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Inhibitory Effect of Angiostatins on Activity of the Plasminogen/Plasminogen Activator System

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Abstract—Angiostatins, kringle-containing fragments of plasminogen, are potent inhibitors of angiogenesis. Effects of three angiostatin forms, K1-3, K1-4, and K1-4.5 (0-2 μM), on the rate of native Glu-plasminogen activation by its physiological activators in the absence or presence of soluble fibrin were investigated *in vitro*. Angiostatins did not affect the intrinsic amidolytic activities of plasmin and plasminogen activators of tissue type (tPA) and urokinase type (single-chain scuPA and two-chain tcuPA), but inhibited conversion of plasminogen to plasmin in a dose-dependent manner. All three angiostatins suppressed Glu-plasminogen activation by tcuPA independently of the presence of fibrin, and the inhibitory effect increased in the order: K1-3 < K1-4 < K1-4.5. The inhibitory effects of angiostatins on the scuPA activator activity were lower and further decreased in the presence of fibrin. Angiostatin K1-3 (up to 2 μM) had no effect, while 2 μM angiostatins K1-4 and K1-4.5 inhibited the fibrin-stimulated Glu-plasminogen activation by tPA by 50 and 100%, respectively. The difference in effects of the three angiostatins on the Glu-plasminogen activation by scuPA, tcuPA, and tPA in the absence or presence of fibrin is due to the differences in angiostatin structures, mechanisms of action, and fibrin-specificity of plasminogen activators, as well as due to the influence of fibrin on the Glu-plasminogen conformation. Angiostatins *in vivo*, which mimic plasminogen-binding activity, can inhibit plasminogen activation stimulated by various proteins (including fibrin) of extracellular matrix, thereby blocking cell migration and angiogenesis. The data of this work indicate that the inhibition of Glu-plasminogen activation under the action of physiological plasminogen activators by angiostatins can be implicated in the complex mechanism of their antiangiogenic and antitumor action.

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Key words: plasminogen, plasminogen activator, plasmin, angiostatin, inhibition of plasminogen activation

The process of angiogenesis including endothelial cell migration and proliferation and blood vessel formation plays a crucial role in tumor growth and metastasis [1]. The switch to the angiogenic phenotype occurs in imbalance between angiogenesis stimulators (basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), fibrinogen, fibronectin, etc.) and inhibitors (endostatin, angiostatin, thrombospondin, PAI-1, α_2-AP, etc.) [2].

Degradation of extracellular matrix, which is necessary for tumor cell invasion and metastasis formation, occurs by the action of plasmin and some matrix metal proteinases (MMPs). Plasmin is a product of plasminogen/plasminogen activators (Pg/PAs) system activation. This system is active in virtually all tumor types, whereas various MMPs are selectively active in different tumor types [3, 4]. Plasminogen (Pg), two-chain urokinase-type plasminogen activator (tcuPA) and its single-chain zymogen (scuPA), tissue-type plasminogen activator (tPA), plasminogen activator inhibitor-1 (PAI-1), specif-

Abbreviations: AFK-pNA, HCO-Ala-Phe-Lys *p*-nitroanilide; 6-AHA, 6-aminohexanoic acid; α_2-AP, α_2-antiplasmin; Glu-Pg and Lys-Pg, Glu- and Lys-form of plasminogen, respectively; LBS, lysine-binding center; MMPs, matrix metal proteinases; NTP, NH_2-terminal peptide; PA(s), plasminogen activator(s); PAI-1, plasminogen activator inhibitor-1; Pm, plasmin; scuPA, single-chain urokinase-type plasminogen activator (pro-urokinase); tcuPA, two-chain urokinase-type plasminogen activator (urokinase); tPA, tissue-type plasminogen activator; SK, streptokinase; S-2444, Glp-Gly-Arg *p*-nitroanilide; S-2288, H,D-Ile-Pro-Arg *p*-nitroanilide.

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ic surface cell receptor of tcuPA (uPAR), and plasmin inhibitor (α_2-antiplasmin, α_2-AP) take part in regulation of plasmin activity [5, 6]. There is some difference in tcuPA, scuPA, and tPA specificity. It is generally assumed that tcuPA is chiefly implicated in extracellular degradation, tissue remodeling, and tumor invasion [6, 7], whereas tPA is chiefly implicated in fibrinolysis [8] and plays an important role in central nervous system physiology and pathology [9]. TcuPA is expressed in the majority of malignant cell types, whereas tPA is expressed in a limited number of tumors, such as melanomas and neuroblastomas, and elevated plasminogen levels are found in a series of tumors [10-12].

Native Glu-plasminogen (Glu-Pg) is a single-chain 93 kDa glycoprotein containing an NH_2-terminal peptide (NTP), five regions of internal sequence homology known as kringles, and a protease domain [13, 14]. The cleavage of the Arg561–Val562 peptide bond by plasminogen activators converts plasminogen into two-chain plasmin [15]. The light chain (protease domain) of plasmin is linked by two disulfide bonds with the heavy chain containing five kringle domains (K1-5) that contain lysine-binding sites (LBS) [16, 17]. Plasminogen binding with fibrin, matrix proteins, and cell receptors via LBS leads to drastic increase in the rate of its conversion into plasmin under the action of activators [18-20]. The degradation of extracellular matrix by the formed plasmin results in release of tumor cells from their primary locality to form metastases [18].

It was first found in 1994 that the plasmin heavy chain fragment containing the first four kringle domains (K1-4) and named angiostatin suppresses both angiogenesis and tumor growth [21]. This discovery has given an impetus to intensive studies on properties of various fragments of plasminogen kringle structure. Several angiostatins representing different fragments of the plasminogen kringle structure were prepared in a series of laboratories by plasminogen proteolysis or plasmin autolysis under various conditions. Biochemical characteristics of these angiostatin forms are summarized in [22]. The studies have shown that the angiostatin containing the first three kringles (K1-3) is a weaker inhibitor of endothelial cell proliferation than is angiostatin K1-4 [23]. On the other hand, the recombinant forms of angiostatins K1-3 and K1-4 demonstrated comparable antitumor activity *in vivo* [24]. Another angiostatin form containing kringle-domains 1-4 and 85% of kringle 5 of plasminogen and named as K1-4.5 or K1-5 suppressed both angiogenesis and tumor growth at a concentration that was 50-fold lower than the effective concentration of K1-4 [25]. The first stage of clinical trials of recombinant K1-3 is now completed.

