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MULTIPLE EFFECTS OF ANGIOSTATINS IN INJURED CORNEA

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Prolonged inflammation and excessive neovascularization of the cornea due to severe injury can impair optical clarity and lead to vision impairment. Plasminogen kringle (K) fragments, known as angiostatins (AS), play a well-established role as inhibitors of neovascularization by suppressing pro-angiogenic signaling. However, AS effects in the cornea, beyond inhibiting the angiogenesis, are still unexplored. In this study, we estimate the protective effect of two AS variants (K1-3 and K5) against alkali burn injury induced in rabbit and rat corneas. AS K1-3 in the single doses of 0.075 or 0.75 μg (0.1 or 1.0 μM, respectively) or 0.3 μg of AS K5 (1.0 μM) were applied locally as eye drops daily for 14 days after the injury. A significant regression of corneal vessels in-growth in injured eyes treated with AS was revealed. Western blot analysis of corneal tissue lysates revealed that injury-induced overexpression of protein markers of hypoxia (HIF-1α), angiogenesis (VEGF), tissue remodeling and fibrosis (MMP-9), autophagy (beclin-1) and endoplasmic reticulum stress (GRP-78) was significantly reduced under AS treatment. Besides, the level of tight junctions protein ZO-1 was shown to be up-regulated after the treatment of the damaged cornea with AS K1-3. Summarizing, our study uncovered novel biological functions of the kringle-containing plasminogen fragments indicating its beneficial effects during corneal healing in the experimental model of alkali burn. The data obtained can be helpful for the development of novel efficient formulations to manage complications of ocular surface injuries.

K e y w o r d s: angiostatins, cornea, alkali burn, neovascularization, HIF-1α, VEGF, beclin-1, MMP-9, ZO-1.

The cornea is an optically transparent, normally avascular tissue that plays several pivotal roles within the eye. It serves as a shield to protect the inner content of the eye from debris, infections and other foreign objects and acts as the main refracting lens to provide perfect light transmission [1]. However, the cornea is very susceptible to injuries that can be caused by physical traumas, foreign objects, dust and other abrasives, chemical irritants, thermal burns, dry eye syndrome, surgery, uncontrolled wearing of contact lenses, viral infections, and ultraviolet light. Corneal injuries may lead to worsening or even loss of vision while more severe cases make corneal transplantation necessary. Corneal blindness has been reported as the second leading cause of blindness, after cataract. Globally, the incidence rate of corneal injuries of various origins accounts for 1.4 million people per year, 12% of whom suffer subsequent vision troubles [2].

When the eye surface is damaged, corneal neovascularization is being induced as an adaptive response to preserve corneal tissue from hypoxic impact. However, when an imbalance between angiogenesis activators and inhibitors is developed, the invasion of new blood vessels into the cornea occurs accompanied by persistent inflammation, massive tissue remodeling, and fibrosis. During severe corneal injuries or traumas, neovascularization can have a significant negative impact on vision [3]. Hypoxia inducible factor-1α (HIF-1α) has been considered the major transcriptional factor, which governs the adaptive response to hypoxia and, therefore, to be the main contributor to the angiogenic response. Vascular endothelial growth factor (VEGF), a crucial participator in stimulating the mitosis of endothelial cells and the formation of new blood vessels, is under the regulation of HIF-1α [4]. Furthermore, the expression of matrix metalloproteinases (MMPs), well-known proangiogenic and profibrotic enzymes via extracellular matrix (ECM) degradation, tissue remodeling, and cell migration, is also activated by HIF-1α [5]. Mild corneal injuries can be self-repaired, while greater injuries or metabolic impacts often lead to various detrimental processes, such as endoplasmic reticulum (ER) stress [6] and dysregulated autophagy [7] resulting in corneal haze, fibrosis, and scar formation that are implicated to pathophysiology of cornea diseases and vision loss.

In healthy conditions, peripheral epithelial cells migrate via epithelial to mesenchymal transition (EMT) closing the defects to facilitate corneal wound healing [8]. A cytoskeletal intermediate protein, vimentin, is overexpressed in epithelial cells with highly motile phenotype and serves as a well-established marker of EMT not only for cancerous neoplasia but also for EMT during wound healing [9]. Zonula occludens (ZO)-1 is a component of epithelial tight junctions, which contributes to the barrier function of corneal epithelium [10]. However, defects can occur, which lead these cells to undergo apoptosis or autophagy, resulting in abnormal repair, loss of regular optical surface, and disruption of barrier function thus aggravating deterioration of vision [11]. Cumulative data from experimental and clinical studies suggest that hypoxia, ECM remodeling, angiogenesis, and autophagy are tightly intertwined and regulate the homeostasis in the cornea during normal reparative processes and may contribute to the development of corneal diseases.

