

Cleavage of Surfactant-Incorporating Fibrin by Different Fibrinolytic Agents

Kinetics of Lysis and Rescue of Surface Activity

Andreas Günther, Phillip Markart, Marc Kalinowski, Clemens Ruppert, Friedrich Grimminger, and Werner Seeger

Department of Internal Medicine, Justus-Liebig-University, Giessen, Germany

Incorporation of surfactant into polymerizing fibrin causes loss of surface activity and marked retardation of clot lysis by plasmin (Günther and colleagues, *Am. J. Physiol.* 1994;267:L618–L624). We compared the efficacy of tissue-type plasminogen activator (t-PA), urokinase-type plasminogen activator (u-PA), activated anisoylated streptokinase–plasminogen activator complex (APSAC), and plasmin to dissolve surfactant-incorporating fibrin. Alveofact was employed as a natural surfactant source, and plasminogen was coinorporated into the fibrin matrix at a physiologic ratio to fibrin. Fibrinolysis was quantified by the release of tracer from ¹²⁵I-labeled fibrin, and the pattern of split products was characterized by sodium dodecyl sulfate polyacrylamide gel electrophoresis. In addition, we investigated the fibrinolysis-related restoration of surface activity by measurement in the pulsating bubble surfactometer. Concentrations of all fibrinolytic agents were chosen to effect \approx 40% lysis of clot material in the absence of surfactant (control). When incorporated into the fibrin matrix, but not when admixed after clot formation, surfactant inhibited the cleavage of fibrin by all fibrinolytic agents in a dose-dependent manner. Interestingly, t-PA and u-PA were significantly less inhibited than was plasmin or APSAC. The pattern of arising fibrin scission products was identical for all fibrinolytic approaches and was independent of surfactant incorporation. Adsorption and minimum surface tension–lowering properties of Alveofact were almost completely lost upon incorporation into fibrin, but surface activity was fully restored upon sustained clot lysis with all fibrinolytic agents. We conclude that the fibrinolytic capacity of all agents investigated is markedly inhibited by surfactant incorporation in fibrin, but this inhibition is significantly less pronounced in the agents employing preincorporated plasminogen (t-PA and u-PA), as compared with plasmin and APSAC. The plasminogen activators may thus proffer to “rescue” pulmonary surfactant function by induction of fibrinolysis in the alveolar compartment. **Günther, A., P. Markart, M. Kalinowski, C. Ruppert, F. Grimminger, and W. Seeger. 1999. Cleavage of surfactant-incorporating fibrin by different fibrinolytic agents: kinetics of lysis and rescue of surface activity. *Am. J. Respir. Cell Mol. Biol.* 21:738–745.**

Surfactant abnormalities are assumed to represent a key event in acute lung injury (1, 2) and possibly also in interstitial lung disease. Indeed, increase in minimum surface tension has been documented in patients with fully established adult respiratory distress syndrome (ARDS) or being at risk for ARDS (3), in patients with severe pneu-

monia necessitating mechanical ventilation (4), and in patients with idiopathic lung fibrosis. Changes in the surfactant composition were consistently noted and comprised altered phospholipid and fatty acid profiles, reduction of surfactant apoproteins A and B, and imbalance of large to small surfactant aggregates. In addition, inhibition of surfactant properties by inflammatory mediators and plasma-derived proteins such as albumin, C-reactive protein, hemoglobin, and fibrinogen leaking into the alveolar space has been suggested as representing a major contributor to impaired surface activity (5, 6). It was recently discovered that polymerizing fibrin effects a severe loss of surfactant function by incorporating hydrophobic surfactant constituents such as phospholipids and surfactant protein B (7). It was noted that the surfactant inhibitory strength of polymerizing fibrin surpassed that of fibrinogen and soluble fibrinmonomer by more than two orders of magnitude, thus representing the most effective surfac-

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Address correspondence to: Andreas Günther, M.D., Dept. of Internal Medicine, Justus-Liebig-University Giessen, Klinikstraße 36, 35385 Giessen, Germany. E-mail: andreas.guenther@innere.med.uni-giessen.de

Abbreviations: activated anisoylated streptokinase–plasminogen activator complex, APSAC; adult respiratory distress syndrome, ARDS; plasminogen activator inhibitor, PAI; sodium dodecyl sulfate polyacrylamide gel electrophoresis, SDS-PAGE; standard error of the mean, SEM; tissue-type plasminogen activator, t-PA; urokinase-type plasminogen activator, u-PA.

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tant inhibitory mechanism hitherto described for plasma proteins.

