurkan S. A., Tcherkassova J. R., Lesnaya N. A.* The role of alpha-fetoprotein receptor in the delivery of targeted preparations in oncology. *Russian J. Oncol.* 2017, 22 (1), 4–14. https://doi.org/10.18821/1028-9984-2017-22-1-4-14 (In Russian).

4. *Nikolaeva L. B., Ushakova G. A.* The first pregnancy and first birth: a guide for doctors. *Moskva: GEOTAR-Media*. 2013, 264 p. (In Russian).

5. *Fryer A. A., Jones P., Strange R., Hume R., Bell J. E.* Plasma protein levels in normal human fetuses: 13 to 41 weeks' gestation. *Br. J. Obstet. Gynaecol.* 1993, 100 (9), 850–855.

6. *Hsu J. J., Hseih T. T., Chiu T. H., Liou J. D., Soong Y. K.* Alpha-fetoprotein levels of paired samples between the amniotic fluid and maternal serum from 16 to 18 weeks' gestation in Chinese women. *J. Formos. Med. Assoc.* 1994, 93 (5), 374–378.

7. *Mizejewski G. J.* Alpha-fetoprotein structure and function: relevance to isoforms, epitopes, and conformational variants. *Exp. Biol. Med.* 2001, V. 226, P. 377–408.

8. *Naval J., Villacampa M. J., Goguel A. F., Uriel J.* Cell-type specific receptors for alpha-

fetoprotein in a mouse T-lymphoma cell line. *Proc. Natl. Acad. Sci. USA.* 1985, V. 82, P. 3301–3305.

9. *Suzuki T., Sasano H., Aoki H., Nagura H., Sasano N., Sano T., Saito M., Watanuki T., Kato H., Aizawa S.* Immunohistochemical comparison between anaplastic seminoma and typical seminoma. *Acta Pathol. Jpn.* 1993, 43 (12), 751–757.

10. *Moldogazieva N. T., Terentiev A. A.* Alpha-fetoprotein and growth factors. Structure-function relationships and analogies. *Usp. Biol. Chim.* 2006, No 46, P. 99–148. (In Russian).

11. *Bogdanov A. Yu., Bogdanova T. M., Ilin A. I.* Endocytic pathway of alpha-fetoprotein in mice bone marrow hematopoietic stem cells: molecular characterization and role in biological activity modification. *Cytology and Genetics.* 2014, 48 (1), 25–40. (In Russian).

12. *Shmagel K. V., Chereshnev V. A.* Alpha-fetoprotein: structure, function and role in embryogenesis. *Akusherstvo i ginekologiya.* 2002, No 5, P. 6–8. (In Russian).

13. *Clark G. F., Dell A., Morris H. R., Patankar M., Oehninger S., Sepp l M.* Viewing AIDS from a glycobiological perspective: potential linkages to the human fetoembryonic defence system hypothesis. *Mol. Hum. Reprod.* 1997, 3 (1), 5–13.

14. *Filella X., Molina R., Alcover J., Coca F., Zarco M. A., Ballesta A. M.* Influence of AFP, CEA and PSA on the in vitro production of cytokines. *Tumour Biol.* 2001, 22 (2), 67–71.

15. *Czokalo M., Wishnewski L.* Culture conditions modify the effects exerted by human fetal AFP on some lymphocyte functions *in vitro*. *Exp. Pathol.* 1981, 20 (4), 233–238.

16. *Keel B. A., Eddy K. B., Cho S., May J. V.* Human alpha-fetoprotein purified from amniotic proliferation *in vitro*. *Mol. Reprod. Dev.* 1991, 30 (2), 112–118.

17. *Leal J. A., May J. V., Keel B. A.* Human alpha-fetoprotein enhances epidermal growth factor proliferative activity upon porcine granulosa cells in monolayer culture. *Endocrinol.* 1990, 126 (1), 669–671.

