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Direct-acting fibrinolytic enzymes in shark cartilage extract
Potential therapeutic role in vascular disorders

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Abstract Fibrinogen and fibrin are molecules with overlapping roles in blood clotting, fibrinolysis, wound healing, inflammation, matrix and cellular interactions and neoplasia. There is currently much interest in the possible use of fibrinolytic agents in human therapeutics. In this study, we report the presence of fibrinolytic activities in shark cartilage extract (SCE). In vitro, SCE at 100 μg/ml completely degraded fibrin gel in an aprotinin-insensitive manner, suggesting a non-plasmin molecular nature. SCE was able to cleave all chains of fibrinogen and fibrin and the cleavage was completely inhibited by 1,10-phenanthroline, suggesting an essential role for metalloprotease(s) in this process. Using fibrinogen zymography, we show that SCE contains two plasmin-independent fibrinolytic activities and that these activities are correlated with the presence of 58 and 62 kDa proteases in the extract. SCE-fibrinolytic activities are inhibited by dithiothreitol, suggesting that disulfide bonds are necessary for the protease structure. Finally, using thromboelastography, SCE markedly induced retraction of human platelet-rich plasma (PRP) clot, this process being completely abolished by 1,10-phenanthroline. These data suggest the presence of novel non-plasmin fibrinolytic activities within SCE. This extract may thus represent a potential source of new therapeutic molecules to prevent and treat vaso-occlusive and thromboembolic disorders.

Abbreviations: EACA, ε-amino-*n*-caproic acid (6-aminohexanoic acid); PAGE, polyacrylamide gel electrophoresis; PRP, platelet-rich plasma; SCE, shark cartilage extract; PAI-1, plasminogen activator inhibitor type-1.

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Introduction

Fibrinogen and its conversion to fibrin play essential roles in blood clotting, wound healing, inflammation, matrix and cellular interactions and neoplasia [1]. These events are regulated by fibrin formation itself and by interactions between specific binding sites on fibrin(ogen) and on other molecules including clotting factors, cell receptors, proenzymes and enzyme inhibitors. Fibrinogen is comprised of two sets of three polypeptides chains, termed Aα, Bβ and γ, which are joined by disulfide bridging within the N-terminal E domain [2]. These domains contain constitutive binding sites that participate in the conversion of fibrinogen to fibrin, fibrin assembly, cross-linking and platelet interactions [3]. Polymerization of fibrinogen monomers is initiated by thrombin after cleavage of fibrinopeptides A and B from Aα chains. Fibrin generation leads to intermolecular association, resulting in linear fibrils. Intermolecular covalent ε-(γ glutamyl) lysine bonds are introduced into these polymers by factor XIIIa, creating γ dimers. Later, cross-links form between complementary sites on α chains and among γ dimers, completing the mature network structure [4].

Fibrin clots are dissolved by the serine protease plasmin, which is generated from its zymogen plasminogen by the action of plasminogen activators, e.g., tPA and uPA. tPA is assumed to be primarily responsible for plasmin formation during fibrinolysis [5] whereas uPA is thought to mediate plasmin-dependent extravascular tissue degradation [6]. During intravascular fibrinolysis, endothelial-derived tPA converts plasminogen to the active protease plasmin, which is able to degrade fibrin. Endogenous tPA is rapidly neutralized by plasminogen activator inhibitor type-1 (PAI-1), which binds to the active site of tPA. Thus, PAI-1 plays a regulatory role in fibrinolysis by limiting the production of plasmin. Under conditions where PAI-1 plasma levels are elevated, the action of tPA is depressed. Thus, PAI-1 is well established as a contributing prothrombotic/antifibrinolytic factor and induces resistance to fibrinolysis not only in chronic and acute cardiovascular diseases but also in other arterial thromboembolic disorders like cerebral [7] and peripheral artery diseases [8], as well as in venous thromboembolic disorders, such as deep vein thrombosis and pulmonary embolism [9] and primary and secondary pulmonary hypertensions [10].

Cartilage is a unique tissue that is avascular and thus relatively tumor-resistant. Previous work has demonstrated that the cartilage contains angiogenic inhibitors [11,12] such as TIMPs [13] and inhibitors of VEGF-mediated signaling events [14], as well as an endothelial-specific proapoptotic activity [15]. It has also been reported that shark cartilage contains substances that strongly inhibit the growth of new blood vessels toward solid tumors [16,17]. However, there is no report showing the presence of fibrinolytic enzyme(s) in shark cartilage extract (SCE).

The present study was undertaken to characterize the presence of fibrinolytic activity in shark cartilage extract. Using fibrinolysis assay and fibrinogen zymography, we demonstrate that shark cartilage extract contains plasmin-independent fibrinolytic activities and that these enzymes consist mainly of two metallo-dependent proteases of 58 and 62 kDa. SCE-associated fibrinolytic activities cleave all chains of fibrin(ogen). Finally, thromboelastography studies demonstrate that SCE did not affect the clotting time or kinetics parameters whereas it decreased the clot firmness.

Materials and methods

Materials

Shark cartilage extract (Neovastat®, AE-941) was obtained from Aeterna Laboratories (Québec City, QC, Canada) [18].