Intra-alveolar accumulation of clot material ("hyaline membranes") is a hallmark of acute or chronic inflammatory lung diseases, including ARDS (8, 9). Leakage of fibrinogen due to increased capillary-endothelial and alveolo-epithelial permeability, as well as a shift of the alveolar hemostatic balance toward a predominance of procoagulant factors, apparently contribute to this finding (10-12). It has been suggested that the delayed clearance of fibrin will result in consecutive invasion of this provisional matrix by fibroblasts. Surfactant impairment by protein leakage, alveolar collapse, persistent "fibrin gluing" of apposed alveolar septae, and subsequent fibroblast invasion may thus represent important sequelae in lung fibrosis, a process previously termed as "collapse induration" (13). Bearing this aspect in mind, a timely dissolution of alveolar fibrin might be reasonable to restore surface activity and, additionally, to prevent onset of fibrotic events.

In a previous study we demonstrated that pulmonary surfactant, when incorporated in a fibrin matrix, may be recovered by fibrinolysis with plasmin, with far-reaching restoration of surface activity upon dissolution of the clot material (14). In the present study we asked whether such an approach might also be reproducible under a more natural condition, when a plasminogen activator has to "meet" plasminogen incorporated with surfactant in the fibrin matrix, to result in the local generation of plasmin. For this purpose, the relative fibrinolytic efficacy of different plasminogen activators and activator-plasminogen complex on natural surfactant-harboring clot material was characterized. Apart from plasmin, we employed urokinase-type plasminogen activator (u-PA), tissue-type plasminogen activator (t-PA), and the activated anisoylated streptokinase-plasminogen activator complex (APSAC). Both u-PA- and t-PA-induced fibrinolysis were found to be less sensitive toward inhibition due to incorporated surfactant material than that effected by APSAC or plasmin. The u-PA-induced fibrinolysis of surfactant-containing clots could not be further augmented by coapplication of high plasminogen doses. However, all proteolytic approaches were capable of restoring full surfactant activity upon sustained lysis; in the cases of u-PA and plasmin, a clear dose-dependency was encountered.

Materials and Methods

Materials

Calf lung surfactant extract (Alveofact) was generously set at our disposal by H. Weller (Thomae, Biberach, Germany). Purified human fibrinogen (> 95% clottability; containing minor amounts of F XIII) and lyophilized bovine thrombin (specific activity, 1,250 U/mg) were kindly provided by Prof. N. Heimburger (Behring, Marburg, Germany). An activated anisoylated plasminogen streptokinase complex (Eminase) was generously provided by Smith Kline & Beecham (München, Germany). Human plasmin (specific activity, 8 U/mg) was purchased from Boehringer Mannheim (Mannheim, Germany). ^{125}I -labeled human fibrinogen (133 $\mu\text{Ci}/\text{mg}$ fibrinogen; > 90% clottability) was obtained from Amersham Buchler (Braunschweig,

Germany), and recombinant t-PA (Actilyse) was from Thomae. The u-PA (Actosolv) was delivered by Behring. Human plasminogen was ordered from Chromogenix (Möln dal, Sweden; 21.7 CU/mg protein). Immunoplates (MAXISORP) were purchased from Nunc (Wiesbaden, Germany). All other chemicals were obtained from Merck AG (Darmstadt, Germany).

Fibrin Plate Assay

Proteolysis of fibrin clots was assayed by use of microtiter plates as previously reported (14). The final volume in each well was 250 μl . All concentrations refer to this final volume, and all components were dissolved in 3 mM Ca^{2+} containing saline. First, unlabeled (6 mg/ml) and labeled (≈ 0.2 nCi/ml) fibrinogen were mixed with various concentrations of Alveofact (0 to 30 mg/ml) and human plasminogen (0.025 mg/ml). Aliquots of this solution were then introduced into the microtiter wells at a volume of 30 μl . Complete clotting was achieved by incubation with thrombin predissolved in 20 μl buffer fluid (0.02 U/ml, 37°C, 1.5 h). The plates were spun at $1,485 \times g$ for 10 min (Univapo 150H centrifuge) for further consolidation of the clot material in the wells. Next, 200 μl saline/ Ca^{2+} , plasmin (0.156 U/ml), u-PA (5,000 U/ml), or t-PA (0.05 mg/ml) was carefully layered onto the surface of the clot, and incubation was performed while gently vortexing (100 \times/min) the plates at room temperature. In experiments with APSAC (1 U/ml), a p-anisoyl group was added to all buffer solutions to avoid rapid deacylation of the catalytic site. In a set of parallel experiments the Alveofact was added together with the previously mentioned proteases instead of being mixed with fibrinogen before coagulation.

At 10, 30, 60, and 120 min (plasmin, u-PA, and t-PA) or 24 h (APSAC) after onset of protease or sham incubation, 150 μl of the supernatant was aspirated and counts were measured in a Canberra Packard γ -counter.

In further experiments the fibrin clot was generated as previously detailed at a constant Alveofact concentration (20 mg/ml), and fibrinolysis was performed by application of 5,000 U/ml u-PA and increasing amounts of plasminogen (0 to 2 mg/ml). In this case, counts were measured after 120 min incubation.