18. *Li M., Li H., Li C., Wang S., Jiang W., Liu Z., Zhou S., Liu X., McNutt M. A., Li G.* Alpha-fetoprotein: a new member of intracellular signal molecules in regulation of the PI3K/AKT signaling in human hepatoma cell lines. *Int. J. Cancer.* 2011, 128 (3), 524–532. https://doi.org/10.1002/ijc.25373

19. *Zheng L., Gong W., Liang P., Huang X., You N., Han K. Q., Li Y. M., Li J.* Effects of AFP-activated PI3K/Akt signaling pathway on cell proliferation of liver cancer. *Tumour Biol.* 2014, 35 (5), 4095–4099. https://doi.org/10.1007/s13277-013-1535-z

20. *Tomashevsky A. I., Yuversky V. N.* A new scheme for the isolation and purification of human alpha-fetoprotein. *Bioorg. chem.* 1999, 25 (6), 412–417. (In Russian).

21. *Lin B., Peng G., Feng H., Li W., Dong X., Chen Y., Lu Y., Wang Q., Xie X., Zhu M., Li M.* Purification and characterization of abioactive alpha-fetoprotein produced by HEK-293 cells. *Protein Expr. Purif.* 2017, V. 136, P. 1–6. https://doi.org/10.1016/j.pep.2017.05.008

22. *Terentev A. A., Kazimirsky A. N., Lychkova A. E., Salmasi J. M.* Lymphocyte apoptosis enhancement by the synthetic peptide of human alpha-fetoprotein (afp 14-20) in experimental ulcer. *Exper. Clin. Gastroenterol.* 2014, 110 (10), 50–52. (In Russian).

23. *Fuller B. J., Lane N., Benson E. E.* Life in the frozen state. *London: CRC Press.* 2004, 672 p.

24. *Shabunin S. V., Vostrilova G. A., Shabanov I. E.* Screening of biologically active substances depending on the technological parameters of cryogenic fractionation of the placenta. *Problems of cryobiology.* 2005, 15 (3), 306–309. (In Ukrainian).

25. *Osetskiy A. I., Grischenko V. I., Snurnikov A. S., Shabanov I. Ye., Babijchuk G. A.* Cryosublimation fractionating of biological material. *Problems of cryobiology.* 2006, 16 (2), 230–240. (In Ukrainian).

26. *Mizejewski G. J.* Therapeutic use of human alpha-fetoprotein in clinical patients: Is a cancer risk involved? *Int. J. Cancer.* 2011, V. 128, P. 239–249. https://doi.org/10.1002/ijc.25292

27. *Dudich E.* MM–093, a recombinant human alpha-fetoprotein for the potential treatment of rheumatoid arthritis and other autoimmune diseases. *Curr. Opin. Mol. Ther.* 2007, No 9, P. 603–10.

28. *Mizejewski G.* Mapping of structure-function peptide sites on the human alpha-fetoprotein amino acid sequence. *Atlas Genet. Cytogenet. Oncol. Haematol.* 2010, 14 (2), 169–216. https://doi.org/10.4267/2042/44695

29. *Uversky V. N., Narizhneva N. V., Ivanova T. V., Tomashevski A. Y.* Rigidity of human alpha-fetoprotein tertiary structure is under ligand control. *Biochem.* 1997, V. 36, P. 13638–13645.

30. *Dudich I. V., Semenkova L. N., Tatulov E., Korpela T., Dudich E.* Improved delivery of poorly water soluble drugs with alphafetoprotein stabilized with metal ions. *International Bureau PCT. WO2015075296 A1.* 05. 28. 2015.

31. *Pak V. N.* The use of alpha-fetoprotein for the treatment of autoimmune diseases and cancer. *Ther Deliv.* 2018, 9 (1), 37–46. https://doi.org/10.4155/tde-2017-0073

32. *Li M., Liu X., Zhou S., Li P., Li G.* Effects of alpha fetoprotein on escape of Bel 7402 cells from attack of lymphocytes. *BMC Cancer.* 2005, No 5, P. 96.

33. *Li M., Zhou S., Liu X., Li P.* Alpha-fetoprotein shield hepatocellular carcinoma cells from apoptosis induced by tumor necrosis factor-

related apoptosis-inducing ligand. *Cancer Lett*. 2007, V. 249, P. 227–234.

34. *Li M., Li H., Li C., Guo L., Zhou S.* Cytoplasmic alpha-fetoprotein functions as a co-repressor in RA-RAR signaling to promote the growth of human hepatoma Bel 7402 cells. *Cancer Lett*. 2009, V. 285, P. 190–199.

