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The primary plasminogen-activator inhibitors in endothelial cells, platelets, serum, and plasma are immunologically related

(vascular fibrinolysis/thrombosis/reverse fibrin autography/complex formation/immunodepletion)

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ABSTRACT **Monospecific antiserum to an unusually stable M_r 50,000 plasminogen-activator inhibitor (PAI) purified from cultured bovine aortic endothelial cells was employed in conjunction with reverse fibrin autography to determine whether human platelets, serum, and plasma contain immunologically related inhibitors. Reverse fibrin autography revealed the presence of a M_r 50,000 inhibitor in the platelet and serum samples but not in normal plasma. However, a M_r 50,000 inhibitor was detected in plasma obtained from individuals with increased PAI activity. In each case, treatment of the sample with the anti-inhibitor serum removed the M_r 50,000 inhibitor. The inhibitor present in each sample neutralized exogenously added tissue-type plasminogen activator in a rapid manner. Inhibition was associated with the formation of a $NaDodSO_4$-resistant enzyme–inhibitor complex of M_r 120,000. Again, treatment of the samples with the anti-inhibitor serum removed both the inhibitory activity and the component in these samples that binds to tissue-type plasminogen activator. Thus, the rapidly acting PAI present in human platelets, serum, and patient plasma is immunologically related to the PAI synthesized by cultured bovine aortic endothelial cells. This molecule may be the physiologically relevant inhibitor of plasminogen activator in the vascular system and, as such, may serve an important role in regulating the initiation of vascular fibrinolysis.**

Vascular thrombolysis may be initiated by tissue-type plasminogen activator (tPA; refs. 1–3) or urokinase-like plasminogen activator (4–6), both of which convert the plasma zymogen, plasminogen, into the active, fibrin-degrading enzyme, plasmin. Conceptually, the activity of such plasminogen activators (PAs) may be regulated by specific inhibitors in the blood. Although plasminogen-activator inhibitor (PAI) activity has been detected in cultured endothelial cells (7–9), platelets (10, 11), and serum (12, 13), initial reports failed to demonstrate its presence in normal human plasma (14–20). However, several recent reports have now established the presence of such an activity in plasma from individuals at risk to develop thrombotic problems (21–25). The relation between this plasma PAI and those previously detected in endothelial cells (7–9), platelets (10, 11), and serum (12, 13) has not been determined.

The objective of this study was to establish the biochemical and immunological relationship of these PAIs. Reverse fibrin autography (RFA; ref. 26) was employed to determine the molecular weights of the fibrinolytic inhibitors in the various samples, and antiserum to a PAI recently purified from bovine aortic endothelial cells (BAEs; ref. 27) was used to establish their immunologic relationship. The results show that samples of human plasma obtained from individuals with a tendency to develop thrombotic problems (21–25) contain a M_r 50,000 PAI that is related immunologically to the primary PAIs in BAEs, human platelets, and human serum.

MATERIALS AND METHODS

Preparation of Serum, Plasma, and Platelets. Blood was collected from healthy volunteers into glass tubes and allowed to clot at 37°C for 1 hr. The clot was removed by centrifugation at 1800 × *g* for 20 min at 4°C, and the resulting serum was collected and stored at −70°C until used. To prepare platelet-rich plasma, the blood was collected into polypropylene tubes containing 3.8% (wt/vol) sodium citrate (pH 7.4) and was centrifuged at 200 × *g* for 15 min at 23°C. The platelet-rich plasma was chilled to 4°C for 15 min and then centrifuged at 1800 × *g* for 30 min at 4°C to obtain platelet-poor plasma. Serum was prepared from these plasma samples by the addition of calcium and thrombin as described (11). Plasma samples collected from patients and from healthy volunteers were obtained from B. Wiman (Karolinska Hospital, Stockholm, Sweden), and were prepared according to the methods used by Chmielewska *et al.* (22). The results presented here were obtained using plasma obtained from individuals with obstetric complications (e.g., pre-eclampsia) and are representative of the results obtained using plasma samples collected from individuals at risk to develop thrombotic disease (21–25). These samples contained 8–11 units of inhibitory activity/ml of plasma, whereas normal samples contained little or no inhibitory activity (22). Serum and patient plasma were "albumin-depleted" by precipitation with ammonium sulfate (25% saturation). The precipitate was resuspended in electrophoresis sample buffer (see below) to the original sample volume (100 μl) and then analyzed. Less than 20% of the total amount of albumin was precipitated under these conditions as determined by Coomassie blue staining of $NaDodSO_4$/polyacrylamide gels. Both gel-filtered platelets and the "releasates" obtained from thrombin-treated gel-filtered platelets (10^9 per ml) were prepared as described (11).