At least three receptors localized on the surface of endothelial cells are involved in angiostatin binding: ATP-synthase, integrin $\alpha v\beta 3$, and actin. The binding of angiostatin by the first two receptors is likely required for antiangiogenic signal transmission into the cell [26]. It is supposed that different cell components or signaling pathways are involved in intermediation of the inhibitory effect of angiostatins. The inhibitory mechanism of angiostatins is complex and is not completely elucidated.

The antiangiogenic effect of angiostatins might also be associated with their direct influence on generation of plasmin from plasminogen under the action of plasminogen activators because angiostatins – due to presence of LBS in kringles – can sorb onto the plasminogen-binding centers. The goal of this work was to study effects of angiostatins K1-3, K1-4, and K1-4.5 on the rate of activation of native Glu-plasminogen by its physiological activators *in vitro*.

MATERIALS AND METHODS

Two-chain urokinase-type activator (54 kDa, 100,000 IU/mg protein) was from Green Cross (Korea); the recombinant single-chain urokinase-type plasminogen activator (50 kDa, 100,000 IU/mg protein) was obtained from the Laboratory of Gene Engineering, Institute of Experimental Cardiology, Moscow, Russia; tissue-type plasminogen activator (72 kDa, 500,000 IU/mg protein) and batroxobin from pit viper *Bothrops atrox* venom were from NIBSC (UK); streptokinase (47 kDa, 6300 IU/mg dry weight) was from Reyon Pharmaceutical Co. Ltd. (Korea); elastase (pancreatic, from porcine pancreas) was from ICN Biomedicals, Inc (Germany); human fibrinogen and HCO-Ala-Phe-Lys *p*-nitroanilide (AFK-pNA) were from Technology-Standard (Russia); Glp-Gly-Arg *p*-nitroanilide (S-2444), H,D-Ile-Pro-Arg *p*-nitroanilide (S-2288), and phenylmethylsulfonyl fluoride (PMSF) were from Sigma (USA); aprotinin (Gordox) was from Gedeon Richter (Hungary); 6-aminohexanoic acid (6-AHA) was from Merck (Germany); Lys-Sepharose 4B, Sephadex G-25, and Sephadex G-75 were from GE Healthcare (Sweden); frozen human blood plasma was from the Hematological Research Center of the Russian Ministry of Healthcare, Moscow.

Main buffer solutions—0.1 M Tris-HCl, pH 7.4 (buffer A); 0.05 M Tris-HCl, pH 8.5 (buffer B); 0.02 M Tris-HCl, pH 8.0 (buffer C); and 0.1 M phosphate buffer, pH 8.0 (buffer D)—were supplied with some or other components required for each particular experiment. Other buffer solutions were used singly and specified in the methods below.

Native Glu-plasminogen was isolated from human blood plasma by affinity chromatography on Lys-Sepharose 4B at 4°C in the presence of 20 KIU/ml aprotinin and purified as described in [27]. The zymogen level in the purified specimen was 90-95% from the data of plasmin (recovered from Glu-plasminogen with streptokinase) titration by aprotinin.

Plasmin was prepared by activation of 1-3-μM Glu-plasminogen solution with streptokinase taken at catalytic concentration ([Pg]/[SK] = 100 : 1 (M/M)) in the buffer A containing 0.15 M NaCl and 20% glycerol (v/v) at 25°C. The completeness of zymogen-to-enzyme conversion was determined from maximum amidase activity of samples.

Fibrin monomer (des-AA-fibrin) was prepared by treatment of human fibrinogen (20 mg/ml) with a thrombin-like enzyme, batroxobin (0.05 IU/ml), followed by dissolution of the formed non-cross-linked polymeric clot in 7 M urea.

Angiostatins K1-3 and K1-4 were prepared by hydrolysis of Glu-plasminogen with elastase according to the method described in **[**23, 28] with some modifications. Glu-plasminogen (3 mg/ml) was incubated with elastase at the ratio [plasminogen]/[elastase] = 50 : 1 (M/M) in buffer B containing 0.15 M NaCl and 200 KIU/ml aprotinin for 5 h at room temperature. The reaction of elastolysis was terminated by triple addition of PMSF to maintain its concentration at the level of ~1 mM for 40-50 min. Then the reaction mixture was separated on a column with Sephadex G-75 for removal of both low and high molecular mass impurities. Protein fractions of the second peak containing K1-3, K1-4, and miniplasminogen were applied onto an affinity column with Lys-Sepharose 4B equilibrated with buffer B containing 0.15 M NaCl. Following elution of miniplasminogen, which is not bound with the sorbent, the sorbed fragments K1-3 and K1-4 were eluted with 0.2 M 6-AHA in the same buffer, dialyzed against buffer C, and applied onto a column with heparin-agarose equilibrated with the same buffer. Following elution of the unbound fragment K1-4 with the equilibrating buffer, the fragment K1-3 was eluted with 0.25 M KCl in the same buffer. The purified fragments K1-3 and K1-4 were dialyzed against water and lyophilized.

Angiostatin K1-4.5 was prepared by the method described in [25] with some modifications. Glu-plasminogen (10 mg/ml) was activated with urokinase (600 IU/ml) in 0.05 M phosphate buffer, pH 9.0, containing 0.02 M L-lysine and 0.1 M NaCl at 37°C. The completeness of conversion of plasminogen into plasmin was determined from increase in amidase activity of the solution to its maximum. The solution of plasmin was mixed with an equal volume of 0.2 M glycine buffer, pH 12.0, and incubated for 18 h at 25°C and final pH value of 10.5. The reaction mixture was fivefold diluted with buffer D containing 40 KIU/ml aprotinin and applied onto a column with Lys-Sepharose 4B equilibrated with the same buffer. Following elution of microplasmin, the sorbed angiostatin K1-4.5 was eluted with 0.2 M 6-AHA in the buffer D, dialyzed against water, and lyophilized.