The liberation of radiolabel in the presence of proteases was corrected at each time point for baseline release of ^{125}I activity detected in the parallel control experiments with incubation of saline. This baseline release of tracer persistently ranged < 10% of total radioactivity, even for the longest incubation period of 24 h. All data of protease-induced liberation of radiolabel from the insoluble clot are given in percent of total activity applied to each well. On the basis of preliminary dose-response studies, the presently employed protease concentrations were chosen to yield an $\approx 40\%$ release of radiolabeled split products after 2 h (plasmin, t-PA, and u-PA) or 24 h (APSAC).

Control experiments ascertained complete conversion to fibrin by the used thrombin incubation protocol. Moreover, as proven by means of ^{14}C -dipalmitoylphosphatidylcholine, more than 95% of surfactant phospholipids were shown to be incorporated into the fibrin matrix under these experimental conditions.

Electrophoretic characterization of the after-fibrinolytic split products as obtained with all proteases was undertaken.

Biophysical Studies

Surface properties were assessed in the pulsating bubble surfactometer as previously described (14). In brief, samples were transferred to the sample chamber and adsorption was measured over the initial 12-s period (the γ -ads value given refers to the surface tension after 12 s adsorption). Next, pulsation was started with a cycling rate of 20 times per minute, and minimal and maximum surface tension were recorded over 5 min (the γ -min value given here refers to the minimal surface tension value obtained after 5 min of pulsation). Surface properties were assessed for the following protocols:

Influence of fibrin clot formation on Alveofact function.

Filter passage for separation of clot material from the soluble phase was performed as described (7). Briefly, 4 mg/ml Alveofact was mixed with 1, 2, 4, 8, or 16 mg/ml fibrinogen and incubated with 5 mU/ml thrombin for 1 h at 37°C. The entire mixture was centrifuged through a 150- μ m nylon gauze to separate polymerized fibrin from the soluble phase. As previously shown (7), phospholipids are incorporated into the clot material and lost from the soluble phase under these conditions. Filtrate samples were transferred directly to the pulsating bubble surfactometer.

Assessment of Alveofact function after fibrinolysis.

The amounts of 1, 2, 4, 8, and 16 mg/ml fibrinogen in saline/ Ca^{2+} , mixed with 0.025 mg/ml plasminogen and 4 mg/ml Alveofact, were clotted by incubation with 5 mU/ml thrombin (12 h at 37°C). Plasmin (0.156 U/ml), t-PA (0.05 mg/ml), u-PA (5,000 U/ml), or APSAC (1 U/ml) were then added and the samples were incubated for another 36 h at 37°C. Samples were then directly transferred to the pulsating bubble surfactometer.

In further experiments, 8 mg/ml fibrinogen mixed with 0.025 mg/ml plasminogen and 4 mg/ml Alveofact were clotted by incubation with 5 mU/ml thrombin (2.5 h at 37°C). Increasing amounts of plasmin (0 to 400 mU/ml) or u-PA (0 to 5,000 U/ml) were then added to the fibrin clot and incubation was performed for 2.5 h at 37°C. Filter passage for separation of clot material from the soluble phase

was performed as detailed earlier and the soluble phase was then directly transferred to the pulsating bubble surfactometer.

Polyacrylamide Gel Electrophoresis

Nonreducing, discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (15), using separation gels with 6, 8, 12, and 16% polyacrylamide, stacking gel with 4% polyacrylamide, and well-forming gel and sample-top gel with 8% polyacrylamide. Gel buffer was Tris 0.375 M, pH 9; and electrophoresis buffer was Tris 0.025 M, pH 9, with 0.1% SDS. The gels were stained with a Coomassie blue-containing mixture of H_2O /methanol/glacial acetic acid (11:9:2; vol/vol/vol), and destained with the same solution in the absence of coloring. All samples were adjusted to a protein concentration of 4 mg/ml before application to the gel; for protein quantification based on the Biuret technique, a commercially available kit (Pierce, Rockford, IL) was used.

Statistics

All data are given as means \pm standard error of the mean (SEM). The statistical difference between the surfactant-induced inhibition of either u-PA- or t-PA-based versus plasmin- or APSAC-based fibrinolysis and between the surfactant inhibition curves by fibrin versus the fibrinolysis split products generated by either t-PA, u-PA, APSAC, or plasmin were assessed by application of Friedman's analysis of variance, followed by the Wilcoxon test. Significance level was set at 0.01.