Plasminogen-free human fibrinogen and human plasmin were obtained from Calbiochem (La Jolla, CA). ^{125}I-labeled fibrinogen was from Amersham Biosciences (Baie d'Urfé, QC, Canada). Aprotinin, Pefabloc and E64 were from Roche (Laval, QC, Canada). Pepstatin A, ε-amino-*n*-caproic acid (6-aminohexanoic acid; EACA), EDTA, alpha-2 macroglobulin and 1,10-phenanthroline were obtained from Sigma (St. Louis, MO).

Kaolin and $CaCl_2$ were obtained from Haemoscope (Niles, IL). All products for electrophoresis were purchased from Bio-Rad (Hercules, CA).

Fibrinolysis assay

To monitor fibrinolytic activity of SCE and/or plasmin, radiolabeled fibrinogen was incorporated into a fibrin gel and solubilized fibrin was quantified by γ-scintillation counting. In the well, fibrin gel (3 mg/ml final concentration) was prepared by mixing 5 μl of plasminogen-free human fibrinogen (6 mg/ml) containing ^{125}I radiolabeled fibrinogen (0.05 μCi) and 5 μl of human thrombin solution (2 U/ml final concentration). The gels were incubated at 37 °C for 30 min to allow complete gelling. After washing, media (250 μl) containing SCE (100 μg/ml final concentration) or plasmin (≈1 mU) were

added to the fibrin gel. After a 24-h incubation at 37 °C, radiolabeled solubilized fibrin degradation products contained in the media were quantified by γ-scintillation counting. Spontaneous release of ^{125}I-fibrin from gels incubated in the absence of SCE or plasmin was quantitated and used as a control. To assess the effect of protease inhibitors on the fibrinolytic activity, SCE and/or plasmin were preincubated in the presence or absence of aprotinin (200 μg/ml final concentration in water), Pefabloc (20 μM), ε-amino-*n*-caproic acid (10 mM), alpha-2 macroglobulin (10 nM), 1,10-phenanthroline (1 mM in methanol), E64 (10 μM), Pepstatin A (5 μM in DMSO) for 75 min at room temperature. SCE was also incubated in methanol or DMSO to assess the effect of the vehicles for 1,10-phenanthroline and Pepstatin A, respectively.

To assess the effects of differents agents on fibrinolytic activity, SCE (1 mg/ml final concentration) was incubated for 2 h at 37 °C in 4 M Urea, 5 mM EDTA, 0.1% sodium dodecyl sulphate (SDS) or 10 mM dithiothreitol (DTT), followed by dialysis against several changes of buffer (10 mM Tris—HCl, pH 8). To assess the effects of heating, SCE was incubated for 20 min at 90 °C and the sample was allowed to cool down to room temperature prior to the assay.

Fibrinogen and fibrin digestion

Fibrinogen solution or fibrin gel was prepared by mixing 16 μl of plasminogen-free human fibrinogen (2.5 mg/ml) and 4 μl of buffer or human thrombin solution (37.5 U/ml final concentration). Fibrin gels were incubated at 37 °C for 20 min to allow complete gelling. SCE (85 μg in 20 μl buffer) was added to every clot and incubated at 37 °C for 18 h. Then, 8 μl of denaturing solution was added and the mixture was incubated 3 min at 100 °C before 7.5% SDS-polyacrylamide gel electrophoresis (PAGE) electrophoresis. To assess the effect of protease inhibitors, SCE was preincubated for 60 min at room temperature in the presence or absence of inhibitors (aprotinin, 1,10-phenanthroline, E64 and Pepstatin A).

Fibrinogen zymography assay

Fibrinogenolytic activity of SCE was detected by fibrinogen zymography. SCE and plasmin (positive control) were resolved under nonreducing conditions on 7.5% SDS-PAGE gels containing 1 mg/ml plasminogen-free fibrinogen. Gels were rinsed two times in 2.5% Triton X-100 for 30 min at room temperature to remove SDS. The gels were further incubated at 37 °C for 48 h in 20 mM NaCl, 5 mM $CaCl_2$, 0.02% Brij-35, 50 mM Tris—HCl buffer, pH 7.6. Gels were stained with Coomassie Blue and destained in 10% (v/v) acetic acid, 30% methanol. Areas of fibrinogenolytic activity were visualized as transparent bands. To test the effects of 1,10-phenanthroline and EDTA on fibrinogenolytic activity, SCE was preincubated with these agents before electrophoresis and the gels were incubated for 48 h at 37 °C in the presence or absence of these agents (10 mM 1,10-phenanthroline, 50 mM EDTA). SCE was also incubated in the presence of methanol to assess the effect of vehicle.

Thromboelastography (TEG)

The principle of TEG is based on the measurement of the physical viscoelastic characteristics of blood clots. Clot formation was monitored at 37 °C in an oscillating plastic cylindrical cuvette ("cup") and a coaxially suspended stationary piston ("pin") with a 1-mm clearance between the surfaces by use of a computerized thrombelastograph (TEG model 5000, Haemoscope; Fig. 1).