Purity of prepared specimens of plasminogen and angiostatins was checked by SDS-PAGE in 12% gel under non-reducing conditions according to the method of Laemmli [29].

Amidase activities of plasmin, tcu-PA, and t-PA were determined by spectrophotometry from the initial rates of hydrolyses of AFK-pNA (0.6 mM), S-2444 (0.4 mM), and S-2288 (2.0 mM), respectively, in buffer A containing 0.15 M NaCl and 0.01% Triton X-100 at 37°C. The final enzyme concentrations in the measuring cell were 1-20 nM. Effects of angiostatins on amidase activities of plasmin and activators of plasminogen were studied in the reaction mixture containing 1 μM of either K1-3 or K1-4 or K1-4.5 (each experiment was repeated twice).

Effects of angiostatins on Glu-plasminogen activation by tPA, tcuPA, or scuPA were studied by the method described in [30] with slight modifications.

Non-stimulated plasminogen activation. Aliquots (150 μl) of buffer A containing 0.15 M NaCl, 0.8 mM AFK-pNA, 0.013% Triton X-100, 0.4 μM Glu-plasminogen, and varied concentrations of angiostatins were placed into microplate wells. Following thermostatting in an Elmi ST-3 thermostatic shaker (Elmi, Estonia) at 37°C, 50 μl of either 0.056 nM tPA, or 0.184 nM tcuPA, or 2.48 nM scuPA in buffer A were placed into the experimental wells and 50 μl of buffer A only into the control wells. Concentrations of angiostatins K1-3, K1-4, and K1-4.5 in the reaction medium were varied from 0-2 μM.

Stimulated plasminogen activation. Experiments were carried out under the same conditions, but all experimental and control solutions additionally contained 0.06 μM fibrin monomer as a stimulator of the reaction.

Kinetics of plasminogen activation was monitored from release of the product of AFK-pNA hydrolysis by formed plasmin at 37°C measured every 3-5 min by absorbance at 405 nm (A_{405}) using a computerized Anthos 2020 microplate reader (Austria). The A_{405} values of experimental solutions were adjusted for changes in control (A_{405}^{o}) if the latter were detectable. Each experiment was repeated four times.

RESULTS

The data of the literature point to significant variations in molecular masses and C- and N-terminal amino acid residues of K1-3, K1-4, and K1-4.5 plasminogen fragments prepared by either plasminogen hydrolysis with various proteases (elastase, MMPs, procathepsin D, metalloelastase, etc.) or plasmin autolysis under various conditions [22, 31].

In this work, we prepared fragments K1-3 and K1-4 by elastolysis of Glu-plasminogen followed by separation of the two fragments, and fragment K1-4.5 was prepared by plasmin autolysis at pH 10.5 in the absence of sulfhydryl donors. We optimized experimental conditions for all stages of preparation of each angiostatin form: plasminogen proteolysis, plasmin autolysis, proteolysis termination, and isolation and purification of target products. Elastolysis of Glu-plasminogen (93 kDa) and

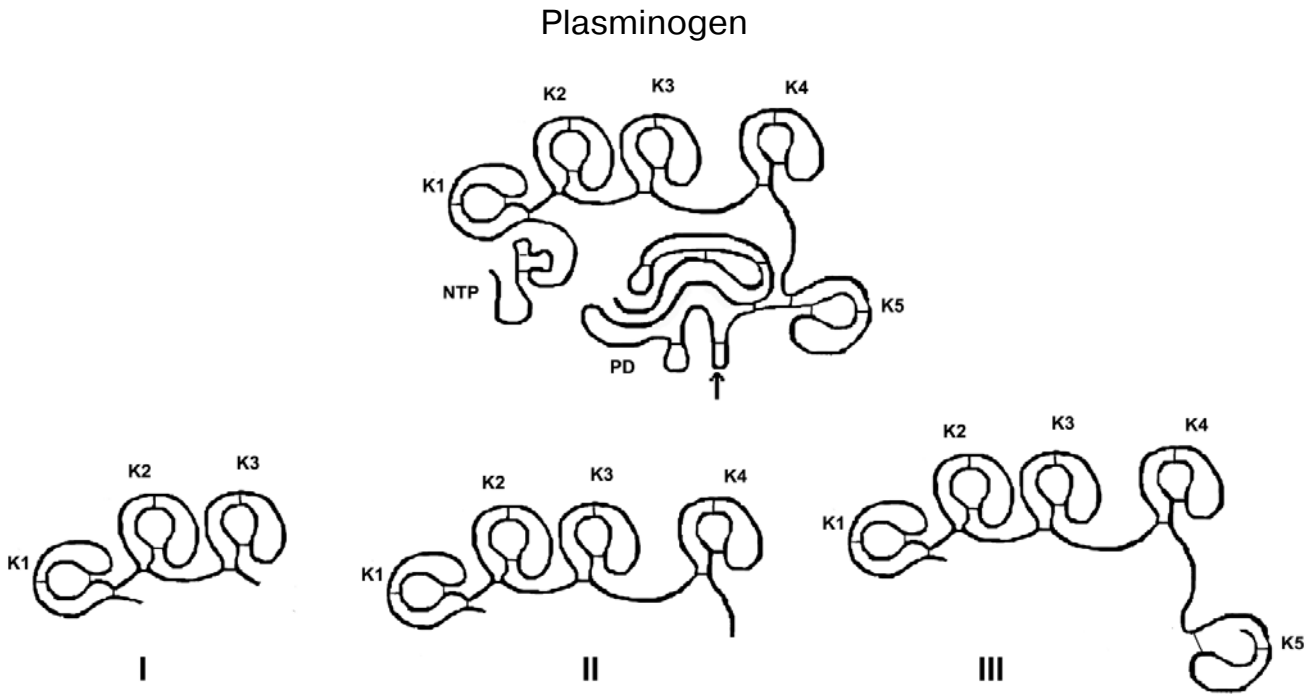

Fig. 1. Schematic representation of structures of Glu-plasminogen and fragments K1-3 (I), K1-4 (II), and K1-4.5 (III). Designations: NTP, NH_2-terminal peptide; PD, protease domain; ↑, site of peptide bond cleavage by plasminogen activator.

