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REVIEW ARTICLE

Structure and Function of Plasminogen/Plasmin System

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Abstract—The main physiological function of plasmin is blood clot fibrinolysis and restoration of normal blood flow. To date, however, it became apparent that in addition to thrombolysis, the plasminogen/plasmin system plays an important physiological and pathological role in a number of other essential processes: degradation of the extracellular matrix, embryogenesis, cell migration, tissue remodeling, wound healing, angiogenesis, inflammation, and tumor cell migration. This review focuses on structural features of plasminogen, regulation of its activation by physiological plasminogen activators, inhibitors of plasmin, and plasminogen activators, and the role of plasminogen binding to fibrin, cellular receptors, and extracellular ligands in various functions performed by plasmin thus formed.

Keywords: plasminogen, plasmin, plasminogen activators, inhibitors, angiostatins, fibrinolysis, inflammation, angiogenesis, oncogenesis

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A zymogen of plasmin (EC 3.4.21.7), plasminogen, circulates in blood. Plasminogen is synthesized in liver and many organs and tissues and considerable amounts of plasminogen are found in extravascular fluids [1, 2]. Activation of plasminogen into plasmin is regulated by tissue-type plasminogen activators (tPA, EC 3.4.21.68), urokinase-type plasminogen activators (uPA, urokinase, EC 3.4.21.31), plasminogen activator inhibitors types 1 and 2 (PAI-1 and PAI-2), and inhibitors of plasmin (α_2-antiplasmin (α_2-AP) and α_2-macroglobulin (α_2-MG)). Plasminogen activators (tPA and uPA) specifically cleave the single activation bond Arg561–Val562 in a single-chain molecule of plasminogen, which results in formation of a two-chain enzyme plasmin. Recently, data on the ability of certain proteases, such as kallikrein and factors XIa and XIIa, to activate plasminogen have appeared [3, 4]. To understand the mechanisms of plasmin involvement in various physiological and pathological processes, structural features of plasminogen, its activators, activator inhibitors, and plasmin inhibitors, all involved in regulation of the plasminogen/plasmin system activity, and their interactions with each other upon binding fibrin, cell receptors, and extracellular ligands, are to be analyzed.

In this paper, we use the term "plasminogen/plasmin system" to refer to the complex effect of plasminogen, its activators, activator inhibitors, and plasmin inhibitors (Table 1); that is, the role of plasmin in not only fibrinolysis, but also a number of other physiological and pathological processes, in which fibrin either is not involved or does not dominate, is considered.

The next sections of the review are dedicated to the analysis of structure of individual key components of the plasminogen/plasmin system and the effect of their binding to fibrin, cell receptors, and ligands of the extracellular endothelial matrix (ECM) on their specific interactions.

PLASMINOGEN

Structure of plasminogen. Native Glu-plasminogen is a single-chain glycoprotein (Glu is the *N*-terminal amino acid; 2% carbohydrates; 93 kDa), which contains the *N*-terminal peptide (NTP), five homologous kringle domains (K1–K5), and the protease domain (PD) (Fig. 1). Each kringle domain contains approximately 80 aa connected by three disulfide bonds [7, 8]. In the presence of trace amounts of plasmin, NTP (residues 1–77) is cleaved and Lys-plasminogen is formed (85 kDa, Lys-Pg); *N*-terminal amino acid of plasmin is lysine or methionine. After cleavage of the Arg561–Val562 specific bond by plasminogen

Abbreviations: PA, plasminogen activators; 6AHA, 6-aminohexanoic acid; ACE, angiotensin-converting enzyme; α_2-AP, α_2-antiplasmin; ECM, extracellular endothelium matrix; uPA, urokinase-type plasminogen activator; PAI-1 and PAI-2, plasminogen activator inhibitors types 1 and 2; K, kringle domain; LBS, lysine-binding site; α_2-MG, α_2-macroglobulin; MMPs, matrix metalloproteinases; scuPA, single-chain urokinase-type plasminogen activator; F, finger-like domain; Pm, plasmin; Pg, plasminogen; Glu-Pg and Lys-Pg, Glu- and Lys-forms of plasminogen; ProMMPs, prometalloproteinases; RAS, renin–angiotensin system; uPAR, urokinase and pro-urokinase receptor; VEGF, vascular endothelium growth factor; NTP, *N*-terminal peptide; tPA, tissue plasminogen activator; *t*AMCHA, *trans*-(4-aminomethyl)cyclohexane carboxylic acid (or tranexamic acid); PDGF, platelet-derived growth factor; FGF, fibroblast growth factor; EGF, epidermal growth factor-like domain.

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Table 1. Biochemical characteristics of the major components of plasminogen/plasmin system [5, 6]

Component	Aa	Mol. weight, kDa	Plasma concentration	Plasma half-life time	Active site
Plasminogen	791	93	0.2 mg/mL	53 h (Glu-Pg) 19 h (Lys-Pg)	His603 Asp646 Ser741
Tissue plasminogen activator (tPA)	530	70	5–10 ng/mL	3–5 min	His322 Asp371 Ser478
Urokinase plasminogen activator (uPA)	411	55/33	1 ng/mL	5–10 min	His204 Asp255 Ser356
α_2-Antiplasmin (α_2-AP)	452	67	0.07 mg/mL	50 h	Arg364 Met365
α_2-Macroglobulin (α_2-MG)		4 × 160	2.5 mg/mL	–	–
Plasminogen activator inhibitor type 1 (PAI-1)	379	52	50 ng/mL	5–7 min	Arg346 Met347
Plasminogen activator inhibitor type 2 (PAI-2)	393	46/60	<5 ng/mL	–	Arg358 Thr359

activators (PA) with simultaneous cleavage of NTP, single-chain Glu-plasminogen is transformed into the two-chain plasmin, in which the heavy A chain (60 kDa) and the light B chain (25 kDa) are connected by two disulfide bonds. The active center of plasmin containing the amino acid triad of Ser741, His603, and Asp646 is located in the light B chain (in PD) [7].

A specific feature of plasmin(ogen) is the presence of lysine-binding sites (LBS) in its kringles, which provides for plasmin(ogen) binding to fibrin, α_2-antiplasmin, cell receptors, and extracellular ligands, affinity to which differs among the kringles. Individual kringles K1, K4, and K5 and combinations thereof are considered as a binding unit between plasminogen and various cell types [9, 10] and fibrin [11]. Kringle 1 has high affinity to 6-aminohexaenoic acid and aminohexyl ligands [12], as well as to α_2-antiplasmin [13] and histidine-rich glycoproteins [14]. Affinity to intact fibrin containing only internal lysines is low for kringle 1, moderate for kringle 2, and high for kringle 5, while kringle 4 possesses practically no affinity to intact fibrin, although it can interact with partially degraded fibrin containing *C*-terminal lysines [11]. Kringle 5 possesses the highest affinity to human endothelial cells, that is, plasminogen interaction with endothelial cells proceeds mainly by means of kringle 5 [10].

Depending on the environment, plasminogen molecule can adopt different conformations. Glu-plasminogen can be in a compact closed α-conformation, which is held together by two intramolecular interactions (between LBS of kringle 5 and *N*-terminal peptide and between LBS of kringle 4 and a ligand in kringle 3), semi-open β-conformation, when one of the interactions is retained, and completely open γ-conformation, when both lysine-dependent intramolecular interactions are destroyed [15]. Lys-plasminogen devoid of the *N*-terminal peptide can adopt only β- or γ-conformations and is activated faster than Glu-plasminogen [16].

