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 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 or streptokinase therapy and after myocardial infarction. In vitro studies demonstrated that only the PB form was present after one day in the culture medium of the human cell line Hep G2, while the NPB form appeared after 11 days. Clearance after inhibition of synthesis by L-asparaginase therapy revealed a more rapid decrease in the PB form relative to the NPB form in blood, demonstrated by a change in the PB-NPB ratio from 2.86 ± 0.55 to 1.74 ± 0.24 (mean of 8, SD). An apparently spontaneous first order conversion from the PB to NPB form, with an apparent half-life of about eight days, was demonstrated at 37°C in plasma and serum in vitro. The conversion was found to be temperature dependent and uninfluenced by the fibrinolytic components fibrinogen, fibrin, and plasminogen. 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.

MATERIALS AND METHODS

Materials. Unless otherwise specified, reagents were of analytical grade and obtained from Merck, Darmstadt, FRG. “Agarose for electrophoresis” (lot No. 33006) and iodoacetamide were obtained from BDH Chemicals Ltd, Poole, England. Carbobax 6000 was from Fluka AG, Buchs, Switzerland. Antiserum raised in rabbits against human alpha-2-antiplasmin was a gift from Dr I. Clemen- sen, Copenhagen. Lys-plasminogen was prepared from human Cohn fraction III by affinity chromatography on lysine-agarose, followed by gel filtration on Sephadex G-150. Trasylol (5880 KIU [kallikrein units]/mg) was a gift from Bayer AG, Wuppertal, FRG, through the courtesy of Dr E. Philipp. The synthetic tripeptide H-D-Val-Leu-Lys-pNA (S-2251) for plasmin was from AB Kabi, Stockholm.

Ellagic acid, actin was from Dade Diagnostics Inc, Miami. Dextran sulphate, sodium salt (moll wt 500,000) was obtained from Pharmacia Ltd, Uppsala, Sweden. Active Cls-esterase was prepared from outdated plasma according to Vroon et al and Haines and Lepow. Trypsin inhibitor from soybean type I-S, from turkey egg white type II-T and diisopropylphosphofluoridate (DFP) were from Sigma Chemical Co, St Louis. Lima bean trypsin inhibitor, ovoinhibitor, and ovomucoid were from Worthington Biochemical Corp, Freehold, NJ. Whole extracts of granu-locytes were prepared as described before. Ellastatin, pepstatin, and chymostatin (see ref. 21) were gifts from Dr H. Umezawa, Microbiological Chemistry Research Foundation, Tokyo.

Plasma. Platelet-poor citrated human plasma and pooled plasma were prepared as described by Kluft et al; serum was prepared by incubation of nonanticoagulated blood at 37°C for four hours in plastic tubes before centrifugation. Factor XII- and factor XIII-deficient plasma were obtained from George King Bio-Medical Inc, Overland Park, Kansas. Plasma depleted in plasma urokinase...
was prepared by chromatography on column coupled antibodies to urokinase as described by Kluit et al. 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 Friburger et al. Modified crossed immunoelectrophoresis with added lys-plasminogen was carried out as described by Kluit and Los. 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 antisemur 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 antisemur had a comparable titer for both forms of alpha-2-antiplasmin as checked by assay of mixtures of the PB and NPB forms.

The activity assay of alpha-2-antiplasmin, the immediate plasmin inhibition test, was performed as described by Kluit et al. and measured 1.00 PB + 0.14 NPB. 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. 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.

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

Changes in the conversion rate of plasma in vitro of the two forms of alpha-2-antiplasmin were assessed by incubation of the plasmas 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 Cls-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 Kluit et al. 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 antisemur against plasminogen was introduced. In some samples, the precaution proved necessary. Three patients from a larger study 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. were studied before the start, at the end, and six to ten days after discontinuation of l-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.

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. 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 (○; n = 19), patients with a stable liver cirrhosis (△; n = 5), and heterozygotes for congenital alpha-2-antiplasmin deficiency (©; n = 15). The total amount of alpha-2-antiplasmin is determined by the Laurell technique (abscissa).
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 immunochemical 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-antiplasmin 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-
CROSSED IMMUNOELECTROPHORESIS (CIE)

DAY 1          DAY 8          DAY 11

CIE + plasminogen

DAY 1          DAY 11

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.

cess 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

Fig 5. Decay of activity in the immediate plasmin inhibition test by incubation of citrated plasma (with azide) of eight apparently healthy volunteers for the indicated periods (abscissa) at 37 °C. The results of the inhibition assay are recorded in percentage of that of pooled plasma (ordinate, log scale). The activity decay shows the conversion of PB to NPB because of the reduced expression of NPB relative to PB (14%; ref. 25) in the activity assay.

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

Fig 6. Decay of alpha-2-antiplasmin activity determined by the immediate plasmin inhibition assay (expressed in percentage of pooled plasma, ordinate, log scale) after incubation of citrated pooled plasma (with addition of azide) at various temperatures. The insert shows a typical example of the change from the PB to the NPB form during the incubation. (The exhibit gels are not run under identical conditions for the first dimension.)

Fig 7. The conversion of PB to NPB in citrated pooled plasma by incubation at 37 °C for the indicated periods (abscissa). PB and NPB (log scale) are calculated from the following data from each sample: modified crossed immunoelectrophoresis gives PB/NPB; the immediate plasmin inhibition test gives PB + 0.14 NPB. Results were in accord with direct assay of PB in one run of the modified crossed immunoelectrophoresis for t = 0, 12, and 20 days.
conversion was no different in normal serum or plasma deficient in factor XII or factor XIII, or in plasma depleted in plasminogen (by sepharose-lysine\(^{16}\)) or plasma urokinase (by immunoabsorption\(^{25}\)). 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 l0 \(^{-4}\) mol/L) or dextran sulphate (1:1 with 25 \(\mu\)g/mL), or by addition of C1s-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 \(\mu\)g/mL), Trasylol (100 KIU/mL), benzamidine (100 \(\mu\)g/mL), lima bean trypsin inhibitor (100 \(\mu\)g/mL) ovomucoid (20 \(\mu\)g/mL), ovoinhibitor (100 \(\mu\)g/mL), elastatin (1 \(\mu\)g/mL), pepstatin (1 \(\mu\)g/mL), chymostatin (1 \(\mu\)g/mL), iodoacetamide (100 mmol/L), \(\beta\)-mercaptoethanol (0.1 mmol/L), HgCl\(_2\) (1 mmol/L), and ZnCl\(_2\) (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 2:1. 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 L-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 vivo. 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 L-asparaginase is evidence for its formation there as well. If we assume the in vitro conversion of PB to NPB to be representative for the in vivo process, we can calculate that k\(_3\) 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\(^{12}\) 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 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 k\(_1\), k\(_2\), or k\(_3\).
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 ($Q_m = 2$ to $4$). The recent report of Sasaki et al$^{33}$ 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 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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