Although topical formulations of steroid and non-steroid anti-inflammatory drugs or anti-VEGF agents is the first-line treatment for individuals with corneal injuries, these substances are only partially effective and may cause several side effects [3]. Therefore, seeking natural remedies that may combine high efficacy in treating excessive neovascularization and have desirable pleiotropic effects to target crucial pathological patterns in order to restore the transparency of an injured cornea is still an actual goal of applied pharmacology. During the last two decades, internal fragments of plasmin(o)gen known as angiostatins (AS) have attracted considerable attention as promising candidates for the suppression of neovascularization [12]. Several proteases can cleave the plasminogen/plasmin molecules at different sites to generate diverse AS species, which contain various number of plasminogen kringle (K) domains (K1-3, K1-4, K1-4.5, K5, etc.). All of these AS-related polypeptides inhibit proliferation and/or migration of endothelial cells to suppress growth of new blood vessels, though with various degrees of effectiveness depending on their K content [13]. Originally isolated from urine of mice bearing Lewis lung carcinoma, AS have been shown to effectively suppress tumor-induced angiogenesis and metastasis growth [14]. Lately, it has been demonstrated that AS variants generated in various tissues contribute to regulation of the angiogenic balance in an organism and are involved in the pathogenesis of various diseases such as cancer, autoimmune disorders, diabetic retinopathy, cardiovascular events, etc. [15-18]. Apart from playing a role of potent endogenous inhibitors of vessel outgrowth via VEGF counteracting mechanisms, AS have been recently reported to be novel anti-inflammatory regulators [19]. After AS had been discovered to be formed in the corneal tissue [20], attempts were made to apply exogenous plasminogen fragments as subconjunctival injections or eye drops for alleviating pathological vascular growth and corneal ulceration in experimental animals [21]. Although principal therapeutic effects of AS toward regression of corneal neovascularization have already been described by several research groups [22, 23], molecular mechanisms, which may underlie multifacial activities of AS in the injured cornea, except well-established inhibition of endothelial cell activity, have not been elucidated. Here, we first addressed the question of whether AS can be beneficial through restricting hypoxia- and inflammation-driven processes in the injured cornea. Alkali burn has been used extensively as an animal model to test efficacy of novel anti-angiogenic drugs to reduce neovascularization in the cornea [24]. Thus, the aim of the current study was to investigate the effects of two AS variants, K1-3 and K5, on the levels of HIF-1α and various key protein regulators related to angiogenesis, ECM remodeling, ER stress, and autophagy in the rat cornea injured by alkali burn. In parallel, we designed the study assessing AS efficacy to inhibit vascular growth in a model of chemical burn-induced corneal injury of rabbit eye.

Materials and Methods

Reagents. All chemicals were purchased from Sigma Aldrich Co. (St Louis, MO, USA) unless otherwise stated. All other reagents were of analytical grade and were provided by commercial suppliers.

Angiostatin production. Native, or Glu-, form of human plasminogen was isolated by the affine chromatography on lysine-Sepharose from the fresh citrated donor plasma as described earlier [25]. AS K1-3 was produced by limited proteolysis of plasminogen with the use of porcine pancreatic elastase followed by gel-filtration and affine chromatography techniques as described elsewhere [26]. Mini-plasminogen, a by-product of plasminogen elastolysis, was used for producing AS K5 by means of pepsin fragmentation followed by purification with the use of aminohexyl (AH)-sepharose affinity chromatography as reported earlier in details [27]. Prepared proteins were electrophoretically pure and did not contain any proteolytic activities.

Animals and models of corneal alkali injury. Wistar rats (6-month-old males, 190-220 g) were kept and handled in compliance with the guidelines of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The experimental protocol has been approved by the Institutional Bioethical Committee (protocol no. 4, 2021/06/08). The rats were anesthetized by intraperitoneal injection of ketamine (50–100 mg/kg) and received topically a drop of tetracaine. A 4 mm diameter Whatman no. 1 filter paper soaked in 1 N NaOH was placed on the center of the corneal surface of both eyes for 30 sec, and then the eyes were thoroughly washed with 10 ml of saline. After the injury, the rats were randomly divided into five groups (n = 6 in each group): I) intact control (saline); II) burn control (saline); III) burn + K1-3 (1.0 μM); IV) burn + K1-3 (0.1 μM); V) burn + K5 (1.0 μM). Topical administration (25 μl) of AS dissolved in sterile buffered saline to the injured eye was done daily for 14 days. Thus, single dose of 1.0 μM solution of K1-3 contains 0.75 μg (respectively, 0.075 μg in the case of 0.1 μM solution), while a single dose of 1.0 μM solution of K5 contains 0.3 μg of this angiostatin. K5 was used in one concentration because of its limited amount available. All rats were thereafter euthanatized.