Plasmin inhibitors (L-lysine and structurally similar ω-amino acids, 6-aminohexaenoic (6AHA) and tranexamic (*t*AMCHA) acids) at low concentrations enhance Glu-plasminogen activation by uPA and tPA and inhibit it at high concentrations [17], as well as by recombinant staphylokinase [18]. The reason for the stimulatory effect of low concentrations of ω-amino acids, which are bound to LBS of kringle 5, is the destruction of one of the intramolecular bond and the

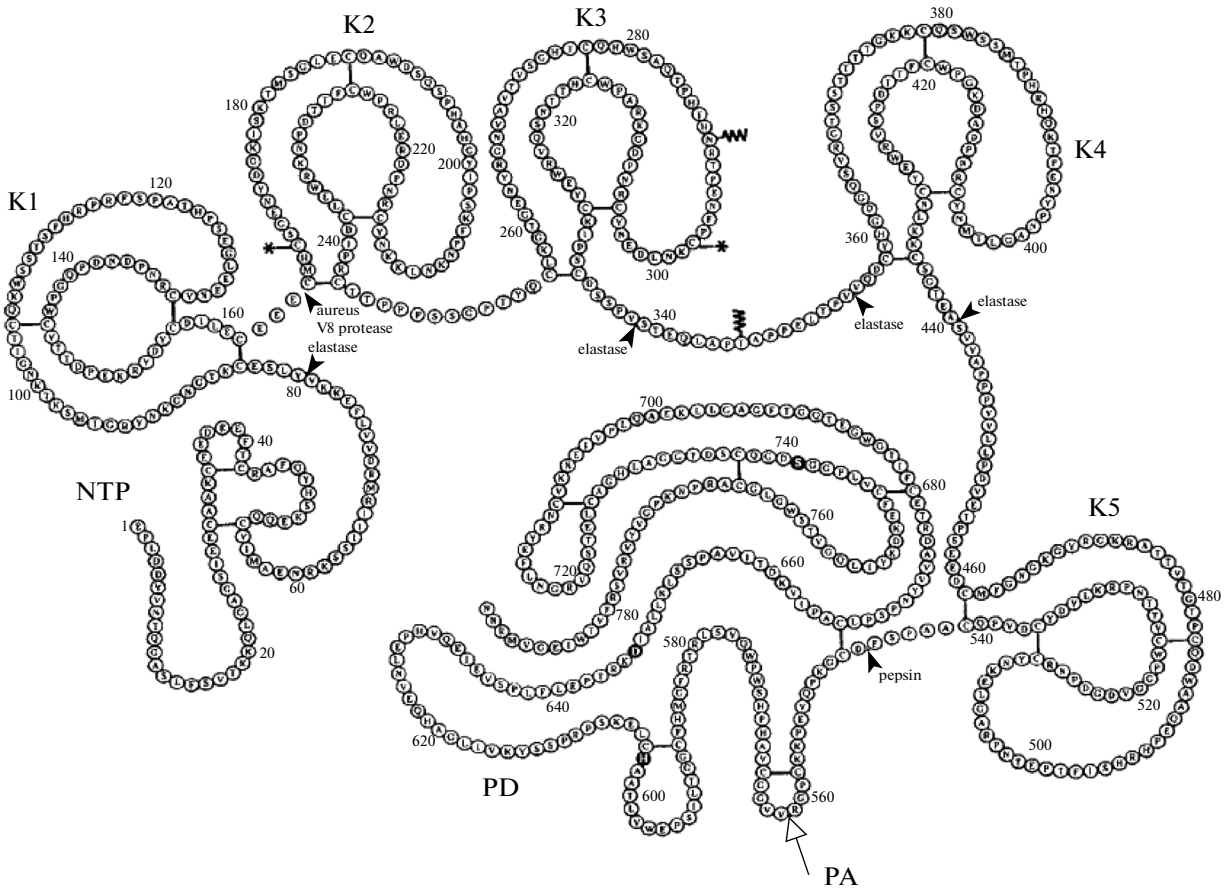

Fig. 1. Structure of Glu-plasminogen molecule [8]: PD, plasmin protease domain (residues Val562–Asn791); K1–K5, kringle domains; NTP, *N*-terminal peptide (residues 1–77); ⁓, glycosylation sites; big arrow marked as PA indicates the Arg561–Val562 bond cleaved by plasminogen activators; small arrows are cleavage sites by other proteases; *, cysteine residues forming the S–S bond between K2 and K3; ●, amino acid residues of the enzyme active site: H603, D646, and S741.

resulting transformation from closed conformation of Glu-plasminogen to semiclosed conformation. On the other hand, ω-amino acids cause dose-dependent inhibition of fibrinolysis induced by uPA, tPA [17], staphylokinase [18], and streptokinase [19], which is explained by saturation of the high-affinity LBS of plasminogen by these inhibitors and plasminogen replacement from the fibrin surface. Therefore, the binding of plasminogen through kringles with high and low molecular weight ligands results in a more open conformation of the molecule, which is easier to activate.

In blood plasma, plasminogen is present in two glycoforms with the same amino acid sequences [7]. Glycoform 1 contains one *O*-bound and one *N*-bound carbohydrate chains, and glycoform 2, only the *O*-bound carbohydrate chain. *N*-bound oligosaccharide is located in kringle 3 (at Asn289), and *O*-bound one, between kringles 3 and 4 (at Thr346) (Fig. 1). Glycoform 1 is activated under the effect of uPA and tPA more slowly than glycoform 2 [20]. Using the example of Glu- and Lys-plasminogen activation by staphylokinase, we demonstrated that the lower rate of glycoform 1 activation, if compared to glycoform 2, is caused by the increased value of K_{Pg} of glycoform 1 activation at identical values of k_{Pg} for glycoforms 1 and 2 [21]. Fibrin stimulates activation of glycoform 2 of both plasminogens (decrease of K_{Pg}) more than activation of glycoform 1. Therefore, *N*-glycosylation of kringle 3 in plasminogen causes steric hindrances for both the enzyme–substrate complex formation in solution and changes of the complex conformation on the fibrin surface. There by, functional properties of plasminogen are determined not only by the specificity of its kringles to certain ligands and the molecule conformation, but also the glycosylation type.

Plasminogen receptors exist on the surface of various cell types, such as monocytes, macrophages, platelets, fibroblasts, endothelial, tumor cells, etc. α-Enolase, annexin II, actin, p11, αIIβ3, etc. were found to be specific plasminogen receptors on the cell surface [16]. These receptors recognizing the LBS of plasminogen kringles may be of protein and nonprotein nature. A specific feature of the receptors is the

Table 2. Structural modules of the enzymes of plasminogen/plasmin system [5]

Symbol	Modules	Function
	Kringle domain (K)	Binding of Lys and Arg residues
	Epidermal growth factor (EGF)-like domain	Binding to receptor
	Vitamin K-dependent Ca^{2+}-binding domain	Binding calcium
	Finger-like domain (F)	Binding to fibrin
	Protease domain (PD)	Cleavage of Lys- and Arg-bonds in substrates

relatively low affinity to LBS of plasminogen and extraordinarily high density on many cells. Plasminogen also binds to tetranectin of the extracellular matrix [22]. Plasminogen binding to cell receptors and extracellular ligands promotes its activation due to the conformational changes accompanying the binding and protects thus formed bound plasmin from inactivation by plasmin inhibitors.

PLASMINOGEN ACTIVATORS

Structure of plasminogen activators (tPA and uPA). tPA is synthesized by endothelium cells and oocytes, and uPA, by leukocytes, tumor cells, macrophages, and fibroblasts [5]. Plasminogen activators are expressed in the form of their single-chain precursors tPA-1 and scuPA, which under the effect of plasmin and kallikrein, respectively, are rapidly transformed into two-chain molecules of tPA and uPA. They specifically cleave the Arg561–Val562 activation bond of plasminogen, thus forming plasmin.

All three proteases—plasmin, tPA, and uPA—are trypsin-like enzymes, but in contrast to trypsin, together with the protease domain (or light B chain) localized in the *C*-terminal region of their molecules, they also possess other domain structures. These domains located in the heavy A chain of the molecules are modules with notable structural and functional autonomy having preserved their structure in the course of evolution [5, 23] (Table 2). Insertion, doubling, or deletion of these domains—the processes that may be explained by an exon shift—changed the specificity of the enzymes, thus creating the evolutionary tree of serine proteases. Structural modules of heavy A chains of tPA and uPA molecule differ considerably from plasmin heavy chain modules (Fig. 2).

