PRODUCTION AND APPLICATIONS OF M... AND BISPECIFIC MONOCLONAL ANTIBO... FOR FIBRINOLYSIS STUDIES

ROGIER BOS

PRODUCTION AND APPLICATIONS OF MONO- AND BISPECIFIC MONOCLONAL ANTIBODIES FOR FIBRINOLYSIS STUDIES

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STELLINGEN

behorende bij het proefschrift
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1. In de studies naar de versnellende werking (van carboxyterminale lysineresiduen) van fragmenten van fibrinogeen op de plasminogeen aktivatie door één-ketenig urokinase-type plasminogeen aktivator (scu-PA), is het onjuist (de carboxyterminale lysineresiduen die zouden ontstaan na plasmine digestie van) het fibrinogeen fragment FCB2 te benaderen zoals (de carboxyterminale lysineresiduen zoals voorkomend in) het fibrinogeen fragment D.
J. Liu and V. Gurewich, J. Clin. Invest. 1991; 88: 2012-2017

2. Het idee dat de zogenaamde 'fibrine cuffs' rond open wonden het herstel zouden vertragen door een verminderde oxygenatie van het omliggende weefsel, gaat voorbij aan de fysische eigenschappen van fibrine en de diffusiesnelheid van zuurstofmoleculen daarin.
N.L. Browse and K.G. Burnand. Lancet 1982; ii: 243-245

3. De conclusie door Koopman et al., dat bij patiënten met een bepaalde mutatie in het fibrinogeen, albumine-fibrinogeen complexen in de circulatie voorkomen, wordt door hun experimenten niet voldoende onderbouwd.
J. Koopman. Proefschrift: 57-70. Rijksuniversiteit Leiden 1992

4. Gezien het feit dat het nu al enige jaren bekend is dat zogenaamde hoge-Tc supergeleiders zeer anisotroop zijn, en gezien de veelal onduidelijke gevolgen hiervan op de fysische eigenschappen, is het verrassend dat niet meer experimenten worden uitgevoerd met kunstmatig gelaagde systemen van conventionele supergeleiders waar de anisotropie een variëerbare parameter is.

5. Het gebruik van een fibrinemonolaag, gevormd door behandeling van geïmmobiliseerde fibrinogeen moleculen met trombine, als model voor de bestudering van de fibrinolytische aktiviteit, houdt onvoldoende rekening met de essentiële rol van een correcte fibrine polymerisatie voor een optimale plasminogeen aktivatie door weefsel-type plasminogeen aktivator.
D. Rouy and E. Anglés-Cano, Biochem. J. 1990; 271: 51-57

6. Justitie maakt steeds vaker gebruik van forensische methodieken voor haar waarheidsvinding; hierdoor neemt voor haar de betrouwbaarheid van het bewijs weliswaar toe, maar door de complexiteit van deze technieken neemt de inzichtelijkheid en dus de acceptatie door justitiabelen en de zittende magistratuur af.

7. Bij de bepaling van de uiteindelijke effectiviteit van cholesterol-verlagende medicijnen, wordt te weinig rekening gehouden met het effect van deze medicijnen op andere risicofactoren, zoals de plasma-fibrinogeen concentratie.
F. Vallés et al. Atherosclerosis 1991; 91: 3-9
Y. Biegel and J. Fuchs. European Society of Atherosclerosis, Lissabon, mei 1991: 135 (kopie op aanvraag verkrijgbaar)

8. Aan de lijst van "-topen", zoals epitoop, cryptotoop, mimotoop, neotoop, en paratoop, die alle de bindingsplaats van antistof en antigeen beschrijven, dient ook een '-toop' toegevoegd te worden die het biologische of chemische effect van een antistof op het antigeen na binding beschrijft (ergotoop).
M.W. Fanger et al. Immunol Today 1989; 10: 92-97
A. Tramontano et al., Science 1986; 234: 1566-1573.

9. Het verdient aanbeveling te komen tot afspraken voor internationaal eenduidige afkortingen voor 'monoclonal antibody' en 'bispecific monoclonal antibody'.

10. Natuurbeheer is per definitie niet natuurlijk.

11. De verklaring voor het ogenschijnlijk kortzichtige vangstbeleid van de (inter)nationale visserij-industrie, ligt in het al te letterlijk nemen van de spreuk 'après nous le déluge'.

12. De houding tegenover allochtonen in Nederland mag niet leiden tot een klassificatie van de bevolking in Nederlanders, Medelanders en Minderlanders.

13. Gezien de verdergaande automatisering, kan het geen kwaad zich vaker te beraden of het gebruik van zogenaamde tijdbesparende computerprogramma's juist niet meer tijd zullen gaan kosten.

14. Eigenwijs is ook wijs.

Rogier Bos, Leiden, 3 juni 1992.

PRODUCTION AND APPLICATIONS OF MONO- AND BISPECIFIC MONOCLONAL ANTIBODIES FOR FIBRINOLYSIS STUDIES

PROEFSCHRIFT

ter verkrijging van de graad van Doctor aan de Rijksuniversiteit Leiden, op gezag van de Rector Magnificus Dr. L. Leertouwer, hoogleraar in de faculteit der Godgeleerdheid, volgens besluit van het college van dekanen te verdedigen op woensdag 3 juni 1992 te klokke 15.15 uur

door

ROGIER BOS

geboren te Vlaardingen in 1960

Promotiecommisie:

Promotor:	Prof.dr. P. Brakman
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Overige leden:	Prof.dr. P.J. Hoedemaeker Prof.dr. E.K.J. Pauwels Dr. E.J.E.G Bast (Rijksuniversiteit Utrecht)

The study in this thesis was performed at the Gaubius Institute-TNO, Leiden, The Netherlands (head Prof.dr. P. Brakman), now part of IVVO-TNO, Gaubius Laboratory, Leiden, The Netherlands (head Prof.dr. D. Knook).

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ik bestudeer de zinloosheid der dingen
en maak al aardig vorderingen

Jan J. Pieterse

Voor Maria
Aan Liesbeth

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CHAPTER 1

SHORT INTRODUCTION

Monoclonal antibodies

The publication by Köhler and Millstein on the production of monoclonal antibodies (MoAb) with a predefined specificity, has had a strong impact on medical and biological sciences [1]. A MoAb can be described as a reagent with a relatively high specificity and affinity for sometimes well-defined structures. Furthermore, MoAb can be produced in almost infinite quantities of constant quality. It was soon understood that these proteins, with such a high selectivity for specific structures, could be employed as powerful tools for numerous applications, such as in structure/function analysis, purification by immunoaffinity chromatography, sensitive diagnostic assays, and the development of new therapeutic bioreagents [2,3,4].

MoAb are produced by B-lymphocytes, usually derived from the spleen of an immunized mouse and immortalized e.g. by fusion with non-secretory murine myeloma cells. The structure of the antibody molecule, or immunoglobulin (Ig), varies with the different (sub)classes. The IgG-type antibody, a good model for the basic structure of an immunoglobulin, consists of four chains, two light-chains and two heavy-chains, held together by hydrophobic interactions and disulphide bonds [5]. Each chain consists of a a variable and one or more constant domains. An IgG contains two antigen binding-sites. Each of the two antigen-binding sites is formed by a combination of two Ig chains, i.e. (the variable part of) an Ig light-chain with (the variable part of) an Ig heavy-chain. The constant domains in the heavy- and light-chains determine the subclass of the molecule, and are responsible for other biological activities important to the immune system, such as complement fixation or monocyte binding. Other Ig-isotypes, e.g. IgA and IgM, consist essentially of two and five of such basic structures, respectively.

Since the MoAb are produced by a cell line, called hybridoma, originally derived from one single cell (*monoclonal*), all MoAb are identical in structure and, more importantly, in specificity and affinity. These characteristics explain the superiority in certain applications of MoAb over the more traditional (polyclonal) antisera. However, production of a MoAb is not always simple, especially since the demands on the properties of the MoAb to be produced are increasingly augmented, e.g. recognition of neo-antigenic sites or inhibition of enzymatic activity or receptor/ligand interaction. Therefore, new methods for MoAb production are still under investigation (e.g. *in vitro* immunisation; synthetic peptides as immunogen; and antigen-directed electrofusion).

Fibrinolysis

The fibrinolytic system is mainly responsible for the degradation of fibrin, an important constituent of blood clots, and involves the intricate and concerted actions of several (pro-)enzymes, activators, inhibitors and potentiators [6]. The end product of the fibrinolytic system is the proteolytic enzyme plasmin. Plasmin is formed from its inactive precursor plasminogen by the action of specific enzymes, so-called plasminogen activators such as tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA). Plasmin, in turn, degrades the insoluble fibrin into soluble fibrin degradation products. The fibrinolytic system is an essential part of the haemostatic balance regulating vascular patency. However, a role (of several components) in various other processes, such as angiogenesis; tumour growth and metastasis; rheumatoid arthritis; and tissue remodelling, has also been postulated [7,8,9,10].