The cup oscillates 4°45′ (1/12 radian) in either direction every 4.5 s, with a 1-s mid-cycle stationary period, resulting in a frequency of 0.1 Hz and a maximal shear rate of 0.1/s. The pin is suspended by a torsion wire that acts as a torque transducer. Upon clot formation, fibrin fibrils physically link the cup to the pin, and the rotation of the cup, as affected by the viscoelasticity of the clot (transmitted to the pin), is displayed online by using an IBM-compatible personal computer and customized software (Haemoscope). The torque experienced by the pin (relative to the oscillation of the cup) is plotted as a function of time. TEG assesses coagulation by measuring various parameters. The following parameters were measured from the TEG tracing:

(1) *R*, the time latency for the initiation of the clot
(2) *K*, the time to initiation of a fixed clot firmness of ≈20-mm amplitude,
(3) the kinetics of clot development as measured by the angle (α)
(4) the clot strength : MA (in millimeters) is the peak rigidity manifested by the clot and is representing primarily platelet function, and to a lesser extent, fibrinogen function.
(5) *G* is the shear elastic modulus (*G*, in dynes per square centimeter). The amplitude on the TEG tracing is a measure of the rigidity of the clot; the peak strength or the shear elastic modulus attained by the clot is a function of clot rigidity and can be calculated from the MA of the TEG tracing.

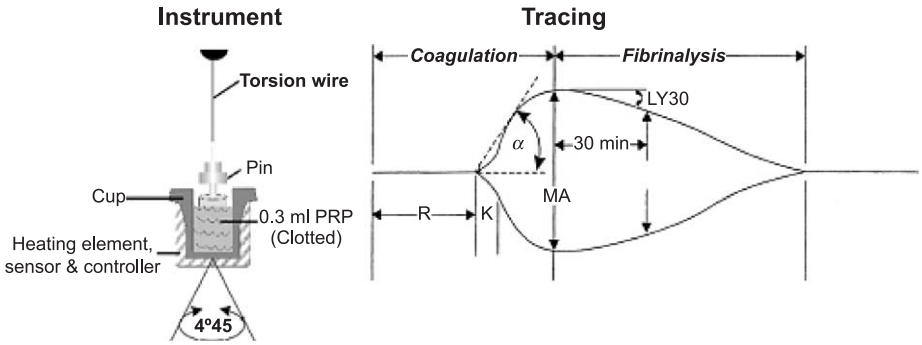

Figure 1 Diagram of TEG and tracing showing the initiation and completion phases of platelet-fibrin clot initiated by Kaolin (500 μg) under shear in human PRP. TEG assesses coagulation and fibrinolysis by measuring various parameters, such as the time latency for the initiation of the clot (*R*), the time to initiation of a fixed clot firmness (*K*) of ≈20-mm amplitude, the kinetics of clot development as measured by the angle (α), the MA of the clot and the clot lysis as measured by the rate of amplitude reduction 30 min after MA (LY30).

(6) the clot lysis : LY30 (in percent) measures lysis at 30 min after MA is reached.

Blood was drawn from consenting volunteers and centrifuged at 1000×*g* for 9 min to obtain platelet-rich plasma (PRP).

To assess the effect of SCE on clot modification, peak clot strength was measured with and without the addition of SCE (600 μg of proteins) in four samples of platelet-rich plasma (PRP; 300 μl), with 30 μl $CaCl_2$ (0.2 M) and 20 μl Kaolin (25 mg/ml). Clot firmness was monitored at 37 °C for 3 h.

The inhibitory effect of 1,10-phenanthroline on clot strength was studied by preincubating this inhibitor with SCE for 20 min at room temperature. PRPs were also incubated with 1,10-phenanthroline alone to assess its effect on clot modification.

Data analysis

Statistical analyses were made with Student's paired *t* test using GraphPad Prism (San Diego, CA). Significant difference was accepted for *P* values less than 0.05.

Results

SCE contains a plasmin-independent fibrinolytic activity

To determine whether SCE exhibits fibrinolytic activity, SCE was incubated with [^{125}I]-fibrin-labeled, cross-linked fibrin gels and solubilization was quantitated. Under these conditions, 100 μg/ml SCE completely solubilized the fibrin gel after a 24-h incubation at 37 °C (Fig. 2A). This effect was not inhibited when aprotinin, Pefabloc or EACA was present in the incubation medium. In contrast, the fibrinolytic activity of plasmin was completely inhibited by any of these three components. Therefore, SCE-induced fibrinolysis was not related to the presence of endogenous plasmin within the SCE. Incubation of alpha-2 macroglobulin with SCE resulted in the complete inhibition of fibrin gel solubilization by plasmin and SCE. In human alpha-2 macroglobulin, several reactive sites, including high-affinity sites for zinc, can mediate reversible or irreversible capture of proteins of diverse biological functions [19]. Alpha-2 macroglobulin may thus interact with and capture the fibrinolytic activity contained in SCE, suggesting the involvement of zinc-dependent protease(s) in SCE-induced fibrinolysis.

To demonstrate that SCE cleaves fibrin(ogen), we incubated fibrino(ogen) with SCE and the digestion products were submitted to SDS-PAGE electrophoresis under reducing conditions. As shown in Fig. 2B, SDS-PAGE analysis of fibrin(ogen) following incubation with SCE demonstrated that all chains of fibrin(ogen) were sensitive to proteolytic attack. Interestingly, the digest fragments of fibrin(ogen) produced by SCE were completely different (in both number and size) from those obtained with plasmin (Fig. 2B), confirming the presence of plasmin-independent fibrinolytic activity in SCE.

