The Mutual Relationship Between the Two Molecular Forms of the Major Fibrinolysis Inhibitor Alpha-2-Antiplasmin in Blood

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Alpha-2-antiplasmin, a major inhibitor of fibrinolysis, is synthesized in the liver and occurs in blood in two molecular forms: a very active plasminogen-binding (PB) form and a less active nonplasminogen-binding (NPB) form. This study investigates the origin and mutual relationship of these two forms in vivo and in vitro. Despite wide variation in plasma concentration of the inhibitor (16% to 138%), the ratio between the two forms in vivo was found to be, in the main, constant among healthy volunteers, heterozygotes for a congenital deficiency of alpha-2-antiplasmin, and patients with a stable liver cirrhosis: PB/NPB = 2.41 ± 0.34 (SD). Resynthesis after depletion or increased synthesis in the acute-phase reaction showed a specific increase of the PB form of the molecule in blood after discontinuation of L-asparaginase therapy. Additions of a variety of enzymes or inhibitors did not interfere with the process. These results demonstrate that the PB form of alpha-2-antiplasmin is produced by the liver and that the NPB form is formed in the circulation.

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was prepared by chromatography on column coupled antibodies to urokinase as described by Kluf et al.23 Plasma depleted in plasminogen was prepared by chromatography on lysine-agarose (cf ref. 17); the depleted plasma showed no response on immunochemical assay for plasminogen (detection limit about 5%) and a residual activity of 1.6% in the streptokinase method of Fribberger et al.24

**Assays.** Modified crossed immunoelectrophoresis was carried out as described by Kluft and Los.16 Briefly, the 1% agarose gel for the first dimension in 0.03 mol/L buffer, pH 8.6, contained 1,000 KIU/mL Trasylol and 0.04 mg lys-plasminogen per milliliter added to the agarose solution just before casting the gel. Before electrophoresis, 5 μL of plasma or serum, 2 μL of lys-plasminogen solution (2 mg/mL), and 1 μL of 10,000 KIU/mL Trasylol were sequentially and rapidly introduced into the punched well. The gel for the second dimension contained antiserum against alpha-2-antiplasmin. The immunoprecipitation peak surface at β-mobility represents the concentration of the PB form of alpha-2-antiplasmin, and at α-mobility, that of the NPB form. The antiserum had a comparable titer for both forms of alpha-2-antiplasmin as assessed by assay of mixtures of the PB and NPB forms.25

The activity assay of alpha-2-antiplasmin, the immediate plasmin inhibition test, was performed as described by Kluft et al.25 and measured 1.00 PB + 0.14 NPB.25 The PB and NPB concentrations can be calculated separately knowing the ratio PB-NPB from the modified crossed immunoelectrophoresis and the result of the immediate plasmin inhibition test using a standard (pooled plasma) with a known ratio and amount of inhibitor calibrated on active-site titrated plasmin.26 Alternatively, PB and NPB concentrations can be determined directly by modified crossed immunoelectrophoresis in simultaneous runs with pooled normal plasma with known amounts of PB and NPB.27

**Alpha-2-antiplasmin determined immunochemically by the technique of Laurell27 was expressed in percentage of pooled normal plasma (1.07 pmol/L).**

Changes in the conversion rate of plasma in vitro of the two forms of alpha-2-antiplasmin were assessed by incubation of the two forms for 18 days at 37 °C and assay of the pattern of the modified crossed immunoelectrophoresis. Depleted or deficient plasmas were compared with starting plasma or pooled normal plasma. Effects of inhibitors (25 μL added to 250 μL plasma) were compared with buffer controls. The effects of CIs-esterase, granulocyte enzymes, dextran sulphate, and ellagic acid on the antiplasmin activity in the immediate plasmin inhibition test were studied after 15 minutes' incubation at 37 °C in 1:1 mixtures.

**Volunteers and patients.** Normal volunteers were from the institute. Heterozygotes with congenital alpha-2-antiplasmin deficiency were from a family described by Kluft et al.4 Patients with advanced liver cirrhosis were in a stable period. The patient receiving local streptokinase for a thrombus was treated with a bolus injection of 5,000 units and a maintenance dose of 5,000 U/h for 24 hours. The dosage was increased to 10,000 U/h until the therapy was stopped after 97 hours. Samples obtained during and shortly after therapy were analyzed in the modified crossed immunoelectrophoresis assay in a special way. To account for the possible presence and interference by plasmin–antiplasmin complexes at β-mobility, an intermediate agarose gel layer containing antiserum against plasminogen48 was introduced. In some samples, the precaution proved necessary. Three patients from a larger study48 of patients with chest pain admitted to hospital with a diagnosis of myocardial infarction, later confirmed by electrocardiography and enzyme studies (AMI <48 hours), were further investigated after demonstration of a rise in activity in the immediate plasmin inhibition test.