Due to the relatively small size of the rat's eye surface that technically limits morphological observations, we additionally developed a rabbit corneal alkali burn model in order to monitor the anti-angiogenic activity of K1-3 (1.0 μM), the most abundant naturally occurring AS variant. There are overall similarities in anatomical features and matrix structure between rabbit and human corneas, therefore rabbit eye is commonly used as an experimental model to simulate human ocular diseases [28]. The model was developed in accordance with earlier reported recommendations for the standardization of corneal alkali burn methodology in rabbits based on the observations that 1 N NaOH produces greater stromal fibrosis and warrants neovascularization in individual corneas [29]. In general, we reproduced above-mentioned rat model of alkali burn corneal injury and divided Chinchilla rabbits (2-month-old, 1,800–2,200 g) into the three groups: I) intact control (n = 2); II) alkali burn group (n = 3); III) alkali burn + K1-3 (n = 3). In a rabbit model, alkali burn was induced only in the right eye, while the left eye obtained an equal volume of saline drops. To determine if tested AS polypeptide has any side effects in the non-injured rabbit eye, K1-3 solution at the same concentration was dropped to the left eye of rabbits from group III daily for entire experimental period. On the 14th day after the injury, bulbar redness as a measure of corneal neovascularization was estimated according to the Efron grading scale (ranging from 0 = normal to 4 = severe) [30] with the assistance of an experienced ophthalmologist in a masked fashion. After the end of the experimental period, the rabbits were returned to their home cages for routine care.

Cornea isolation and tissue lysate preparation. After eye enucleation, rat corneas were slightly rinsed for 5 min in the ice-cold phosphate-buffered saline (PBS). Both corneas of each rat were pooled, ground in liquid nitrogen, and homogenized in RIPA-buffer (0.05 M Tris-HCl, pH 7.4, 0.15 M NaCl, 1% Triton X-100, 0.1% SDS) supplemented with the Pierce™ protease and phosphatase inhibitor cocktail (ThermoScientific, USA, cat. no. A32961). Tissue to buffer ratio was 1:5 (m/v). After additional sonification (ultrasound disintegrator Sartorius, Labsonic®M, Göttingen, Germany), homogenates were centrifuged at 16,000 g for 45 min at 4°C. Concentrations of total protein in supernatants were evaluated spectrophotometrically, using the measurements of absorbance at the wavelengths of 280 and 260 nm as described earlier [31]. Supernatants were then mixed with an equal volume of 2 × reducing Laemmli sample buffer.

Western blot. Sample aliquots were loaded onto 10% SDS-PAGE (50 μg protein per well) and electrophoresed in a vertical gel electrophoresis chamber (BioRad, USA). After electrophoresis, proteins were transferred from the gel onto nitrocellulose membrane (GE Healthcare, Amersham Bioscience, UK, RPN 203D) with 0.45 μm pore diameter by electroblot. After 1 h blocking in a 5% solution of a skimmed milk powder in PBS, the blots were overnight incubated at 4°C with the following primary antibodies to the proteins of interest: rabbit anti-HIF-1α (Sigma Aldrich, USA, cat. no. HPA001275, 1:2,500 diluted), mouse anti-VEGF (Invitrogen, USA, cat. no. MA5-12184, 1:3,000 diluted), rabbit anti-MMP-9 (Sigma Aldrich, USA, cat. no. AV33090, 1:2,000 diluted), mouse anti-vimentin (Santa Cruz Biotechnology, Inc, USA, clone V9, cat. no. sc-6260, 1:500 diluted), mouse anti- BECN1/Beclin-1 (Santa Cruz Biotechnology, Inc, USA, clone E-8, cat. no. sc-48341, 1:1,000 diluted), rabbit anti-GRP-78 (Abcam, USA, cat. no. ab21685, 2,000 diluted), mouse anti-ZO-1 (Invitrogen, USA, cat. no. ZO1-1A12, 1:2,500 diluted), and β-actin (Invitrogen, USA, cat. no. MA5-15739, 1:5,000 diluted). After washing each membrane five times with PBS supplemented with 0.05% Triton X-100 (PBST), the blots were incubated with HRP-conjugated goat anti-rabbit IgG (1:6,000 diluted) or HRP-conjugated goat anti-mouse IgG (1:8,000 diluted) purchased from Invitrogen, USA (cat. nos. G-21234 and 31430, respectively) for 2 h at 37°C. Non-specifically bound antibodies were washed away with PBST, and thereafter specific immunoreactivity was developed by enhanced chemiluminescence (ECL) with the use of p-coumaric acid, luminol, and hydrogen peroxide. The molecular weight of each protein band was determined by comparing their migration with the location of coloured markers Ruler™ Plus Prestained Protein Ladder 10-230 kDa (ThermoScientific, Lithuania, cat. no. 26619). Each band immunostaining was quantified by measuring optical density values with the use of densitometry software TotalLab TL120 (Nonlinear Inc., USA), normalized to the β-actin level as a loading control, and expressed as arbitrary units (a. u.).

Statistical analysis. The results are presented as mean ± SEM. Analysis of variances (ANOVA) followed by post-hoc Tukey's Multiple Comparison was used to verify significant difference between group means. *P* value of less than 0.05 was considered significant.

