

EDITORIAL REVIEW**Fast-acting Plasmin Inhibitor in Human Plasma**

By D. Collen and B. Wiman

The fast-acting and physiologically most important inhibitor of plasmin in human plasma is a recently discovered and purified α_2 -glycoprotein with a molecular weight of 65,000–70,000 daltons occurring at a concentration of 1 μ M. The inhibitor rapidly forms a completely inactive 1:1 stoichiometric complex with plasmin through reaction with the B chain (light chain) of the enzyme, which contains the active center. It also reacts with trypsin and very slowly with uro-

kinase and with some other enzymes in purified systems, but its role in vivo as an inhibitor of proteases other than plasmin seems negligible. Antiplasmin is the only plasma protein that can inhibit the fibrinolysis associated with transformed or malignant cells. The plasmin-antiplasmin complex contains neoantigenic structures not present in the parent molecules that may form the basis of immunochemical methods for detecting activation of the fibrinolytic system in blood.

RECENTLY, a new and physiologically important inhibitor of plasmin was identified in human plasma by three different groups.^{1,4} This inhibitor has been called antiplasmin,^{1,2,5} α_2 plasmin inhibitor,³ or primary plasmin inhibitor.⁴

Upon activation of plasminogen in plasma, the formed plasmin is first preferentially bound to antiplasmin. Only upon complete activation of plasminogen (concentration in plasma 1.5 μ M), resulting in saturation of the antiplasmin (concentration in plasma 1 μ M), is the excess plasmin neutralized by α_2 -macroglobulin.² In the presence of normal concentrations of these two inhibitors, the other plasma protease inhibitors do not play a role in the inactivation of plasmin.^{2,6}

During the last year, a number of studies have been performed on the characterization and function of this inhibitor. These studies will be briefly reviewed here.

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Identification

Plasma and serum exert a very important inhibitory effect on plasmin. There are at least five well-defined plasma proteins that inhibit plasmin in a purified system, namely, α_2 -macroglobulin, α_1 -antitrypsin, inter- α -trypsin inhibitor, antithrombin III-heparin complex, and C_1 -esterase inhibitor. However, the role of these inhibitors in the inactivation of plasmin formed in its natural environment, plasma, is not well understood. It has long been accepted that there are essentially two functionally important plasmin inhibitors in plasma, an immediate reacting one and a slow-reacting one,⁷ identical with α_2 -macroglobulin and α_1 -antitrypsin, respectively.^{8,9}

Müllertz^{6,10} studied the activation products of human plasminogen in post-mortem and urokinase-activated human plasma and found a compound that reacted with antiplasminogen antisera but not with antisera against the known protease inhibitors. One of the explanations he suggested was that this compound might represent plasmin in complex with a strong (unknown) inhibitor. Müllertz and Clemmensen⁴ later purified this compound and provided unequivocal evidence for the existence of a plasmin inhibitor in plasma, different from the known protease inhibitors, that they called the primary plasmin inhibitor.

Aoki and von Kaulla¹¹ identified a fibrinolytic inhibitor in human serum that they thought acted mainly at the level of plasminogen activation. Later work by Moroi and Aoki³ indicated, however, that this inhibitor acted primarily at the level of formed plasmin, and it was therefore called α_2 -plasmin inhibitor.

During our studies on the turnover of labeled plasminogen during fibrinolytic therapy, we observed the formation in vivo of radioactive complexes that were initially assumed to represent plasmin- α_2 -macroglobulin and plasmin- α_1 -antitrypsin complexes.¹² Further work, however, indicated that the presumed plasmin- α_1 -antitrypsin complex did not react with antisera against α_1 -antitrypsin. By isolating this complex and producing antibodies against its inhibitor part, we obtained positive evidence for the existence of a new fast-acting plasmin inhibitor in human plasma, which we called antiplasmin.^{1,2}

With the use of this antiserum it has since been shown that antiplasmin and the proteins purified by Moroi and Aoki³ and by Müllertz and Clemmensen⁴ are identical.

Gallimore¹³ partially purified a fibrinolytic inhibitor from human serum that he called inter- α -antiplasmin. Recent data¹⁴ indicate that this preparation is a mixture of antiplasmin and the inhibitor of plasminogen activation described by Hedner,¹⁵ which in turn is different from antiplasmin.¹⁶

Purification

To date four methods have been described for the purification of antiplasmin from plasma.