Results

Incubation of the preformed, plasminogen-containing clot with the different proteases resulted in a progressive release of labeled split products. At the concentrations chosen, \approx 40% of total radioactivity was liberated within 2 h (plasmin, u-PA, and t-PA; Controls in Figures 1–3) or 24 h (APSAC; Control in Table 1). When these proteases were applied together with increasing concentrations of Alveofact (2 to 30 mg/ml), comparable kinetics of cleavage were obtained for each agent (Table 2). In contrast, preincorporation of the surfactant material into the fibrin matrix re-

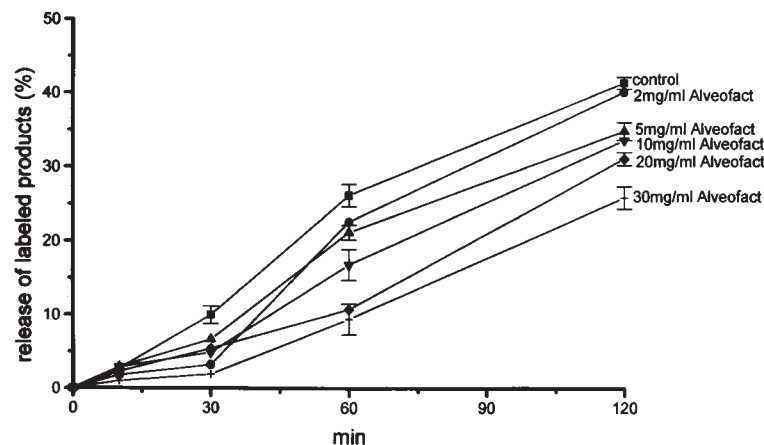


Figure 1. Influence of Alveofact on the kinetics of fibrinolysis by the plasminogen activator t-PA. ^{125}I -labeled fibrinogen mixed with plasminogen was clotted in microtiter plates, and the time course of the tracer release induced by subsequent incubation with t-PA is given in percent of total radioactivity (Control, squares; for details, see MATERIALS AND METHODS). In parallel studies, increasing concentrations of Alveofact (2 mg/ml, circles; 5 mg/ml, triangles; 10 mg/ml, inverted triangles; 20 mg/ml, diamonds; 30 mg/ml, plus sign) were admixed to the fibrinogen before clotting, and addition of t-PA was performed accordingly. Each point reflects the mean \pm SEM of four independent experiments; error bars are missing when falling into symbol.

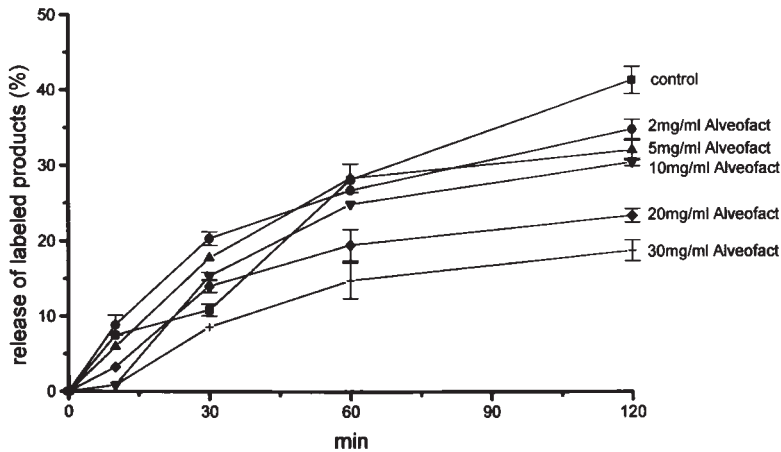


Figure 2. Influence of Alveofact on the kinetics of fibrinolysis by the plasminogen activator u-PA. ^{125}I -labeled fibrinogen mixed with plasminogen was clotted in microtiter plates, and the time course of the tracer release induced by subsequent incubation with u-PA is given in percent of total radioactivity (Control, squares; for details, see MATERIALS AND METHODS). In parallel studies, increasing concentrations of Alveofact (2 mg/ml, circles; 5 mg/ml, triangles; 10 mg/ml, inverted triangles; 20 mg/ml, diamonds; 30 mg/ml, plus sign) were admixed to the fibrinogen before clotting, and addition of u-PA was performed accordingly. Each point reflects the mean \pm SEM of four independent experiments; error bars are missing when falling into symbol.

sulted in a marked and dose-dependent inhibition of the fibrinolysis induced by all agents. The impact of different concentrations of preincorporated Alveofact on the kinetics of t-PA- and u-PA-induced clot lysis is displayed in