Results

In the present study, we assessed the primary biological activity of plasminogen fragment AS K1-3 to reduce blood vessel outgrowth by utilizing a commonly used model of chemically induced corneal neovascularization in rabbits. Overall corneal neovascularization was scored using a system of scaling from 0 to 4 (Efron scale), where 0 = no neovascularity, 1 – slight neovascularity, 2 – moderate neovascularity, 3 – severe neovascularity, 4 – complete neovascularity. As shown in Fig. 1, there was no significant difference in neovascularity grading between intact control and isolated K1-3 dropped corneas.

This observation means that tested AS does not have irritating hypoxic action in the healthy cornea. However, intense vessel outgrowth was seen in all rabbit corneas after NaOH-induced burn, the severity of which was scored to be 3.67±0.56 according to Efron scale. Significant regression of corneal neovascularization was observed on the 14th post-injury day in rabbits, which obtained K1-3 1 μM solution as eye drops. It is worth noting that chemical burn induced corneal opacification in the limbal region, while AS treatment did not diminish opacity extent during the period of observation. The obtained findings clearly prove that produced AS (plasminogen fragment K1-3) has an expectable physiological activity in terms of angiogenesis inhibition. Due to extremely low amounts of AS K5 obtained for the experiments, its effect on overall corneal neovascularization in chemically induced corneal burn of rabbit eye was not explored in the current research,

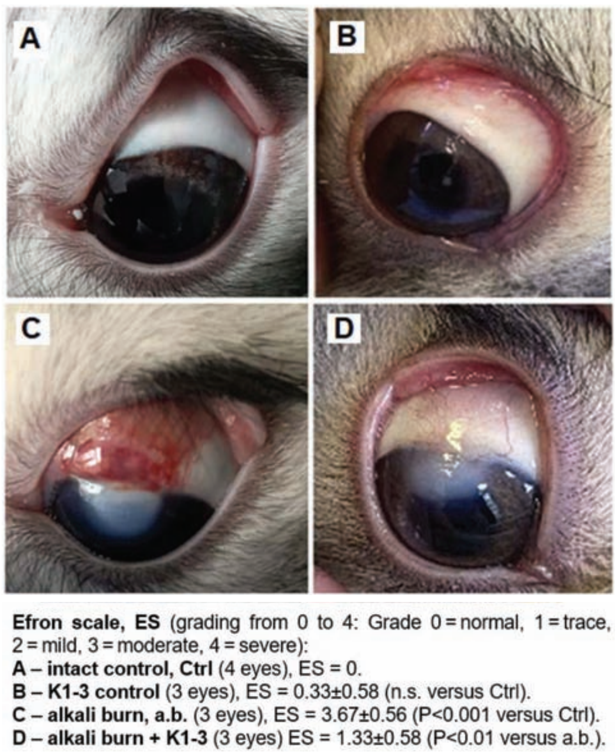

Fig. 1. Topical application of angiostatin K1-3 reduces corneal neovascularization induced by alkali burn in rabbits: ***A*** *– intact control;* ***B*** *– K1-3 control;* ***C*** *– alkali burn;* ***D*** *– alkali burn + K1-3 treatment (P < 0.05 considered statistically significant difference; n.s. – non-significant difference)*

however, the results of immunochemical analysis described below demonstrate that the effects of K5 on the expression of the studied biomarkers were comparable with those of K1-3 applied at the same dose.

To verify a hypothesis that AS may play multiple roles in cornea healing processes, aside from angiogenesis suppression, we further investigated their effects on the expression of the key regulatory proteins related to hypoxia, ECM remodeling, reepithelization, EMT, and autophagy. Western blot results on the quantification of the corresponding markers are depicted in Fig. 2. It was shown that burn injury caused statistically significant elevation of HIF-1α (by two folds vs. control), VEGF (both monomeric and dimeric forms, by 4.5 and 2 folds, respectively, vs. control), MMP-9 (by 2.7 folds vs. control), GRP-78 (by 3.4 folds vs. control), and beclin-1 (by 2.6 folds vs. control) protein levels on the 14th post-injury day. As well, there was a two-fold decrease in the protein level of ZO-1 ($P < 0.05$ vs. control), which is related to tight junctions, indicating persistent epithelial defects and slow epithelization

