Minireview

Understanding the enzymology of fibrinolysis and improving thrombolytic therapy

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Abstract Cardiovascular disease is responsible for 17 million deaths per year but acute myocardial infarction and stroke can be treated with thrombolytics ("clot busters"), which are plasminogen activators. However, despite many years of study and huge investment from the pharmaceutical industry, clinical trials of new drugs have often been disappointing. Part of the problem may be our incomplete understanding of the regulation of plasminogen activation in vivo. We have developed precise in vitro methods and with the application of computer simulations, we hope to improve our understanding of plasminogen activation to facilitate improvements in thrombolytic therapy.

Keywords: Cardiovascular disease; Fibrinolysis; Thrombolytic; Plasminogen activator

1. Introduction

WHO figures highlight cardiovascular disease (CVD) as the single biggest cause of mortality worldwide, estimated at 17 million deaths per annum [1]. Estimates of disability adjusted life years for men and women, which include the burden of the debilitating effects of myocardial infarction and stroke, are also headed by CVD. CVD affects high income nations (for example there are 270 000 heart attacks in Britain every year) and increasingly CVD is a problem for low and middle income nations. Developing countries accounted for 60% of the total burden of coronary heart disease in 2002, and this figure is rising rapidly. Thrombolytic therapy is a cost effective way of improving survival from myocardial infarction in developed [2] and developing countries [3]. Fibrinolysis is the biochemical process underlying thrombolysis since both processes depend on the degradation of the fibrin network that holds the blood clot together in a coronary or cerebral artery in the case of myocardial infarction (heart attack) or ischaemic stroke, respectively. In a simplified form, fibrinolysis can be represented as a two-step process according to Scheme 1.

The two stages are: (I) the activation of plasminogen to plasmin by a plasminogen activator (PA) and (II) is the degradation of the structural protein, fibrin, to fibrin degradation products (FDP). The participants in stage (I) are assembled on the surface of fibrin fibres and the propagation of plasmin action in the circulation is limited by the potent plasma inhibitor system. Thrombolytic therapy, which was introduced in the 1980s, is essentially the delivery of PA into the circulation. From the point of view of developing and improving thrombolytic therapy, the characteristics of the PA need to be optimized to promote rapid clot dissolution. The only way of doing this is to maximize the generation of plasmin (while minimizing the side effects) and this is why the characteristics of PAs are of overriding importance for successful therapy. However, despite many years and huge sums of money spent on improving thrombolytic therapy though clever design of recombinant proteins (to date mostly serine proteases and some plasminogen binding proteins), improvements in survival rates have been hard fought for and so small that they are only observable in huge, and hugely expensive, clinical trials. Is it possible that some of this lack of progress is due to our incomplete understanding of the basic enzymology and regulation of the process of fibrinolysis? Approaches to exploring this question will be discussed below.

2. A brief history of thrombolytic proteins

Historically, the first generation of thrombolytics (e.g., streptokinase and urokinase) were systemic activators of plasminogen giving rise to large scale plasmin production in the circulation and concomitant loss of circulating plasminogen, fibrinogen and the plasma inhibitors. Streptokinase (and staphylokinase, see below) belong to a group of plasminogen binding proteins secreted by invasive bacteria in order to generate plasmin and facilitate "bacterial metastasis" [4]. Urokinase is a naturally occurring protease that can be isolated on industrial scales from human urine, although it is present in circulation as an inactive, single chain pro-enzyme. Second generation thrombolytics with fibrin binding domains were produced by genetic engineering with the seemingly rational aim of targeting the PA to a fibrin clot. Thus, the development of tissue plasminogen activator (tPA), for example, was driven by the theory that since tPA has to bind to fibrin to express significant activity, administered tPA would be targeted at the clot and systemic plasmin generation would be limited. The idea was that this would be a good thing as systemic loss of plasminogen substrate would be reduced; and possibly bleeding complications (for example intracranial hemorrhage

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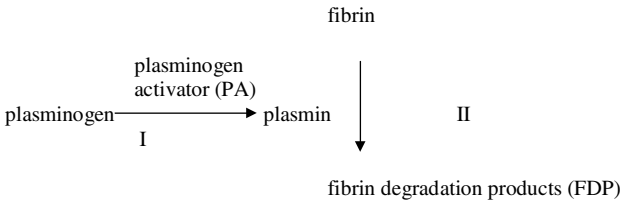

Scheme 1.

(ICH), which occurs in up to 3% of patients receiving treatment) might be reduced.

Following on from tPA, the so-called third generation of thrombolytics were engineered. The goal was improved properties in terms of longer plasma half life, or resistance to natural inhibitors, or improved fibrin binding and "zymogenicity", making them even more fibrin specific (e.g., reviewed [5,6]). Many of these variants used the basic tPA template but domains were deleted or modified with the stated aim of improving properties. The domain arrangement of a selection of these molecules is shown in Fig. 1, including tPA (pharmaceutical name: alteplase). In spite of the huge and rising global burden of CVD and concomitant massive financial investment by the pharmaceutical industry, protein engineering of second and third generation thrombolytics may be interpreted as a failure. Basic scientific considerations, in vitro data and animal studies have not been translated into significant improvements in outcomes in clinical trials [7,8]. This lack of clear improvement and the much increased cost of second and third generation thrombolytics means that streptokinase is still the most widely used thrombolytic for the treatment of acute myocardial infarction in many parts of the world, including the UK for example, as it has been since the earliest uses of thrombolytic therapy.