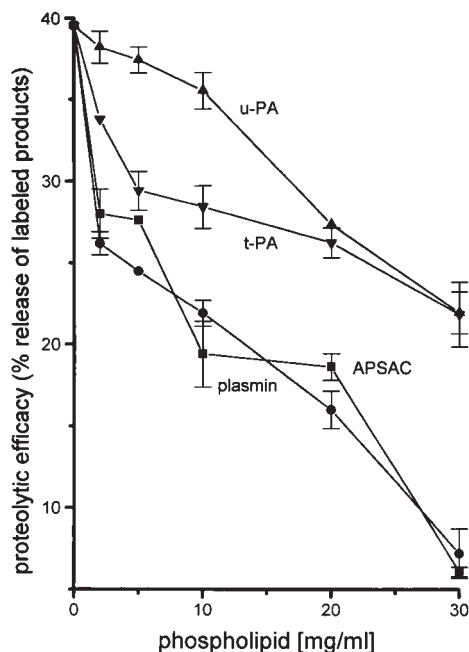


Figure 3. Direct comparison of the inhibitory capacity of surfactant incorporation on plasminogen activator-induced and plasmin-induced fibrinolysis. Concentrations of t-PA (inverted triangles, 0.05 mg/ml), u-PA (triangles, 5,000 U/ml), plasmin (circles, 0.156 U/ml), and APSAC (squares, 1 U/ml) were found to result in $\approx 40\%$ tracer release from ^{125}I -labeled fibrin within 2 h (t-PA, u-PA, and plasmin) and 24 h (APSAC) in control experiments in the absence of surfactant. Dose-inhibition curves for different concentrations of Alveofact, preadmixed to the fibrin clot, are shown. Each point reflects the mean \pm SEM of four independent experiments; error bars are missing when falling into symbol. The dose-inhibition curves for t-PA and u-PA differed significantly from those for both plasmin and APSAC, with P at least ≤ 0.01 .

Figures 1 and 2. At 30 mg/ml Alveofact, the rapidity of appearance of split products was approximately halved. As depicted in Table 1, the APSAC-induced fibrinolysis displayed an even higher sensitivity to the surfactant-related inhibitory mechanism: at 30 mg/ml Alveofact, incubation with APSAC for 24 h resulted in the release of only $\approx 6\%$ radioactively labeled split products, as compared with $\approx 40\%$ in controls (Table 1). The same applied for plasmin: at 30 mg/ml Alveofact the plasmin-induced clot lysis was reduced to $< 5\%$ within 2 h as compared with $\approx 40\%$ in controls. Figure 3 summarizes the inhibition of fibrinolysis of surfactant-containing clots induced by the various agents (parallel experiments): the surfactant-induced inhibition of fibrinolysis was more pronounced in plasmin and APSAC versus u-PA- and t-PA-mediated fibrinolysis. The inhibition of u-PA-induced fibrinolysis was not influenced by the additional application of increasing amounts of plasminogen (0 to 2 mg/ml; not depicted).

Notwithstanding the marked impact of surfactant incorporation on the kinetics of clot lysis, the pattern of split products obtained from these different fibrinolytic approaches was not affected. As evident from the SDS-PAGE depicted in Figure 4, appearance of the terminal fibrin split (Fs) products FsD, FsE, and D-Dimer was noted

TABLE 1
*Influence of Alveofact on the fibrinolysis induced by the APSAC**

Incorporated Surfactant	Release of Labeled Products (%)
0 mg/ml	37.4 ± 1.1
2 mg/ml	28.0 ± 0.8
5 mg/ml	27.6 ± 2.5
10 mg/ml	19.4 ± 0.9
20 mg/ml	18.6 ± 1.4
30 mg/ml	6.1 ± 3.05

* ^{125}I -labeled fibrinogen, mixed with plasminogen and various concentrations of Alveofact, was clotted in microtiter plates, and the tracer release after incubation with APSAC for 24 h is given in percent of total radioactivity and in dependence of the concentration of preadmixed Alveofact. Each point reflects the mean \pm SEM of four independent experiments.

TABLE 2

*Influence of Alveofact on the fibrinolysis by plasmin, APSAC, t-PA, or u-PA when administered simultaneously with the proteases on a preformed fibrin clot**

Surfactant Added	Release of Labeled Products (%)			
	Plasmin	APSAC	t-PA	u-PA
0 mg/ml	37.4 ± 1.1	37.4 ± 1.1	37.4 ± 1.1	37.4 ± 1.1
10 mg/ml	44.3 ± 1.1	31.9 ± 0.5	39.7 ± 1.5	46.4 ± 0.5
20 mg/ml	43.8 ± 0.4	37.8 ± 1.2	33.3 ± 0.4	38.3 ± 0.9
30 mg/ml	32.9 ± 0.9	45.1 ± 2.5	30.0 ± 1.4	32.9 ± 0.3

* 125 I-labeled fibrinogen, mixed with plasminogen, was clotted in absence of surfactant in microtiter plates, and the tracer release after incubation with plasmin, t-PA or u-PA (2 h), or with APSAC (24 h), is given in percent of total radioactivity and in dependence of the concentration of Alveofact being coapplied with the proteases. Each point reflects the mean ± SEM of four independent experiments.

for all protocols of lysis of a surfactant-harboring fibrin matrix, and this pattern did not differ from that observed in the absence of surfactant (not depicted).