autolysis of plasmin (85 kDa) were monitored by dynamics of disappearance of electrophoretic bands corresponding to the zymogen or enzyme and intensification of bands with masses about ~ 38, 45, and 55 kDa corresponding to K1-3, K1-4, and K1-4.5, respectively. Schematic structures of Glu-plasminogen and the three prepared fragments of its kringle structure are presented in Fig. 1.

The data of SDS-PAGE of purified specimens of Glu-plasminogen and the three angiostatins in 12% gel under non-reducing conditions is presented in Fig. 2. One can see that plasminogen proteolysis and plasmin autolysis result in formation of two angiostatin isoforms: K1-3 (M_r ~ 33 and 39 kDa), K1-4 (M_r ~ 42 and 45 kDa), and K1-4.5 (M_r ~ 52 and 55 kDa). This data is also indicative of the absence of residual admixture of intact plasminogen or plasmin molecules in the purified angiostatin specimens.

We checked for possible contamination of angiostatin with degraded C-terminal plasminogen or plasmin fragments of similar molecular masses. To do this, we carried out hydrolysis of a specific plasmin substrate, AFK-pNA (0.6 mM), in buffer A containing 0.15 M NaCl and 0.01% Triton X-100 at 37°C in presence of 1.0 μM of angiostatin K1-3, K1-4, or K1-4.5 (before and after their incubation with streptokinase). The absence of amidase activity before and after activation of the specimens with streptokinase indicated the absence of detectable mini- or

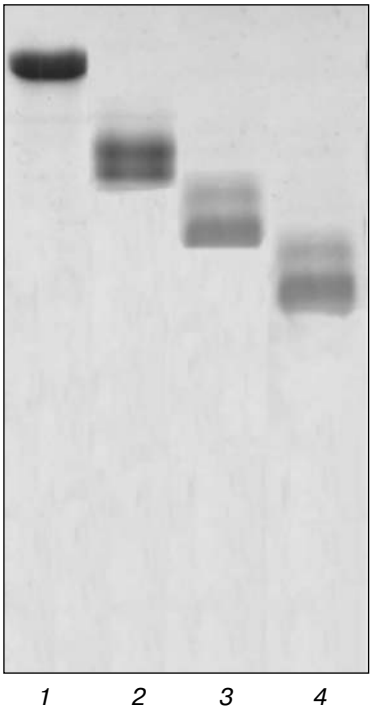

Fig. 2. SDS-PAGE in 12% gel under non-reducing conditions: *1*) Glu-plasminogen; *2*) angiostatin K1-4.5; *3*) angiostatin K1-4; *4*) angiostatin K1-3.

microplasmin(ogen) amounts in the purified angiostatin specimens.

Effects of angiostatins on plasminogen activation were evaluated using a coupled method based on simultaneously occurring processes of plasminogen (Pg) activation by plasminogen activator (PA) and hydrolysis of plasmin substrate (S) by the produced plasmin (Pm) [30]:

$$PA + Pg \overset{K_{Pg}}{\rightleftarrows} PA\text{-}Pg \overset{k_{Pg}}{\longrightarrow} PA + Pm, \quad (1)$$

$$Pm + S \overset{K_m}{\rightleftarrows} Pm\text{-}S \overset{k_{cat}}{\longrightarrow} Pm + p\text{-}NA. \quad (2)$$

The production of *p*-nitroaniline (p-NA) (if [S] >>K_m, [Pg] > K_{Pg}, [PA] << [Pg] = const) is described by the equation:

$$A_{405} = 0.5 \cdot \varepsilon_{M\ 405} \cdot k_{cat} \cdot k_{Pg} \cdot [PA] \cdot t^2, \quad (3)$$

and the plasminogen activation rate is proportional to the activator concentration:

$$v_{act} = A_{405}/t^2 = 0.5 \cdot \varepsilon_{M\ 405} \cdot k_{cat} \cdot k_{Pg} \cdot [PA]. \quad (4)$$

Under these conditions, we evaluated the degree of angiostatin impact on plasminogen activation by tPA, tcuPA, or scuPA from alteration of v_{act} in the presence of the different angiostatins.

Effects of angiostatins on the rate of plasminogen activation determined by coupled method might result from their influence on the intrinsic activities of plasmin, plasminogen activator, or both enzymes. So, we determined individual amidase activities of plasmin, tcuPA, and tPA in the presence of the three angiostatins at concentrations more than 50-100 times exceeding those of the enzymes. We did not evaluate the effects of angiostatins on activity of scuPA because of its low intrinsic amidase activity. The data presented in Table 1 show that angiostatins do not influence the intrinsic activities of plasmin, tcuPA, and tPA.