SCE-induced fibrinolysis is metalloprotease-dependent

We next further characterized the nature of the SCE-associated fibrinolytic activity. First, four different types of protease inhibitors, each blocking proteolytic activity against one of the four classes of proteases (metallo-, serine, cysteine and acidic proteases), were tested. The protease inhibitors

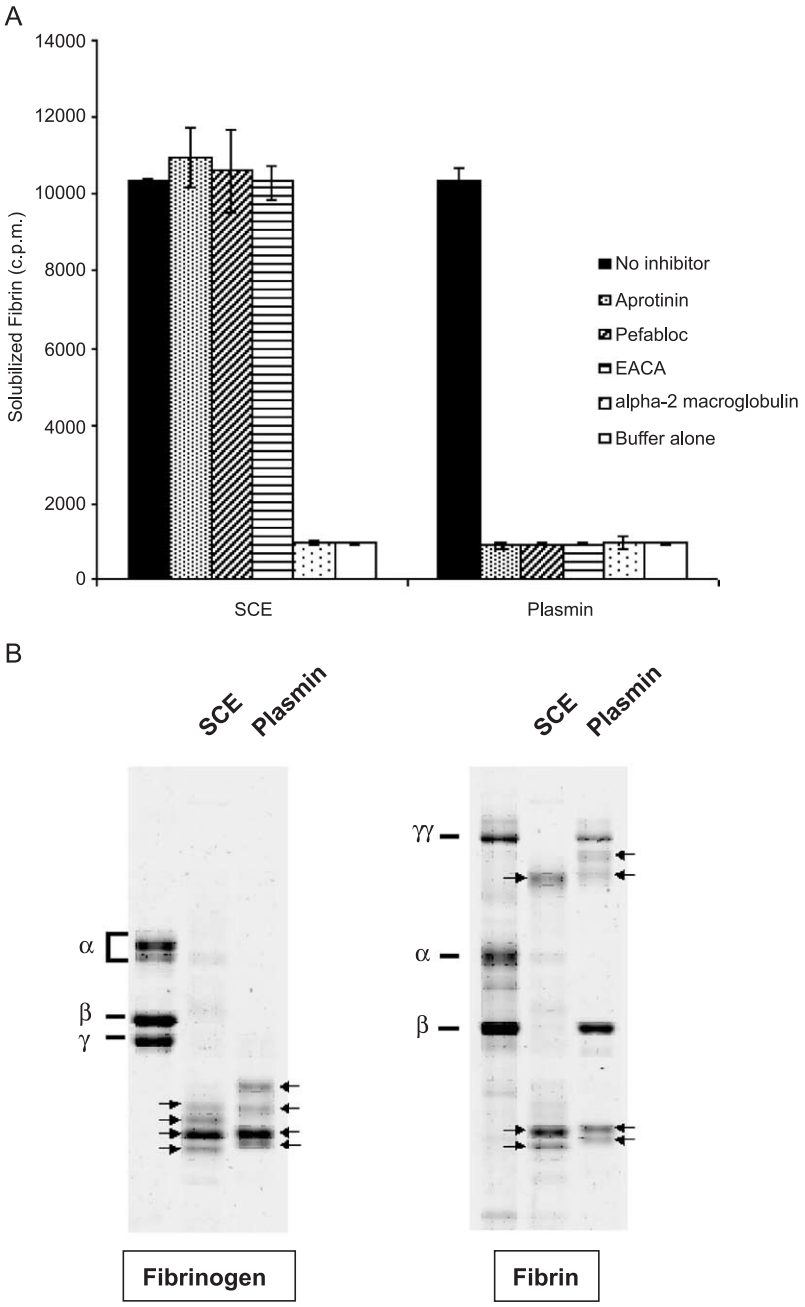

Figure 2 Plasmin-independent fibrinolytic activity in SCE. (A) Fibrinolytic activity of SCE or plasmin in the presence or absence of aprotinin (200 μg/ml), Pefabloc (20 μM), EACA (10 mM) or alpha-2 macroglobulin (10 nM). The portion of each bar represents the number of counts recovered in the incubation media. Results are expressed as the mean±S.E.M. cpm released from [^{125}I]-fibrin-labeled gels. (B) SDS-PAGE analysis of fibrinogen or fibrin (40 μg each) after an 18-h incubation alone, with SCE (85 μg) or plasmin. Aα, Bβ and γ chains of fibrinogen and γγ, α and β chains of fibrin are indicated to the left of each gel.

used were 1,10-phenanthroline (metalloprotease inhibitor), aprotinin (serine protease inhibitor), E64 (cysteine protease inhibitor) and pepstatin A (acidic protease inhibitor). SCE (100 μg/ml) was preincubated with the different protease inhibitors and the media were added to fibrin gels. As can be seen in Fig. 3A, the serine, cysteine and acidic protease inhibitors had no effect on the fibrinolytic activity of SCE while 1,10-phenanthroline completely inhibited fibrinolysis. Fibrinolytic activity of SCE was however unaffected by TIMP-2 (1 μg/ml final concentration) or BB94 (10 μM final concentration; data not shown), suggesting that members of the MMP family are not involved in this process.