Immunoadsorption of the PAI. Rabbit antiserum was prepared against purified BAE PAI according to published procedures (27). This antiserum was shown to be monospecific for the BAE PAI both by immunoprecipitation of ^3H-labeled BAE conditioned medium and by analysis of BAE conditioned medium by two-dimensional gel electrophoresis and immunoblotting (27). It does not recognize α_2-antiplasmin, the primary inhibitor of plasmin in blood. Either the antiserum (40 μl), nonimmune serum (40 μl), or buffer alone [40 μl, phosphate-buffered saline (P_i/NaCl, 0.14 M NaCl/10 mM sodium phosphate, pH 7.2)] was added to protein A-coated Sepharose CL-4B beads (80 μg; Pharmacia, Uppsala, Sweden) that had been rehydrated and washed ac-

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Abbreviations: PA, plasminogen activator; tPA, tissue-type PA; PAI, plasminogen-activator inhibitor; RFA, reverse fibrin autography; BAE, bovine aortic endothelial cell.

cording to the manufacturer's instructions. Samples of platelet releasate (100 μl), serum (35 μl), or patient plasma (35 μl) were diluted into buffer, added to the beads, and then incubated at 23°C for 1 hr in a total volume of 250 μl. The beads were removed by centrifugation, the supernatant fluid was collected, and the beads were washed three times by centrifugation with 1 ml of P_i/NaCl and then extracted for 1 hr at 37°C with 0.25 M Tris·HCl, pH 6.8/2.2% $NaDodSO_4$/20% (vol/vol) glycerol/0.025% bromophenol blue/2.5% (vol/vol) 2-mercaptoethanol. The resulting immunosupernatant fluids and/or the extracts from the immunoprecipitates then were assayed for PAI activity.

Assays for PAI. RFA was performed on $NaDodSO_4$/polyacrylamide slab gels (9% resolving gel) as outlined previously (7, 26, 28). Molecular weight standards were obtained from Bio-Rad and included carbonic anhydrase (M_r 31,000), ovalbumin (45,000), bovine serum albumin (66,200), and β-galactosidase (116,250). These protein standards were fractionated electrophoretically in a lane of the gel separated from the other applied samples. This lane was then excised and stained with 0.1% Coomassie brilliant blue (Bio-Rad).

The following protocol was developed to examine the tPA-binding capacity of the samples. Platelet releasate, serum, and patient plasma, as well as the supernatant fluids obtained from the immunoprecipitations described above, were incubated for 10 min with ^{125}I-labeled tPA. The mixtures then were fractionated by $NaDodSO_4$/PAGE, and the gels were fixed in 10% acetic acid, dried, and subjected to autoradiography on Kodak X-Omat film for various times at −70°C. To quantitate the distribution of the ^{125}I-labeled tPA, the individual lanes in the dried gel were removed and cut into 1-mm slices, and the radioactivity in each slice was determined by using a gamma counter (Micromedic Systems, Horsham, PA). The tPA was purified according to published procedures (2, 29) and was labeled with ^{125}I (Amersham) by the Iodogen method (30), modified so that the labeling time was 3.5 min at 4°C. The specific activity of the final product was ≈5.3×10^6 cpm/μg of protein.

The inhibitory activity of the various samples was determined in the ^{125}I-labeled fibrin plate assay by adding exogenous tPA (25 μl, 2.5 milliunits) to 75 μl of appropriately diluted releasate, serum, or plasma. The mixtures were preincubated for 15 min, plasminogen was added (900 μl; final concentration, 4 μg/ml), and then the amount of fibrinolytic activity was determined as described (11). The fibrinolytic activity in these mixtures was compared to that of control samples containing no inhibitor (i.e., containing tPA and plasminogen alone).