However, we found that the three angiostatins inhibit in a dose-dependent manner the Glu-plasminogen-to-plasmin conversion induced by physiological plasminogen activators. Figure 3 displays plots of the rates of Glu-plasminogen activation by tcuPA (I) and scuPA (II) versus concentration of the angiostatins in absence (a) or presence (b) of fibrin. In the absence of fibrin, the inhibitory effect of the angiostatins on plasminogen activation by tcuPA was rather more expressed

Table 1. Effects of angiostatins (1 μM) on amidase activities of plasmin (10 nM), tcuPA (18 nM), and tPA (0.72 nM) determined from the hydrolysis rates of their specific substrates, as described in "Materials and Methods" ($p < 0.001$)

Enzyme	Rate of p-NA formation, μmol/min			
	without angiostatins	K1-3	K1-4	K1-4.5
Plasmin	3.48 ± 0.12	3.26 ± 0.20	3.58 ± 0.18	3.52 ± 0.15
tcuPA	3.91 ± 0.09	4.02 ± 0.22	3.86 ± 0.19	4.10 ± 0.09
tPA	2.60 ± 0.18	2.85 ± 0.20	2.75 ± 0.15	2.39 ± 0.14

Table 2. Inhibitory effects of angiostatins (2 μM) on the rate of Glu-plasminogen (0.3 μM) activation by its physiological activators in the absence or presence of soluble fibrin monomer (0.06 μM) ($p < 0.001$)

Plasminogen activator	Fibrin monomer	Inhibition, %		
		K1-3	K1-4	K1-4.5
scuPA (0.62 nM)	–	25	38	68
	+	7	20	76
tcuPA (0.046 nM)	–	31	42	75
	+	29	38	90
tPA (0.014 nM)	+	0	51	100

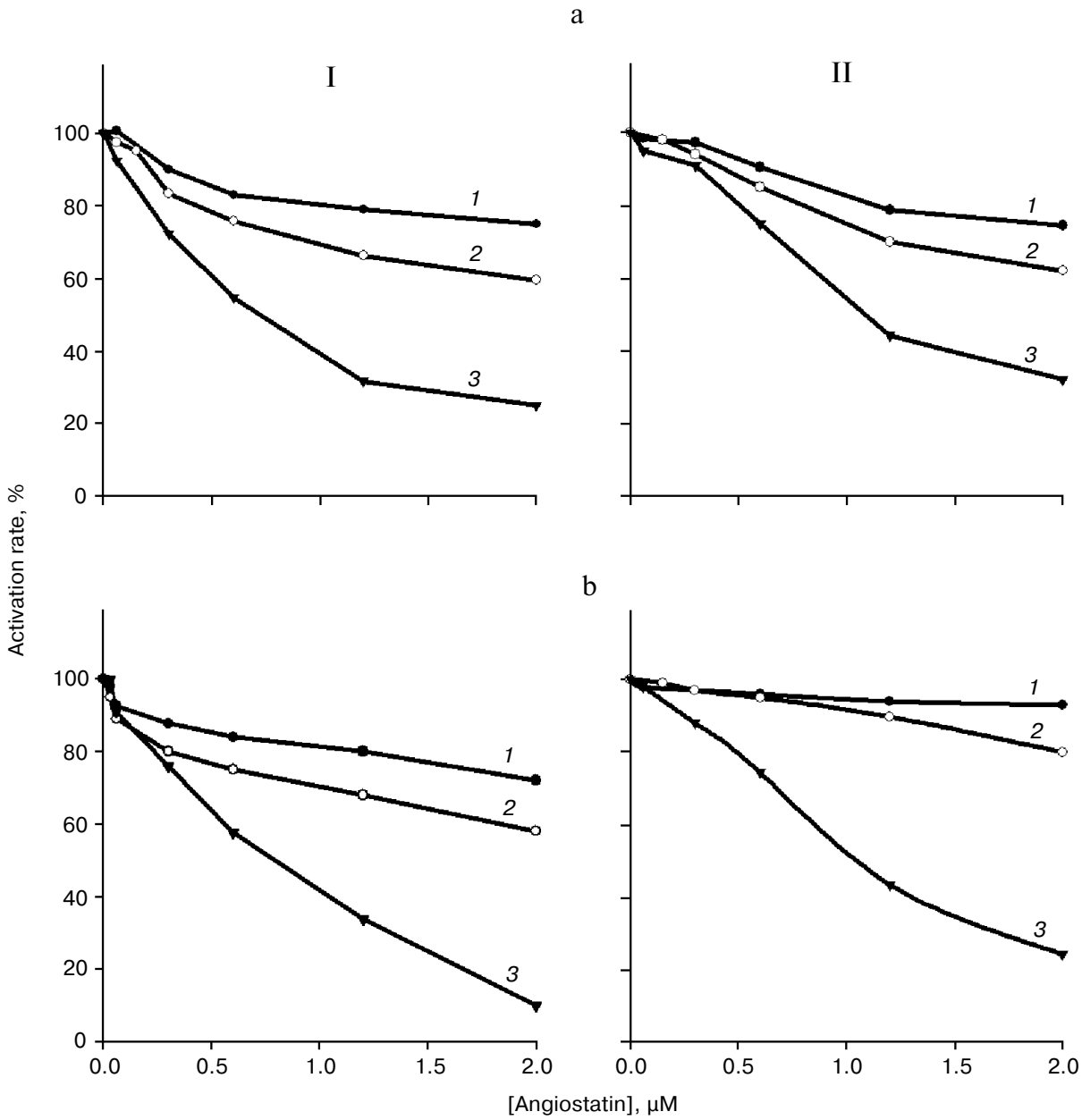

Fig. 3. Effects of angiostatins K1-3 (*1*), K1-4 (*2*), and K1-4.5 (*3*) on the rate of Glu-plasminogen activation by tcuPA (I) and scuPA (II) in absence (a) or presence (b) of fibrin ($p < 0.001$).

(Table 2) than that on plasminogen activation by scuPA. Addition of fibrin led to conspicuous decrease in the inhibitory effects of angiostatins K1-3 and K1-4 on the plasminogen activator activity of scuPA, whereas the activator activity of tcuPA changed only slightly. However, fibrin elevated the inhibitory effect of angiostatin K1-4.5 on the activating activities of both tcuPA and scuPA. Angiostatin K1-4.5 compared with angiostatins K1-3 and K1-4 (lacking most of the fifth kringle) was a more potent inhibitor of plasminogen activation by both the activators, both in the absence and presence of fibrin.