In accordance with previous studies, incorporation of Alveofact into the fibrin matrix caused a dramatic loss of surface tension-reducing properties, being attributable mainly to the loss of surface active material from the aqueous phase ($\geq 95\%$ under the present conditions). However, long-term incubation for 36 h of such surfactant-loaded fibrin clots with either u-PA, t-PA, plasmin, or APSAC resulted in a far-reaching restoration of surface activity, indicating the release of functionally intact surfactant compounds. This proved right for the reduction of surface tension under static conditions (adsorption experiments; Figure 5A) as well as under dynamic film compression and re-expansion (minimum surface tension, Figure 5B). In short-term experiments using various concentrations of plasmin and u-PA for 2 h, a dose-dependent restoration of surface properties was ascertained with clearly improved surface activity (γ -min ≈ 10 mN/m) at 100 mU/ml plasmin and 50 U/ml u-PA, and almost-baseline surface tension values (γ -min < 5 mN/m) in the presence of 400 mU/ml plasmin and 5,000 U/ml u-PA (Figure 6). In accordance with the previously reported low protein sensitivity of the presently used surfactant material used in the current study (Alveofact), the differently generated Fs products did not exert any major inhibitory effect on the surface properties of Alveofact in the actual-dose range.

Discussion

Incorporation of pulmonary surfactant into polymerizing fibrin was recently shown to interfere with the fibrinolytic potency of proteases acting directly on the fibrin matrix, such as plasmin, trypsin, or elastase (14). In the present study we reproduced more physiologic conditions by adding plasminogen before the process of clot formation at a naturally occurring plasminogen-fibrinogen ratio, which was thus incorporated into the fibrin clot with and without the simultaneously present surfactant material. Using the plasminogen activators u-PA and t-PA as well as APSAC, the clot enrichment with natural surfactant was again

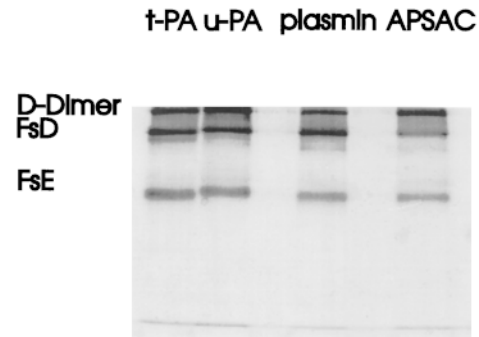


Figure 4. SDS-PAGE of fibrinolysis products generated by the plasminogen activators u-PA and t-PA, plasmin, or APSAC. Fibrinogen was mixed with Alveofact (30 mg/ml) and clotted in microtiter plates. Proteolysis was performed with t-PA, u-PA, and plasmin (2 h), and APSAC (24 h). Aliquots of the supernatants were then adjusted to identical protein content and applied to discontinuous, nonreducing SDS-PAGE. The profiles of scission products did not differ between t-PA, u-PA, plasmin, and APSAC. FsD, fibrin split product D; FsE, fibrin split product E.

noted to inhibit the process of fibrinolysis in a dose-dependent manner, without changing the pattern of arising scission products. However, a marked difference in the sensitivity of the various proteolytic approaches to the inhibitory capacity of surfactant incorporation was noted: the directly acting agents plasmin and APSAC were significantly more strongly blocked than were the agents employing the preincorporated plasminogens, t-PA and u-PA. These findings add a piece of knowledge to the understanding of the hemostatic balance in the alveolar space, and may be relevant for therapeutic attempts aiming at clot lysis in this compartment.

The molecular mechanisms underlying the capacity of clot-embedded pulmonary surfactant to inhibit fibrinolysis are presently not fully settled. The present finding of differences in susceptibility between u-PA and t-PA versus plasmin and APSAC may help to elucidate these mechanisms. The following aspects are to be discussed.

First, interaction of the proteases with surfactant compounds might occur independent of their incorporation into the fibrin matrix. To address this aspect, separate control experiments were performed (data as given in Table 2). Clotting was performed in the absence of surfactant, then proteases plus surfactant were added and the kinetics of fibrinolysis were assessed by release of labeled products. None of the fibrinolytic agents currently employed was inhibited under these conditions. Thus, a direct inhibitory effect of surfactant compounds on plasmin, t-PA, u-PA, or APSAC is largely excluded, but an "intimate" association of the surfactant compounds with the fibrin matrix, known to occur only when surfactant is present during the formation of the fibrin network, is obviously required.