Six patients with acute lymphocytic leukemia described by Velenga et al.29 were studied before the start, at the end, and six to ten days after discontinuation of t-asparaginase intravenous therapy. The daily dosage varied from 5,500 to 7,496 U/mL. The total duration of the therapy varied from seven to 17 days and resulted in reduction in the immediate plasmin inhibition test to 5% to 30% of normal, reverting to normal again after discontinuation.29

Informed consent was obtained.

**Cell culture.** The established cell line Hep G2 derived from a human liver tumor was obtained from Dr B.B. Knowles (Wistar Institute of Anatomy and Biology, Philadelphia) and is known to produce alpha-2-antiplasmin.27 The cells were cultured at 37 °C in 25-cm² flasks containing 0.1 to 0.2 mL/cm² (Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (Flow Laboratories, Irvine, Scotland), penicillin (100 IU/mL), and streptomycin (0.1 mg/mL) under a 5% CO₂/air atmosphere. For the experiments on alpha-2-antiplasmin, cells were grown to a density of 3 mg protein/25 cm². After two washings with DMEM, 2 mL of fresh medium were added to each flask. Media were harvested after one and eight days of incubation at 37 °C. During the incubation of 11 days, 1 mL of fresh medium was supplied on day 8. The media were centrifuged to remove cellular debris and stored at −20 °C. For the purpose of analysis, the medium was ten times concentrated on a Minicon concentrator B 15 (Amicon BV, Oosterhout, The Netherlands).

**RESULTS**

**Steady state.** As shown in Fig 1, the ratio PB-NPB is not significantly dependent on the synthesis rate of alpha-2-antiplasmin under steady state conditions, eg, in stable individuals. The data was obtained from apparently normal individuals, from heterozygotes for congenital alpha-2-antiplasmin deficiency with an approximately half normal plasma concentration, and from patients with liver cirrhosis with an apparently impaired synthesis of the inhibitor in a stable period. (It was recently demonstrated that no enhanced catabolism occurs in such patients.26) The total amount of alpha-2-antiplasmin (PB + NPB) was determined by Laurell immunoassay. The ratio PB-NPB in this group (Fig 1) was 2.41 ± 0.34 (SD), well in accord with experience in a larger group of 29 apparently healthy volunteers showing 2.33 ± 0.29 (SD).

![Fig 1. The ratio PB-NPB determined by the modified crossed immunoelectrophoresis technique for healthy volunteers (O; n = 19), patients with a stable liver cirrhosis (x; n = 5), and heterozygotes for congenital alpha-2-antiplasmin deficiency (C; n = 15). The total amount of alpha-2-antiplasmin is determined by the Laurell technique (abscissa).](image-url)
Acute phase reaction. The alpha-2-antiplasmin activity as well as the PB and NPB forms were determined in three patients who showed an acute-phase reaction after myocardial infarction. During the acute phase, the increase in alpha-2-antiplasmin activity coincided with an increase in PB antiplasmin, while the NPB form remained virtually constant (Fig 2).

Streptokinase therapy. During streptokinase therapy of a patient (see Fig 3), alpha-2-antiplasmin was almost consumed by the active process. After treatment, the alpha-2-antiplasmin activity was restored to almost normal by resynthesis at the first blood sampling (indicated as “3” in the figure). At this particular time, as shown by the modified crossed immunoelectrophoresis pattern, the PB alpha-2-antiplasmin had almost exclusively reappeared.

L-asparaginase therapy. During L-asparaginase therapy, synthesis of alpha-2-antiplasmin was inhibited. A gradual, functional, and immunochromatographic lowering of the plasma concentration with a half-life of approximately five days was observed in six patients. At the discontinuation of therapy, alpha-2-antiplasmin was found to be 26% ± 9% of normal (immunoassay). A concomitant significant change was observed in the ratio of PB-NPB for all patients: from 2.86 ± 0.55 at the start to 1.74 ± 0.24 at the end of treatment. This indicates a selective reduction in PB alpha-2-antiplasmin. After treatment, resynthesis resulted in recovery of normal alpha-2-antiplasmin levels (92% ± 12%, after about eight days) and an increased PB-NPB ratio of 4.65 ± 1.32 (n = 6), indicating selective increases in PB alpha-2-antiplasmin.