To further demonstrate that the cleavage of all fibrin(ogen) chains by SCE is metalloprotease-

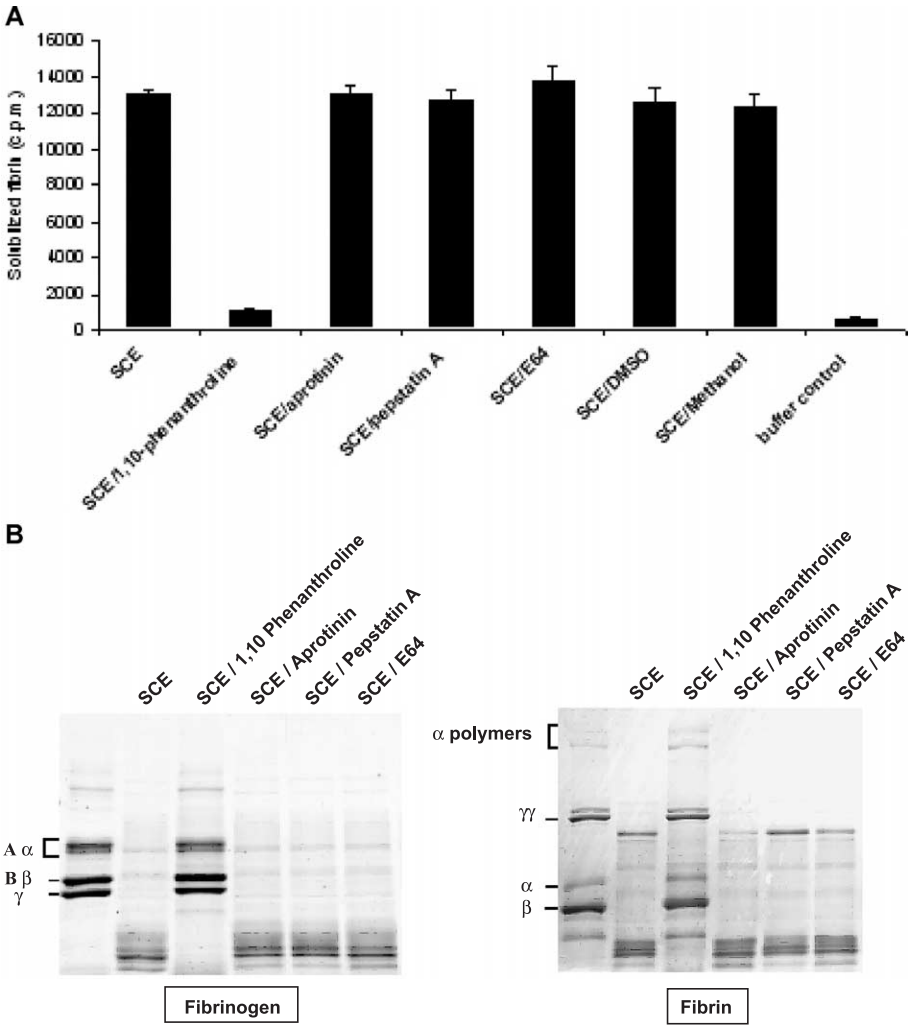

Figure 3 Effect of protease inhibitors on SCE-induced fibrinolysis. (A) Fibrinolytic activity of SCE in the presence or absence of 1,10-phenanthroline (1 mM), aprotinin (200 μg/ml), pepstatin A (5 μM) or E64 (10 μM). The size of each bar represents the number of counts recovered in the incubation media. Results are expressed as the mean±S.E.M. cpm released from [^{125}I]-fibrin-labeled gels. (B) SDS-PAGE analysis of fibrinogen or fibrin (25 μg each) after an 18-h incubation alone, with SCE (85 μg) or SCE and either 1,10-phenanthroline (1 mM), aprotinin (200 μg/ml), pepstatin A (5 μM) or E64 (10 μM). Aα, Bβ and γ chains of fibrinogen and γγ, α and β chains of fibrin are indicated to the left of each gel.

dependent, we incubated SCE with the four types of protease inhibitors and examined fibrin(ogen) cleavage by SDS-PAGE. As shown in Fig. 3B, the serine, cysteine and acidic protease inhibitors had no effect on the proteolytic attack of fibrin(ogen) chains by SCE whereas 1,10-phenanthroline completely inhibited degradation, confirming an essential role for metalloprotease(s) during SCE-induced fibrinolysis.

Fibrinolytic activity of SCE is associated with ≈62 and ≈58 kDa metalloproteases

To investigate whether the fibrinolytic activity of SCE is due to one or to several molecular entities, we analyzed the fibrinogenolytic activity of SCE by fibrinogen zymography. As shown in Fig. 4, two fibrinogenolytic areas were detected in the presence of SCE with apparent molecular weights of about 58 and 62 kDa. We next tested the ability of 1,10-phenanthroline and EDTA to inhibit these fibrinogenolytic activities. As can be seen in Fig. 4, 1,10-phenanthroline and EDTA completely inhibited fibrinogen degradation induced by SCE at 10 and 50 mM, respectively, whereas these components had no effect on plasmin-induced fibrinogen degradation.

