A new approach to thrombolytic therapy

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Introduction

The blood fibrinolytic system which plays a role in the removal of fibrin from the vascular tree consists of three main components: the pro-enzyme plasminogen, which can be activated by limited proteolysis to plasmin; plasminogen activators which may be of different origin (blood activator, endothelial activator, tissue activator or intrinsic plasminogen activator) and α₂-antiplasmin which rapidly neutralizes plasmin. The biochemical and biological properties of plasminogen and of α₂-antiplasmin, the main inhibitor of plasmin have been studied in detail (for references see ref. 1). Much less is however known about the “extrinsic” plasminogen activators which are released into the blood, presumably from the vascular wall.

During the past few years specific molecular interactions between plasminogen activator and fibrin, between plasminogen and fibrin and between plasmin and α₂-antiplasmin have been described, on the basis of which a molecular model for the regulation of fibrinolysis in vivo was proposed1). In addition it has recently become possible to purify "extrinsic" plasminogen activator in sufficient amounts to study its biological properties in more detail.

Regulation and control of fibrinolysis

The regulation and control of fibrinolysis appears to occur at several levels: release of plasminogen activator from the vascular wall, fibrin-associated activation of plasminogen and inhibition of formed plasmin by α₂-antiplasmin.

Release of plasminogen activator. The mechanisms controlling the release of plasminogen activator from the endothelial cells are virtually unknown. Cash has speculated that plasminogen activator release may be under neurohumoral control3). Higher neurogenic centers or specific peripheral afferent organs might stimulate the release of a plasminogen activator releasing hormone (PARH) with a structure similar to vasopression from the neurohypophysial region. This PARH would constitute the major pathway for the release of plasminogen activator from the endothelial cells, whereas the catecholamine pathway would only be involved in severely stressful situations3). An alternative pathway for the release of

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plasminogen activator in vivo involving activated protein C has recently been proposed\(^4\).

**Molecular mechanism of physiological fibrinolysis.** Physiological fibrinolysis appears to be regulated by specific molecular interactions between plasminogen activator and fibrin, between plasminogen and fibrin and between plasmin and \(\alpha_2\)-antiplasmin.

As described in more detail below extrinsic plasminogen activator has a weak affinity for plasminogen in the absence of fibrin (\(K_M=65\,\mu M\)) but a much higher affinity in the presence of fibrin (\(K_M\) between 0.15 and 1.5 \(\mu M\)). This increased affinity appears to be the result of a "surface assembly" of plasminogen activator and plasminogen on the fibrin surface. In this reaction plasminogen binds to fibrin primarily via specific structures called the "lysine-binding site". Thus one way of regulating fibrinolysis is at the level of plasminogen activation localized at the fibrin surface.

Plasmin is extremely rapidly inactivated by \(\alpha_2\)-antiplasmin (\(k_1=10^7\,M^{-1}s^{-1}\))\(^5\); the half-life of free plasmin in the blood is therefore estimated to be approximately 0.1s. Plasmin with an occupied lysine-binding site is however inactivated 50 times slower by \(\alpha_2\)-antiplasmin. Reversible blocking of the active site of plasmin with substrate also markedly reduces the rate of inactivation by \(\alpha_2\)-antiplasmin. From these findings one can extrapolate that plasmin molecules generated on the fibrin surface, which are bound to fibrin through their lysine-binding sites and involved in fibrin degradation, are protected from rapid inactivation by \(\alpha_2\)-antiplasmin. Plasmin released from the fibrin surface would however be rapidly inactivated by \(\alpha_2\)-antiplasmin.

**Studies on human extrinsic plasminogen activator**

The molecular model for the regulation of fibrinolysis described above has important consequences for the development of thrombolytic agents. Indeed, the presently available thrombolytic agents streptokinase and urokinase have no specific affinity for fibrin and therefore activate circulating and fibrin-bound plasminogen relatively indiscriminately. Consequently plasmin formed in circulating blood will initially be neutralized very rapidly by \(\alpha_2\)-antiplasmin and be lost for thrombolysis. Once the inhibitor becomes exhausted residual plasmin will degrade several plasma proteins (fibrinogen, factor V, factor VIII, etc.) and cause a serious bleeding tendency. This may explain why treatment with streptokinase or urokinase has only a limited efficacy and is associated with serious, sometimes life-threatening side effects.

From this reasoning it appears that specific thrombolysis will only be possible if the activation process of plasminogen can be localized at and confined to the fibrin surface. According to the present concepts this can only adequately be
achieved with the use of an activator which, like the physiological activator, adsorbs to the fibrin surface and becomes active in loco. Such plasminogen activators have been isolated from tissues (human uterus, pig ovaries, pig heart, etc.). The yield of these procedures is however extremely low (to obtain 1 mg pure activator 5 kg of human uterus is required), so that it is practically impossible to foresee any therapeutic application of such substances.