Hep G2 cell culture. The hepatoma cell line Hep G2 produces alpha-2-antiplasmin22 linearly in increasing amounts, with the incubation time as shown in Fig 4 by normal crossed immunoelectrophoresis. In the modified crossed immunoelectrophoresis, significant amounts of PB alpha-2-antiplasmin only were present at day 1, while on day 11 the NPB form had also appeared.

Incubations in vitro. Incubation of citrated pooled normal plasma in vitro was found to result in a slow decay of alpha-2-antiplasmin activity. Figure 5 shows that this pro-
Fig 4. Production of alpha-2-antiplasmin in culture media of Hep G2 cells. Normal crossed immunoelectrophoresis shows an increased production with time (upper panel). Modified crossed immunoelectrophoresis (CIE + plasminogen) of medium (concentrated to comparable alpha-2-antiplasmin concentrations) of day 1 and day 11 are given in the lower panel.

The process exhibits individual variation that, for the initial rate, was estimated to show a range of ± 20% around the mean. As shown in Fig 6 for pooled normal plasma, the process is moderately temperature dependent, with an increase in rate of a factor of 2.4 and 1.9 for 0°C to 25°C and 25°C to 37°C, respectively.

As shown in the insert of Fig 6, the decay of activity is accompanied by a conversion from PB to NPB alpha-2-antiplasmin. Combining the activity assay and the modified crossed immunoelectrophoresis to determine PB and NPB separately (see Materials and Methods), it was demonstrated (as shown for pooled normal plasma in Fig 7) that the PB form shows a first-order decay with a half-life of eight days.

The conversion of PB to NPB on in vitro incubations was found to be uninfluenced by a number of circumstances. The
conversion was no different in normal serum or plasma deficent in factor XII or factor XIII, or in plasma depleted in plasminogen (by sepharose-lysine) or plasma devided (by immunoabsorption in factor XII or factor XIII, or in plasma depleted in plasminogen (by sepharose-lysine) or plasma devided (by immunoabsorption). The conversion was not influenced by EDTA (1 mmol/L), by addition of granulocyte enzymes, by contact activation by either ellagic acid (1:1, with 10⁻⁴ mol/L) or dextran sulphate (1:1 with 25 μg/mL), or by addition of CI₈-esterase (375 U/mL). The addition of the following inhibitors (highest final concentrations tested) did not prevent the conversion, as judged from the conversion after 18 days in the modified crossed immunoelectrophoresis: DFP (1 mmol/L), soya bean trypsin inhibitor (100 μg/mL), Trasylol (100 KIU/mL), benzamidine (100 μg/mL), lima bean trypsin inhibitor (100 μg/mL) ovomucoid (20 μg/mL), ovoinhibitor (100 μg/mL), elastatinal (1 μg/mL), pepstatin (1 μg/mL), chymostatin (1 μg/mL), iodoacetamide (100 mmol/L), β-mercaptoethanol (0.1 mmol/L), HgCl₂ (1 mmol/L), and ZnCl₂ (1 mmol/L).

DISCUSSION

Using modified crossed immunoelectrophoresis, the two forms of alpha-2-antiplasmin, PB and NPB, could be separately determined and both were present in all plasmas studied. The ratio between the forms was found to be rather constant among individuals, with a mean molar ratio of PB-NPB of 2:4:1. This was irrespective of the largely variable synthesis rate of alpha-2-antiplasmin, which resulted in corresponding plasma levels of total alpha-2-antiplasmin of 16% to 138% obtained by studying patients with stable liver cirrhosis, heterozygotes for alpha-2-antiplasmin, and normal individuals.

In situations where synthesis of new or additional alpha-2-antiplasmin was observed, such as after depletion by streptokinase or t-asparaginase therapy, or in acute-phase conditions after myocardial infarction, primarily the PB form of alpha-2-antiplasmin was found to be produced. Accordingly, in cultures of the hepatoma cell line Hep G2, the PB form was found to be initially produced, and only later was the NPB form also detected in the culture media. This demonstrates that the liver produces primarily (probably exclusively) the PB form of alpha-2-antiplasmin. As to the origin of the NPB forms of alpha-2-antiplasmin, it was demonstrated that the PB form apparently spontaneously converts to the NPB form in vitro. This was demonstrated in platelet-poor citrated plasma and indicates the involvement of plasma components; alternatively, the conversion involves an intrinsic property of alpha-2-antiplasmin. Assuming the exclusive synthesis of the PB form, the occurrence of the NPB form in vivo is evidence for its formation there as well. In addition, the data showing a more rapid disappearance of the PB form relative to the NPB form (decrease in the PB-NPB ratio) during therapy with t-asparaginase is compatible with and explained by an in vivo conversion of PB to NPB.