Certain components of the fibrinolytic system, i.e. the plasminogen activators, are employed as therapeutic agents [11]. When the normal balance between coagulation and fibrinolysis is disturbed, pathological fibrin deposits may form and plasminogen activators are administered to activate endogenous plasminogen to plasmin (thrombolytic therapy). Plasmin then degrades the fibrin matrix of the thrombus, and this leads to reperfusion of the occluded blood vessel. Preferably, only clot-associated plasminogen is activated, since circulating plasmin may readily degrade other, essential plasma proteins. Since most plasminogen activators are rapidly cleared from the circulation and/or are inhibited by fast acting inhibitors, which occur naturally in plasma, large doses of t-PA or u-PA are necessary for effective thrombolytic therapy. Combined with the limited fibrin-specificity of exogenously administered t-PA and especially u-PA, this increases the chance for systemic plasminogen activation. A more comprehensive overview of the fibrinolytic system with special reference to thrombolytic therapy is given in chapter 2.

Monoclonal antibodies and fibrinolysis

Study of the processes involved in the fibrinolytic system is complicated by the intricacy of the mechanisms involved, and the sometimes limited understanding of the functions of identified (and yet to be identified) plasma proteins. Monoclonal antibodies are powerful tools for the study of these questions. They are used for the purification of components of the fibrinolytic system, in order to study their interactions in a purified system [12,13]; or for the development of diagnostic assays. These tests may be used to determine the concentration of components of the fibrinolytic system for an improved diagnosis of thrombotic disorders; to assess risk-factors; or to monitor patients during thrombolytic therapy [14,15]. Furthermore, MoAb can be applied for the immunoscintigraphic imaging of thrombi *in vivo* [16]. Additionally, the effect of monoclonal antibodies on specific functions and/or characteristics of some components can be studied *in vitro* or *in vivo*, in order to unravel their physiological function

(structure/function analysis) [8,17,18]. Finally, monoclonal antibodies can be used to target plasminogen activators to their site of action, i.e. the thrombus (antibody-targeted thrombolytic therapy) [19,20].

AIMS OF THE STUDY

As mentioned above, the success of t-PA and u-PA as thrombolytic agents is limited due to some of their negative properties *in vivo*. It was our ultimate goal to increase the efficacy of t-PA and u-PA in thrombolytic therapy. One approach to achieve this, is by linking t-PA or u-PA to a MoAb specific for fibrin, the major constituent of thrombi (antibody-targeted thrombolytic therapy) [19,20]. In principle, however, such a conjugate would be expected to diminish only one negative property of t-PA or u-PA, i.e. their limited or non-existent fibrin affinity.

We aimed at not only increasing the fibrin-affinity but at *simultaneously* diminishing some other negative property of t-PA or u-PA, e.g. the fast hepatic clearance and/or the inhibition by inhibitors such as PAI-1. Therefore, we chose to target the plasminogen activators to fibrin by means of so called bispecific monoclonal antibodies (bs-MoAb). Ideally, such a bs-MoAb would have one binding-site for fibrin, whereas the other binding-site would bind to t-PA or u-PA, such that the plasminogen activator would be cleared more slowly from circulation, or would no longer be inhibited by PAI-1. Hybridoma cell lines producing fibrin-specific MoAb (Y22) [16] or u-PA-specific MoAb [21], were available for our studies in producing such bs-MoAb. Hybridoma cell lines producing t-PA-specific MoAb with the desired biological effect were not available and were produced and characterized as part of this study.

In this thesis the production, purification and characterization *in vitro* of mono- and bispecific monoclonal antibodies is described, as part of the necessary steps towards our goal.

Chapters 2 to 8 have already been published, are in press, or are submitted for publication.

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CHAPTER 2

THE POTENTIAL IMPROVEMENT OF THROMBOLYTIC THERAPY BY TARGETING WITH BISPECIFIC MONOCLONAL ANTIBODIES; WHY THEY ARE USED AND HOW THEY ARE MADE

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ABSTRACT

The generation of the proteolytic enzyme plasmin from its inactive precursor plasminogen, mediated by so called plasminogen activators, is the essential step in thrombolytic therapy. Plasmin is responsible for the degradation of the insoluble fibrin, the major component of a thrombus, to soluble fibrin degradation products. Sofar, the use of the more recently developed thrombolytic agents single-chain urokinase-type plasminogen activator (scu-PA) and tissue-type plasminogen activator (t-PA) were disappointing, mainly due to some of their negative properties *in vivo*, i.e. rapid inhibition and/or hepatic clearance. Besides some background information on the haemostatic balance; t-PA and scu-PA structure; and mechanisms of action, we here review some reported attempts to improve on these agents for thrombolytic therapy following various strategies. One of the more potential strategies, antibody-targeted thrombolytic therapy using bispecific monoclonal antibodies, is discussed somewhat more extensively, as are the several procedures that can be followed for bispecific antibody preparation.

INTRODUCTION

The haemostatic balance

Under normal conditions, a balance exists between the two processes of coagulation and fibrinolysis, often referred to as the haemostatic balance. The coagulation system is a fast-acting and potent defence mechanism which protects the body against excessive blood loss after vascular damage. One of the late products of the coagulation system is thrombin, that is formed via a cascade of enzymes from prothrombin. Thrombin then initiates the polymerization of fibrin to form, together with activated platelets, a haemostatic plug. The precursor of fibrin, and substrate for thrombin, is the soluble plasma protein fibrinogen. Fibrinogen is a large glycoprotein with a molecular weight of 340.000 dalton, consisting of 2 Aα-, 2 Bβ- and 2 γ-chains linked together by several disulphide bonds. Thrombin cleaves the fibrinopeptides A and B from fibrinogen, and this results in the formation of so-called fibrin monomers. Up to a certain concentration these fibrin molecules are kept in solution by complexing with fibrinogen. These complexes are often referred to as soluble fibrin. Beyond a critical concentration the fibrin monomers will align in a half-staggered overlap and polymerize to form fibres. Eventually the fibres will aggregate side-to-side to form a three-dimensional gel (Fig. 1). The structure of the gel is further stabilised by activated Factor XIII, that chemically cross-links the γ-chains and, at a somewhat slower rate, the α-chains of adjacent fibrin molecules [reviewed in 1].

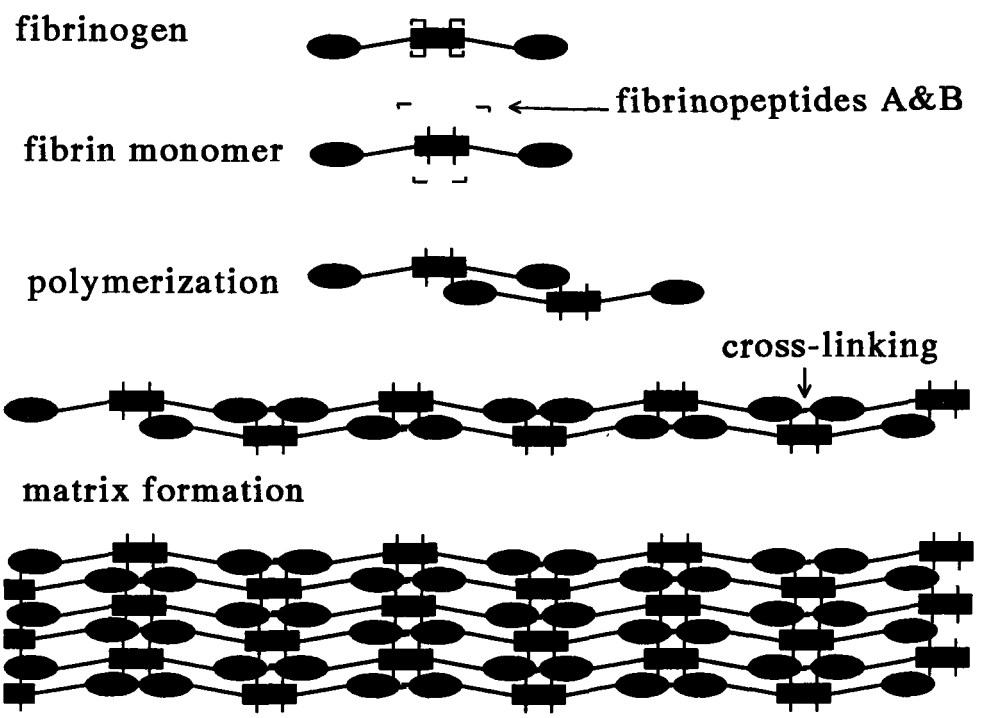

Figure 1. Schematic representation of the formation of a fibrin gel, as originally proposed in [1]. Fibrinogen is here depicted as a trinodular molecule. Thrombin cleaves the fibrinopeptides A and B from fibrinogen to form fibrin monomers. This is followed by the polymerization of the fibrin monomers into fibres. Subsequently, the fibres will aggregate to form a three-dimensional gel, wich is stabilized by factor XIIIa, a transglutaminase, which forms isopeptide bonds between adjacent fibrin molecules.