We have found that a stable cell line derived from a human melanoma, produced a large amount of a plasminogen activator which appeared to be very similar or identical to the physiological plasminogen activator. We have developed a relatively simple purification procedure with high yield, which has enabled us for the first time to produce significant quantities of tissue plasminogen activator. Thus on a laboratory bench scale we have produced approximately 200 mg purified plasminogen activator in 1981. This has allowed us to initiate a whole series of studies, not only on the biochemical and biological properties of this activator, but also on its therapeutic possibilities.

The plasminogen activator, isolated from the culture fluid of human melanoma cells, appeared to be a single chain protein with a molecular weight of approximately 70,000, which is indistinguishable from the plasminogen activator isolated from human uterus on the basis of molecular weight, amino acid composition, kinetic properties towards synthetic substrates and immunochemical properties.

A kinetic analysis of the activation of plasminogen by tissue plasminogen activator obeyed Michaelis-Menten kinetics with $K_M=65 \mu M$ and $k_{cat}=0.06 \text{s}^{-1}$ in the absence of fibrin and with $K_M=0.16 \mu M$ and $k_{cat}=0.1 \text{s}^{-1}$ in the presence of fibrin. These findings indicate that fibrin enhances the activation rate of plasminogen by tissue plasminogen activator by increasing the affinity of plasminogen for fibrin-bound tissue plasminogen activator and not by influencing the catalytic efficiency of the enzyme. These data support and provide a molecular explanation for the hypothesis that fibrinolysis is both triggered by and directed towards fibrin.

As a preliminary to the study of the thrombolytic properties of tissue plasminogen activator in vivo we have investigated the mechanism of inhibition of this activator in human plasma and its turnover following intravenous injection in rabbits. Our studies on the inhibition of tissue plasminogen activator in human plasma revealed that it is slowly inhibited by $\alpha_2$-antiplasmin ($t_1/2 \approx 60 \text{ min}$) and by $\alpha_2$-macroglobulin ($t_1/2 \approx 120 \text{ min}$). We found no evidence for the existence of another significant inhibitor in plasma.

Following intravenous injection of mixtures of $^{125}$I-labeled and unlabeled tissue plasminogen activator in rabbits the activator activity disappeared with a $t_1/2$ of 2 min. This appeared not to be due to inhibition but to cleaning by the
In in vitro systems composed of mixtures of purified components as well as in total plasma, this activator appeared to have a much higher specific fibrinolytic activity than urokinase. In particular it appeared to be possible to dissolve blood clots hanging in circulating plasma specifically without causing breakdown of the coagulation system in the plasma; this appeared not to be possible with urokinase.

The thrombolytic effect of tissue plasminogen activator was investigated in rabbits with an experimental pulmonary embolus. From this study it appeared that on a molar basis tissue plasminogen activator has a higher thrombolytic effect than urokinase and that (partial) thrombolysis can be obtained without systemic fibrinolytic activation and breakdown of the hemostasis system. The thrombolytic effect of tissue plasminogen activator was also investigated in dogs with an experimental thrombosis of the femoral vein. Urokinase infusion at a rate of 2,500 IU per kg per hour for four hours did not induce significant lysis (17.4 ± 3.7 percent) as compared to saline infusion (16.3 ± 3.8 percent). Significant lysis was obtained with 25,000 IU of urokinase per kg per hour for four hours (40.6 ± 4.8 percent) but this was associated with defibrinogenation. Infusion of 2,500 urokinase equivalent units of plasminogen activator per kg per hour for four hours caused significant lysis (33.5 ± 7.8 percent with single chain activator and 60.1 ± 10.8 percent with two chain activator) without causing any fibrinogen breakdown.

In summary it appears that recent studies have led to a better insight in the regulation and control of the fibrinolytic system in vivo and to the purification and characterization of a plasminogen activator from human cell cultures, which is most probably identical with the physiological plasminogen activator in the blood. This plasminogen activator has the biochemical and biological properties of a specific thrombolytic agent and appears to be effective in experimental thrombosis in animals. Therefore it seems possible and even probable that eventually extrinsic (tissue type) plasminogen activator might constitute a more selective thrombolytic agent than the presently employed urokinase and streptokinase.

Acknowledgement

The recent studies on plasminogen activator from our laboratory which are reported in this review were carried out in collaboration with Drs. D. C. Rijken (Leuven, Belgium), O. Matsuo (Miyazaki, Japan), M. Hoylaerts (Leuven, Belgium), H. R. Lijnen (Leuven, Belgium), C. Korninger (Vienna, Austria) and with the technical assistance of A. van Nuffelen, J.-M. Stassen, M. Verstreken, E. Demarsin and G. Lemmens.
References