Miscellaneous. Protein was determined by the method of Bradford (31), with bovine serum albumin used as a standard. Plasminogen was prepared by affinity chromatography on lysine-Sepharose (32). Human α-thrombin was a gift from J. Fenton (New York State Department of Health, Albany, NY).

RESULTS

Comparison of the Molecular Weights of the Fibrinolytic Inhibitors in Platelets, Serum, and Plasma. Analysis of various blood-derived samples by RFA (Fig. 1) revealed the presence of fibrinolytic inhibitors in whole blood serum (lane 2) and in the plasma from a patient with pre-eclampsia (lane 5). However, these inhibitors exhibited lower apparent molecular weights than the inhibitor detected in platelet "releasate" (lane 1). This difference was attributed to the large amount of albumin present in the serum and plasma, since the inhibitor in these samples comigrated with the M_r 50,000 inhibitor in platelets when the albumin was removed (lanes 3 and 6). Thus, the inhibitors detected in human platelets, serum, and patient plasma had the same apparent molecular weight as the BAE PAI (7, 27). This inhibitor was not detected in platelet-poor plasma obtained from healthy volunteers (lane 4).

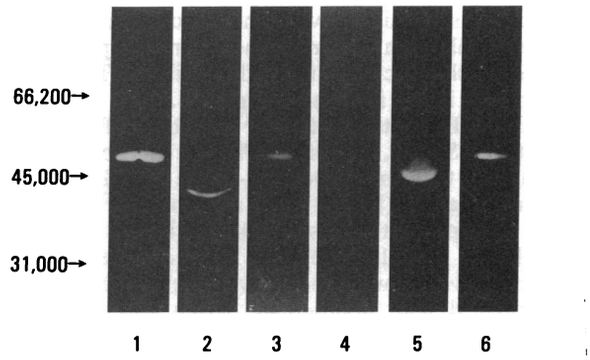

FIG. 1. Comparison of the molecular weights of the fibrinolytic inhibitors detected in platelets, serum, and plasma. Human blood-derived samples were fractionated by $NaDodSO_4$/PAGE and then analyzed by RFA. Lanes: 1, platelet releasate (75 μl); 2, whole blood serum (20 μl); 3, albumin-depleted serum (40 μl); 4, normal plasma (20 μl); 5, patient plasma (20 μl); 6, albumin-depleted patient plasma (40 μl). Positions of molecular weight standards are indicated at left.

Removal of the Fibrinolytic Inhibitors in Platelets, Serum, and Patient Plasma with Antiserum to the BAE PAI. The immunologic relationship between the inhibitors in human platelets, serum, and patient plasma and the PAI recently purified from BAEs (27) was examined in immunoprecipitation experiments using antiserum directed against the BAE PAI (Fig. 2). In each case, the M_r 50,000 inhibitor present in these samples (Fig. 2 *A–C*, lanes 1) was removed by the antiserum (lanes 2) and recovered in the immunoprecipitates (lanes 3). No significant amount of inhibitor activity was immunoprecipitated from any of the samples when protein A-Sepharose containing nonimmune serum was used (data not shown). Thus, the inhibitors detected in platelets, serum, and patient plasma were related immunologically to the PAI synthesized by BAEs (7, 27). Whereas immunoprecipitation did not alter the mobility of the platelet inhibitor (Fig. 2*A*, lanes 1 and 3), it did alter the mobilities of the serum (*B*, lanes 1 and 3) and plasma (*C*, lanes 1 and 3) inhibitors. In both instances, the immunoprecipitated inhibitors migrated more slowly than the inhibitors in the starting materials. Moreover, they now comigrated with the platelet inhibitor. Again, this

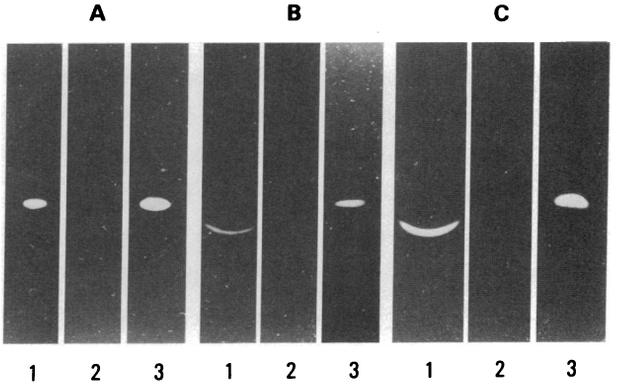

FIG. 2. Immunoprecipitation of the inhibitors in platelets, serum, and patient plasma. Human blood-derived samples were immunoadsorbed with the antiserum against the BAE PAI. The resultant immunosupernatant fluids and immunoprecipitates were then subjected to $NaDodSO_4$/PAGE and RFA. (*A*) Platelet releasate (50 μl per lane). (*B*) Serum (20 μl). (*C*) Patient plasma (20 μl). Lanes: 1, starting material; 2, immunosupernatant fluid; 3, immunoprecipitate.