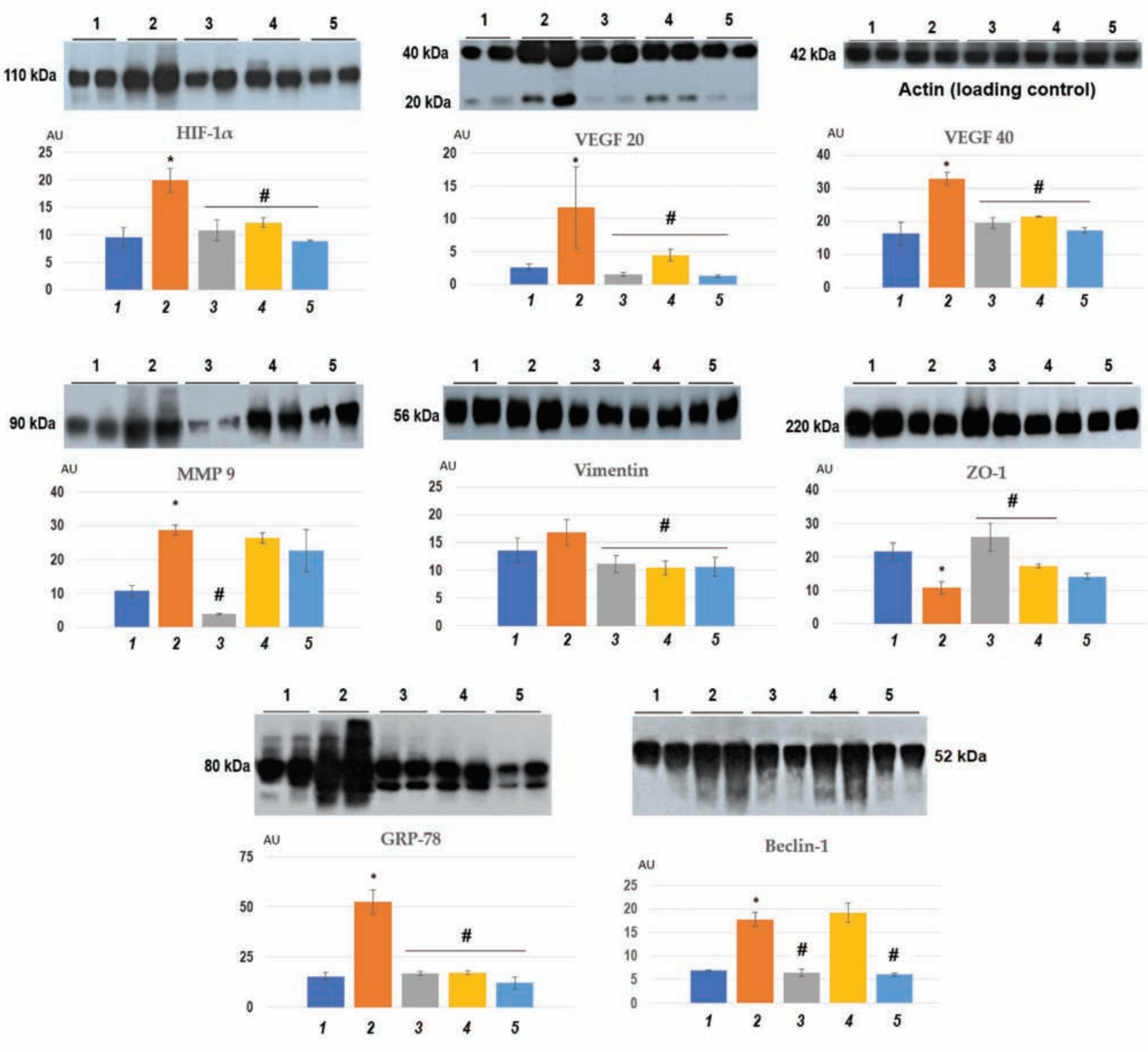

*Fig. 2. Angiostatin application modulates levels of regulatory proteins in the rat cornea after alkali burn (western blot data): 1 – intact control; 2 – alkali burn; 3 – burn + K1-3 (1.0 μM); 4 – burn + K1-3 (0.1 μM); 5 – burn + K5 (1.0 μM). The results of western blot were analyzed by scanning densitometry and expressed as arbitrary units (AU). *P < 0.05 vs. control; #P < 0.05 vs. alkali burn*

in burned corneas. The content of EMT marker, vimentin, had a tendency to increase in burned untreated corneas, but these changes did not reach the level of statistical significance.

It was found that both variants of AS applied as eye drops almost equally decreased burn-induced overexpression of HIF-1α, VEGF, vimentin, and GRP-78. Of note that K1-3 and K5, both taken in the concentration of 1.0 μM appeared to be more potent suppressors of VEGF expression that 0.1 μM K1-3 solution. Furthermore, K1-3 in the highest concentration was the only treatment regime, which was able to reduce MMP-9 level in the injured corneal tissue (by 7.55 fold as compared with burn group, $P < 0.05$). Similarly, the highest dose of K1-3 recovered ZO-1 level, which was reduced after alkali burn, by the normal value. AS, either 1.0 μM K1-3 or K5, significantly reduced up-regulated expression of beclin-1 in the injured cornea that proves a dose-dependent manner of AS efficacy.