35. *Zhang W., Liu J., Wu Y., Xiao F., Wang Y., Wang R., Yang H., Wang G., Yang J., Deng H., Li J., Wen Y., Wei Y.* Immunotherapy of hepatocellular carcinoma with a vaccine based on xenogeneic homologous alpha fetoprotein in mice. *Biochem. Biophys. Res. Commun*. 2008, 376 (1), 10–14. https://doi.org/10.1016/j.bbrc.2008.08.061

36. *Semenkova L. N., Dudich E. I., Dudich I. V.* Induction of apoptosis in human hepatoma cells by alpha-fetoprotein. *Tumour Biol*. 1997, V. 18, P. 261–273.

37. *Li M., Li H., Li C., Zhou S., Guo L., Jiang W.* Alpha fetoprotein is a novel protein-binding partner for caspase-3 and blocks the apoptotic signaling pathway in human hepatoma cells. *Int. J. Cancer*. 2009, V. 124, P. 2845–2854. https://doi.org/10.1002/ijc.24272

38. *Laderoute M. P., Pilarski L. M.* The inhibition of apoptosis by alpha-fetoprotein (AFP) and the role of AFP receptors in anti-cellular senescence. *Anticancer Res*. 1994, No 14, P. 2429–2438.

39. *Wang X., Wand Xu B.* Stimulation of tumor-cell growth by alpha-fetoprotein. *Int. J. Cancer*. 1998, V. 75, P. 596–599.

40. *Wang X. W., Xie H.* Alpha-fetoprotein enhances the proliferation of human hepatoma cells in vitro. *Life Sci*. 1999, V. 64, P. 17–23.

41. *Pak V. N.* α-fetoprotein-binding toxins and teratogens against cancer. *Ther Deliv*. 2019, No 1, P. 1–3. https://doi.org/10.4155/tde-2018-0068

42. *Hirano K., Watanabe Y., Adachi T., Ito Y., Sugiura M.* Drug binding properties of human alpha-fetoprotein. *Biochem. J*. 1985, V. 231, P. 189–191.

43. *Belyaev N. N., Abdolla N., Perfilyeva Y. V., Ostapchuk Y. O., Krasnoshtanov V. K., Kali A., Tleulieva R.* Daunorubicin conjugated with AFP selectively eliminates MDSCs and inhibits experimental tumor growth. *Cancer Immunol. Immunother*. 2018, 67 (1), 101–111. https://doi.org/10.1007/s00262-017-2067-y

44. *Gabrilovich D. I.* Myeloid-derived suppressor cells. *Cancer Immunol. Res*. 2017, 5 (1), 3–8. https://doi.org/10.1158/2326-6066.CIR-16-0297

45. *Dudich E.* MM-093, a recombinant human alpha-fetoprotein for the potential treatment of rheumatoid arthritis and other autoimmune diseases. *Curr. Opin. Mol. Ther*. 2007, 9 (6), 603–610.

46. *Aussel C., Fehlmann M.* Alpha-fetoprotein stimulates leukotriene synthesis in P388D1

macrophages. *Cell. Immunol*. 1986, V. 101, P. 415–424.

47. *Nakanishi M., Rosenberg D. W.* Multifaceted roles of PGE2 in inflammation and cancer. *Semin. Immunopathol*. 2013, 35 (2), 123–137. https://doi.org/10.1007/s00281-012-0342-8

48. *Mizejewski G. J.* Alpha-fetoprotein (AFP) and inflammation: Is AFP an acute and/or chronic phase reactant? *J. Hematol. Thrombo. Dis*. 2015, 3 (1), 191–199. https://doi.org/10.4172/2329-8790.1000191

49. *Murgita R. A.* Recombinant human alpha-fetoprotein as an immunosuppressive agent. *Pat. USA. US7423024*. 09. 09. 2008.

50. *Kusner L. L., Sengupta M., Aguilo-Seara G., Sherman I.* Preclinical pilot study of alpha-fetoprotein on moderation of MG weakness. *Abstracts of the 13th International Conference on Myasthenia Gravis and Related Disorders. NY: The New York Academy of Sciences*. 2017.