Stability of SCE-fibrinolytic activity

We next investigated the sensitivity of the fibrinolytic activity to different treatments. SCE was

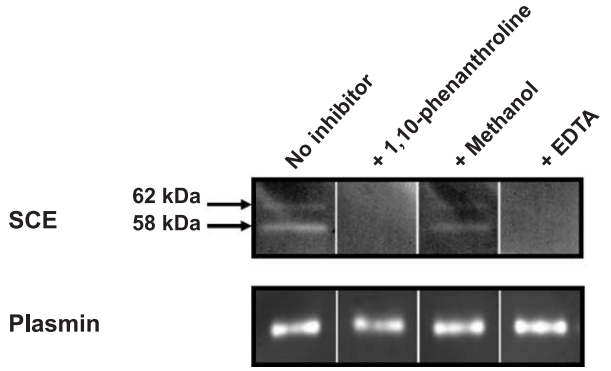

Figure 4 Fibrinogenolytic activity of SCE. Two fibrinogenolytic activities were detected in the presence of SCE with apparent molecular weight of about 58 and 62 kDa. Fibrinogenolytic activities of SCE were completely inhibited by 1,10-phenanthroline and EDTA at 10 and 50 mM, respectively, whereas these components had no effect on plasmin-induced fibrinogen degradation. Note that methanol had no effect on plasmin-induced fibrinogenolytic activity.

submitted to a variety of treatment including heat, addition of sulfydryl reagent or detergent for 2 h before testing its fibrinolytic activity. As shown in Fig. 5, short exposure of SCE to 90 °C completely inhibited fibrinolytic activity. A decrease in fibrinolytic activity was also observed following incubation of SCE in various chaotropic conditions such as 0.1% SDS or in the presence of 4 M Urea. Moreover, the chelating agent EDTA strongly inhibited the fibrinolytic activity, confirming its metalloprotease nature. Fibrinolytic activity of SCE was completely lost following incubation with dithiothreitol, suggesting that disulfide bonds are necessary for maintaining its structure.

Effect of SCE on strength of human PRP clot

Thromboelastography provides a global assessment of hemostasis, giving sequential information on the time to initial clot formation, the clot strength, and the degree of fibrinolysis [20]. Because native thromboelastography is slow to provide information, the addition of Kaolin has been proposed for reducing the time to trace generation. Kaolin, like celite or ellagic acid, is used as the contact activating agent to activate the intrinsic pathway of coagulation, causing the blood to clot [21].

Kaolin-triggered TEG was performed with different amounts of SCE proteins (0, 200, 400, 600 μg). Under these conditions, the addition of SCE resulted in dose-dependent effects on clot formation and lysis (data not shown).

The effect of SCE (600 μg of proteins) on the different clot parameters measured by computerized TEG in human PRP is shown in Fig. 6, and a tracing illustrating the effect of SCE on clot formation and lysis under shear is shown in Fig. 7.

As shown in Fig. 6, SCE induced a significative increase in clot lysis (measured by LY30 in %, 30 min after MA is reached) and the addition of 1,10-phenanthroline resulted in complete inhibition of the SCE-induced PRP clot lysis. 1,10-phenanthroline alone had no effect on the different clot parameters.

SCE (600 μg of proteins) did not alter the clotting time or kinetics parameters (*R*, *K* and α). SCE had, however, an effect on the resistance to deformation (measured by MA and *G*, elastic modulus) and significantly reduced the clot strength (in dynes per square centimeter) in the presence of PRP. However, the addition of 1,10-phenanthroline did not

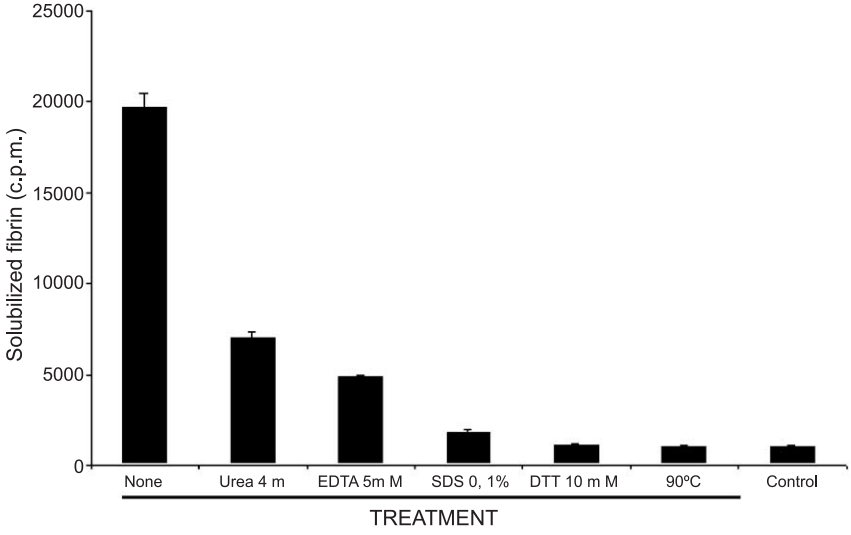

Figure 5 Pretreatment of SCE modulates its capacity to degrade fibrin gel. The fibrinolysis assay was performed as described in Materials and methods, except that SCE was either untreated or pretreated for 2 h at 37 °C with the indicated conditions. The results are means±S.D. of two distinct experiments performed in duplicate.