Discussion

In the present report, we describe for the first time that AS, which are well- known to perform a "canonical" role as the most potent inhibitors of neovascularization *in vivo*, are able to target multiple cellular events related to the post-traumatic processes in the chemically injured cornea, beyond angiogenesis inhibition. The development of an adequate animal model of the corneal injury is pivotal to gaining valuable insights into the underlying pathogenesis of human ocular diseases. As opposed to acids, which are able to bind with proteins located on the outer layer of the cornea, the hydroxyl ions, which are produced by alkaline compounds, cause saponification of fatty acids, denaturation or dissolution of stromal collagen, and can destroy the underlying ECM [32]. Corneal neovascularization is considered a sight-threatening condition that introduces vascular pathology into the normally avascular corneal tissue, therefore, regression of vessel outgrowth is thought to be emerging treatment concept in the management of neovascular ocular diseases associated with eye traumatization. Nevertheless, besides neovascularization, many other individual processes occur as serious complications of corneal chemical injury that include chronic inflammation, stromal fibrosis, persistent epithelial defects or slow epithelization, corneal perforation and opacity, which can be potential targets for novel therapeutics [2].

Although several steroid and non-steroid drugs are widely used for the management of ocular surface diseases, they exert some side effects such as elevated intraocular pressure, cataract or glaucoma formation, and increased risk of concomitant infections [3]. From this point of view, purified plasminogen fragments have raised a recent wave of interest as useful therapeutic tools for treating of corneal injuries. AS in a wide range of concentrations have neither toxic nor immunogenic capacities and do not exert known undesirable effects. Since the protection of the ocular surface is a vital component in the treatment of corneal injuries, the results of our study give ground to regard tested proteins as possible therapeutics to provide a wide spectrum of beneficial effects in the injured cornea, including control for neovascularization and inflammation-related complications, but simultaneously keeping ocular surface from affecting the healing process.

Human plasminogen, a zymogen of fibrinolytic enzyme plasmin (EC 3.4.21.7) and a parent molecule for AS formation, is an abundant protein in blood circulation. Its plasma concentration is approximately 200 mg/l. It is a glycoprotein with an apparent molecular mass of 92 kDa, containing 2% carbohydrate and consisting of 5 kringle domains with a total of 24 disulfide bonds. Early studies have shown that AS have properties distinct from the precursor protein. During proteolytic cleavage, removal of a segment of plasminogen facilitates conformational changes in the resulting molecules that confers their unique binding properties and biological activities compared with the plasminogen. All known variants of AS can be produced by limited proteolysis of plasminogen by various proteases, including by plasmin auto-proteolysis. Among them, K5 displays the most potent inhibitory activities toward both endothelial cell proliferation and migration [33]. K1-3 plasminogen fragment, which is formed by elastolysis of plasminogen molecule and comprises its Cys84-Cys333 region, possesses potent anti-proliferative activity toward endotheliocytes, while exerting relatively weak anti-migratory effects [34]. Because of its high therapeutic efficacy and short amino acid sequence, K5 has considerable potential in the treatment of ocular neovascular diseases. An extra potential benefit of K5 could be the possibility of its production as a recombinant molecule [35].

There are several circumstances that prompted our laboratory to further explore the use of AS proteins as modulatory agents in the model of chemical corneal injury. Firstly, plasminogen is locally synthesized in the corneal tissue in physiologically relevant concentrations to play a role of AS local source. Coppini et al. [20] have shown that AS are constantly produced in the cornea by cysteine proteases cathepsins and critically involved in the maintenance of angiogenic balance by counteracting VEGF. Ambati et al. [22] have proved biological activity of AS isolated from tumor tissue to regress neovascularization in any tissue, including cornea. Secondly, Sack et al. [36] have shown that tear fluid collected after overnight eye closure contains various isoforms of AS in amounts necessary to counteract VEGF-mediated pro-angiogenic signaling during hypoxic condition. In our most recent study [37], we detected enhanced levels of AS in the tear fluid of patients with non-penetrating ocular traumas. It is thought that AS overproduction in the injured eye can provides an adaptive mechanism aimed at protecting cornea against traumatic injury-induced excessive neovascularization. Thirdly, AS has been reported to display potent anti-inflammatory capacities, except being powerful anti-angiogenic agents [19]. Perri et al. [38] have demonstrated that human AS K1-3 protein almost completely blocks murine and human macrophage migration via disruption of actin cytoskeleton but does not affect macrophage viability. Naïve cornea is devoid of blood and lymphatic vessels, and few immune cells are present in different parts of cornea. However, corneal injury is accompanied by infiltration and accumulation of macrophages, which play a dual role in tissue repair. During chronic inflammation, macrophages may permanently produce a plethora of biologically active molecules such as growth factors, cytokines, MMPs, reactive oxygen species, and other paracrine factors that support cell migration, tissue remodeling, and angiogenesis, but when uncontrolled, they may aggravate pathology. As a result of acute injury, angiogenic privilege of the cornea might be overwhelmed by inducing disequilibrium between angiogenic and anti-angiogenic regulators due to a surplus of macrophage-produced proangiogenic factors, like VEGF [39]. It has been reported earlier [40] that VEGF is the most significant pro-angiogenic factor in neovascular corneal diseases. Both, hypoxia and inflammatory stimuli have been shown to overexpress and stabilize HIF-1α, a major transcriptional factor, which is required for the induction of VEGF synthesis [41]. In response to hypoxia, the expression of VEGF increases 30-fold within minutes [42]. The critical role of HIF-1α in regulating VEGF expression in macrophages already at an early stage of the post-injury period and acquiring the inflammatory phenotype of corneal macrophages is well documented [43]. Overexpression of HIF-1α in parallel with up-regulation of pro-angiogenic and pro-fibrotic factors (VEGF and MMP-9, respectively), which is demonstrated by the results of western blot analysis, indicates the development of hypoxia state in the injured cornea. Since increased expression of HIF-1α and VEGF by macrophages greatly contributes to the process of formation of new vessels, which invade the cornea with the consequent loss of transparency, targeted modulation of macrophage activity may represent a promising approach to diminish corneal neovascularization. There are several reports indicating the inhibition of HIF-1α using siRNA to treat corneal neovascularization [4]. Moreover, double-target interference for VEGF and HIF-1α appeared to be effective in inhibiting neovascularization in rabbits following corneal alkali burn [44]. It is possible that decreased HIF-1α and VEGF expression, in parallel with vessel regression, observed in animals with corneal alkali burn after topical AS application can be a result of inhibitory action of the tested proteins on macrophage activity.