difference is consistent with the conclusion that the large amount of albumin present in the serum and plasma distorts the electrophoretic mobility of the inhibitor. This albumin is not present in the immunoprecipitates.

Complex Formation Between ^{125}I-Labeled tPA and the PAI in Platelets, Serum, and Patient Plasma. The ability of the PAI in platelets to form $NaDodSO_4$-stable complexes with tPA was investigated (Fig. 3). The addition of ^{125}I-labeled tPA (lane 1) to platelet releasate resulted in the formation of a complex of M_r ≈120,000 (lane 2). A complex of the same molecular weight was evident when releasate that had been preadsorbed with nonimmune serum was used (lane 3). However, no M_r 120,000 complex was apparent when the releasate had been immunoadsorbed with antiserum against the BAE PAI (lane 4). Quantitation of the radioactivity in this gel indicated that the total counts (distributed as either free or complexed tPA) were essentially the same in each lane (data not shown).

When ^{125}I-labeled tPA (Fig. 4*A*, lane 1) was added to serum (*B*, lane 1) or patient plasma (*C*, lane 1), M_r 120,000 complexes also formed (arrow). Again, complex formation was not affected by preadsorbing the samples with nonimmune serum (Fig. 4 *B* and *C*, lanes 2) but was completely prevented by immunoadsorbing the samples with the antiserum against the BAE PAI (*B* and *C*, lanes 3). The electrophoretic mobility of the free ^{125}I-labeled tPA added to the serum and patient plasma was distorted by other proteins (e.g., albumin) present at high concentration in these samples. This conclusion is based on the finding that this same distorted pattern was apparent when serum was added to the labeled tPA under conditions that did not allow complex formation (i.e., in the presence of electrophoresis sample buffer; compare lanes 1 and 2 in Fig. 4*A*). In spite of this distorted profile, the total radioactivity recovered from each lane was essentially the same (data not shown). No M_r 120,000 complex formed when either normal plasma or the serum derived from it was assayed in this manner (data not shown). Interestingly, a higher M_r complex (170,000–190,000) was also evident, but only in the immunodepleted samples (Fig. 4 *B* and *C*, lanes 3). Based on previous reports (33, 34), it seems likely that this complex contains tPA and $C\bar{1}$ (first component of complement) inhibitor. These results indicate that in platelet releasate, serum, and the patient plasma, the component that rapidly complexes with tPA is removed by the antiserum against the BAE PAI. Removal of this component coincides with the removal of the M_r 50,000 inhibitor detected in these samples by RFA (see Fig. 2).

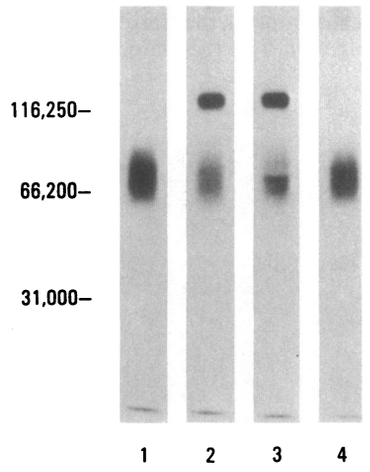

FIG. 3. Autoradiogram showing complex formation between ^{125}I-labeled tPA and the PAI in human platelets. ^{125}I-labeled tPA (15 ng, ≈60,000 cpm; lane 1) was incubated for 10 min at 37°C with 40 μl of platelet releasate (lane 2), or with releasate that had been immunoadsorbed with nonimmune serum (lane 3), or with the anti-PAI serum (lane 4). The resulting mixtures were subjected to $NaDodSO_4$/PAGE and autoradiography. The film was developed after 19 hr of exposure at −70°C. Positions of molecular weight standards are indicated.