Second, the susceptibility of fibrin toward proteolytic approaches might be influenced by the three-dimensional clot structure. Investigators have repeatedly claimed that changes in the fibrin architecture (e.g., clot retraction induced by platelets or fibroblasts) result in reduced suscep-

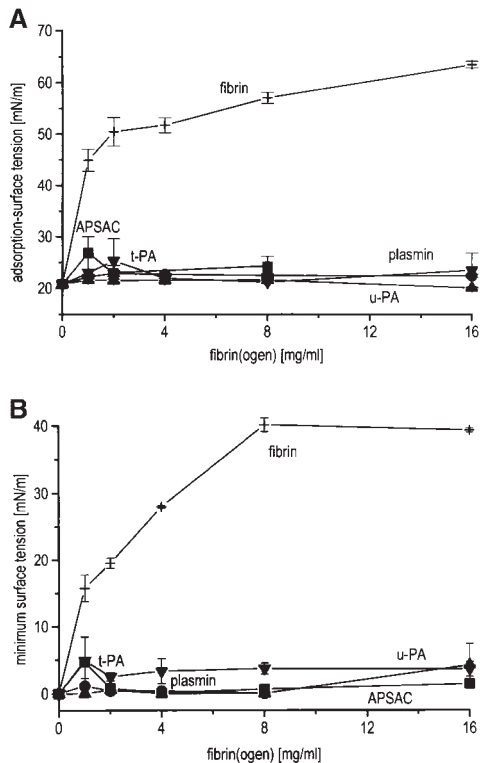


Figure 5. Influence of fibrin and variously generated fibrinolysis products on the surface activity of Alveofact. A total of 4 mg/ml Alveofact was mixed with increasing concentrations of fibrinogen and plasminogen and coagulation was induced by addition of thrombin. After clotting, t-PA (*inverted triangles*), u-PA (*triangles*), plasmin (*circles*), APSAC (*squares*), or saline (fibrin; *plus sign*) were added to the fibrin matrix and allowed to incubate for 36 h at 37°C. Filter-passage of the samples ascertained retention of insoluble clot material on the filter. Clot-free filtrates were transferred directly to the pulsating bubble surfactometer. Minimum surface tension after 5 min of oscillation (γ -min, *B*) and surface tension after 12 s of adsorption (γ -ads, *A*) are given. Each point reflects the mean \pm SEM of four independent experiments; *error bars* are missing when falling into symbol. The dose-inhibition curves in the presence of all proteases differ from that in the absence of lysis (fibrin), with $P \leq 0.001$.

tibility of the clot toward proteolytic attacks, and a decrease in the mean pore size is regarded as an underlying mechanism (16–18). Interestingly, the incorporation of surfactant into fibrin clots was recently shown to exert pronounced effects on the elastic behavior and the structure of the fibrin network, yielding a greater mean pore size of the fibrin matrix compared with control clots (19). To address this aspect more directly, another series of control experiments was performed. After formation of a fibrin clot in the presence of Alveofact, the incorporated surfactant compounds were washed out by detergent treatment as described (19), and the “post-surfactant” fibrin matrix was exposed to proteolytic attack by the different agents. No inhibition of fibrinolysis was noted under these conditions (data not given in detail). Therefore, the surfactant-induced changes in clot architecture are unlikely to be responsible for the inhibition of fibrinolysis, but

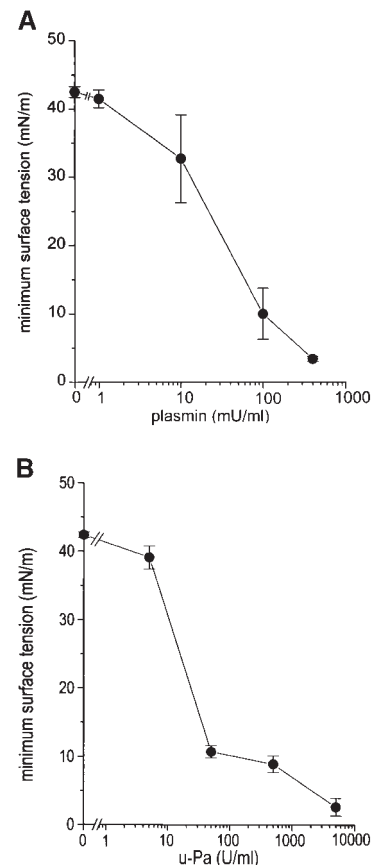


Figure 6. Influence of increasing amounts of plasmin (*A*) or u-PA (*B*) on the restoration of surface activity of a clot-embedded calf lung surfactant extract. A total of 4 mg/ml Alveofact was mixed with 8 mg/ml fibrinogen and 0.025 mg/ml plasminogen, and coagulation was induced by addition of thrombin. After clotting, increasing amounts of u-PA or plasmin were added to the fibrin matrix and incubated for 2 h at 37°C. Filter passage of the samples resulted in the retention of insoluble clot material on the filter membrane. Clot-free filtrates were transferred directly to the pulsating bubble surfactometer, and minimum surface tension after 5 min of oscillation (γ -min) is shown. Each point reflects the mean \pm SEM of four independent experiments.

the presence of surfactant compounds within the clot is evidently a prerequisite for the inhibitory mechanism.