It is generally considered that early reparative phase (8 to 21 days) is a critical post-injury period, which is accompanied by dynamic changes in inflammation, neovascularization, and re-epithelialization. Since slow epithelization or complete failure of reepitalization, resulting in persistent epithelial defects or corneal perforations, are among the most serious complications of chemical injury, treatment of corneal defects at this stage is critical [45]. ZO-1 protein is a component of epithelial tight junctions, which is expressed in superficial and subsuperficial cell layers of the corneal epithelium and contributes to its barrier function. Earlier, it was been shown that hypoxia induces a change in the distribution of ZO-1 as well as a disruption of barrier function in cultured human corneal epithelial cells [46]. In the present study, we showed that topical application of K1-3 as 1.0 μM solution restored ZO-1 protein content in the burned cornea near the basal level. This observation suggests that AS may contribute to the formation of tight junctions and play an important role in the differentiation of corneal epithelial cells after injury. Further, vimentin, an abundant cytoskeletal protein in cells of mesenchymal origin, has been associated with increased cell motility as a hallmark of EMT during wound healing [9]. In epitheliocytes and keratocytes of uninjured cornea, relatively low levels of vimentin are considered normal, while corneal epithelial cells transiently express vimentin during wound healing. When excessive, vimentin expression may contribute to the development of fibrosis, scarring, and opacity during corneal wound healing [47]. Based on western blot results, AS-mediated ZO-1 up-regulation, together with the tendency of reducing level of the EMT marker and pro-fibrotic factor vimentin, suggest that tested proteins may restore tight junctions and modulate EMT in alkali burned cornea.

Autophagy is an evolutionary conservative catabolic process aimed at supporting cell survival through the recycling of intracellular substances and organelles in response to various stresses. Components of autophagic pathways are constitutively expressed in high levels in various ocular structures, including the cornea, lens, retina, and orbit in the eye. Autophagy could be an important pathway influencing corneal wound healing in various wound repair conditions and/or stages [48]. Dysregulation of autophagy is involved in the pathogenesis of ocular disorders, including corneal disorders. For example, a continuously high level of stress or acute injury may lead to enhanced autophagy. Frost et al. [7] showed that elevated levels of autophagic proteins are associated with corneal endothelial dystrophies. Therefore, the autophagy pathway has attracted considerable attention as a potential therapeutic target in ocular diseases. Excessive autophagy can lead to autophagic cell death, thus the dysregulation of the autophagy pathway can represent potential contributory mechanisms associated with the pathophysiological conditions in the cornea [49]. Beclin-1 is one of the autophagy-related proteins, which is commonly used as a marker to monitor autophagy flux. It initiates and regulates the formation of the nascent autophagosome (phagophore) [50]. We showed beclin-1 overexpression in wounded corneas that means enhanced autophagy flux induced by chemical injury. However, both K1-3 and K5 in the concentration of 1 μM modulates autophagy flux in the injured cornea by reducing it near the control level, but not completely inhibiting this process. It is an important observation because the basal level of autophagy in injured tissue is indispensable for the maintenance of corneal epithelial physiology via degrading and recycling damaged macromolecules and organelles, restoring normal ECM after corneal injury, and providing energetic supply and survival for cells in the stress condition.