On the other side of the balance, the fibrinolytic system is responsible for the subsequent breakdown of the fibrin matrix and dissolution of the clot. The end product of the fibrinolytic system is the serine protease plasmin. Plasmin does not occur as an active enzyme in the circulation, but as an inactive precursor, a zymogen called plasminogen. The conversion of plasminogen into plasmin, by hydrolysis of the Arg560-Val561 peptide bond, is mediated by specific enzymes, so-called plasminogen activators. Activated plasmin in turn can degrade the insoluble fibrin matrix into soluble fibrin degradation products [reviewed in 2]. The fibrinolytic system is regulated at different levels by several inhibitors and potentiators (Fig. 2).

Essentially, there is a delicate equilibrium between the products of the two continously ongoing processes of coagulation (fibrin formation) and fibrinolysis (subsequent fibrin degradation), i.e. thrombin and plasmin. However, when the balance is disturbed, this results either in an increased tendency for bleeding or, vice versa, a pathological fibrin deposition; a clot or thrombus. Our interest lies primarily in the

removal of these thrombi. To remove such a thrombus, the fibrinolytic system is often enlisted to degrade the fibrin matrix of the thrombus. This is referred to as thrombolytic therapy.

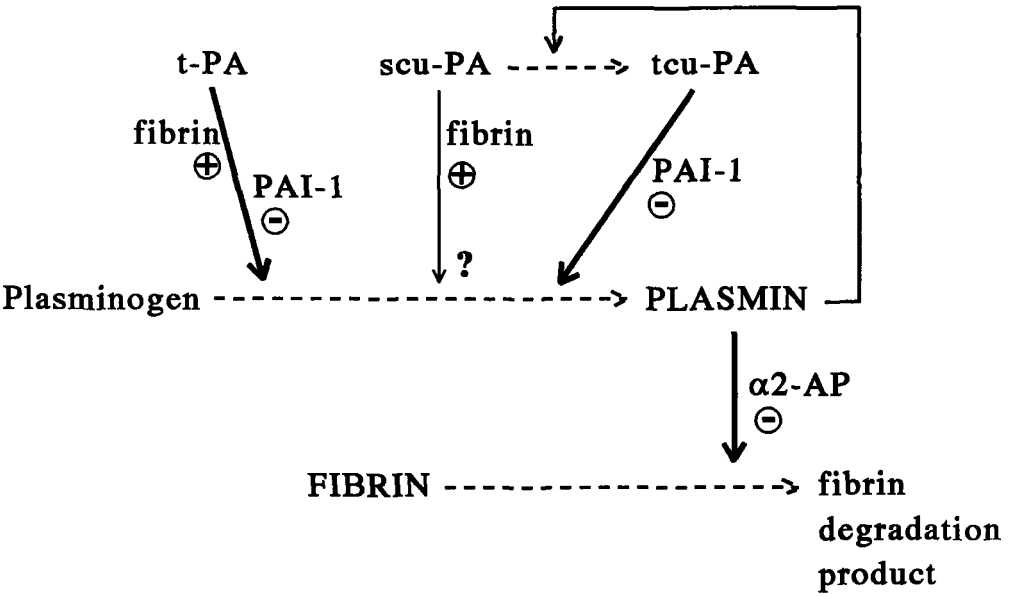

Figure 2. The interactions between some main components of the fibrinolytic system. Plasminogen is converted to plasmin by the action of plasminogen activators. Plasmin, in turn, degrades the insoluble fibrin to soluble fibrin degradation products. Enzymatic activity is shown as a solid arrow, substrate conversion as a dashed arrow. Inhibitors (-) or potentiators (+) of the reactions are placed near the appropriate arrows. The ability of scu-PA to activate plasminogen at physiological conditions is disputed, hence the question mark.

The essential step in thrombolytic therapy is the activation of plasminogen into plasmin by exogenously-administered plasminogen activators. Preferably, this step is restricted to the site of the thrombus, since plasmin has a broad substrate-specificity and will readily degrade other proteins. Systemic plasminogen activation, resulting in the formation of free plasmin, causes depletion of the major plasmin-activity-down-regulator alfa2-antiplasmin (α2-AP), and the subsequent degradation by plasmin of several crucial plasma proteins such as fibrinogen and factors V and VIII, increasing the risk for bleeding complications. When plasmin is formed at the site of the thrombus, the relative abundance of fibrin as a substrate for plasmin prevents this. Furthermore, fibrin-associated plasmin is relatively resistant to inhibition by α2-AP and can thus efficiently degrade the fibrin in a thrombus [3].

Agents for thrombolytic therapy

At present, there are five thrombolytic agents either in use or under clinical investigation. These are streptokinase, anisoylated plasminogen/streptokinase complex (APSAC), tissue-type plasminogen activator (t-PA), two-chain urokinase (tcu-PA or UK) and its precursor single-chain urokinase (scu-PA or pro-UK). Studies have shown that the success of these agents in thrombolysis may be limited; no or only partial reperfusion, reocclusion and significant bleeding events have been reported [4,5,6].

One common factor contributing to the low success rate of all these agents in thrombolytic therapy, is the lack of susceptibility of clots to exogenously-administered plasminogen activators. This is partly explained by the natural role of the fibrinolytic system within the haemostatic balance. It was proposed that t-PA has a prophylactic function and should be present at the time of clot formation. It thus preconditions the clot for eventual lysis. This is confirmed by the findings that a small increase in t-PA levels, when present during clot formation, strongly decreases the clot lysis-time [7]. When administered afterwards, t-PA, as well as streptokinase and tcu-PA, are far less effective for inducing clot lysis. This prophylactic/preconditioning theory is supported by the observed effect of thrombin on vascular endothelial cells; thrombin is a very potent inducer of t-PA release [8]. In the case of a thrombus, this preconditioning evidently failed, and we now enlist plasminogen activators to perform a task somewhat distinct from their original purpose.

Streptokinase, APSAC and tcu-PA are agents that lack any thrombus specificity and will activate both fibrin-bound and free-circulating plasminogen equally well. The use of these agents, therefore, results in the systemic activation of plasminogen. The two physiological plasminogen activators, t-PA and scu-PA, activate plasminogen preferentially at a fibrin surface (see below). Despite the relative fibrin specificity of t-PA and scu-PA, their use as thrombolytic agents has also been disappointing. Both activators are removed very fast from the circulation by an efficient and rapid hepatic clearance. Furthermore, t-PA activity is inhibited by the fast-acting plasminogen activator inhibitor type-1 (PAI-1). However, at the present dosages used, PAI-1 inhibition is of minor importance since the excess t-PA over PAI-1 results in a rapid elimination of most PAI-1 activity. Scu-PA, as a zymogen, is resistant to PAI-1 inhibition.

Because of the high clearance and/or neutralisation rates of t-PA and scu-PA, administration of relatively high dosages of the agents, over long infusion periods, is required for an effective reperfusion of the occluded blood vessel. Combined with the limited thrombus specificity of exogenously-administered t-PA and especially scu-PA, this may lead to systemic plasminogen activation. Reported strategies on the further improvement of these activators are aimed at increasing their thrombus specificity and/or at decreasing their clearance and/or inhibition rates. In this summary several of these strategies will be described in more detail.

Tissue-type plasminogen activator

Tissue-type plasminogen activator (t-PA) is a serine protease with an apparent molecular weight of 65.000 dalton [9]. It is secreted by endothelial cells initially as a single-chain molecule, that can be converted by plasmin to a two-chain molecule by hydrolysis of the Arg275-Ile276 peptide bond [10]. The two chains are held together by a single disulphide bond between Cys264 and Cys395. The protein can be glycosylated at Asn117 (high-mannose oligosaccharides), Asn184 and Asn448 (complex oligosaccharides). Glycosylation patterns may vary, especially between recombinant t-PA expressed in different cell types [11,12]. The molecule can be divided into several structural domains based on homologies with other proteins [reviewed in 13 and 14]. These domains include, from the amino-terminal, the finger (F) domain, the epidermal growth factor (EGF) domain, two kringle (K1 and K2) domains and the serine protease (P) domain (Fig. 3). The A- or heavy-chain contains the first four domains and a connecting peptide, while the B- or light-chain comprises the P-domain. Studies on the gene structure of t-PA showed that each domain is encoded by one or two adjacent exons [15]. It was proposed that these domains behave functionally as autonomous modules [16,17].