51. *Mizejewski G. J.* Biological role of alpha-fetoprotein in cancer: prospects for anticancer therapy. *Expert. Rev. Anticancer Ther*. 2002, 2 (6), 709–735. https://doi.org/10.1586/14737140.2.6.709

52. *Chereshnev V. A., Rodionov S. Iu., Vasil'ev N. V., Orlov O. A., Cherkasov V. A.* Alpha-fetoprotein immunotherapy as a stage of combined treatment of cancer patients. *Vopr. Onkol*. 2005, 51 (1), 86–92.

53. *Posypanova G., Severin S.* Alpha-fetoprotein and recombinant alpha-fetoprotein fragments as drug delivery tools. In: Alpha-fetoprotein: functions and clinical applications. Lakhi N., Moretti M. (Eds). *Nova Science Publishes, NY, USA*. 2016, P. 277–300.

54. *Pak V. N.* The use of alpha-fetoprotein for the delivery of cytotoxic payloads to cancer cells. *Ther. Deliv*. 2014, 5 (8), 885–892. https://doi.org/10.4155/tde.14.59

55. *Sharapova O. A.* Recombinant fragments of human alpha-fetoprotein for creating targeted drug delivery (Ph. D. dissertation). *Available from the Scientific Electronic Library of theses and abstracts*. 2012.

56. *Bereznikova A. B., Posypanova G. A., Makarov V. A., Antipova O. V., Severin E. S.* AFP octapeptide is a promising peptide vector for targeted delivery of cytostatics to tumor cells. *Vopr. biol. med. farm. chemistry*. 2012, No 5, P. 15–21. (In Russian).

57. *Sharapova O. A., Pozdnyakova N. V., Severin S. E., Laurinavichyute D. K., Yurkova M. S., Andronova S. M., Fedorov A. N., Severin E. S., Posypanova G. A.* Isolation and characterization of the recombinant human α-fetoprotein fragment corresponding to the c-terminal structural domain. *Bioorganic chem*. 2010, 36 (6), 696–703. (In Russian).

58. *Garc a-Fruit s E., Ar s A., Villaverde A.* Localization of Functional Polypeptides in

Bacterial Inclusion Bodies. *Appl. Environ. Microbiol.* 2007, 73 (1), 289–294. https://doi.org/10.1128/AEM.01952-06

59. *Gil-Garcia* M., *Navarro S., Ventura S.* Coiled-coil inspired functional inclusion bodies. *Microb. Cell Fact.* 2020, 19 (1), 117. https://doi.org/10.1186/s12934-020-01375-4

60. *J ger V. D., Lamm R., K sters K., l c G., Oldiges M., Jaeger K. E., B chs J., Krauss U.* Catalytically-active inclusion bodies for biotechnology-general concepts, optimization, and application. *Appl. Microbiol. Biotechnol.* 2020, 104 (17), 7313–7329. https://doi.org/10.1007/s00253-020-10760-3

61. *Colligan S. H., Tzetzo S. L., Abrams S. I.* Myeloid-driven mechanisms as barriers to antitumor CD8+T cell activity. *Mol. Immunol.* 2020, V. 118, P. 165–173. https://doi.org/10.1016/j.molimm.2019.12.012

62. *Weiss J. M., Subleski J. J., Back T., Chen X., Watkins S. K., Yagita H., Sayers T. J., Murphy W. J., Wiltrout R. H.* Regulatory T cells and myeloid-derived suppressor cells in the tumor microenvironment undergo Fas-dependent cell death during IL-2/αCD40 therapy. *J. Immunol.* 2014, 192 (12), 5821–5829. https://doi.org/10.4049/jimmunol.1400404

63. *Atretkhany K. N., Drutskaya S.* Myeloid-derived suppressor cells and proinflammatory cytokines as targets for cancer therapy. *Biochemistry (Mosc).* 2016, 81 (11), 1274–1283. https://doi.org/10.1134/S0006297916110055

64. *Belyaev N. N.* Myeloid-derived suppressor cells (MDSC) as a main tumor induced negative regulators of cancer immunity and possible ways for their elimination. *KazNU Bulletin Biol. Series.* 2014, 1 (60), 79–83.