Since tPA is an effective plasminogen activator only in presence of fibrin [32], we evaluated dependences of fibrin-stimulated Glu-plasminogen activation by tPA on concentrations of the three angiostatins (Fig. 4). One can see that angiostatin K1-4.5 is a potent inhibitor of plasmin generation from plasminogen under the action of tPA. The inhibitory effect of angiostatin K1-4 on the activation activity of tPA was about twofold lower than that of angiostatin K1-4.5, whereas angiostatin K1-3 had virtually no effect on rate of plasminogen activation by tPA.

A comparison of decelerations of Glu-plasminogen activation by its physiological activators in the presence of

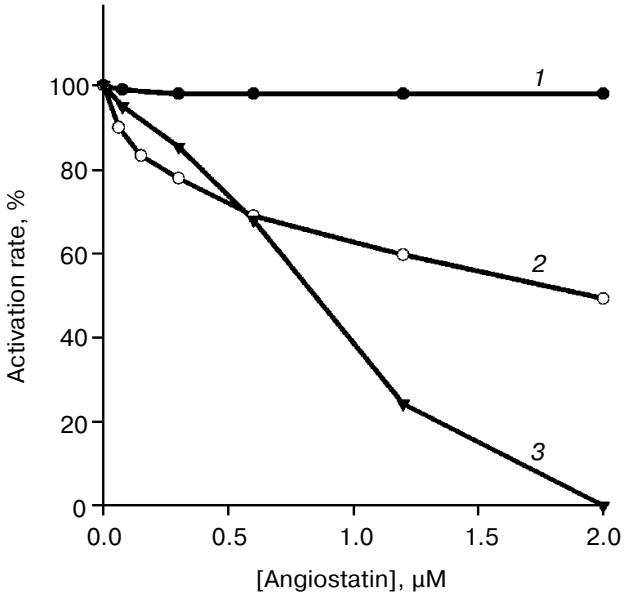

Fig. 4. Effects of angiostatins K1-3 (*1*), K1-4 (*2*), and K1-4.5 (*3*) on the rate of fibrin-stimulated activation of Glu-plasminogen by tPA ($p < 0.001$).

equal concentrations of the three angiostatins demonstrates (Table 2) that the inhibitory efficiency of angiostatin K1-4.5 is considerably higher than that of angiostatins K1-4 and K1-3, independently from the plasminogen activator type.

The data show that angiostatins K1-3, K1-4, and K1-4.5 do not influence the intrinsic activities of the Pg/PAs system enzymes, but they inhibit conversion of plasminogen to plasmin under the action of plasminogen activators.

DISCUSSION

The Pg/PAs system is implicated in multiple plasmin-dependent processes, such as blood clot dissolution, tissue remodeling, and extracellular proteolysis, which are necessary for tumor metastasis. The binding of plasminogen and its activators with cell receptors and extracellular matrix proteins sharply elevates the rate of plasminogen activation by its activators. The formed plasmin degrades the extracellular matrix and activates MMPs, thus facilitating angiogenesis and tumor invasion [18-20]. Formation of new blood vessels from existing vessels of surrounding tissue is required for the growth of primary tumor (2-3 mm^2) [2]. Angiostatins inhibit proliferation and migration of endothelial cells, angiogenesis, and metastasis [33]. Angiostatins can bind with the same centers that can plasminogen due to the presence of LBS in kringles, but, because of the absence of the protease domain of plasminogen, they cannot be transformed into the active plasmin. Angiostatins can reduce the rate of plasmin production from plasminogen due to replacement of plasminogen in its biding centers, which might be one of the mechanisms of their antiangiogenic effect. To test this supposition, we compared the effects of angiostatins differing in contents of kringle domains on the rate of the activation of native Glu-plasminogen by three physiological activators, scuPA, tcuPA, and tPA, having different mechanisms and fibrin-specificities of action.

Three angiostatin forms – K1-3, K1-4, and K1-4.5 – were used for this study. Electrophoresis under non-reducing conditions showed that the purified angiostatin specimens do not contain residual impurity of intact plasminogen or plasmin molecules; however, K1-3, K1-4, and K1-4.5 migrate as double bands with M_r (kDa) 33 and 39, 42 and 45, or 52 and 55, respectively (Fig. 2). The presence of similar electrophoretic bands in the purified specimens of angiostatins K1-3 and K1-4 prepared by plasminogen elastolysis was reported in [34, 35]. It is established by now that elastase hydrolyzes peptide bonds between kringles 3 and 4 and kringles 4 and 5, as well as the Val79–Tyr80 bond in the Glu-plasminogen molecule to form fragments K1-3, K1-4, and K4, miniplasminogen, and pre-activation NTP [23, 28]. Autolysis of plasmin at alkaline pH values is preferably associated with cleavage of the plasmin heavy chain. Since the disulfide bonds Cys512–Cys536 and Cys462–Cys541 fastening the fifth kringle of the heavy chain are accessible to solvent, they are disrupted by hydroxyl ions [22, 31]. This facilitates autoproteolysis of the Arg530–Lys531 bond in the fifth kringle. Moreover autolytic removal of NTP (Glu1-Lys77) of the enzyme occurs. As a result, fragment K1-4.5 (Lys78-Arg530), which contains K1-4 and most of the fifth kringle (69 of 80 residues), and microplasmin are produced [22, 25, 31].

Native Glu-plasminogen is known to exist in two glycoforms: type I (~33%), which is glycosylated at Asn289 and Thr346, and type II (~67%), which is only glycosylated at Thr346 [36]. The presence of angiostatin isoforms is attributed both to formation of the two glycoforms (with and without N-bound carbohydrate chain at Asn289 [37]) and to proteolytic cleavage of several bonds in those regions of the polypeptide chain that link kringles [34, 35]. It is likely that these chain regions in plasminogen glycoform II are more easily hydrolyzed by elastase or plasmin than those in glycoform I carrying the N-bound carbohydrate chain on kringle 3, which can result in formation of two or three forms of angiostatin. The isoforms of angiostatin K1-3 prepared by elastolysis of plasminogen have identical N-terminal residue and C-terminal regions of different length: Tyr80-Val338 and Tyr80-Val354 [34]. Despite a slight difference, the angiostatin isoforms identically inhibited proliferation of endothelial cells [35].