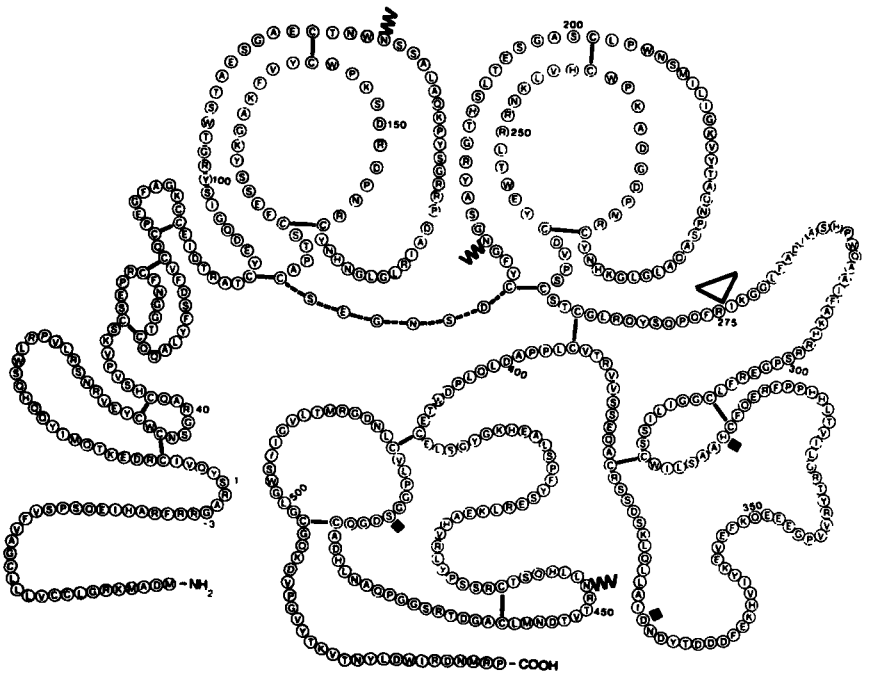

Figure 3. Two-dimensional model of the t-PA molecule, including from the amino-terminal the signal peptide, prosequence, F-, EGF-, K1-, K2-, connecting peptide and P-domain; adapted from [14]. The solid bars indicate potential disulphide bonds, the zig-zag lines indicate possible N-glycosylation sites, the active-site amino acid residues are indicated by solid squares, and the arrow indicates the plasmin cleavage site for generation of two-chain t-PA.

One characteristic of t-PA that contributes to its relative fibrin specificity is its ability to bind to fibrin. There is still some debate on the sites involved. However, the F- and K2-domain are now generally accepted to be responsible for the affinity of t-PA for fibrin [16,17,18,19]. There is evidence for a lysine binding site in the K2-domain binding to carboxyl-terminal lysine residues which are exposed by limited plasmin digestion of fibrin and competed for by 6-aminohexanoic acid [20]. There are also indications of an aminohexyl-site in the K2-domain, similar to that found in plasminogen, which binds to intrachain lysine residues [21,22]. It can be concluded that the F-domain and K2-domain in t-PA are involved in the binding of t-PA to intact fibrin, and limited digestion of fibrin by plasmin increases t-PA binding [23].

In the absence of fibrin, t-PA is capable of activating plasminogen, though slowly and inefficiently (low k_{cat} and high K_m). However, the presence of (soluble fragments of) fibrin induces a strong decrease in the K_m (about 20-fold) and an increase in the k_{cat} (about 30-fold) of the plasminogen activation by t-PA, probably by the formation of a cyclic ternary complex between fibrin, t-PA and plasminogen [24,25,26]. This results in a considerably increased catalytic efficiency (k_{cat}/K_m) of t-PA. It can be concluded that t-PA is a poor plasminogen activator in the absence of fibrin, but is potentiated in the presence of fibrin or soluble fibrin fragments [reviewed in 27]. Fibrin thus acts as a cofactor of its own breakdown. The fibrin-induced acceleration of the t-PA-mediated activation of plasminogen, at least partially explains the fibrin specificity of t-PA. Contrary to other serine proteases, both the single and the two-chain form of t-PA are enzymatically active, though single-chain t-PA is somewhat less active towards low molecular weight substrates. In the presence of fibrin there is no difference in the activity towards plasminogen between the two forms [28]. It has been proposed that a site in the K2-domain of t-PA is necessary for the rate-enhancing effect of fibrin on t-PA mediated plasminogen activation [22].

The rapid clearance of t-PA by the liver is mediated partly by liver endothelial cells and partly by liver parenchymal cells [29]. The half-life of 'wildtype' t-PA in the circulation is in the order of a few minutes. The receptor for the liver endothelial cell mediated uptake of t-PA is apparently a mannose-receptor [30]. The receptor on the liver parenchymal cell is still to be identified. There is no direct evidence for the role of the carbohydrates in the clearance of t-PA, especially via the high-mannose carbohydrate at Asn117, though deglycosylated mutants of t-PA have been reported to have a prolonged half-life [31]. Extensive research using mutants of t-PA has pointed to the F-domain, and probably the EGF-domain, as potential sites for hepatocyte uptake [32,33].

Single-chain urokinase

The scu-PA protein is synthesized and secreted by endothelial cells, and has a molecular weight of 54.000 [10]. There is a single carbohydrate present at Asn302. It can be

converted, by plasmin or kallikrein, to the two-chain form (tcu-PA) by hydrolysis of the Lys158-Ile159 peptide bond. Scu-PA is a real zymogen in the sense that it has hardly any enzymatic activity towards low molecular weight substrates or plasminogen, and it is resistant to inhibitors which are very active towards tcu-PA [34]. The conversion of scu-PA into tcu-PA has drastic effects on the enzymatic activity of the protein, i.e. tcu-PA is a very potent plasminogen activator and is sensitive to inhibitors. The two chains in tcu-PA are held together by a single disulphide bond between Cys148 and Cys279 [reviewed in 35].

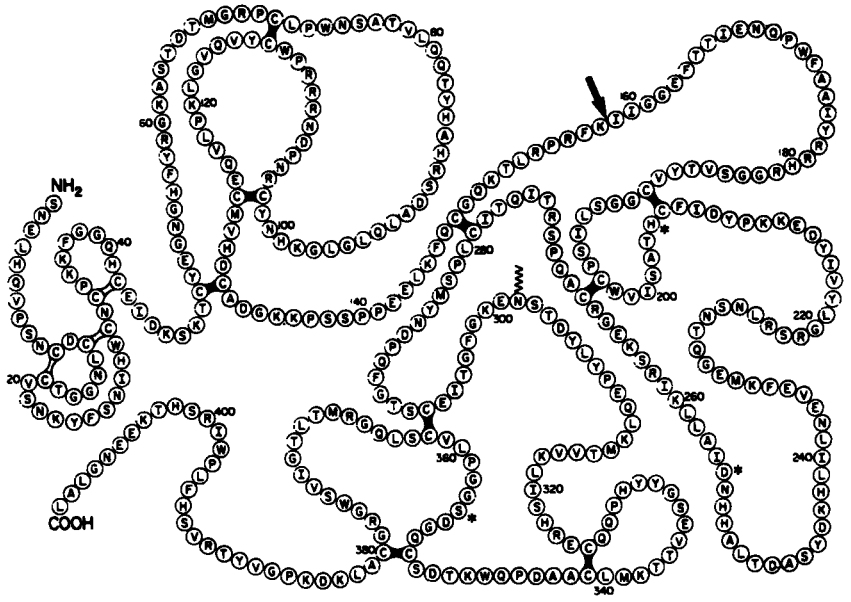

Figure 4. Two-dimensional model of the scu-PA molecule, including from the amino-terminal the F-, K-, connecting peptide and P-domain; adapted from [35]. The solid bars indicate potential disulphide bonds, the zig-zag line indicates a possible N-glycosylation site, the active-site amino acid residues are marked with an asterix, and the arrow indicates the plasmin cleavage site for generation of tcu-PA.

As with t-PA, the structure of sc(tc)u-PA can be divided in several structural domains for their homology to other proteins. These include, from the amino-terminal, an EGF-domain, a K-domain and a connecting peptide (that constitute the A- or light-chain), and a P-domain (B- or heavy-chain) (Fig. 4). Thrombin can also convert scu-PA to a two-chain variant of the protein by hydrolysis of the Arg156-Phe157 peptide bond [36]. This molecule is inactive, but can be activated by plasmin, though not as efficiently as intact scu-PA [37]. Apparently, exposure of the amino-terminal Ile159 of the B-chain is essential for a proper conformation of the active site.

Scu-PA shows some remarkable enzymatic properties. As a zymogen, scu-PA has little enzymatic activity; and it has no affinity for fibrin. Yet it shows some fibrin specific activation of plasminogen [34,38,39]. Several hypothesis were suggested to explain this phenomenon. The most intriguing one is that scu-PA has no activity towards free plasminogen. However, fibrin-associated (conformationally-changed) plasminogen becomes a good substrate for scu-PA [40,41]. Another hypothesis is that the fibrinolytic activity of scu-PA is mediated entirely by the conversion of scu-PA to tcu-PA by plasmin, present in low concentrations at the fibrin surface [34,39]. There is conflicting evidence regarding the necessity of the latter step for effective fibrinolysis, though this positive plasmin-mediated feedback will evidently improve the fibrinolytic capacity of scu-PA [42,43]. The major advantage of scu-PA as a thrombolytic agent is its relative inactivity towards circulating plasminogen and its resistance to PAI-1 inhibition.

Little is known about the mechanisms involved in the fast hepatic clearance of sc(tc)u-PA from the circulation. Liver parenchymal cells seem to play an important role.