The difference in plasminogen activating functions between tPA and uPA is determined by structural modules of their heavy A chains. The tPA molecule binds to fibrin through the finger-like domain (F) and kringle 2 (there are no data on kringle 1 function) [24], and epidermal growth factor (EGF)-like domain is responsible for tPA binding to cell receptors. Similar to plasminogen, tPA possesses a high affinity to fibrin. The rate of plasminogen activation under the action of tPA in solution is very low but increases sharply in the presence of fibrin. The uPA molecule contains no finger domain, and uses the growth factor-like domain to bind the receptors. In contrast to kringles of tPA and plasmin(ogen), the only kringle of uPA (or scuPA) has no affinity to fibrin [25]. Therefore, fibrin exerts practically no influence on the plasminogen activating activity of uPA. Figure 3 presents our experimental data demonstrating that with increasing concentration of soluble fibrin, the rate of plasminogen activation under the action of tPA increases considerably, while fibrin does not affect the rate of activation of plasminogen under the action of uPA.

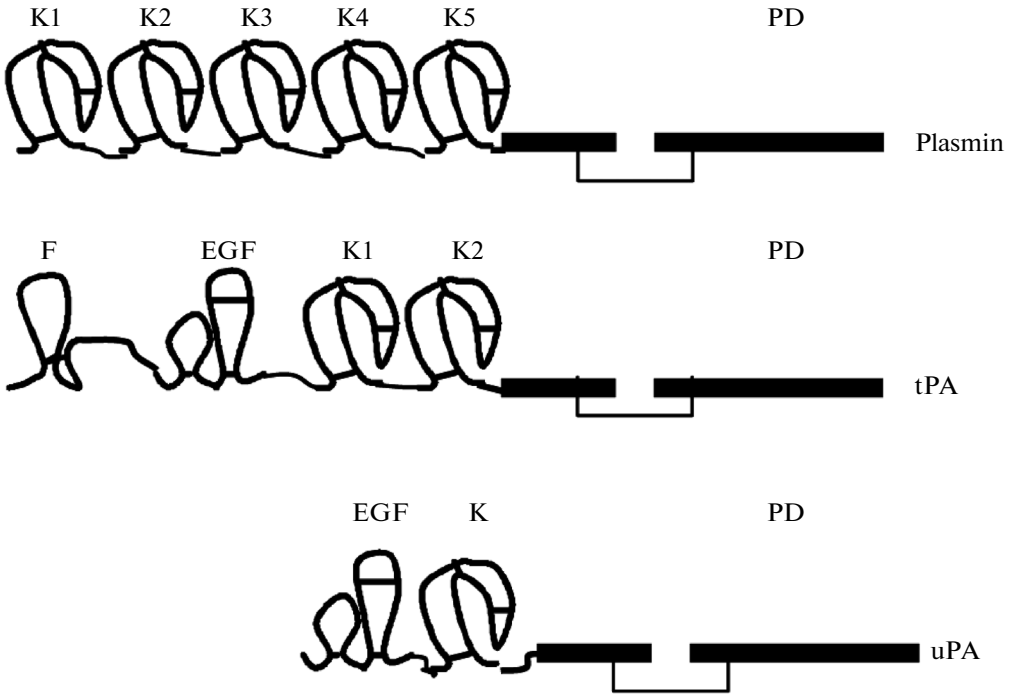

Fig. 2. Schematic representation of two-chain serine proteases of the plasminogen/plasmin system.

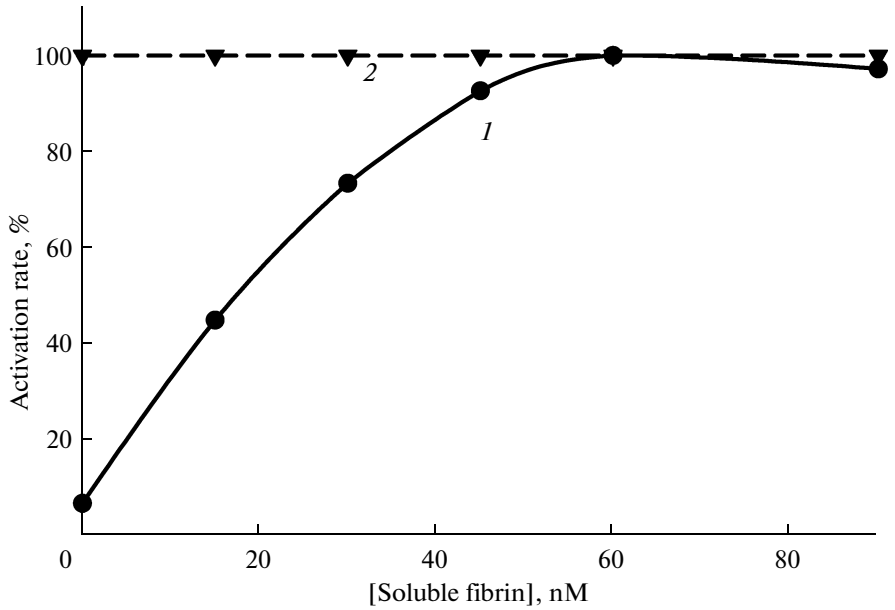

Fig. 3. The rate of Glu-plasminogen (1 μM) activation initiated by 0.25 IU/mL tPA (*1*) and 0.25 IU/mL uPA (*2*) in function of concentration of soluble fibrin (37°C, pH 7.4) ($p < 0.01$) [65].

tPA receptors. tPA receptors contained on the surface of various cell types play either a pro-fibrinolysis function or the function of the activator clearance from circulation. Vascular endothelium cells are rich with receptors that localize tPA on their surface. Some binding sites on the cell surface are common for tPA and plasminogen (for example, the membrane protein annexin II), which enhances the rate of plasminogen activation and plays an important role in the maintenance of the fluid state of the blood [26]. The main organ involved in rapid clearance of tPA ($\tau_{1/2} = 3–5$ min) is the liver, with its endothelial and parenchymal cells con-

taining heterogeneous receptors recognizing various regions of tPA [27]. The plasminogen activating activity of tPA increases considerably also upon its interaction with ECM proteins, such as collagen-IV, laminin-I, and thrombospondin, which may promote proteolysis of the connective tissue [28].

uPA receptors. A specific receptor of uPA and scuPA, uPAR, has been found on monocytes and tumor cells. Both forms of the activator bind to the receptor through a growth factor-like domain located in the *N*-terminal region of the molecule. Binding of uPA and scuPA to uPAR ($K_d \sim 10^{-9}$–10^{-10} M [29]) stimulates transformation of the slightly active scuPA form into highly active uPA and of plasminogen into plasmin. Activation of plasminogen in the extracellular matrix under the action of uPA bound to uPAR on the cell surface plays the key role in processes of ECM degradation, proliferation and migration of cells, and angiogenesis [30–32]. After neutralization of uPA bound to uPAR on the cell surface with a specific PAI-1 inhibitor, uPA–PAI-1 complex is rapidly internalized by cells and degraded in lysosomes [33]. On surfaces of hepatocytes, macrophages, and fibroblasts, there is another receptor, a protein-bound low-density lipoprotein receptor/α_2-macroglobulin receptor (LRP), which also plays an important role in uPA internalization. LRP can function in concert with uPAR.

INHIBITORS OF PLASMINOGEN ACTIVATORS AND PLASMIN

The decisive role in neutralization of tPA and uPA activity is played by the rapidly acting plasminogen activator inhibitor PAI-1 (concentration of PAI-2 in plasma is lower than that of PAI-1 by an order of magnitude). The main and rapidly acting inhibitor of plasmin is α_2-AP, while the less specific α_2-MG starts to inactivate plasmin only after exhaustion of α_2-AP pool. Physicochemical properties of the inhibitors are presented in Table 1. PAI-1 and α_2-AP regulating the activity of fibrinolysis system are referred to the class of serpins. Inhibition of tPA, uPA, and plasmin by serpins occurs through the interaction of the active center of the proteases with exposed mobile reactive loops of the inhibitors. The mechanism of protease (E) inhibition by serpins (I) comprises two stages:

$$E + I \underset{k_{-i}}{\overset{k_i}{\rightleftarrows}} E^*I \overset{k_2}{\rightleftarrows} E{-}I. \qquad (1)$$

Plasminogen activator inhibitor type 1. Interaction of PAI-1 with uPA or tPA first leads to formation of noncovalent stoichiometric complex uPA*PAI-1 or tPA*PAI-1 ($k_i > 10^7$ M^{-1} s^{-1}) [34]. At the second stage, Arg346–Met347 (P_1-P_1') bond in the reactive center of PAI-1 is cleaved with formation of a covalent bond between the Arg346 residue of the inhibitor and serine active sites of uPA (Ser356) or tPA (Ser478) resulting in tight covalent complexes uPA–PAI-1 and tPA–PAI-1 [33]. PAI-1 inactivates single- and two-chain tPA with equal power, but inhibits uPA much more strongly than scuPA.