The three angiostatin specimens which we have prepared in this work did not contain any trace amounts of

mini- or microplasmin(ogen)s judging by the absence of hydrolysis of a specific plasmin substrate in the presence of the angiostatins, both before and after their incubation with streptokinase in the control experiment. We preliminarily checked the possible influence of angiostatins on the rates of specific substrate hydrolyses by plasmin, tcuPA, and tPA. The tests showed that angiostatins taken at 50-100-fold excess over the enzymes did not influence the intrinsic amidase activities of plasmin and activators of plasminogen (Table 1).

Dependences of the rates of Glu-plasminogen activation by scuPA, tcuPA, and tPA on concentrations of the three angiostatins, which are presented in Figs. 3 and 4, distinctly show that the angiostatins inhibit the production of plasmin from plasminogen in a dose-dependent manner. However, the inhibitory effects of the three angiostatins on non-stimulated and fibrin-stimulated plasminogen activation by scuPA, tcuPA, and tPA are different, which can be explained by difference in the angiostatin structures (in the number of kringle domains) and in mechanisms and fibrin-specificities of action of plasminogen activators, as well as by conformational changes in the Glu-plasminogen molecule in the presence of fibrin.

Glu-plasminogen can exist in a closed compact α-conformation, which is supported by two intramolecular interactions (between LBS of kringle 5 and NTP and between LBS of kringle 4 and a ligand on kringle 3), half-open β-conformation with only one of the two interactions, and open γ-conformation when both lysine-dependent intramolecular interactions are disrupted [38]. Lys-plasminogen (Lys-Pg) devoid of NTP exists in either β- or γ-conformation depending on conditions, and it is activated quicker than is Glu-plasminogen [39]. Glu-plasminogen is supposed to take α-conformation in the presence of chloride ions, β-conformation when bound to intact fibrin, and γ-conformation when bound to partially degraded fibrin [38]. Three LBS are localized in the plasminogen kringles: two high-affinity LBS in kringles 1-3 and 5 and one low-affinity LBS in kringle 4. However, plasminogen possesses only two fibrin-binding sites localized in kringles 1-3 and 5, whereas kringle 4 does not appreciably bind with fibrin. Kringle 5 was shown to possess the highest affinity to intact fibrin [40].

The known difference in mechanisms of plasminogen activation by its physiological activators is mainly due to variations in their structures. For instance, tPA and tcuPA have homologous light B-chains containing the active center, but their regulatory heavy A-chains are different. The tPA A-chain contains a fibronectin-like finger domain, two kringle domains, and an epidermal growth factor (EGF) domain [41]. The EGF domain is involved in tPA binding with receptors, and the kringle 2 and finger domains are responsible for tPA binding with fibrin. Efficacy of tPA, which is a poor plasminogen activator in solution, becomes three orders higher in the presence of fibrin [32, 42]. The tcuPA A-chain contains the EGF domain important for its binding with cellular uPAR receptor and one kringle domain. Unlike tPA, tcuPA does not bind to fibrin because it has no finger domain, and its single kringle domain has no affinity to fibrin [41]. Therefore, tcuPA activates plasminogen in solution and on fibrin with equal rates [43]. ScuPA containing the same domains at its NH_2-terminus as does tcuPA has no direct affinity to fibrin. Nevertheless, unlike tcuPA, it is a fibrin-selective plasminogen activator. High affinity of scuPA to plasminogen (K_{Pg} = 0.8 μM) is the key feature differing scuPA from tcuPA (K_{Pg} = 50 μM) [44]. In the presence of fibrin, both tPA and tcuPA can activate Glu-plasminogen molecules attached to the intra-chain lysine residues of intact fibrin, whereas scuPA selectively activates Glu-plasminogen molecules bound with the C-terminal lysine residues of partially degraded fibrin, which are formed under the action of plasmin at the initial stage of plasminogen activation [45]. Therefore, the activator activity of scuPA substantially increases in the presence of fibrin.

On the basis of contemporary data on the mechanisms of scuPA, tcuPA, and tPA action and the influence of fibrin on the conformation of Glu-plasminogen, the observed effects of the three angiostatins on the activity of the Pg/PAs system can be explained as follows. In the absence of fibrin, all three of the angiostatins inhibit Glu-plasminogen activation by tcuPA and scuPA, and the inhibitory effect increases in the order: K1-3 < K1-4 < K1-4.5 (Fig. 3a), which is indicative of the involvement of plasminogen kringle domains in formation of binary enzyme–substrate complexes tcuPA·Pg and scuPA·Pg. Probably, the affinity of the angiostatins to plasminogen activators increases with increasing number of kringle domains, which leads to more effective plasminogen displacement from tcuPA·Pg and scuPA·Pg enzyme–substrate complexes and, as a consequence, to a decrease in the plasmin formation rate. Kringle 5 plays a dominant role in the binding of Glu-plasminogen to fibrin [40]. The Glu-plasminogen molecule therewith takes the more open β-conformation due to the destruction of the bond between LBS of kringle 5 and NTP, which can lead to some enhancement of the rate of its conversion to plasmin. On one hand, plasmin induces the formation of C-terminal lysine residues on fibrin – new centers for sorption of Glu-plasminogen, which is necessary for scuPA binding, and on the other hand, it converts scuPA into the more active tcuPA. Some decrease in inhibitory effects of angiostatins K1-3 and K1-4 on the scuPA activator activity in the presence of fibrin (Fig. 3b (II) and Table 2) can be explained by inverse stimulating effect of fibrin on plasminogen activation by scu-PA. As indicated earlier, fibrin has no effect on the rate of plasminogen activation by tcuPA. Therefore, the inhibitory effects of angiostatins K1-3 and K1-4 on the activator activity of tcuPA are identical in absence (a) and in presence (b) of fibrin