IMPROVEMENTS ON THE POTENCY OF t-PA AND sc(tc)u-PA

Mutants of t-PA

Based upon the information derived from structure-function analysis of the domains of t-PA, and the idea that these domains behave as functionally-autonomous modules, attempts have been made to improve the efficacy of t-PA as a thrombolytic agent by modifications of the t-PA molecule. Mutants of t-PA were produced with domain-deletions, -substitutions and -insertions, or by site-directed mutagenesis. A large variety of t-PA mutants was thus prepared and characterized for their fibrinolytic or thrombolytic potential. Most attempts were aimed at the modification of one specific property of the molecule, either by increasing its fibrin affinity [44,45,46,47,48], or by decreasing its hepatic clearance [32,33,49,50,51,52,53,54]. Though it was proposed that the domains behaved as functionally-independent moieties, it soon became evident that redesigning one part of the t-PA molecule had drastic effects on other functional properties of the molecule. Many mutants showed a decreased enzymatic activity or fibrin affinity, or an impaired fibrin-stimulation, although the mutations were not always made in the domains reported to be involved in these functions (see above).

The most promising results using this strategy, were obtained with mutants of t-PA that lacked the F- and EGF-domain of t-PA. These mutants had a markedly increased half-life *in vivo*. Thus smaller doses are required for effective reperfusion of the occluded bloodvessel, as compared with wildtype t-PA, even though enzymatic activity and especially fibrin affinity were impeded [32,33]. The modified agent, therefore, can be given as a single intravenous bolus injection. Similar, but more pronounced results were

obtained with t-PA modified by site-directed mutagenisis in the carboxyl-terminal part of the F-domain. This t-PA variant had a fibrinolytic activity similar to that of wildtype t-PA, but a markedly decreased plasma clearance. *In vivo* this resulted in a significantly improved thrombolytic efficacy [55]. Mutation of only a few amino acids apparently is less likely to interfere with the intricate structure of other domains than more extensive mutations [17,56].

Targeting via other molecules

Another strategy to improve the efficacy of certain drugs is by complexing them to specific carrier proteins, i.e. molecules with a selective affinity for the site of action. Attempts have been reported on the targeting of t-PA or sc(tc)u-PA to the thrombus, i.e. to fibrin or to activated platelets. An elegant approach was to chemically cross-link tcu-PA to fibrinogen, a molecule with an assumed affinity for a (growing) thrombus. This procedure somewhat increased clot specific fibrinolytic efficacy of tcu-PA *in vitro* [57].

Antibodies are proteins that, by nature, have a very high affinity for specific structures. There have been reports of attempts to target t-PA or sc(tc)u-PA to a thrombus via these molecules. Several conjugates have been reported between monoclonal antibodies (MoAb) specific for fibrin or platelets and t-PA or sc(tc)u-PA. These conjugates were prepared by chemical conjugation of the two proteins. This procedure results in a heterogeneous mixture of molecules. Furthermore, chemical conjugation invariably leads to loss of antibody and/or enzymatic activity, and batch to batch variations are hard to prevent. Nevertheless, such conjugates have been shown to greatly enhance the fibrin (platelet) specificity and thrombolytic potency of t-PA and sc(tc)u-PA [58,59,60].

Conjugates between anti-fibrin antibodies and plasminogen activators were also prepared using more sophisticated molecular biological techniques. In this approach chimaeric proteins were constructed by linking the gene coding for the first two domains and the hinge region of an antibody heavy-chain molecule to the gene that codes for (parts of) t-PA or scu-PA, including the proteolytic domain. Expression of this recombinant gene in a cell that also expresses the appropriate antibody light chain, leads to the production of a chimaeric protein with the affinity of a MoAb and the enzymatic activity of a plasminogen activator [61,62]. It is crucial for this approach that the presence of the antibody moiety does not interfere with the correct folding of the plasminogen activator moiety, and vice versa. Furthermore, the correct steric assembly (mutual orientation) on the fibrin surface of the active site of the activator moiety and its substrate plasminogen is likely to be affected.

A more elegant approach is to use bispecific monoclonal antibodies (bs-MoAb) as carrier proteins. Bispecific antibodies combine the antigen binding sites (Fab'-fragments) of two different MoAb; one directed against the target, i.e. fibrin, the other directed

against the thrombolytic agent, i.e. t-PA or sc(tc)u-PA (Fig. 5). Thus, binding of the agent to its carrier is predefined and uniform. This strategy can circumvent problems such as loss of activity due to chemical conjugation while positive characteristics of the plasminogen activator, e.g. the rate-enhancing effect of fibrin on t-PA-mediated plasminogen activation, can remain intact. Furthermore, because of the flexible nature of the antibody molecule, and the likely preservation of the wildtype configuration of the plasminogen activator, chances for an impeded plasminogen activation at the fibrin surface are probably reduced.

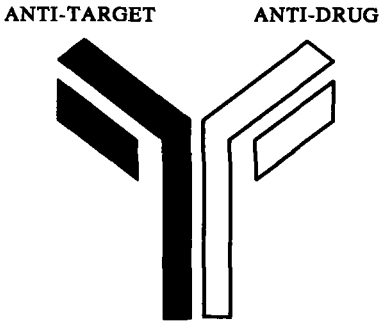

Figure 5. Schematic representation of a bispecific antibody reactive to both target (i.e. fibrin) and drug (i.e. t-PA or sc(tc)u-PA), as used for antibody-targeted thrombolytic therapy.

Results currently achieved in antibody-targeted thrombolytic therapy using bispecific monoclonal antibodies, show that the fibrin-specificity and thrombolytic potency of both t-PA and sc(tc)u-PA are strongly improved [63;64,65,66,68,69]. The authors have shown that bs-MoAb induce an increased affinity of t-PA or sc(tc)u-PA for fibrin, resulting in a notably lower dose of plasminogen activator in the presence of bs-MoAb to achieve similar fibrinolytic activity *in vitro* or thrombolytic activity *in vivo*, as compared with in the absence of bs-MoAb. Dependent on the assay used, enhancement-factors varied between three-fold to infinite. Apparently, the bs-MoAb accumulate t-PA or sc(tc)u-PA rapidly onto the fibrin, with a concomitant increase in fibrin-localized plasminogen activation. Hence, fibrinolysis will be increased and the chance of systemic plasminogen activation will be decreased. Furthermore, the results show that t-PA, immobilised to fibrin as a complex with a bs-MoAb, is capable to associate correctly with fibrin and fibrin-bound plasminogen, i.e. form a cyclic ternary complex (see above). This is an essential aspect of t-PA-mediated plasminogen activation, since t-PA is less active towards plasminogen when not (correctly) bound to fibrin.

Moreover, by a rational choice of MoAb for bs-MoAb production, some adverse properties of the plasminogen activator, such as binding to PAI-1 or the rapid clearance by liver cells, can also be modulated [67,68]. A t-PA or u-PA specific MoAb can be selected which binds to or near a site on the plasminogen activator, which is involved in the interaction with PAI-1 or with the liver cell receptor. Thus, binding of the

thrombolytic agent to such a special bs-MoAb may not only increase its affinity for fibrin, with optimal preservation of its activity, but it may diminish some negative aspects of the agent at the same time.

BISPECIFIC ANTIBODIES

Production

Molecules with bispecific properties can easily be prepared by immunocross-linking two intact MoAb to form tetrameric molecules [70]. Smaller molecules can be prepared by the chemical cross-linking of intact MoAb or separately prepared Fab'-fragments from two different MoAb [71,72]. The latter method however, has the disadvantages inherent to chemical conjugation such as heterogeneous preparations, loss of activity and batch to batch variations mentioned above. Furthermore, it is not always easy to prepare Fab' fragments, especially from isotypes other than IgG1.

These problems can be avoided by producing bs-MoAb using biological methods, involving somatic cell hybridisation [73,74]. In this technique two hybridoma cell lines, each secreting a MoAb of the desired specificity, are fused. This will result in a cell type, called hybrid hybridoma or quadroma, that will produce, amongst others, MoAb with bispecific properties (Fig. 6). Using the quadroma technology, bs-MoAb can be produced in large quantities of consistent quality. Furthermore, it has been suggested that chemically prepared bs-MoAb are more immunogenic than biologically prepared bs-MoAb [66].

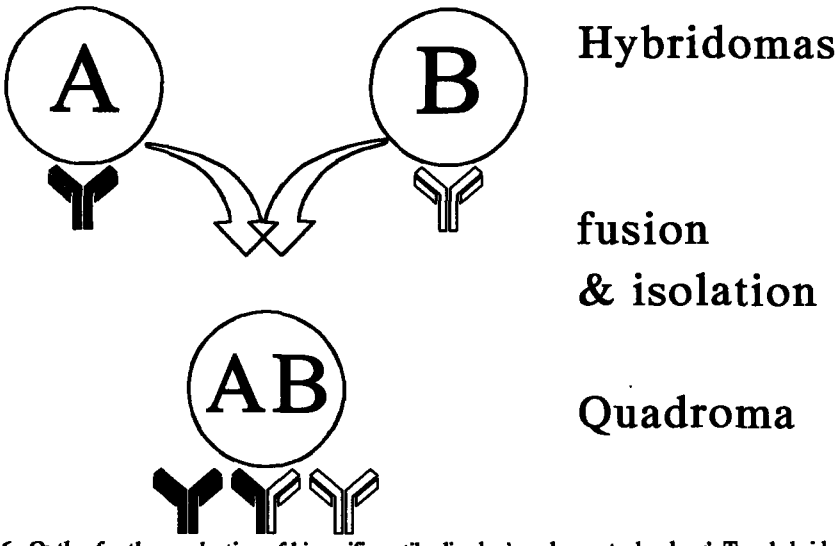

Figure 6. Outlay for the production of bispecific antibodies by 'quadroma technology'. Two hybridoma cell lines, producing MoAb of the desired specificity, are fused. The isolated hybrid hybridoma or quadroma cell line produces, amongst others, MoAb with bispecific properties.