The data on conversion in vitro and in the vivo data agree when related to each other in a minimum hypothesis, which is also based on published data, as shown in Fig 8. First-order reactions are proposed for catabolism (k2, k3) of both forms and, as demonstrated in Fig 8, for the conversion of PB to NPB (k1). The recognition of the PB-NPB conversion implies that previous studies on the catabolism of radiolabeled alpha-2-antiplasmin do not report exclusively on k2. In the proposed model, the plasma half-life is represented by ln 2/(k2 + k3). This has been determined by Collen and Wiman to give a half-life of 2.83 days on injecting healthy subjects with a preparation that, according to our evaluation of a similar one, mainly represents the PB form (≈95%) (courtesy of Drs Knot and Collen; see ref.). Using the additional information that the mean PB-NPB ratio in healthy subjects is 2.33, which represents k3/k1 during steady state, we can conclude that k2 in this model actually corresponds to a half-life of three days. In the model, then, k1 and k3 remain undetermined for the in vivo situation. If we assume the in vitro conversion of PB to NPB to be representative for the in vivo process, we can calculate that k3 has a value corresponding to a half-life of 3.4 days. These values are similar to the half-life of the plasma alpha-2-antiplasmin of 3.3 days, determined by Tamaki et al by infusion of normal fresh plasma (PB + NPB mixture) in a patient with congenital deficiency of alpha-2-antiplasmin. It is not unrealistic to assume that both alpha-2-antiplasmin forms have closely similar catabolic rates, but definite proof awaits a direct determination of the catabolism of the NPB form. Alternatively, the assumption that the catabolic rates of both forms are identical results in a calculated k1 for the in vivo conversion comparable to a half-life of 7.2 days, which is close to the in vitro value of eight days.

It is concluded that the model describes the quantitative aspects of the relationship between the two forms and implies no present need to assume other processes than the ones observed in vitro to describe the mutual relationship between the two forms of alpha-2-antiplasmin. Further considerations are:

1. In the proposed model, the ratio PB-NPB is independent of the synthesis rate of alpha-2-antiplasmin in a steady state, as actually observed (Fig 1).
2. When synthesis is blocked, a relatively rapid decrease in the PB form in relation to the NPB form occurs, resulting in a shift in the PB-NPB ratio, as actually observed during asparaginase therapy.
3. The individual variation in the PB-NPB ratio (Fig 1) can only originate in a variation in k1, k2, or k3. According
to the data in Fig 6, it can originate in the variation in $k_1$, which shows a comparable variability to that of the PB-NPB ratio.

The nature of the conversion process is not elucidated by this study, but it has been demonstrated that it concerns a temperature-dependent process in vitro, with a more moderate temperature dependence than enzymatic reactions ($Q_{10} = 2$ to 4). The recent report of Sasaki et al.\(^3\) that a C-terminal peptide has plasminogen-binding properties (cf. ref. 34) would suggest the possibility of splitting off such a peptide. Our attempts to inhibit the conversion by a spectrum of protease inhibitors (directed to various serine and thiol proteases) was unsuccessful, and a number of proteases tested (added or generated in the plasma) were incapable of achieving or enhancing the conversion. It is significant that our experiments practically exclude the involvement of the known hemostasis components: fibrinogen, fibrin, factor XIII, plasminogen, plasma prourokinase, factor XII and its related processes. This strongly suggests the involvement of a previously unknown component or of an intrinsic property of the alpha-2-antiplasmin; the latter may be relatively less likely in view of the individual variation in the conversion (Fig 5).

The significance of the conversion of PB to NPB alpha-2-antiplasmin and that of the NPB form has still to be established. It appears that the NPB form represents partial inactivation of the alpha-2-antiplasmin as it is a much less reactive inhibitor with a substantially less avid plasmin inhibition and does not participate in plasminogen binding and not significantly in binding by the fibrin stabilizing factor (factor XIII) to fibrin. One of the important mechanisms to direct fibrinolytic processes or actions of plasminogen activators to fibrin lies in the particular features of the PB form of alpha-2-antiplasmin.

Because of the low spontaneous velocity of the conversion process, the conversion can only be involved in slow processes. This might be the case in setting free fibrinolytic enzymes in aging thrombi, in cavities with a slow flow, such as the synovium or cystes, and in parts of the extracellular space; however, the conversion may be enhanced, possibly locally.

Discovery of the mechanism, or hypothetical factors or cofactors, in the conversion process and manipulation of the conversion by these or related components might present an intriguing possibility for fibrinolytic therapy and might help to establish the importance of the process, especially if it proves to be liable to local variation.

REFERENCES


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