PAI-1 is synthesized in various types of cells, including hepatocytes, platelets, endothelial cells, smooth-muscle cells, monocytes, and macrophages. It is present in plasma and ECM. A unique feature of PAI-1 is the absence of disulfide bonds, therefore it circulates in plasma in three forms: active, inactive, and latent [35]. Secreted in the active form, PAI-1 molecule is easily ($\tau_{1/2}$ = 5 min) transformed into latent inactive form due to conformational changes that result in the reactive center being shielded by a molecule loop [36]. Latent form can be reactivated under the action of negatively charged phospholipids. The third, inactive form PAI-1 is formed as a result of its cleavage by proteases via the substrate pathway. In plasma, in complex with vitronectin, as well as in the matrix, PAI-1 remains active much longer than in the free state [33].

Under pathological conditions, including thrombosis, inflammatory processes, etc., PAI-1 expression by various cells may be stimulated by cytokines, angiotensin-II, growth factor, hormones, lipids, aldosterol, interleukin-1, tumor necrosis factor, etc. [35], which leads to local concentrating of the inhibitor. This is particularly important in the case of platelets, which contain large amounts of PAI-1 in latent form [34]. Upon activation and aggregation of platelets, PAI-1 is released; it binds to fibrin, which explains the relative resistance to lysis of platelet-rich clots. Fibrin contains two types of PAI-1-binding sites: small number of high-affinity sites with $K_d < 1$ nM and high number of low-affinity sites with $K_d = 3.8$ μM [37].

α_2-Antiplasmin. At the first stage of plasmin (Pm) reaction with α_2-AP, a noncovalent enzyme–inhibitor complex Pm*α_2-AP ($k_i = 2$–4×10^7 M^{-1} s^{-1}) is formed due to interactions between lysine residues in *C*-terminal domain of α_2-AP molecule with the complementary lysine-binding site in kringle 1 of plasmin molecule [13]. At the second stage, Arg364–Met365 (P_1-P_1') bond is cleaved in the reactive center of the inhibitor with formation of a covalent bond between Arg364 residue of the inhibitor and Ser741 residue of plasmin active site. Since *C*-terminal domain of the inhibitor molecule remains noncovalently bound to kringle 1 of plasmin, an extremely stable covalent complex Pm–α_2-AP is formed, in which plasmin has at least one open LBS [38].

α_2-AP is synthesized in liver and platelets [34]. It has regions of specific binding with plasmin and fibrin [38]. In contrast to PAI-1, α_2-AP is cross-linked with fibrin and approximately 20% of the plasmin inhibitor is incorporated into a clot [34]. *N*-Terminal domain of α_2-AP binds to fibrin; the middle domain, containing a loop with reactive center, interacts with plasmin active site, while *C*-terminal domain comprising 51 aa is unique for serpins and serves for binding to the heavy chain of

plasmin. α_2-AP regulates fibrinolysis via three mechanisms: (1) neutralizes plasmin in plasma; (2) inhibits plasmin(ogen) binding to fibrin; and (3) increases the stability of clot to lysis being incorporated in the fibrin network with the aid of factor XIIIa (a transglutaminase), catalyzing the formation of peptide bonds between Gln14 residue of α_2-AP *N*-terminal domain and *C*-terminal region in fibrin α-chain [39].

BIOLOGICAL FUNCTIONS OF PLASMINOGEN/PLASMIN SYSTEM

Plasmin specifically cleaves peptide bonds formed by carboxylic groups of L-lysine and L-arginine in proteins [40]. Apart from fibrin, active plasmin can destroy fibrinogen, factors V, VIII [41], and X [42] and activate some zymogens, including tPA-1, scuPA [43], and matrix prometalloproteinases (proMMPs) [44, 45]. Binding of plasminogen through LBS in its kringles to fibrin and cell receptors promotes its activation into plasmin and protects the bound enzyme thus formed from inactivation with α_2AP. Localization of plasminogen on surface of cells performing various biological functions leads to formation of cell surfaces with a wide range of plasmin proteolytic activity [46]. Therefore, the plasminogen/plasmin system is involved in multiple physiological and pathological processes, including thrombolysis, degradation of extracellular matrix, embryogenesis, cell migration, tissue remodeling, wound healing, angiogenesis, inflammation, oncogenesis, and metastasis [47, 48].

Plasminogen/plasmin system and fibrinolysis. A zymogen of plasmin, plasminogen, circulates in blood at a concentration of 2 μM. The main physiological function of plasmin is degradation of fibrin. Fibrin is not a passive substrate of plasmin. On one hand, binding to fibrin transforms the closed conformation of plasminogen into the open, easily activated one, and on the other, fibrin protects plasmin thus formed from inhibition by α_2-AP. When a blood clot (thrombus) is formed, blood cells and proteins are cross-linked with fibrin fibers. Under physiological conditions, fibrin formation initiates activation of plasminogen by its activators, which specifically interact with each other on the clot surface, generating plasmin, which in turn efficiently breaks the fibrin clot down to soluble fragments. The effect of fibrin is manifested the most upon plasminogen activation by tPA, which, similarly to plasminogen, possesses high affinity to fibrin. Such a colocalization on the fibrin surface considerably (by a factor of 1000) increases the rate of plasmin activation by tPA [24, 49]. Besides, the plasmin generated by lysing fibrin creates on its surface an increasing number of *C*-terminal lysines—new sites for plasminogen and tPA binding, which in turn leads to a considerable increase in their concentration on fibrin [50].

The main role in thrombus lysis is played by tPA [51], while uPA, possessing no affinity to fibrin, activates plasminogen preferably in plasma and ECM [29]. Formation of high concentrations of plasmin in circulation under conditions of fibrinolysis inhibitors' deficiency may lead to the degradation of fibrinogen, factors V, VIII, and X, causing severe coagulation defects and, consequently, uncontrollable bleeding. In the case of insufficient expression of plasminogen activators or high level of their inhibitors leading to plasmin deficiency, thrombus can block the vessel lumen partially or completely, which is the reason for myocardial infarction, stroke, and other thromboembolic diseases.

Relationship between plasminogen/plasmin and renin–angiotensin (RAS) systems in circulation. Angiotensin converting enzyme (ACE) is the key enzyme of RAS catalyzing the transformation of inactive angiotensin-1 (AT-1) decapeptide into a powerful vasoconstrictor AT-II. ACE inhibitors are known to be used as hypotensive agents in therapy of cardiovascular diseases (myocardial infarction, ischemia, and atherosclerosis). There are data on some indirect links between plasminogen/plasmin system and RAS in circulation. Through binding to receptor type I, AT-II induces vessel narrowing and PAI-1 secretion by endothelial cells, thus promoting thrombosis progression [52]. Another main substrate of ACE is bradykinin, which causes vessel widening and stimulates tPA secretion by endothelial cells, thus enhancing the fibrinolytic potential [53]; degradation of bradykinin under the action of ACE increases the risk of thrombosis. ACE inhibitors decrease AT-II production and prevent bradykinin degradation and platelet aggregation [53, 54].

We performed an in vitro investigation of cross effects of synthetic plasmin inhibitors (6AHA and *t*ANCHA) and ACE (captopril, lisinopril, and enalapril) used in medicine as antifibrinolytic and hypotensive agents, respectively, on the activity of the two systems [55]. ACE inhibitors were found to be capable of inhibition, stimulation, or producing no effect on plasminogen activation and plasmin activity. The modulatory effect depended on the type of side groups in the inhibitor molecule and specificity to fibrin of the plasminogen activator [55]. Therapeutic concentrations of ACE inhibitors in plasma are relatively low (<0.01 mM) and do not affect plasminogen activation system. However, as a result of binding with circulating proteins or cell surfaces upon prolonged administration, ACE inhibitors can accumulate in certain tissues and directly influence plasmin generation from plasminogen. On the other hand, plasmin inhibitors suppressed ACE activity in a dose-dependent manner [55] (Fig. 4). This result probably explains the hypotensive side effect of these antifibrinolytic agents mentioned in the literature [56].