Third, clot-embedded surfactant compounds may interfere with the access of the proteases to their fibrin binding sites, and the differential employment of the preincorporated plasminogen may be responsible for the differences in susceptibility between plasmin and APSAC on the one side and t-PA and u-PA on the other. In the case of plasmin- and APSAC-induced clot lysis, the bulk of protease attack is directed against the surface of the clot. To exert proteolysis, both plasmin and the streptokinase–plasmin complex must have access to the fibrin knots D and E via their lysin binding sites (12, 20). Should these fibrin knots be “hidden” by incorporated surfactant compounds, marked suppression of proteolysis is well conceivable. Plasmin may not profit from the fact that the proenzyme plasminogen is already present within the clot, as plasmin

is not capable of cleaving the ARG⁵⁶⁰-VAL⁵⁶¹ peptide bond of the sc-plasminogen and therefore cannot activate the proenzyme (20). APSAC is basically capable of cleaving plasminogen to plasmin, but with much lower affinity compared with the plasminogen activators u-PA and t-PA (21). Within this line of reasoning, the kinetics of plasmin- and APSAC-induced fibrinolysis were not retarded when plasminogen was omitted from the clot (data not provided in detail). In contrast, t-PA and u-PA are strictly dependent on the preadmixture of plasminogen to the fibrin matrix. For the generation of plasmin and thus fibrinolytic activity, formation of the ternary complex between fibrin, plasminogen, and the plasminogen activator is required (22). It is well imaginable that in contrast to "exogenously" provided plasmin, the preincorporated plasminogen provided in parallel to Alveofact has better access to the fibrin binding sites, despite the presence of surfactant compounds—which may explain the lower susceptibility of the plasminogen activators to the inhibitory capacity of surfactant.

How relevant are the presently used concentrations of fibrinolytic agents for a putative fibrinolysis *in vivo*? In general, there are only few data concerning the concentration or activity of these factors in the alveolar lining layer. The mean level of urokinase activity in the bronchoalveolar lavage fluid (BALF) from healthy individuals was reported to range between 45 and 198 mU/ml, but was found to be depressed to levels of 0 to 8 mU/ml in ARDS patients (10). At present, it is impossible to determine the exact urokinase activity in the alveolar lining layer, but in view of an approximated BALF dilution factor of ≈ 100 , u-PA activity levels of ≈ 15 U/ml in the healthy individual and 0.5 U/ml in an ARDS patient can be assumed. In addition, high levels of α_2 -antiplasmin and plasminogen activator inhibitor (PAI)-1 are to be expected in ARDS and may contribute to the downregulation of the fibrinolytic activity. The alveolar fibrinogen concentration might approximate that of plasma (≈ 4 mg/ml) under conditions of ARDS, and the alveolar surfactant concentration was suggested to range around 10 mg/ml. In our experiments, 50 U/ml urokinase sufficed to largely restore the surface activity of 4 mg/ml Alveofact embedded in 8 mg/ml fibrin. Thus, the presently effective u-PA dosage may surpass only slightly that of the endogenous u-PA pool under physiologic conditions and may serve as a good basis for the calculation of u-PA quantities needed to dissolve the surfactant-containing alveolar fibrin under conditions of ARDS.

In conclusion, the most obvious explanation for the inhibition of fibrinolysis by preincorporated surfactant is its interference with the access of plasmin(ogen) to the lysin residues of the fibrin molecule involved in the binding of this (pro-)enzyme. Membranous interposition of the surfactant material within the solvent channels of the fibrin matrix and hydrophobic interaction between surfactant compounds (phospholipids, hydrophobic surfactant apoproteins) and lipophilic clusters within the fibrin(ogen) molecule may be regarded as underlying mechanisms. The lower susceptibility of t-PA- and u-PA-effected compared with plasmin- and APSAC-effected fibrinolysis to inhibition by preincorporated surfactant may result from the fact that, in contrast to plasmin and APSAC, both being admixed af-

ter coagulation, the plasminogen activators employ the preadmixed plasminogen, for which the access to the fibrin binding sites is less restricted by the surfactant compounds. Markedly increased procoagulant and depressed fibrinolytic activity within the alveolar compartment has repeatedly been demonstrated in acute inflammatory lung diseases such as ARDS and was mostly ascribed to increased tissue factor/F VII and decreased u-PA concentrations, in concert with highly increased PAI-1 and α_2 -antiplasmin (8–11, 23). In contrast, plasminogen levels appear to remain constant under these pathophysiologic conditions (10, 11). Thus, the presently demonstrated superiority of plasminogen activators in cleavage of surfactant-harboring fibrin clots favorably suits the clinical situation. By antagonizing the high alveolar burden of PAI-1 and taking the advantage of a largely unchanged plasminogen pool, systemic or preferably local (aerosol technique) administration of plasminogen activators might offer a therapeutic approach to enhance fibrinolysis in the alveolar space and to restore surface activity, if future studies confirm the important role of alveolar fibrin for persistent atelectasis, gas exchange disturbances, and subsequent progressive fibrosis.

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