65. *Alizadeh D., Trad M., Hanke N. T., Larmonier C. B., Janikashvili N., Bonnotte B., Katsanis E., Larmonier N.* Doxorubicin eliminates myeloid-derived suppressor cells and enhances the efficacy of adoptive T-cell transfer in breast cancer. *Cancer Res.* 2014, 74 (1), 104–118. https://doi.org/10.1158/0008-5472.CAN-13-1545

66. *Vincent J., Mignot G., Chalmin F., Ladoire, S., Bruchard M., Chevriaux A., Martin F., Apetoh L., R b C., Ghiringhelli F.* 5-Fluorouracil selectively kills tumor-associated myeloid-derived suppressor cells resulting in enhanced T cell-dependent antitumor immunity. *Cancer Res.* 2010, 70 (8), 3052–3061. https://doi.org/10.1158/0008-5472.CAN-09-3690

67. *Sevko A., Michels T., Vrohlings M., Umansky L., Beckhove P., Kato M., Shurin G. V., Shurin M. R., Umansky V.* Antitumor effect of paclitaxel is mediated by inhibition of myeloid-derived suppressor cells and chronic inflammation in the spontaneous melanoma model. *J. Immunol.* 2013, 190 (5), 2464–2671.

68. *Bracci L., Schiavoni G., Sistigu A., Belardelli F.* Immune-based mechanisms of cytotoxic chemotherapy: implications for the design of novel and rationale-based combined treatments against cancer. *Cell Death Differ.* 2014, 21 (1), 15–25. https://doi.org/10.1038/cdd.2013.67

69. *Wang Z., Liu Y., Zhang Y., Shang Y., Gao Q.* MDSC-decreasing chemotherapy increases the efficacy of cytokine-induced killer cell immunotherapy in metastatic renal cell carcinoma and pancreatic cancer. *Oncotarge.* 2016, 7 (4), 4760–4769. https://doi.org/10.18632/oncotarget.6734

70. *Pak V. N.* Magic bullet and immunotherapy against metastasis. *J. Cancer Prev. Curr. Res.* 2016, 6 (3), 00206. https://doi.org/10.15406/jcpcr.2016.06.00206

71. *Qin H., Lerman B., Sakamaki I., Wei G., Cha S. C., Rao S. S., Qian J., Hailemichael Y., Nurieva R., Dwyer K. C., Roth J., Yi Q., Overwijk W. W., Kwak L. W.* Generation of a new therapeutic peptide that depletes myeloid-derived suppressor cells in tumor-bearing mice. *Nat. Med.* 2014, 20 (6), 676–681. https://doi.org/10.1038/nm.3560

72. *Arshad N. M., In L. L., Soh T. L., Azmi M. N., Ibrahim H., Awang K., Dudich E., Tatulov E., Nagoor N. H.* Recombinant human alphafetoprotein synergistically potentiates the anti-cancer effects of 1'-S-1'-acetoxychavicol acetate when used as a complex against human tumours harbouring AFP-receptors. *Oncotarget.* 2015, 6 (18), 16151–16167. https://doi.org/10.18632/oncotarget.3951

73. *Dominguez G. A., Condamine T., Mony S., Hashimoto A., Wang F., Liu Q., Forero A., Bendell J., Witt R., Hockstein N., Kumar P., Gabrilovich D. I.* Selective targeting of myeloid-derived suppressor cells in cancer patients using DS-8273a, an agonistic TRAIL-R2 antibody. *Clin. Cancer Res.* 2016, 23 (12), 2942–2950. https://doi.org/10.1158/1078-0432.CCR-16-1784

74. *Wilkerson A., Kim J., Huang A. Y., Zhang M.* Nanoparticle systems modulating myeloid-derived suppressor cells for cancer immunotherapy. *Curr. Top. Med. Chem.* 2017, 17 (16), 1843–1857. https://doi.org/10.2174/1568026617666161122121412

75. *Wang H. F., Ning F., Liu Z. C., Wu L., Li Z. Q., Qi Y. F., Zhang G., Wang H. S., Cai S. H., Du J.* Histone deacetylase inhibitors deplete myeloid-derived suppressor cells induced by 4T1 mammary tumorsin vivoand *in vitro. Cancer Immunol. Immunother.* 2017, 66 (3), 355–366. https://doi.org/10.1007/s00262-016-1935-1

76. *Pak V. N.* Selective targeting of myeloid-derived suppressor cells in cancer patients through AFP-binding receptors. *Future Sci. OA.* 2018, 5 (1), FSO321. https://doi.org/10.4155/fsoa-2018-0029