(Fig. 3, I). Unlike angiostatins K1-3 and K1-4, the inhibitory effect of angiostatin K1-4.5 on the activator activities of tcuPA and scuPA (curves *3*) in the presence of fibrin (b) was higher than in its absence (a). Probably, the more effective plasminogen displacement both from the fibrin surface and from the binary enzyme–substrate complex by angiostatin K1-4.5 containing a large share of kringle 5 leads to more substantial inhibition of plasminogen activation by both activators. These data suggest a significant contribution of plasminogen kringle 5, as compared with its other kringles, to the formation of enzyme–substrate complexes and their realignment on the surface of fibrin. The fact that the inhibitory effect of angiostatin K1-4.5 on the scuPA activator activity on the fibrin surface is lower (Table 2) than on tcuPA activator activity is also probably associated with the stimulating effect of fibrin on Glu-plasminogen activation by scuPA.

The effects of three angiostatins on Glu-plasminogen activation by tPA (Fig. 4) differed from their effects on tcuPA and scuPA activator activities (Fig. 3). The rate of fibrin-stimulated tPA-induced activation of Glu-plasminogen decreased by 50 and 100% in the presence of 2 μM of angiostatins K1-4 and K1-4.5, respectively, whereas angiostatin K1-3 taken at the same concentration had no effect on the tPA activator activity (Table 2). The observed effects are due to the direct binding of tPA, unlike tcuPA and scuPA, to fibrin via its finger domain and kringle 2 [42]. The tPA A-chain was earlier reported to contain a plasminogen-binding center different from the active center of the B-chain [20]. Plasminogen and tPA binding by fibrin favors their convergence and reorientation, which leads to the formation of effective Pg–tPA enzyme–substrate complex on fibrin and to substantial acceleration of the activation of the zymogen [32]. Both fibrin-binding centers of plasminogen (the weak one in kringles 1-3 and the strong one in kringle 5) interact with the same sites on fibrin. However, the binding of plasminogen kringle 5 to fibrin plays an important role in stimulation of plasminogen activation by tPA [34]. The strong inhibition of fibrin-stimulated tPA-activation of plasminogen by angiostatin K1-4.5 (Fig. 4, curve *3*) can be mainly due to its competition with plasminogen for binding with fibrin and, to a lesser extent, for binding with tPA. Since angiostatin K1-4.5 contains both the weak (within kringle 1-3) and the strong (within kringle 5) fibrin-binding centers, it can displace plasminogen from the surface of fibrin in a dose-dependent manner. A complete inhibition of plasminogen activation by tPA observed at concentrations of angiostatin K1-4.5 6-7 times exceeding that of plasminogen indicates that it completely displaces plasminogen from fibrin. Contrariwise, angiostatin K1-3 containing only the weak fibrin-binding center cannot compete with plasminogen for its binding to fibrin and, therefore, has no effect on the rate of fibrin-stimulated activation of plasminogen by tPA (Fig. 4, curve *1*). On the other hand, angiostatin K1-4, which also contains only the weak fibrin-binding center localized in kringles 1-3, induces dose-dependent inhibition of tPA-catalyzed plasminogen activation (curve *2*), although to substantially lesser extent than does angiostatin K1-4.5 (curve *3*). Possibly, the affinity of angiostatin K1-4, containing the additional kringle, to fibrin or tPA is higher than that of K1-3. Owing to this fact, angiostatin K1-4 hinders the formation of effective tPA·Pg–fibrin ternary complex to a greater degree than does angiostatin K1-3, and thereby significantly decelerates the activation of the zymogen. This assumption is in accordance with the fact that the rate of tPA-catalyzed activation of miniplasminogen containing a protease domain and K5 is significantly lower in the presence of fibrin as compared with Glu-plasminogen activation [34]. Therefore, other plasminogen kringles, along with kringle 5, are necessary for the fibrin-stimulated activation of plasminogen by tPA.

The results obtained *in vitro* demonstrate that angiostatins K1-3, K1-4, and K1-4.5 differently inhibit Glu-plasminogen activation by tcuPA and scuPA, both in the absence and presence of fibrin, whereas fibrin-stimulated activation of Glu-plasminogen by tPA is only inhibited by angiostatins K1-4 and K1-4.5. The binding of plasminogen and its activators to other proteins of extracellular matrix and cell surface receptors, apart from fibrin, can promote plasminogen activation *in vivo*. At present, plasminogen is found to bind with tetranectin of extracellular matrix [46] and with specific receptors on the cell surface (α-enolase, annexin II, actin, and others) [18, 33, 47]. The binding of scuPA and tcuPA with the specific receptor uPAR ($K_d \sim 10^{-9}$-10^{-10} M) [6] stimulates conversion of scuPA to tcuPA and plasminogen to plasmin on the cell surface. The resulting view is that tcuPA-induced plasminogen activation plays the key role in processes facilitating tumor progression and the formation of metastases, such as degradation of extracellular matrix and cell proliferation and migration [48]. The plasminogen activator activity of tPA substantially increases with its interaction with fibrin(ogen) and extracellular matrix proteins, such as collagen-IV, laminin-1, and thrombospondin, which can also favor the pathological proteolysis of conjunctive tissue [20]. Thus, the binding of plasminogen and its activators to distinct proteins of extracellular matrix or cell receptors *in vivo* also results in the accelerated formation of plasmin, initiating degradation of extracellular matrix, which is necessary for tumor invasion and metastases formation. Angiostatins simulating the plasminogen-binding activity can inhibit the matrix-stimulated conversion of plasminogen into active plasmin in the cell microenvironment, thus inhibiting cell migration and angiogenesis. The results obtained in the present work demonstrate that the inhibition of Glu-plasminogen activation under the action of physiological plasminogen activators by angiostatins can be implicated in the complex mechanism of their antiangiogenic and antitumor action. However, the

inhibitory effects of various angiostatins on different tumor types expressing chiefly tcuPA or tPA can differ.

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