Isolation

One of the crucial steps in quadroma technology is the post-fusion selection procedure that isolates the desired quadroma cells from the mass of non-fused or incorrectly fused hybridoma cells. To date three methods are frequently used. In one method, each of the two cell lines to be fused is labelled with a different fluorescent dye. Fused cells are isolated by selection for double-stained cells on a fluorescent-activated cell sorter (FACS) [75,76]. A second procedure uses irreversible biochemical inhibitors. In this method each of the cell lines is pre-incubated with an earlier determined lethal dose of a complementary toxic agent. These agents are selected to inhibit a different part of the cell metabolism. Fused cells can survive, since they are metabolically complete [74,77]. A third method involves the use of two genetically-controlled drug resistance markers in the hybridoma cells. Each hybridoma cell line has a resistance to a different drug. Quadroma cells are selected simply by culturing in a medium containing both drugs [78,79,80]. The advantage of using drug resistance markers for quadroma isolation, is that this method selects for functionally-stable synkarions (fused nuclei). The other two methods, isolation of double-fluorescent cells by FACS or of metabolically complete cells, primarily select for heterokaryons (fused cell membranes) which may be genetically unstable. Therefore, a relatively low percentage of cells initially isolated, actually produce bs-MoAb [75,76,80].

Several techniques are available to confer a specific drug resistance marker to a hybridoma cell line. Selection for spontaneous mutants that have acquired some resistance to a certain drug is frequently used. Several drugs are available for this method, but in only a few is the genetic background of the acquired drug resistance fully understood. Furthermore, the isolation of spontaneous drug resistant mutants can be a time-consuming process due to a low frequency of mutation, and can coincide with the loss of antibody production. The most frequently-used drugs select for deficiencies in the purine and pyrimidine salvage pathway, based upon mutations in the genes coding for hypoxanthine guanine phosphoribosyl transferase (HGPRT) or, less efficiently, thymidine kinase (TK). These mutations render the cell sensitive to aminopterin, a recessive selection marker. Other drugs used are ouabain, emitin and actinomycin D. The isolation of spontaneous mutants resistant to these latter drugs is, in our experience, a particularly tedious affair.

An alternative to the selection of spontaneous mutations, is the transfection of hybridoma cells with a bacterium-derived marker-gene coding for a specific (dominant) drug resistance [reviewed in 81]. Commonly-used markers are the genes coding for xanthine-guanine phosphoribosyl tranferase (GPT), rendering the cell resistant to mycophenolic acid [82], and for antibiotic-specific aminoglycoside phosphotransferases, e.g. rendering the cell resistant to the neomycin analogue G418-sulphate (NEO) [83] or hygromycin-B (HM-B) [84].

Several methods for the efficient transfection of hybridoma cells are available. In one method a retroviral shuttle system is used, based upon modified retrovirusses which are incapable of autonomous replication [79]. Though the stable transfection frequency obtained in this method is relatively high, the presence and/or introduction of a replication competent (helper) retrovirus needs to be excluded, necessitating additional (expensive) precautions [85]. A preferred-method of transfecting the hybridoma cell lines is by electroporation of a bacterial plasmid construct. The actual mechanisms involved in the stable integration and expression of the bacterial gene in the eucaryotic genome after electroporation are still debated [reviewed in 86]. However, recent developments in the methodology have reached such a level that an efficient and stable transfection of a hybridoma cell line is now possible [80].

Purification

One major bottle-neck in the quadroma technology is the purification of the bs-MoAb from the other immunoglobulin (IgG) molecules which are produced. The quadroma cell synthesizes IgG heavy- and light-chains from both parental cells. Before secretion, the chains are assembled, supposedly at random, to form a mature IgG molecule, comprising a paired heavy/light-chain combination [73,87]. For an active antigen binding-site, the heavy- and light-chains of one parental type should combine, i.e. homologous pairing should occur. Then, for the formation of a functional *bispecific*-MoAb, a heterologous pairing of the homologous heavy/light-chain combinations is required. All other combinations which are assembled and secreted are either monospecific and/or non-functional MoAb. It is not always simple to separate these closely-related molecules from each other.

Most often ion-exchange or adsorption chromatography is applied, using solid-phases to which the two parental IgG bind differently. The bs-MoAb are expected to elute at conditions intermediate between those of the parental MoAb. Cation or anion exchangers and hydroxylapatite are frequently used as the solid-phase [87]. These methods, however, require that there is a sufficient difference in the binding of the two parental MoAb, rarely an attribute that can be selected for in quadroma production. Furthermore, the major contributors to the binding characteristics of an IgG are the heavy-chain moieties, also present in non-functional heterologously-paired heavy/light-chain combinations, which therefore co-elute with the desired bs-MoAb.

The best procedure for purifying bs-MoAb so far developed is by sequential double affinity chromatography [68,88]. First the IgG is applied on a column with one antigen immobilized on a solid carrier. Bound IgG is eluted, and this material is subsequently applied to a second column with the other antigen immobilized on a solid carrier. Bound material is eluted for a second time, and this fraction contains the pure, functionally-active bs-MoAb. Disadvantages of this method are that the bs-MoAb are exposed to two

elution steps, frequently involving extreme pH-changes. Furthermore, the availability of sufficient amounts of purified antigen for the preparation the affinity-columns can be a problem with most antigens, especially when large-scale purifications for clinical trials are considered.

CONCLUDING REMARKS

For improving the efficacy of t-PA and sc(tc)u-PA in thrombolytic therapy, promising results have been obtained with a) t-PA mutants that have a prolonged plasma half-life, and b) targeting with (biologically-prepared) bispecific antibodies. A combination of these two successful approaches, by using special bs-MoAb which not only increases the thrombus specificity of t-PA and sc(tc)u-PA, but simultaneously decreases their plasma clearance or PAI-1 inhibition in a directed manner, may further improve the efficacy of these agents.

Moreover, it may be feasible that the potency of a bs-MoAb, to increase the thrombus specificity of the agent and, at the same time, increase the plasma half-life of the agents activity, is so effective that administration of only the bs-MoAb, perhaps in combination with a drug that induces the release or stimulates the production of endogenous t-PA, is sufficient for effective thrombolytic therapy.

In this summary combinational therapy was not discussed. In combinational therapy thrombolytic agents are administered in combination with additional agents, i.e. with other thrombolytic agents which may act synergistically, or with other drugs which inhibit coagulation or platelet-aggregation. This is another strategy that may lead to an improved thrombolytic therapy.

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CHAPTER 3

PRODUCTION AND CHARACTERISATION OF A SET OF MONOCLONAL ANTIBODIES AGAINST TISSUE-TYPE PLASMINOGEN ACTIVATOR (t-PA)

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SUMMARY

To generate bispecific monoclonal antibodies, reactive to both fibrin and tissue-type plasminogen activator (t-PA), we planned to generate anti-t-PA monoclonal antibodies (mAb) which eliminate negative aspects of t-PA such as the inhibition by plasminogen activator inhibitor-type 1 (PAI-1) and the rapid hepatic clearance of t-PA. Here we report on the isolation and characterisation of a set of 13 mAb against t-PA, some of which meet the above requirements. Apart from their potential in the production of bispecific antibodies, these and the other mAb can be useful in structure-function analysis and a variety of other applications.

Experiments involving PAI-1 showed that one mAb (12-5-3) reacts only with free t-PA, and prevents the subsequent binding of PAI-1 to mAb-bound t-PA. *In vitro* studies on the receptor mediated uptake of t-PA by hepatic cells, showed that one mAb (1-3-1) specifically inhibited the association of t-PA with liver endothelial cells, cells. Other tests showed that mAb 7-8-4 and 12-5-3, but not 1-3-1, inhibited *in vitro* the enzymatic activity of t-PA.

On the basis of these and other observations, we conclude that especially mAb 1-3-1, and *in vivo* possibly 7-8-4 and 12-5-3 may be good candidates for incorporation in bispecific monoclonal antibodies.