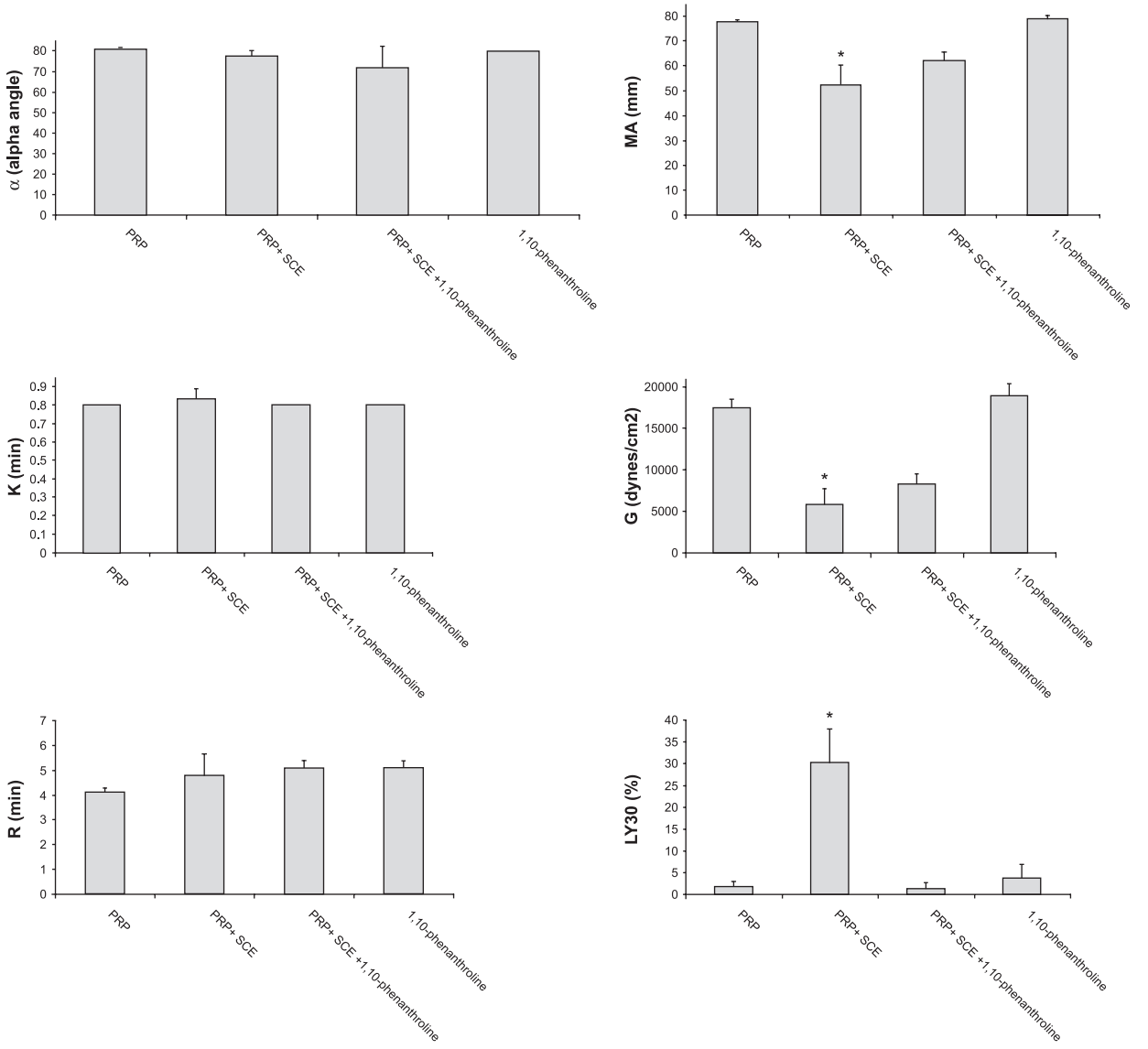

Figure 6 Effect of SCE on different TEG parameters in human PRP. *R* indicates time latency for initiation of clot; *K*, time to initiation of clot firmness of ≈20-mm amplitude; α, α angle; MA, maximum amplitude; and LY30, percentage lysis at 30 min after MA. (*) Statistically significant differences ($P<0.05$; Student's *t* test).

reverse the effect of SCE on the clot strength, suggesting the involvement of metallo-independent compound(s) in SCE in decreasing clot strength. The clot strength, represented by MA, is the peak rigidity manifested by the clot and primarily represents platelet function and to a lesser extent, fibrinogen function. This result suggests that SCE may contain inhibitor(s) of platelet aggregation.