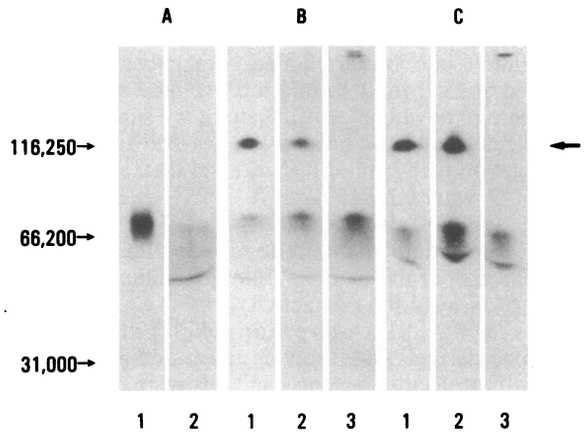

FIG. 4. Complex formation between ^{125}I-labeled tPA and the PAIs in serum and patient plasma. ^{125}I-labeled tPA (0.6 ng, ≈3000 cpm; *A*, lane 1) was incubated with serum containing electrophoresis sample buffer (30 μl; *A*, lane 2), serum alone (30 μl, *B*), or patient plasma (15 μl, *C*) for 10 min at 23°C. The serum and plasma samples shown in *B* and *C*, respectively, were untreated (lanes 1), adsorbed with nonimmune serum (lanes 2), or immunoadsorbed with anti-PAI serum (lane 3). Each mixture then was subjected to $NaDodSO_4$/PAGE and autoradiography. The film was developed after 5 days of exposure at −70°C. Positions of molecular weight standards are indicated. Arrow at right indicates the M_r 120,000 complex (see text).

Immunodepletion of tPA Inhibitory Activity from Platelets, Serum, and Patient Plasma. Although the antiserum directed against the BAE PAI removed both the M_r 50,000 inhibitor detected by RFA and the component that complexes with ^{125}I-labeled tPA, it was not clear how much of the total tPA inhibitory activity present in the various samples could be attributed to this component. Therefore, the total tPA inhibitory activity of the samples was assessed after they had been immunoadsorbed. Samples pretreated with protein A-Sepharose in the presence of buffer or nonimmune serum (Table 1) inhibited tPA-mediated fibrinolysis to a similar extent (70–80%). Samples pretreated with protein A-Sepharose in the presence of antiserum specific for the BAE PAI exhibited considerably less inhibition (10–30%). Thus, removal of most of the tPA inhibitory activity present in each sample coincided with the removal of both the M_r 50,000 inhibitor

Table 1. Immunoadsorption of the tPA inhibitory activity from platelets, serum, and patient plasma

Sample	% inhibitory activity remaining after immunoadsorption		
	Buffer	Nonimmune serum	Anti-PAI serum
Platelet releasate	71	74	8
Serum	66	66	32
Patient plasma	79	82	20

Platelet releasate (10 μl), serum (4 μl), and plasma obtained from a patient having pre-eclampsia (4 μl) were preadsorbed with protein A-Sepharose containing buffer, nonimmune serum, or anti-PAI serum. Resulting supernatant fluids were analyzed for tPA inhibitory activity in the ^{125}I-labeled fibrin plate assay. Values represent the reduction of fibrinolytic activity compared to mixtures containing tPA (2.5 milliunits/ml) and buffer only. Nonadsorbed platelet releasate, serum, and patient plasma inhibited fibrinolysis by 75, 80, and 82%, respectively.

detected by RFA and the component that forms M_r 120,000 complexes with ^{125}I-labeled tPA.