The results demonstrate that, in addition to links between plasminogen/plasmin and RAS systems based on changes in secretion of components of one system under the action of changes in secretion of the

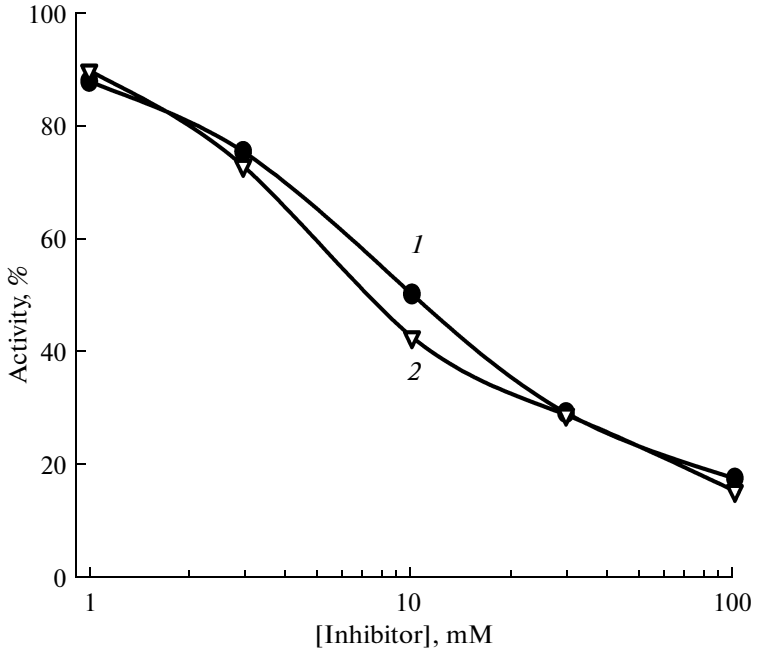

Fig. 4. Dose-dependent effect of plasmin inhibitors 6AHA (*1*) and *t*AMCHA (*2*) on ACE activity [55] ($p < 0.01$).

components of the other system, there may be other links based on direct effects of exogenous inhibitors of one system on the activity of enzymes of the other.

Role of plasminogen/plasmin system in processes of angiogenesis and inflammation. Plasminogen/plasmin system plays an important role in degradation of ECM. Binding to cell receptors and extracellular ligands modifies plasminogen conformation into a more open and easily activated one [16, 22]. tPA also binds with a number of extracellular proteins, which considerably increases its plasmin-generating activity and may promote proteolysis of connective tissue by the formed plasmin [28]. Activation of plasminogen with urokinase bound to uPAR on the cell surface plays the key role in processes of ECM degradation, cell proliferation and migration, and angiogenesis [29–32]. Plasmin is involved in ECM degradation by directly degrading a number of proteins (laminin, fibronectin, thrombospondin, etc.), as well as through activation of matrix prometalloproteinases (proMMPs) [48]. Secreted in the form of zymogens pro-MMPs are activated outside cells by various proteinases [57, 58]. Plasmin was shown to be capable of activation of prostromelisin-1 (proMMP-3), progelatinase B (proMMP-9), and procollagenase-3 (proMMP-1) [59]. Active MMPs with various substrate specificities can degrade most of ECM components. Plasmin also activates and releases vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF), thus promoting angiogenesis [60].

Angiogenesis—or generation of new blood vessels from the existing ones in an organ or a tissue—requires migration and proliferation of endothelial cells, which is promoted by ECM degradation. Plasminogen/plasmin system is involved in normal (physiological) and pathological angiogenesis. Physiological angiogenesis (from the development of a fetus and the birth to formation of normal vessels in a grown-up organism) proceeds with moderate intensity and accelerates during a number of processes, including regeneration of injured tissues, recanalization of thrombi, and scarring. In contrast to normal vascular network, pathological angiogenesis (i.e., in the course of growth and metastasis of a tumor, myocardial infarction, wound healing, chronic inflammatory diseases, etc.) proceeds abnormally: vessels are heterogeneous, irregularly branched, have multiple fenestrations, and are hyperpermeable for plasma proteins [61].

Inflammation and angiogenesis are two tightly linked processes. Neovascularization maintains the conditions for chronic inflammation (including psoriasis, rheumatoid arthritis, granulomatous disease, etc.), since new incomplete capillaries promote leukocyte permeation into the inflammation site. The reason causing the inflammatory response may be of biological, immune, chemical, or metabolic nature. Inflammation is a protective response of an organism to various damages and is aimed at removal of the damage cause, its localization, removal of damaged tissues with subsequent regeneration or restoration of their functions. The plasminogen/plasmin system is involved in all phases of wound healing. In the course of the process, fibrin and other ECM proteins are being degraded, keratinized and inflamed cells migrate, release and/or activation of growth factors

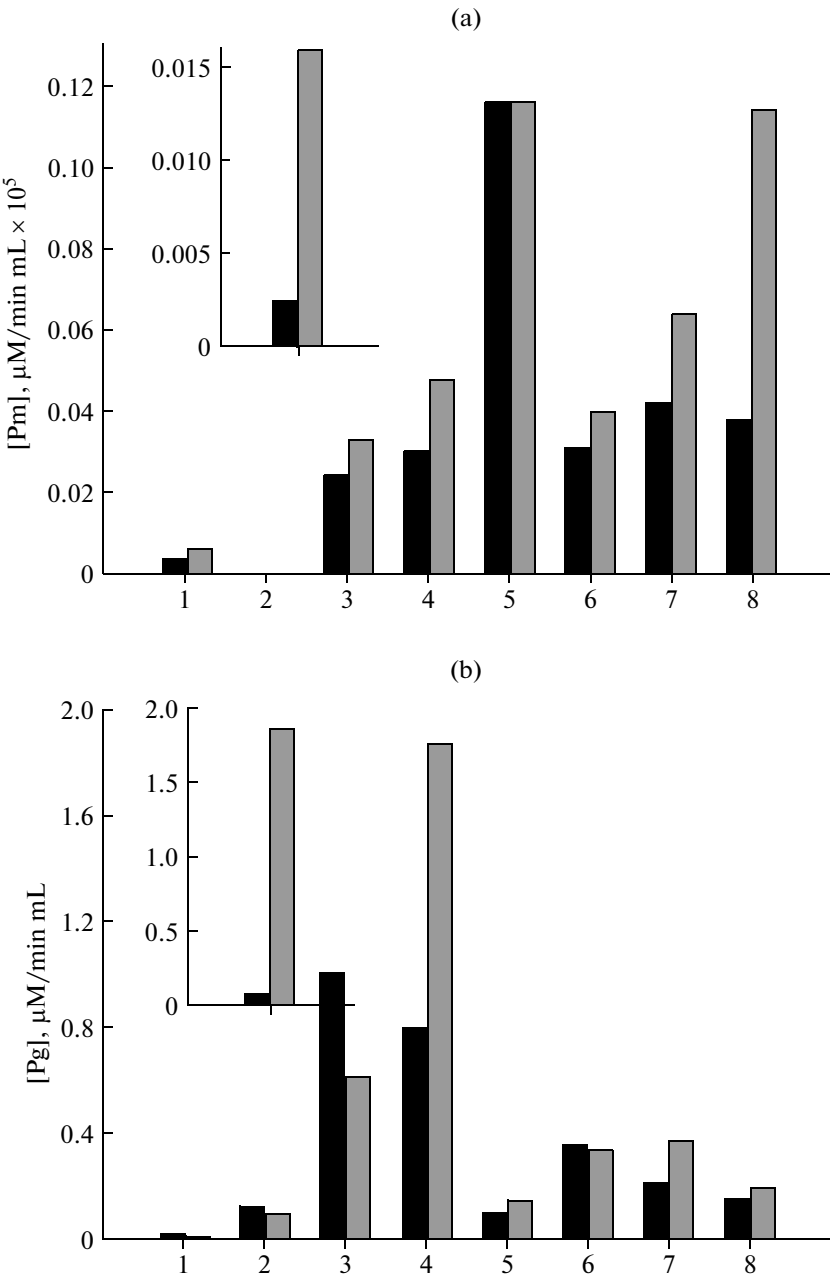

Fig. 5. Specific activity of plasmin (a) and specific potential activity of plasminogen (b) in rabbit eye tissue homogenates: 1, eye lens; 2, vitreous body; 3, iris; 4, ciliary body; 5, retina; 6, choroid; 7, cornea; 8, conjunctiva. In the in-sets, plasmin (a) and plasminogen (b) activity in the anterior chamber liquid. Dark bars correspond to healthy animals and light bars, day 3 after cornea burn [65] ($p < 0.01$).

and proMMPs are regulated, angiogenesis proceeds, and collagen is remodeled [62, 63].