Discussion

We have recently reported that SCE (Neovastat) affects the plasminogen activator (t-PA)/plasmin system. Neovastat markedly stimulates t-PA mediated plasmin generation by increasing both the k(cat) of t-PA as well as its affinity for plasminogen [22]. We also reported that SCE (Neovastat) stimulates t-PA gene transcription in endothelial cells in a TNF alpha-like manner [23]. In this study, we report, for the first time, the presence of plasmin-independent fibrinolytic activities in SCE. Insensitivity of SCE-induced fibrinolysis to serine protease inhibitors (aprotinin, Pefabloc) and plasmin inhibitor (EACA) clearly demonstrated that the plasminogen activator/plasmin system did not participate in SCE-mediated fibrin clot degradation. This datum is consistent with the finding that SCE (Neovastat) did not contain endogenous t-PA or plasminogen

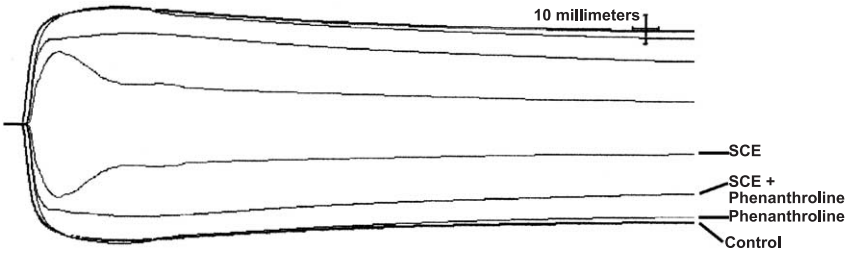

Figure 7 Representative tracing of the TEG curves showing the effect of SCE on the clot formation and lysis. This figure represents an overlay of four TEG curves with a common origin (starting point). The tracing illustrates the effect of SCE on Kaolin-induced clot formation and clot retraction and the inhibitory efficacy of 1,10-phenanthroline on SCE-induced clot lysis with the use of TEG. Note that 1,10-phenanthroline alone had no effect on clot formation.

[22]. The plasmin-independent nature of SCE-fibrinolytic activity was reinforced by different digestion patterns obtained following incubation of fibrinogen or fibrin with SCE or plasmin. Interestingly, incubation of alpha-2 macroglobulin with SCE resulted in the complete inhibition of fibrin gel solubilization. Similar data have been reported for mutalysin II, a zinc endopeptidase from *Lachesis muta muta* snake venom, which is completely inhibited by human alpha-2 macroglobulin [24]. This may suggest the involvement of zinc-dependent protease(s) in SCE-induced fibrinolysis. The metalloprotease nature of SCE-fibrinolytic activity is further supported by its inhibition by only 1,10-phenanthroline but not by inhibitors specific for other classes of proteases (serine, cysteine and acidic proteases).

Using fibrinogen zymography, the fibrinolytic activity consits mainly of two proteases around 58 and 62 kDa. The fibrinogen degradation was inhibited in the presence of chelating agents (EDTA, 1,10-phenanthroline), indicating their metalloprotease nature.

Clot formation is initiated by thrombin-induced cleavage of fibrinopeptide A from fibrinogen. The resultant fibrin monomers spontaneously polymerize to form fibril strands that undergo linear extension, branching and lateral association, leading to the formation of a 3D network of fibrin. A unique property of network structures is that they behave as rigid elastic solids, capable of resisting deforming shear stress. This resistance to shear stress can be measured by elastic modulus, an index of clot strength. In the present study, the utility of TEG in determining the effect of SCE on clot firmness was assessed. Blood clot firmness is an important parameter for in vivo thrombosis and hemostasis because the clot must withstand the shear stress at the site of vascular injury. TEG was used to assess the efficacy of SCE on various factors (coagulation activation, thrombin generation, fibrin formation, platelet activation, platelet-fibrin interaction and fibrin polymerization) involved in clot formation and retraction. TEG allows acquisition of quantitative information, allowing measurement of the maximal strength attained by clots. Our results demonstrated that the addition of SCE (600 μg of proteins) did not alter the clotting time or kinetics parameters (R, K and α) whereas a strong effect was observed on the clot lysis parameter (LY30). This effect was completely inhibited by 1,10-phenanthroline, here again suggesting a role for metalloprotease(s) in clot retraction. Finally, regarding the 1,10-phenanthroline-insensitive effect of SCE on clot strength, it is interesting to speculate that SCE may contain inhibitors of platelet aggregation.

A number of enzymes that interfere with blood coagulation have been previously described and isolated from various snake venoms [25]. Because of the possible therapeutic role of fibrin(ogen)olytic enzymes in dissolving clots, these direct-acting enzymes may prove useful as an alternative to, or for use in synergic combination with, presently used thrombolytic agents. According to the fibrin(ogen) digestion patterns, the SCE enzymes act by a completely different mechanism than the plasminogen activators, the only agents presently approved for clinical use [26,27].

In conclusion, our results suggest that SCE contains fibrinolytic enzymes that through an ability to degrade fibrinogen and fibrin lead to enhanced clot retraction. Thus, SCE may represent a potential source of new therapeutic molecules for vascular disorders. These enzymes may be useful for preventing and treating vaso-occlusive and thromboembolic disorders such as myocardial infarction, restenosis, angiopathic or cerebral thrombosis. Further purification and characterization of the proteins responsible for this effect are currently underway and should provide interesting new information about their relationship with the avascular nature of cartilage. These enzymes might be responsible in part for blocking blood vessel invasion into the cartilage and may represent the "guards" of avascular cartilage's integrity.

Acknowledgments

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