DISCUSSION

In the work described here, we have employed RFA and monospecific antiserum directed against the BAE PAI to examine the relationship between the rapidly acting PAI synthesized by endothelial cells (7–9, 27) and those detected in platelets (10, 11), serum (12, 13), and the plasma of individuals at risk to develop thrombotic disease (21–25). Three conclusions can be drawn from these studies. (*i*) The PAIs present in the blood-derived samples are biochemically similar to each other and to the BAE PAI, since each can be detected after NaDodSO$_4$/PAGE and each has an apparent M_r of 50,000 (Fig. 1; refs. 7 and 27). Moreover, each of these PAIs forms NaDodSO$_4$-resistant complexes with exogenously added ^{125}I-labeled tPA (Figs. 3 and 4; ref. 27). (*ii*) The blood-derived PAIs are immunologically related to the BAE PAI, since each is recognized by antiserum against this molecule, but not by nonimmune serum (Fig. 2). (*iii*) The PAI related to the BAE PAI is the primary inhibitor of tPA in the blood-derived samples. This conclusion is based on the observations that when these samples are depleted of this molecule by incubation with the antiserum against the BAE PAI, both their tPA-binding activity (Figs. 3 and 4) and their rapidly acting PAI activity (Table 1) are removed.

The data presented indicate that treatment of the human blood-derived samples with the anti-BAE PAI serum removed a M_r 50,000 component with tPA-neutralizing capability. The possibility that a second inhibitor, one that is NaDodSO$_4$-sensitive and thus not detectable by RFA, also contributes to the tPA-binding and tPA-inhibiting activities measured here was considered. The results suggest that in the samples analyzed, the contribution of such molecules is low compared to that of the endothelial cell/platelet PAI. This conclusion is based primarily on the observation that immunoadsorption of the M_r 50,000 inhibitor detected in each of the samples by RFA coincided with removal of the tPA-binding activity and the majority of the tPA inhibitory activity as determined by use of assays initiated under nondenaturing conditions (i.e., the complex-formation assay and the ^{125}I-labeled fibrin plate assay). Because the antiserum appears to be specific for the BAE PAI (27), it is unlikely that it also removes other inhibitors present in the samples. Many investigators have demonstrated that platelets, serum, and plasma contain inhibitory activities against plasmin as well as PAIs (11, 14–25, 33–35). Thus, the residual antifibrinolytic activity remaining in the immunodepleted samples may be caused by plasmin inhibitors in addition to any PAIs not removed by the antiserum against the BAE PAI. We propose, therefore, that the M_r 50,000 PAI described in this report is the major inhibitor of tPA in the blood, whereas other PAIs may play important regulatory roles outside the vascular system and/or may be important inhibitors of vascular PA under certain other physiologic or pathologic conditions. Kruithof *et al.* (23) have provided evidence indicating that the primary plasma inhibitory activity against both urokinase and tPA resides in the same molecule. Thus, the PAI described here may also be the primary urokinase inhibitor in human blood.

Although PAI activities have been detected in a variety of human cells (7–9, 11, 36–40) and body fluids (12–25, 33–35), only three of these have been characterized sufficiently to allow a direct comparison with the PAI activity measured here. These include protease nexin (38), the placenta-derived inhibitor (36), and the dexamethasone-induced PAI synthesized by rat hepatoma cells (39). Based on its unusual stability and immunologic properties, the PAI derived from endothelial cells and platelets described in this and other reports (7, 11, 27) clearly is not protease nexin or the placental inhibitor (ref. 27 and unpublished observations). The PAI synthesized by rat HTC cells is both acid- and NaDodSO$_4$-stable (39) and, in these respects, is similar to the BAE PAI (7, 27). Moreover, antiserum to the BAE PAI crossreacts with the HTC PAI (44), indicating that these are also immunologically related molecules. The PAI synthesized by cultured rat granulosa cells is also immunologically related to the BAE PAI (40), suggesting that this class of PAIs may regulate a variety of processes involving plasminogen activation. The relationship of these PAIs to the PAIs elaborated by macrophages (37), uterine cells (41), and tumor cells (42) remains to be determined.

As to the physiologic role in blood of the PAI described here, we (11) and others (43) have suggested that the timely release of a rapidly acting PAI from platelets and/or endothelial cells may cause the environment surrounding a growing thrombus to be quite refractory to fibrinolysis, whether initiated by endogenous or infused PA. The detection of the platelet-derived PAI in serum (11) further suggests that the blood in the vicinity of a developing thrombus may be, at least transiently, antifibrinolytic. The primary vascular PAI may, therefore, prevent the premature lysis of blood clots. In addition, although we still do not know its precise site of origin, the presence of the PAI in the plasma of individuals with a tendency to develop thrombotic disease (21–25) suggests a possible relationship between these conditions and an overall decrease of the fibrinolytic potential of the blood.

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