We studied the involvement of plasminogen/plasmin system in the process of rabbit eye inflammation caused by an alkaline burn of the cornea [64, 65]. Figure 5 demonstrates the increase in plasmin and plasminogen levels in various rabbit eye tissues (vascularized and vessel-free) and in the anterior chamber liquid in the acute phase of inflammatory process observed on day three after the burn [64]. Figure 6 presents the dynamics of changes in the levels of plasmin, plasminogen, and its activators at various time points of the inflammatory process of a rabbit eye in lacrimal fluid, which reflects the metabolic status of both external and internal eye structures [65].

Inflammation started from the destruction of upper layer of epithelium and cornea opacification, which was accompanied by release of tPA, uPA, and plasmi-

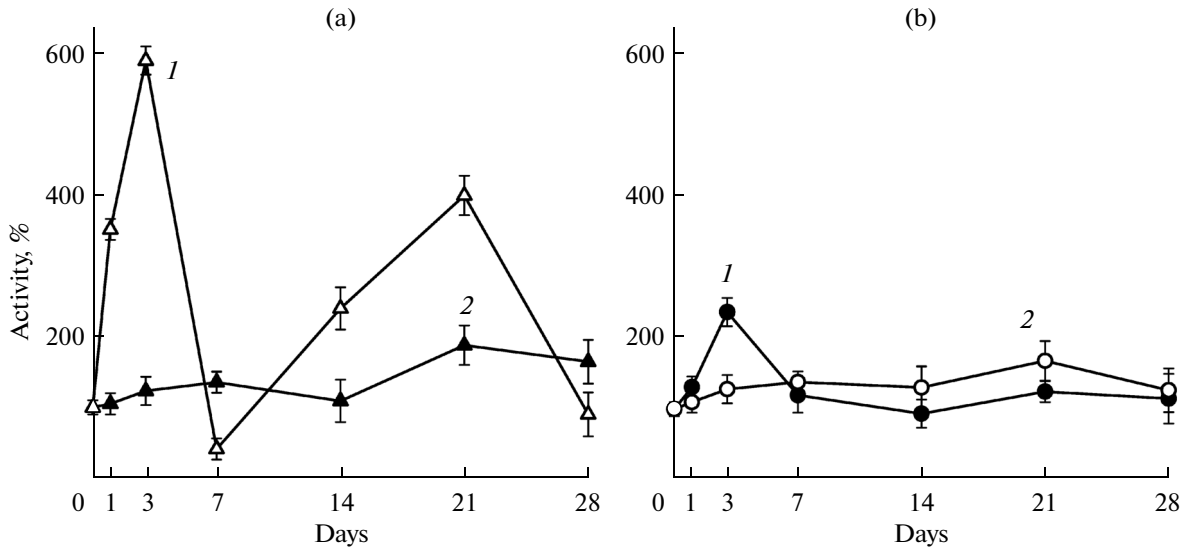

Fig. 6. Dynamics of change of activator levels, tPA (*1*) and uPA (*2*) (a), as well as plasminogen (*1*) and plasmin (*2*) (b) in lacrimal fluid in inflammatory process in rabbit eyes caused by alkaline burn of cornea compared to control levels [65].

nogen as their substrate into the lacrimal fluid, and after 2 days, when the epithelium defect was healed and swelling and permeability of the vessels increased, the first peak of plasminogen activator levels in comparison with their control levels was observed. In the following days, stroma of the cornea was cropped out and the exulceration processes started in it. Peak of cornea exulceration (days 14–21) was accompanied by repeated increase in the levels of all the agents. After 28 days, when inflammation ceased and scar tissue was formed at the burnt site, the levels of the agents dropped to practically initial values. The results are explained as follows: first, plasmin stimulates plasminogen activator release by cells, activates procollagenases, which degrade collagen in the basal membrane and stroma, and degrades fibronectin in the epithelium, which leads to the development of cornea damage. Then, plasmin induces the release of growth factors by blood vessel endothelial cells, which in turn induce angiogenesis, as well as protease and fibronectin inhibitors, which promotes tissue healing. Plasmin is involved in scar tissue removal by cleavage of fibrin and fibronectin that had performed their functions. The results demonstrate that the plasminogen/plasmin system plays an important role not only in inflammation development, but also in wound healing.

Role of plasminogen/plasmin system in oncogenesis. The key process of growth, invasion, and metastasizing of tumors is ECM degradation, in which several proteases are involved, including plasmin and matrix MMPs, some of which are activated by plasmin. It was demonstrated that the plasminogen/plasmin system is active in practically all types of tumors, while various MMPs are selectively active in tumors of different types [29]. uPA bound to uPAR on cell surface has been demonstrated to play the key role in processes of ECM degradation, cell proliferation and migration, and angiogenesis, which promote tumor progression and metastasis [30–32]. It is considered that tPA is engaged mainly in fibrinolysis processes [51, 66]. However, it was noted that uPA and tPA can functionally replace each other to a certain extent. For example, the specific interaction of tPA with annexin on the membrane of pancreatic tumor cells activates plasminogen and promotes invasion of these cells [67], while uPA (in the absence of its receptor or tPA) can efficiently dissolve fibrin [68]. uPA was shown to be expressed by more types of malignant cells, and tPA, by a limited number of tumors (melanomas, neuroblastoma, acute nonlymphoid leukemia, etc.). Besides, tissues of a number of tumors are distinguished by elevated plasminogen concentration [69–71].

So, tumor cells release plasminogen activators and their receptors, thus stimulating plasminogen activation outside cells. The formed plasmin together with activated MMPs cause degradation of ECM proteins, creating the conditions for migration and proliferation of endothelial cells, necessary for novel vessel growth [61, 72]. New vessels promote growth of primary dormant tumor (<3 mm^3), since the process of its growth requires more and more oxygen and nutrients, as well as removal of the metabolism products. Besides, as a result of ECM and basal membrane destruction by plasmin and MMPs, tumor cells escape their primary localization, migrate through the connective tissues, and invade the capillaries. After adhesion to vessel walls