INTRODUCTION

The lysis of fibrin, the protein matrix of blood clots, is mediated by the serine protease plasmin. Plasmin does not occur as such in the circulation, but as a zymogen, plasminogen, which can be activated by so-called plasminogen activators such as tissue-type plasminogen activator (t-PA). Both t-PA and plasminogen have the ability to bind to fibrin. As a result of this binding, a cyclic ternary complex is formed in which plasminogen is activated far more efficiently by t-PA than in the absence of fibrin [1-3]. By this mechanism t-PA mediated plasminogen activation is relatively fibrin-specific, as compared with other plasminogen activators such as urokinase or streptokinase, which do not bind to fibrin.

t-PA consists of an array of structural domains, including from the amino-terminal end: the finger (F), the epidermal growth factor (EGF), two kringles (K1 and K2) and the proteolytic (light) chain (P) [for review see 4 and 5]. These structures are believed to have autonomous functions; the F- and K2-domains of t-PA are generally accepted to be involved in the fibrin binding capacities of t-PA [6,7]; the P-domain contains the active site of t-PA [8]; while the site involved in the rate-enhancing effect of fibrin (and analogues) on plasminogen activation by t-PA is located on the K2-domain [6,7].

The activation of plasminogen to plasmin is the key process in thrombolytic therapy. Because of the fibrin specificity of t-PA, it is frequently used in thrombolytic therapy [9]. However, for an efficient therapy, high dosages of t-PA are necessary, over a longer infusion period. This is due to the short half-life of t-PA activity in the circulation as a result of rapid clearance in the liver and inhibition by the naturally occurring plasminogen activator inhibitor type-1 (PAI-1). The high dosages of t-PA, combined with the limited fibrin affinity of t-PA, can cause adverse side effects, such as the activation of circulating, non-fibrin bound plasminogen to plasmin. In such a situation, plasmin may degrade other plasma proteins such as fibrinogen [10,11]. Reported strategies for the improvement of the specificity and efficacy of t-PA as a thrombolytic agent are based upon increasing the fibrin affinity of t-PA and/or by prolonging the half-life of t-PA *in vivo* [12-16]. This will reduce the required effective dose of t-PA and thereby alleviate the t-PA induced systemic effects.

The high specificity and affinity of a monoclonal antibody (mAb) for a defined structure, make it ideal as a carrier for the site-specific delivery of effective agents (drugs targeting). This strategy requires the stable association of mAb and drug, usually achieved by chemical conjugation. Several investigators, however, have reported on the use of bispecific mAb for targeting of drugs [17-20]. Bispecific mAb combine the antigen-binding sites of two different mAb; e.g. one for the target (i.e. fibrin), the other for the drug (i.e. t-PA). Our approach to improve the fibrinolytic properties of t-PA is to produce bispecific mAb that bind fibrin and particulary t-PA in a special manner. More specifically, we want to select for this purpose an anti-t-PA mAb, that may reduce the activity neutralisation rate of t-PA *in vivo* (i.e. reduce the liver clearance of t-PA and/or prevent the inhibition of t-PA by PAI-1), but which does not affect the fibrinolytic qualities of t-PA. Here we report our results on the experiments to find such a monoclonal antibody.

MATERIALS AND METHODS

Antigens (t-PA, t-PA$_{red}$ and t-PA/PAI-1 complex)

Purified human melanoma t-PA was kindly provided by Dr. J.H. Verheijen of this institute. This preparation contains 75% one-chain and 25% two-chain t-PA, and has a specific activity of 5 x 10^5 IU/mg.

To disrupt secondary and tertiary structures in t-PA, a solution of 0.37 mg t-PA/ml in 0.1 M Tris, 0.15 M NaCl, pH 7.4 (Tris/NaCl) was incubated for 1 hour at room temperature with 0.1 M 2-mercaptoethanol. Free -SH groups, generated in this process, were blocked by adding 0.2 M iodoacetamide and incubating for 1 hour at room temperature. This mixture, designated t-PA$_{red}$, was then dialysed against Tris/NaCl, and

reduction was confirmed by sodium dodecyl sulphate polyacrylamide gel electrophoresis [21].

To obtain t-PA/PAI-1 complex, endotoxin-stimulated (10 μg/ml) human umbilical vein endothelial cells, producing large amounts of active PAI-1 [22], were grown overnight in the presence of 100 μg/ml t-PA. Excess free t-PA was removed from the medium using an immobilised mAb specific for free t-PA (12-5-3, see results below). The remaining t-PA/PAI-1 complex was purified using another immobilised t-PA specific mAb (1-3-1, see results below). Immobilisation of the mAb was done on CNBr activated Sepharose 4B according to the manufacturer's instructions (Pharmacia, Uppsala, Sweden).

Other proteins

Polyclonal goat anti t-PA antiserum (Gat-PA) was kindly provided by Dr. J.H. Verheijen of this institute. The gamma-immunoglobulin fraction (IgG) from this antiserum was purified using Protein G-Sepharose according to the manufacturer's recommendations (Pharmacia, Uppsala, Sweden). Purified polyclonal rabbit anti PAI-1 labelled with horseradish peroxidase (RaPAI-1/HRP) ws kindly donated by M. Voskuilen of this institute, and purified plasminogen (glu-plasminogen) was provided by Dr. D.W. Traas of this institute. Chromogenic substrates S2251 and S2288 were obtained from Kabi (Kabi-Vitrum, Mölndal, Sweden). Stimulator, for enhancement of the plasminogen activation by t-PA, was prepared as described by Nieuwenhuizen et al. [2], by CNBr digestion of fibrinogen (Kabi-Vitrum, Mölndal, Sweden). For active PAI-1 the culture medium of human umbilical vein endothelial cells, incubated overnight with 10 μg/ml endotoxin (HUVECS), was used [22]. This medium, containing concentrations of active PAI-1 upto 750 U/ml, was immediately frozen upon harvesting and stored at -70°C.

t-PA activity measurements

The amidolytic activity of t-PA was determined using the low molecular weight chromogenic substrate S2288 (0.4 mM) in 0.1 M Tris containing 0.1% (w/v) Tween 80, pH 8.0 (Tris/Tween) [23]. The efficacy of t-PA in activating plasminogen was determined by adding the following to final concentration: stimulator (80 μg/ml), plasminogen (0.11 μM) and the plasmin specific chromogenic substrate S2251 (0.3 mM) in Tris/Tween [24]. A calibration curve of t-PA activity was used to determine the amount of t-PA.

Immunisation and fusion

Several female BALB/c mice were immunised (25 μg per intraperitoneal inoculation) using either t-PA, $t\text{-}PA_{red}$ or t-PA/PAI-1 complex as immunogen. For the first immunisation, the immunogen was mixed with an equal volume of Freund complete adjuvant. Second, third and fourth booster injections, mixed with an equal volume of

Freund incomplete adjuvant, were given at 3 to 4 week intervals. The final boost was given intraperitoneally in 0.15 M NaCl, 3 days prior to fusion.

Fusion was performed essentially as described earlier [25,26]. Briefly, mice were sacrificed and the splenocytes were isolated. The splenocytes were fused with either P3X63.Ag8 or SP2/0 Ag.14 myeloma cells at a 4:1 ratio (splenocyte:myeloma) for 60 seconds at 37°C using 50% (w/v) polyethylene glycol 1500 (Boehringer Mannheim, Mannheim, Germany). After dilution of the polyethylene glycol to <1.0% (w/v) with culture medium and a resting period of 30 minutes at 37°C, the cells were washed and seeded in selective culture medium containing hypoxantine, aminopterin and thymidine with 10% (v/v) HUVECS added [25-27], at a concentration of 2.5 x 10^5 splenocytes/well in 96-well microtitre plates (Costar, Cambridge, MA, USA). Cell growth was monitored visually, and culture medium was renewed after approximately 4 and 8 days. Between 10 and 12 days after fusion the culture medium was screened for the presence of mAb reactive with t-PA in an enzyme linked immunosorbent assay (ELISA). Cells from positive wells were cloned twice by limiting dilution [28].

Screening ELISA

To demonstrate the presence of anti-t-PA mAb in the culture medium, the following procedure was adopted: Gat-PA IgG was adsorbed to microtitre plates (Greiner, Frickenhausen, Germany) at 3 μg/ml in 0.01 M phosphate, 0.15 M NaCl, pH 7.4 (PBS) by incubation overnight at 4°C. Prior to use, the plates were washed with PBS containing 0.1% (v/v) Tween 20 (PBST) and t-PA was added to the wells (250 ng/ml in PBST). After incubation for 1 hour at room temperature, the plates were washed and three-fold diluted culture medium in PBST was added to the wells. After an incubation of 1 hour at room temperature, the plates were washed and bound mAb was quantified by incubation with peroxidase-labelled polyclonal goat anti mouse-immunoglobulin (Nordic, Tilburg, the Netherlands), diluted in PBST containing 0.1% (w/v) bovine serum albumin (BSA), and subsequent conversion of the chromogenic substrate 3,3',5,5'-tetramethyl benzidine in the presence of H_2O_2 (TMB/H_2O_2) [29].

Culture media from wells which were positive in the first ELISA, were re-tested in a negative control ELISA, in which the t-PA incubation step was omitted.