To date no further significant progress has been seen with other tPA-based thrombolytics such as reteplase, monteplase, lanoteplase or pamiteplase for example, or chimeras such as amediplase (the fibrin binding kringle 2 of tPA fused with the serine protease domain of uPA which has better catalytic parameters than tPA), or other molecules such as saruplase (single chain urokinase) or staphylokinase [6]. Despite all the detailed investigations of protein biochemistry on these molecules (reviewed [9]) the only advantage of the newer thrombolytics is extended plasma half life enabling the delivery of the drugs as a bolus injection rather than the longer infusion of first and second generation treatments. This may be a significant improvement in reducing the lag time from symptoms to treatment since reteplase [10] may be given by paramedics before arrival at hospital in 2 bolus injections and tenectaplase as a single bolus injection [11,12]. Hence, so far the only improvements in thrombolytic therapy relate to the pharmacokinetics of the drugs, not other properties such as fibrin binding or specificity, or inhibitor susceptibility. Our current understanding of PA activity in fibrin has been inadequate to permit the successful rational design of new molecules or optimized treatment regimes.

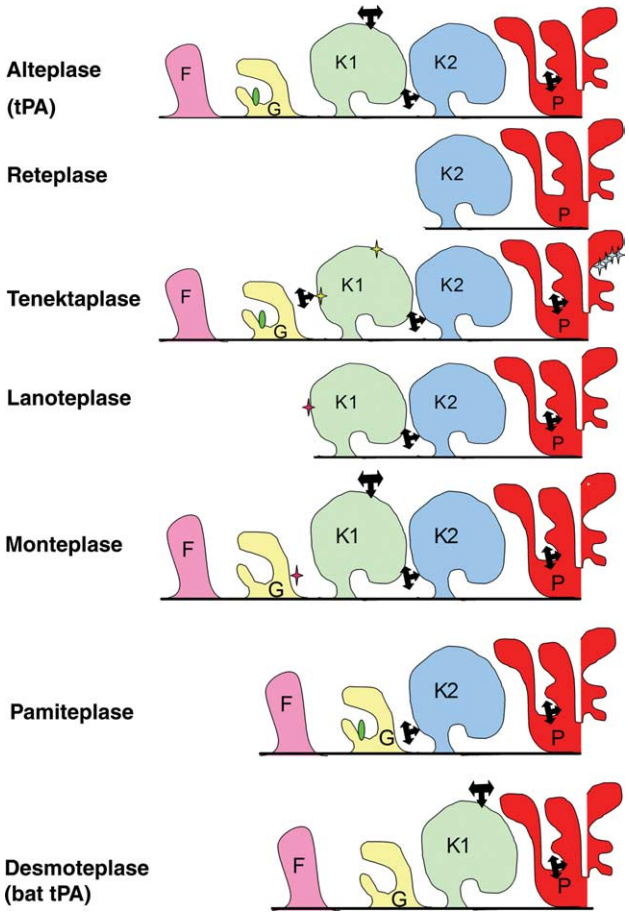

Fig. 1. The domain arrangement of tPA and related variants developed as thrombolytics. Native tPA (alteplase) consists of 527 amino acids in the domains: finger (F), growth factor (G), kringle 1 (K1), kringle 2 (K2), and serine protease (P). The gene was cloned from melanoma cells and is expressed in CHO cells. Fibrin binding is mostly regulated initially via F and subsequently via K2 which binds to newly generated C-terminal lysine residues in degrading fibrin. Glycosylation sites are present at N117 (K1), N184 (K2) and N448 (P), and there is a fucosylation site at T61. Reteplase is a deletion variant of tPA consisting of K2P and is produced in *Escherichia coli* so is not glycosylated. Tenektaplase is modified in 3 sites with the addition of a glycosylation site by substitution at T103N and removal of the glycosylation site at N117Q. These substitutions improve plasma half life while maintaining fibrin binding properties. The third mutation is KHRRR(296-299)AAAA designed to reduce the reactivity with the specific serpin inhibitor PAI-1. Lanoteplase is another deletion variant of tPA lacking FG domains and also the glycosylation site at N117Q to improve plasma half life. Monteplase may be viewed as a protein folding variant of tPA caused by the change of C84S. This substitution leads to an unexpected rearrangement of disulfide bonds, loss of the fucosylation site at T61 and altered carbohydrate at N117. Pamiteplase has K1 deleted, again to improve plasma half life, and a site specific change at R275E designed to maintain the molecule in the single chain form which may improve fibrin binding. Desmoteplase is the DSPAα_1 variant cloned from the vampire bat and lacks the fibrin binding K2 domain (though fibrin binding is still mediated via the F domain).

3. The enzymology of fibrinolysis

Scheme 1, above, is clearly a simplistic overview of the reactions underlying fibrinolysis and includes no suggestion of regulatory mechanisms. A slightly more detailed impression of the steps involved in plasmin generation is shown in Fig. 2. In this case, we have split plasmin generation into 3 parallel processes and may consider this scheme for plasmin generation with reference to tPA as the activator enzyme (PA). In (1), plasminogen activation can take place in solution and does not require