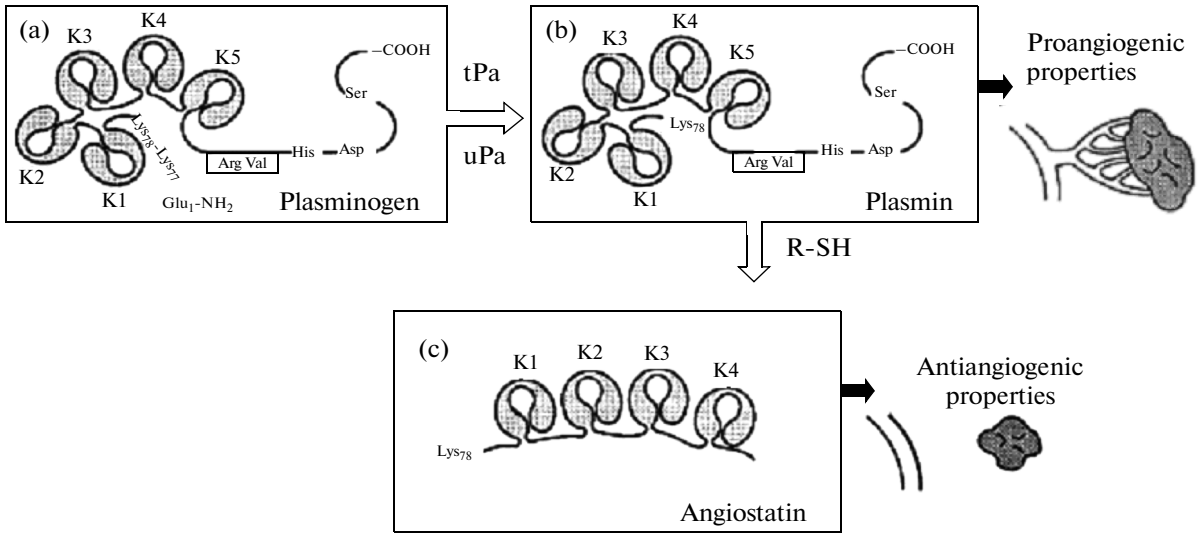

Fig. 7. Tumor-cell mediated mechanism of angiostatin generation. (a) Plasminogen is transformed into plasmin upon cleavage of the Arg561–Val562 peptide bond by plasminogen activators. (b) Being a proangiogenic protease, plasmin is able to degrade ECM proteins, thus promoting migration of endothelial cells and angiogenesis. (c) Due to autolysis in the presence of a sulfhydryl donor, plasmin is transformed into an angiogenesis inhibitor angiostatin [74].

of other organs and tissues from the bloodstream, they invade the tissues and proliferate there, forming metastases. Studies in recent decades have reliably proven the involvement of plasminogen/plasmin system in tumor progression and metastases [29–32, 61, 72].

It should be noted that neovascularization of a dormant tumor starts upon a shift in the balance between stimulators and inhibitors of angiogenesis, which are released by both tumor and stromal cells (fibroblasts, macrophages, leukocytes, platelets, etc.). Proangiogenic switch can be caused by either an increase in the expression of angiogenesis stimulators (VEGF, fibroblast growth factors (bFGF and aFGF), platelet-derived growth factor (PDGF), etc.) or suppression of angiogenesis inhibitors, acting locally (thrombospondin-1 and -2, interferon-α, etc.) or system-wide (PAI-1, α_2-antiplasmin, endostatin, vasostatin, angiostatin, etc.) [61, 72].

ANGIOGENIC AND ANTITUMOR ACTIVITY OF PLASMIN(OGEN) KRINGLE FRAGMENTS

The discovery of angiostatin, an inhibitor of angiogenesis, was a significant event illustrating the link between the plasminogen/plasmin system and angiogenesis regulation. Angiostatin (38 kDa) is a product of plasmin(ogen) degradation containing the first four kringles (K1–4) out of five in its heavy chain; it was first discovered in tumor-bearing mice as a system-wide angiogenesis inhibitor [73]. Later on, in brain and ovarian cancer tissues angiostatin K1–4.5 (52 kDa) was found, containing kringles 1–4 and part of kringle 5 [74]. Various possible mechanisms of angiostatin formation in vivo have been described.

One of the proposed mechanisms of angiostatin K1–4 generation by PC-3 tumor cells is as follows. First, under the effects of uPA or tPA plasminogen is transformed into plasmin, which, by degrading the ECM, stimulates cell migration, angiogenesis, and tumor growth (Fig. 7). Then, plasmin is subjected to autolysis in the presence of a free sulfhydryl donor (R-SH), which, apparently, also participates in changes of plasmin conformation, exposing for proteolysis previously unavailable fragments of the enzyme.

Another mechanism implies that phosphoglycerate kinase (PGK) secreted by fibrosarcoma tissue can reduce plasmin, thus promoting its autolysis. Besides, various matrix MMPs can cleave plasmin(ogen) [75]. In mouse tumors and cell culture fluid, another form of an antiangiogenic plasminogen fragment was found, A_{61} (61 kDa), containing kringles 1–4 and seven residues of kringle 5 (Lys78–Lys468) [75]. Formation of A_{61} was also induced upon incubation of HT1080 fibrosarcoma cells with plasminogen. In this case formation of plasmin from plasminogen, autolysis, and reduction of the Cys462–Cys541 bond of the enzyme are stimulated by a plasmin(ogen) receptor, annexin II tetramer [76].

A mechanism of angiostatin generation by tumor cells without the involvement of SH donors was proposed. Bound to lysine residues of β-actin (another cell receptor of plasminogen) on surface of tumor cells (PC-3 prostate cancer, HT1080 fibrosarcoma, and MDA-MB231 breast cancer) through its kringles, plasminogen is transformed into plasmin under the

Table 3. Inhibitory effects of kringle fragments of plasminogen on proliferation and migration of endothelial cells, tumor activity, and ability to bind 6AHA and *t*AMCHA [75]

Kringles	Proliferation inhibition (IC_{50}), nM	Migration inhibition	6AHA binding, μM	*t*AMCHA binding, μM	Tumor activity inhibition
K1	320	+/-	12	1	?
K2	?	+	560	94	?
K3	460	+	–	–	?
K4	–	+	26	5	?
K5	40	+	140	22	++
K1-4	135	+++	+++	+++	++
K1-3	70	+/–	+++	+++	++
K1-4 + 7rK5	35				
K1-4 + 69rK5	0.05	++	+++	+++	+++
K2-3	–	++	+++	+++	?
recK1-3 and recK1-4	17				

–, no effect; +/–, minimal potentiating effect; +, ++, and +++, different degrees of potentiating effect; and "?", the effect is not known.

action of uPA. As a result of proteolysis of β-actin-bound plasmin in the absence of an SH donor, angiostatin K1–4.5 is formed [77].

The observed antitumor activity of angiostatin K1–4 stimulated intensive studies on plasminogen kringle structure. To date, individual kringles, as well as various forms of natural angiostatins and recombinant variants of some of them (recK1–3 and recK1–4) have been obtained. Angiostatins obtained by plasminogen hydrolysis with various proteases (elastase, procathepsin D, metalloelastase, pepsine, etc.) or autolysis of plasmin under varying conditions exhibited great variation of molecular masses and *C*- or *N*-terminal amino acid residues [75–78]. Table 3 summarizes the properties of both individual kringles of plasminogen and various angiostatins obtained from plasminogen by now. Two forms of angiostatin that in addition to K1–4 contain 7 (K1–4 + 7rK5) or 69 residues of kringle 5 (K1–4 + 69rK5) turned out to be the most efficient inhibitors of cell proliferation. A powerful inhibitory effect has been produced by angiostatins K1–4 and K1–4 + 69rK5 on growth of primary and metastasizing tumors: Lewis lung carcinoma, T241 fibrosarcoma, reticulum cell sarcoma, colon carcinoma, and prostate cancer [77, 79].

It was shown that at least three receptors on endothelial cell surface are involved in angiostatin binding; they include ATP synthase, $\alpha v\beta 3$ integrin, and actin. Binding of angiostatins by the first two receptors is probably necessary for angiogenic signal transmission inside the cell [80]. For example, angiostatin is known to bind subunits of ATP-synthase on the surface of endothelial cells inducing an influx of H^+ ions in cytoplasm and cytolysis [81]. It is assumed that various cell components or signal pathways are engaged in the mediation of the inhibitory effect produced by angiostatin. The mechanism of the antitumor effect of angiostatins is complex and not completely understood.

Since constant transport of nutrients to the tumor by means of the newly formed vessels is required for tumor growth and metastasis, the mechanism of tumor growth and metastasis suppression by angiostatins is, probably, connected with angiogenesis inhibition. We obtained angiostatins K1–3, K1–4, K1–4.5, and K1–5 and studied their properties in vivo and in vitro. It was shown, for example, that angiostatin K1–4.5 inhibits neovascularization of rabbit eye cornea induced by alkaline burn (Fig. 8) [65].