Moroi and Aoki used a combination of ammonium sulfate precipitation, DEAE-Sephadex chromatography, affinity chromatography on insolubilized plasminogen, and hydroxylapatite chromatography of plasminogen-depleted plasma and purified the inhibitor to homogeneity.³ Wiman and Collen developed a modified purification method consisting of chromatography on in-

solubilized plasminogen followed by chromatography on DEAE-Sephadex and insolubilized concanavalin A, which resulted in a higher yield and a more stable inhibitor preparation.⁵ Müllertz and Clemmensen⁴ partially purified the inhibitor by ammonium sulfate precipitation, DEAE-Sephadex chromatography, chromatography on insolubilized concanavalin A, gel filtration, and further DEAE-Sephadex chromatography. Collen et al.¹⁷ isolated the inhibitor (mostly in an inactive form, however) by immunoabsorption chromatography and gel filtration.

Quantitative Determination

The inhibitor can be accurately determined in plasma either with enzymatic assays based on the very fast inhibition of plasmin or with immunochemical assays using specific antisera. In the enzymatic assays, residual plasmin can be measured with natural or with synthetic substrates.¹⁸⁻²⁰ The normal level of the inhibitor in plasma is around 70 mg/liter plasma, or $1 \mu\text{M}$.^{3,5}

Physicochemical Properties

Antiplasmin is a single-chain glycoprotein with a molecular weight of 65,000–70,000 daltons as estimated by sodium dodecyl sulfate (SDS)-gel electrophoresis and ultracentrifugation.^{3,5} On electrophoresis it migrates as an α_2 -globulin. The amino acid composition reported by Moroi and Aoki³ and that reported by Wiman and Collen⁵ correspond well for most amino acids, and the estimated carbohydrate content of the molecule was determined as 11.7% and 13.7%, respectively. The inhibitor is immunochemically different from α_1 -antitrypsin, α_2 -macroglobulin, C₁-esterase inhibitor, antithrombin III, α_1 -antichymotrypsin, and inter- α -trypsin inhibitor^{3,5} and from the inhibitor of plasminogen activation described by Hedner and co-workers.^{5,16} The inhibitor is stable in solution above pH 6.3 but is rapidly inactivated below pH 6.0.⁵ The NH₂-terminal amino acid sequence is Asn-Gln-Glu-Gly,⁵ and the sedimentation constant is 3.4.^{3,5}

Interaction With Enzymes

In purified systems^{3,5} and in plasma,^{2,6} antiplasmin forms a 1:1 stoichiometric complex with plasmin that is devoid of protease or esterase activity and that cannot be dissociated by denaturing agents such as urea, guanidine HCl, or dodecyl sulfate. SDS-gel patterns indicate that complex formation occurs by strong interaction between the light chain of plasmin and the inhibitor. Moroi and Aoki recently claimed that the complex between the inhibitor and the light (B) chain of plasmin is dissociated by hydrazine, indicating that there is an ester bond between the active-site seryl residue of plasmin and antiplasmin.²¹ A polypeptide with a molecular weight of 11,000–14,000 daltons may be released after formation of the complex.²⁻⁴ This finding has led to the assumption that inhibition occurs by hydrolysis of a specific peptide bond in the inhibitor followed by esterification between the newly formed COOH-terminal residue of the inhibitor and the active-site serine residue of plasmin.²⁻⁴ It was shown that the release of this polypeptide is not an essential step in complex formation but the result of hydrolysis of the complex by free plasmin.⁵

Antiplasmin was also shown to react with trypsin and slowly with urokinase in purified systems.³ When trypsin, chymotrypsin, thrombin, and plasmin were added to normal plasma, antiplasmin played a role in the binding of plasmin and, to a lesser extent, trypsin, but not of thrombin or chymotrypsin.²²

Variation and Biologic Function

The normal plasma level of antiplasmin determined either enzymatically or immunologically is between 80% and 120% (Mean \pm 2 SD), 100% being the value obtained for a pool of normal plasma.^{19,20} The concentration may drop to less than 30% in severe cases of liver disease or intravascular coagulation^{19,20} but is normal in patients with cardiovascular, renal, or malignant disease. The inhibitor is temporarily exhausted during thrombolytic therapy with streptokinase.²³

The principal biologic role of antiplasmin seems to be the very fast and irreversible neutralization of plasmin in the circulation. However, antiplasmin also interferes with the binding of plasminogen to fibrin and thus may have an additional antifibrinolytic effect.²⁴

Inhibition by Antiplasmin of Fibrinolysis Associated With Oncogenic Transformation and Neoplasia

There is a close association between neoplasia and increased fibrinolytic activity that is independent of the nature of the transforming stimulus. Neoplastic cells secrete specific proteases that activate plasminogen to plasmin.²⁵ Although the rate and extent of cell growth in culture do not require fibrinolytic activation, this seems to be a necessary element of cell migration.²⁶ Inhibition of fibrinolysis may thus constitute an important natural defense mechanism against invasiveness of tumor cells and might therefore be useful in confining metastasizing cancer.