血栓溶解療法への新しい前進

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血液線溶系の反応とその制御
血管内血栓の溶解に働く血液線溶活性発現にはプラスミノゲンとそのアクリベーター、およびα2-アンチプラスミンの3つの因子が関与するが、この2、3年間にこの方面的検討が著しく進められたなかで、血管壁から放出されるプラスミノゲン・アクリベーターについて、新しくその放出機序と制御機構に関心が集中した。その詳細は未だ明らかでないが、高倉神経中枢と相関解されホルモンの関与を示唆する所見が得られる傾向にある。このアクリベーターのプラスミノゲンに対する作用にはLBS（lysine-binding site）と呼ばれる特異構造を介したフィブリンとの結合が大きな役割を演じ、フィブリン固定斑表面上での本物質とプラスミノゲンの濃縮がおこり、ここでフィブリンの分解が惹起される。プラスミノゲンはα2-アンチプラスミンによって瞬時に失活するが、LBSで固定されているプラスミノゲンはこの阻害物質による失活作用を受けにくい。

ヒト外因性プラスミノゲン・アクリベーター
SKやUKはフィブリンに対して親和性が弱く、血小板粘着中あるいはフィブリンに結合したプラスミノゲンは無差別に活性化し、その結果、血中で生成されたプラスミノゲンはまずα2-APにより瞬時に失活させて血栓溶解には役立たない。もしα2-アンチプラスミンが消費されて、プラスミノゲン凝固因子を働くとフィブリン、第V、第Ⅴ因子などが分解され、出血傾向が惹起される。かくしてフィブリン上でプラスミンの活性化が起こった時にのみ、特異的な血栓溶解が可能となる。このような特異的血栓溶解は生理的な組織アクリベーターを使用することで著明となるが、著者はその組織アクリベーターの大量取得に成功し、その生化学的、生物学的特性および血栓溶解薬としての価値を検討した。

この組織プラスミノゲン・アクリベーターはヒトの子宮、胎児の卵巣、心臓等から得られるが、その収量は例えばヒトの子宮5kgから純粋なアクリベーター1mgが得られる程度である。ここで同様なアクリベーターがヒトの内臓腫からも得られることがわたった。このアクリベーターは分子量7万、一本鎮で、これによるプラスミノゲンの活性化反応はMichaelis-Mentenの法則に従う。そしてこれは生成されたフィブリンがプラスミノゲンの自己への親和性を増加させることにより、上記のようにフィブリンに親和力の強いアクリベーターとの反応を増強することとなる。

この組織アクリベーターはヒト血清中でα2-アンチプラスミンとα2-マクログロブリンにより、緩急両者の阻害作用を受けるが、この組織アクリベーターに対する特異的なヒトヒトはまた血中に存在しない、また家兎にヒトのアクリベーターを投与すると、その活性は2分で半減した。

In vitroで精製反応系および血漿を用いて組織アクリベーターのフィブリン分解能を測定すると、UKよりも特異的に高いフィブリン分解能を示した。特に循環している血漿中に血栓を浮遊した場合は血漿の凝固系に影響を与えること

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となく血栓を溶解し、家兎で成功した実験的
肺栓塞の成績では、この組織アグリマーは
UKよりも血栓溶解能が強力であるのみなら
ず、全身線溶を活性化することがなく、また止
血機構も乱されません。同様の成績はイヌの大
腿静脈血栓症でも確認された。

●葉酸研究の完全なる集大成！
葉 酸 －基礎と臨床

編集 京都大学内科教授 内野治人 岐阜歯科大学内科教授 外林秀紀
専門医18氏分担執筆
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●周産(生)期感染症の臨床指針書！
妊娠・胎児・新生児の感染症

編集 日本周産(生)期医学研究会 専門医91氏分担執筆
B5判 289頁 図102 表175 定価9,800円（税240円）

本書は周産(生)期医学の立場に立って問題の多い妊娠、胎児、新生児の感染症を再検討
し、ウイルス、マイコプラズマ、原虫、細菌を含めて、新しく見出された疾患、従来より
知られた感染症を現代の進歩したウィルス学、細菌学および免疫学より洗い直し、産婦人
科医、小児科医の実地診療上必要な感染症の臨床指針書である。

●内容：【妊娠の感染症】1.妊娠感染症。2.化学療法。3.免疫感染症。4.妊娠糖尿病。【胎児の感
染症】1.胎児感染症の化学療法。2.先天性トキプラズマ症。3.サイトロニガロウィルス感染症。4.HBs
遅延と未熟児感染症。8.感染の母児関係。9.新生児肝炎と産科的異常。10.妊娠予防接種の胎児への影響。
【新生児の感染症】1.新生児感染症。2.新生児と抗生物質。3.新生児感染症の診断と治療。4.先天性免疫
不全症と新生児敗血症。5.子後不良であった新生児細菌感染症。6.未熟児期における感染症の集団発生。
7.新生児期感染症。8.細菌培養と感受性検査。【周産期感染症と免疫】1.妊娠の感染と免疫。2.胎児感染
と免疫。3.新生児感染症と免疫。4.初乳の免疫に関する役割。

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