To verify whether angiostatins can inhibit the activity of plasminogen/plasmin system, we studied the

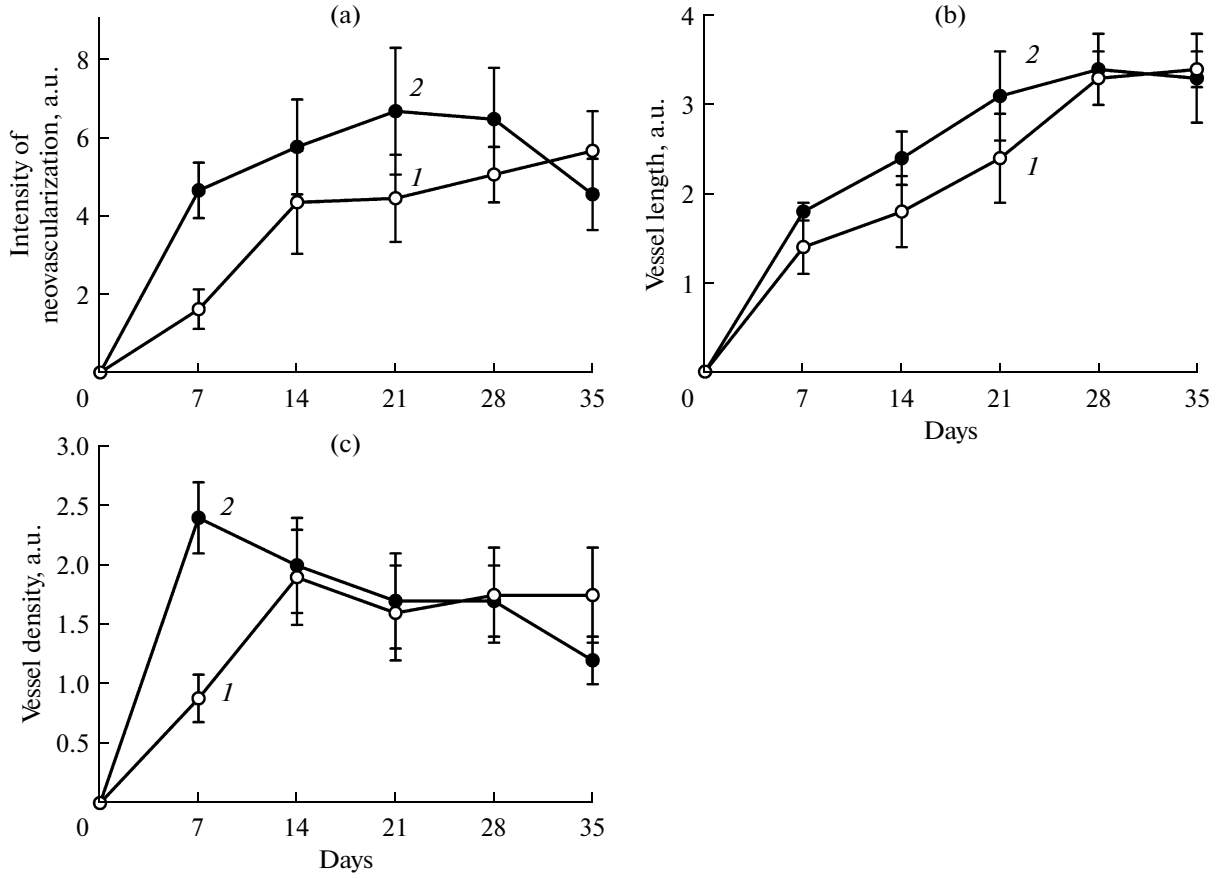

Fig. 8. Effect of angiostatin K1–4.5 on neovascularization of rabbit eye cornea caused by alkaline burn (*1*) and control (*2*) [65].

effects of angiostatins K1–3, K1–4, K1–4.5, and K1–5 on the rate of native Glu-plasminogen activation under the action of uPA and tPA [82]. It was found that angiostatins do not affect inherent amidase activities of plasmin, uPA, and tPA, but inhibit the reaction of plasminogen transformation into plasmin induced by physiological activators in a dose-dependent manner. The inhibitory effect of angiostatins increased in the sequence K1–3 < K1–4 < K1–4.5 < K1–5 (Fig. 9). A study of the effect of angiostatin K1–4.5 on kinetic constants of Glu-plasminogen activation (k_{Pg} and K_{Pg}) by its activators demonstrated that angiostatin inhibits activation by uPA (in solution) in a competitive manner, while the activation by tPA (in the presence of soluble fibrin) is inhibited in a combined manner [83]. Analysis of the results allowed the conclusion that angiostatin containing the same kringles as plasminogen, but possessing no protease domain, competitively replaces plasminogen in its complexes with uPA and tPA, as well as plasminogen and tPA from fibrin surface, thus inhibiting generation of plasmin. The results indicate that inhibition of plasminogen activation under the action of its physiological activators by angiostatins is part of the complex mechanism of their antiangiogenic and antitumor effects. Under in vivo conditions, angiostatins imitate plasminogen-binding activity and can inhibit activation of Glu-plasminogen bound to cell receptors or various ECM proteins (including fibrin) and thus suppress cell migration, angiogenesis, and tumor growth and metastases.

Therefore, the diversity of physiological and pathological processes in which the plasminogen/plasmin system is involved is associated with the unique structure of native plasminogen, which ensures that the activity of a highly efficient enzyme plasmin is under tight control of conformational changes in plasminogen molecule. Binding of plasminogen through LBS in kringles with lysine residues in substrates or receptors causes the transformation of its closed form into an open one. The open form of plasminogen is rapidly activated under the action of tPA or uPA, and the bound plasmin thus formed remains protected from inactivation by α_2-antiplasmin. Upon activation of fibrin-bound plasminogen, plasmin activity focuses on cleavage of its physiological substrate fibrin. Localization of plasminogen on the surface of cells performing various biological functions and binding to extra-

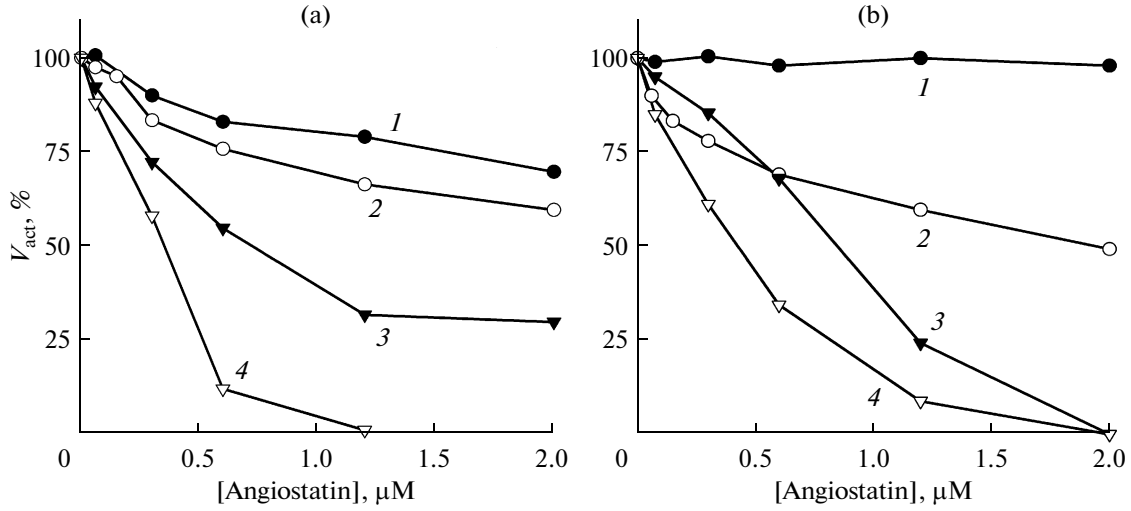

Fig. 9. Dose-dependent effect of angiostatins K1–3 (*1*), K1–4 (*2*), K1–4.5 (*3*), and K1–5 (*4*) on Glu-plasminogen activation under the action of uPA in the absence of fibrin (a) and under the action of tPA in the presence of fibrin (b), $p < 0.05$.

cellular ligands provides grounds for the involvement of plasminogen/plasmin system in such processes as ECM degradation, embryogenesis, cell migration, remodeling of tissues, wound healing, angiogenesis, inflammation, and tumor metastasis. In addition to these functions, upon proteolytic degradation in vivo, plasmin(ogen) is capable of generation of angiostatins (kringle fragments of the heavy chain), which possess antiangiogenic and antitumor properties.

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