Furthermore, the western blot assay showed that GRP-78 expression significantly increased after alkali-induced corneal burn, indicating ER stress development. ER stress is a condition, which is developed due to an overwhelming accumulation of misfolded proteins in the ER, and is implicated in various physiological states, including extreme hypoxic stress. Overactivated ER stress is accompanied by mitochondrial dysfunction and oxidative stress, resulting in cell death [51]. So far, little is known about role of ER stress in ocular diseases. Mohlin et al. [52] reported that autophagy and ER stress contribute to the degeneration of photoreceptors in cultured porcine retina exposed to cyclic dim light illumination. Other authors highlighted involvement of ER stress in diabetes-induced corneal endothelial dysfunction [53], while inhibition of ER stress has been efficient in enhancing human corneal epithelial cell viability under hyperosmotic conditions [6]. It is believed that pharmacological inhibition of ER stress could alleviate hyperglycemia-induced pathological changes in the cornea by improving mitochondrial bioenergetics, preventing endothelial cell loss, and promoting corneal epithelial wound healing. GRP-78, the major chaperone and master regulator of the so-called unfolded protein response that underlie ER stress [54]. Our data indicate that topical application of AS in injured cornea may offer therapeutic potential by reducing GRP-78 expression as a hallmark of attenuation of ER stress. Since autophagy and ER stress are intertwined [55], suppressing ER stress may abrogate over-activated autophagy and improve the healing process in the injured cornea.

The results of our study expand our knowledge on the functional significance of AS in injured corneal tissue, which extends far beyond their well-recognized function to suppress angiogenesis. Obtained results have perspective practical value, and application of AS in ophthalmology may be an effective option for clinicians to consider. It has been documented that 20% of tissue samples obtained from corneal transplants had histological evidence of neovascularization [4]. Since excessive proteolytic activities, including MMPs and lysosomal enzymes, and neovascularization are among the major factors of corneal transplant failure [42, 56], topical AS could be tested as multi-action modulators, alone or synergistically with other drugs or formulations, to decrease risk of graft rejection via inhibiting proteolytic pathways. AS can be applied as an additive to bandage contact lenses, which are used as a therapeutic eye bandage that creates a protective stabilizing shell around the cornea. Such a bandage can be useful for controlling neovascularization and inflammation extent and promoting re-epithelialization of the corneal epithelium after laser vision correction, surgical interventions, or other corneal injuries.

Conclusions. Our study uncovered novel biological functions of kringle-containing plasminogen fragments, AS, which have previously been widely described for their potent anti-angiogenic effects. The model of corneal alkali burn was useful to test the effects of AS on the key patterns of healing process, such as hypoxia-induced vasculogenesis, reepithelization and EMT, tissue remodeling, autophagy, and ER stress, though additional experiments are required for the investigation of subtle molecular mechanisms of the described effects. In the context of ophthalmology, our results may provide a basis for the use of AS in the development of new treatments for severe ocular surface injuries and corneal damage.

Conflict of interest. The authors have completed the Unified Conflicts of Interest form at http://ukrbiochemjournal.org/wp-content/uploads/2018/12/coi_disclosure.pdf and declare no conflict of interest.

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МНОЖИННІ ЕФЕКТИ АНГІОСТАТИНІВ ЗА ПОШКОДЖЕННЯ РОГІВКИ

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Тривале запалення та надмірна неоваскуляризація рогівки, що розвиваються внаслідок гострої травми ока, можуть погіршувати оптичну прозорість рогівки і призводити до погіршення зору. Фрагменти плазміногену, відомі як ангіостатини (AS), які пригнічують проангіогенне сигналювання. Однак ефекти AS на рогівку, окрім інгібування ангіогенезу, залишаються наразі не вивченими. У представленій роботі досліджували ефекти двох варіантів AS (K1-3 і K5) за умов лужного опіку рогівки кроликів та щурів. AS K1-3 у разовому дозуванні 0,075 або 0,75 мкг (0,1 або 1,0 мкМ), та AS K5 – 0,3 мкг (1,0 мкМ) застосовували як очні краплі щоденно протягом 14 днів після пошкодження. Морфологічний тест Ефрона показав, що AS сприяють регресу новоутворених судин у рогівці тварин за лужного опіку. Вестерн-блот аналіз лізатів тканини рогівки показав, що застосування AS сприяло зменшенню рівнів протеїнів-маркерів, асоційованих із гіпоксією (HIF-1α), ангіогенезом (VEGF), ремоделюванням і фіброзом тканин (MMP-9), аутофагією (beclin-1), а також стресом ендоплазматичного ретикулума (GRP-78), як основних ланок патологічного процесу. Крім того, AS K1-3 сприяв зростанню рівня ZO-1, протеїну щільних контактів, що свідчить про перебіг реепітелізації рогівки після хімічного ушкодження. Результати дослідження розкривають нові біологічні функції крингл-вмісних фрагментів плазміногену, що лежать в основі про-

текторного впливу AS під час загоєння рогівки за експериментального лужного опіку. Отримані дані можуть бути корисними для розробки нових ефективних препаратів для лікування ускладнень ушкодженої поверхні ока.

Ключові слова: ангіостатини, рогівка, лужний опік, неоваскуляризація, HIF-1α, VEGF, beclin-1, MMP-9, ZO-1.

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