As yet, no natural protease inhibitor has been found that effectively blocks any of the plasminogen activators.²⁷ The plasmin-mediated fibrinolysis, however, can be neutralized by soybean trypsin inhibitor and bovine pancreatic trypsin inhibitor but not by the plasma protease inhibitors α_2 -macroglobulin, α_1 -antitrypsin, inter- α -trypsin inhibitor, C₁-esterase inhibitor, α_1 -antichymotrypsin, or antithrombin III.²⁸

We studied the role of antiplasmin in the inhibition of fibrinolysis associated with malignant cells.²⁹ When mixed cultures of mouse fibroblasts and mouse fibroblasts transformed with Kirsten murine sarcoma virus were grown in petri dishes and overlaid with casein, the appearance of focal lysis zones due to plasminogen activator released by the malignant cells was inhibited by addition of human plasma. Likewise, lysis of a fibrin clot by the culture fluid of a human melanoma line was inhibited by the addition of human plasma. Plasma from which the antiplasmin was specifically removed by immunoabsorption completely lost its inhibitory activity in both the caseinolytic and fibrinolytic assays. Thus antiplasmin is the only protein in human plasma capable of inhibiting the fibrinolytic activity associated with oncogenic transformation or neoplasia. Whether this effect is due exclusively to inhibition of formed plasmin or also to interference with plasminogen activation remains unclear. To settle this problem will require investigation of the influence of antiplasmin on the

molecular events occurring during plasminogen activation by malignant proteases in purified systems.

Plasmin-Antiplasmin Complex: Its Determination and Value as an Indicator of Activation In Vivo of the Fibrinolytic System

Activation of the fibrinolytic system results in formation of plasmin, which has a short lifespan in the blood, since it is very rapidly bound to and neutralized by antiplasmin. If plasmin-antiplasmin complex formation were associated with the emergence of structural or conformational alterations that render this complex antigenically distinct from the precursor molecules, immunochemical quantitation of such neoantigens could constitute a means of measuring activation of the fibrinolytic system in vivo.

The plasmin-antiplasmin complex was isolated from plasma in which the fibrinolytic system was activated by affinity chromatography and gel filtration.² This purified material was used for the production of antisera in rabbits by conventional immunization procedures. The occurrence of precipitating antibodies to structures present in the complex but not in the precursor molecules was demonstrated by immunodiffusion.³⁰ With the use of a passive hemagglutination inhibition immunoassay using tanned red cells coated with purified plasmin-antiplasmin complex it was shown that it was possible to measure the plasmin-antiplasmin complex directly in human plasma on the basis of immunochemical quantitation of its neoantigens.³¹

For the study of the occurrence in vivo and clinical relevance of this complex, we developed a simple latex agglutination test for its rapid quantitation in plasma.³² In this method, polystyrene (latex) particles are coated with purified gamma globulins from the absorbed sera.

The purified complex was found to cause a clear agglutination of the particles at a concentration of 0.1-0.2 mg/liter; purified plasminogen and antiplasmin were more than 100 times less reactive. Activation of fresh human plasma with urokinase caused progressive generation of agglutinating activity up to a plasma dilution of 1:480. Intravenous injection of streptokinase into patients resulted in an increase of the plasmin-antiplasmin titer to at least 1/240. The titer remained high during the first 3 hr after the injection and was still elevated after 24 hr, indicating that the half-life of this complex in plasma is several hours.

Of 101 male and 23 female control subjects, only 3 males had a plasmin-antiplasmin titer above 1/16. Of 230 hospitalized patients, 25 had plasmin-antiplasmin titers of 1/40 or more. Most of these patients had diseases frequently associated with coagulation or fibrinolysis in vivo, but only one of them showed diffuse intravascular coagulation detectable by routine methods. In those patients without increased plasmin-antiplasmin titers, none of the hemostasis analyses was indicative of coagulation or fibrinolysis in vivo. Of eight patients with diffuse intravascular coagulation due to various causes, seven had plasmin-antiplasmin titers of 1/80 or 1/160.

From these findings it was concluded that activation of the fibrinolytic system in vivo, either directly or secondarily to coagulation in vivo, is associated with the appearance of circulating plasmin-antiplasmin complexes that can be directly assayed in plasma on the basis of their neoantigenic expression.

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