Cell culture, mAb production and purification

Hybridoma cells were cultured in Dulbecco's modified Eagle's medium (Gibco, Paisley, Scotland), containing 10% (v/v) fetal calf serum. Larger quantities of mAb were obtained by *in vivo* (ascites) production in pristane-primed BALB/c mice. The heavy-chain isotype of the mAb was determined by an agglutination assay based on sheep erythrocytes, labelled with mouse Ig-subclass specific rat monoclonal antibodies, performed according to the manufacturer's directives (Serotec, Oxford, England). The

mAb were purified from ascites on either Protein A-Sepharose (Pharmacia, Uppsala, Sweden) in the case of an IgG [30], or by low-salt precipitation for IgM [31,32].

Epitope competition assay

A sandwich-ELISA was adopted to determine whether the mAb react with different or the same antigenic sites (i.e. epitopes) on the t-PA molecule. To this end the mAb were labelled with biotin using sulphosuccinimidyl-6-(biotinamido) hexanoate (NHS-LC-Biotin) according to the manufacturer's instructions (Pierce, Rockford, Ill, USA). The unlabelled mAb was adsorbed to microtitre plates at a concentration of 10 μg/ml in PBS. After incubation overnight at 4 °C, the plates were washed with PBST and t-PA was added in serial three-fold dilutions from 100 to 1.2 ng/ml in PBST. After 2 hours at room temperature, unbound material was washed away, and biotin-labelled mAb, diluted in PBST containing 0.1% (w/v) BSA, were added to duplicate rows. After another 2 hours at room temperature, non-bound mAb was removed by washing, and bound biotin-labelled mAb was assessed by incubation with Streptavidin/HRP (Pierce, Rockford, Ill, USA) diluted in PBST containing 0.1% (w/v) BSA and subsequent conversion of TMB/H_2O_2. A peroxidase-labelled conjugate of Gat-PA (Gat-PA/HRP) was added simultaneously to separate wells as a control on the binding of t-PA.

Activity recovery assay

A two-step assay was developed to determine the effect of the mAb on the enzymatic activity of t-PA, either amidolytic activity or plasminogen activation. For this assay the mAb were adsorbed to microtitre plates at 10 μg/ml in PBS as described above. Prior to use the plates were washed with Tris/Tween, and t-PA was added in serial two-fold dilutions from 100 to 3.1 IU/ml in Tris/Tween. After 2 hours at room temperature, a sample of the supernatant was taken in duplicate, and (non-bound) t-PA activity in these samples was determined as described above. These data were used to calculate the amount of t-PA actually bound to the solid phase mAb, i.e. the difference between added t-PA activity and remnant t-PA activity in the supernatant after incubation. The remaining t-PA solution was removed from the wells, and the plates were washed with Tris/Tween. Then the activity of complexed t-PA, i.e. t-PA activity recovered when bound to the solid phase mAb, was determined as described above.

The t-PA activity actually recovered on the solid phase mAb was plotted against the calculated mAb-bound t-PA activity. A straight line was obtained, and the slope of the line indicates the effect of the mAb on t-PA activity. If full t-PA activity is recovered upon binding of t-PA to the solid phase mAb, the slope of the line should be 1. A slope of <1 indicates an inhibition on the enzymatic activity of t-PA by the mAb. This test was so designed that it ensured all t-PA to be in complex with the mAb, avoiding the possibility that a measured difference in effect of the mAb on the enzymatic activity of

t-PA may be due to a different dissociation constant for t-PA between the mAb. Binding of t-PA antigen, after a 30-minute incubation with the substrate mixture containing 10 KIU/ml aprotinin (Trasylol), was assessed using Gat-PA/HRP, to confirm the continous presence of t-PA to the solid-phase mAb, even when recovered t-PA activity was reduced or absent.

Influence on the binding of PAI-1 to t-PA

The influence of the mAb on the complex formation of t-PA with PAI-1, and the reactivity of the mAb with either free t-PA or t-PA/PAI-1 complex, was determined using an ELISA. Plates adsorbed with mAb at 10 μg/ml in PBS were prepared as described above and t-PA, at a non-saturating concentration of 50 ng/ml PBST, was allowed to react with the mAb for 1 hour at room temperature. After washing the plates with PBST, serial two-fold dilutions of PAI-1 in PBST were added and incubated for 1 hour at room temperature. The binding of PAI-1 to the mAb-bound t-PA was assessed using RaPAI-1/HRP. Control assessment of t-PA binding to the solid phase mAb, after incubation with PAI-1, was done by incubating Gat-PA/HRP in a separate series of wells.

Additionally, t-PA, at a final non-saturating concentration of 50 ng/ml, was pre-incubated for 1 hour at room temperature with serial two-fold dilutions of PAI-1 in PBST, before being added to the mAb coated wells. Binding of both t-PA and PAI-1 to the solid phase mAb was determined, using Gat-PA/HRP and RaPAI-1/HRP respectively, as described above.

Influence on the fibrin binding of t-PA

Binding of t-PA to fibrin, and the possible influence of the mAb thereon, was measured essentially as described by Verheijen et al. [7]. In short, t-PA was radioactively-labelled by incubation with a I^{125}-labelled peptidyl chloromethyl ketone [33,34], and subsequently incubated with a 20-fold molar excess of mAb. The mAb/t-PA immunecomplex was then added to fibrinogen (400 μg/ml) and clotting was induced by the addition of thrombin. The fibrinogen concentration was specifically chosen to achieve a maximal 90% fibrin binding of the added I^{125}-t-PA in the absence of mAb. After 60 minutes at 37°C, clots were spun down at 12,000 x g for 15 minutes, and I^{125}-levels remaining in the supernatant were determined. The relative fibrin binding capacity of t-PA in the presence of a mAb was expressed as a percentage of the maximum amount of fibrin-bound t-PA in the absence of mAb.

To study the effect of the mAb on the F-domain mediated fibrin binding of t-PA separately, identical experiments were performed in the presence of 0.1 M 6-AHA to eliminate the contribution of the K2-domain to the binding of t-PA to fibrin [35]. When 6-AHA is present, the maximal fibrin bound t-PA is reduced to half of that observed

when 6-AHA is absent.

As a control the effect of 6-AHA on the interaction between mAb and t-PA was studied in an ELISA. For this, plates coated with 10 μg/ml mAb were prepared and t-PA was incubated at the non-saturating concentration of 50 ng/ml as described above. Binding of t-PA to the solid phase mAb was assessed after a 20-minute incubation of Tris/Tween with or without 0.1 M 6-AHA, using Gat-PA/HRP.

Influence on liver cell association of t-PA

The effect of the mAb on the specific, receptor mediated association (i.e. binding and internalisation) of t-PA with liver cells, responsible for the short half-life of t-PA in circulation, was determined as described elsewhere in more detail [36]. In short, t-PA was radioactivally labelled using the Iodo-Gen method [37]. Rat endothelial and parenchymal liver cells were isolated, using density gradient centrifugation followed by centrifugal elutriation and differential centrifugation respectively, as described by Kuiper et al. [38]. ^{125}I-t-PA (0.15 nM) was pre-incubated with buffer (control), excess mAb (100 nM), or excess unlabelled t-PA (100 nM) and incubated on the cells for 10 minutes at 37°C. After washing unbound protein from the cells, association of ^{125}I-t-PA with the cells was determined and corrected for cell weight (mg of cell protein). Association of t-PA with the liver cells was expressed as a percentage of the control.

RESULTS

Fusion

Over 24 fusions were performed and, on average, hybridomas were found in approximately 65% of the seeded wells. The yield of anti-t-PA mAb producing hybridoma cell lines was low; only 13 stable anti-t-PA mAb-producing cell lines were eventually obtained. These were cloned twice before further characterisation. Designated codes and some characteristics of the 13 mAb are summarised in Table 1.

Epitope competition

Studies on the potential of the mAb to compete with each other for binding to t-PA, showed that the mAb apparently bound to at least 6 distinct antigenic sites (i.e. epitopes) on t-PA (Table 2, summarised in Table 1). A group of 5 mAb (7-8-4, 10-1-3, 2-1-3, 15-4-2 and 1-1) apparently recognise the same or adjacent epitopes, since they compete for binding to t-PA. This group was designated as 'epitope cluster 1'. mAb 1-1 appears not only to compete with mAb from cluster 1, but also with mAb 1-3-1 (cluster 2), it was therefore assigned to both epitope cluster 1 and 2. The mAb 5-1 and 19-1-3 also competed for the same antigenic site on t-PA (cluster 3). The remaining mAb did not

Table 1. Summary of characteristics of 13 anti-t-PA monoclonal antibodies

mAb-code	antigen	isotype	reactive with t-PA/PAI-1	epitope cluster (Table 2)
7-8-4	t-PA	IgG1	yes	I
10-1-3	t-PA	IgG1	yes	I
12-5-3	t-PA	IgG2a	no	V
1-3-1	$t\text{-}PA_{red}$	IgG1	yes	II
2-1-3	$t\text{-}PA_{red}$	IgG1	yes	I
4-5-1	$t\text{-}PA_{red}$	IgM	yes	NA
15-4-2	$t\text{-}PA_{red}$	IgG1	yes	I
19-1-3	$t\text{-}PA_{red}$	IgG1	yes	III
18-1	$t\text{-}PA_{red}$	IgM	yes	NA
1-1	t-PA/PAI-1	IgG1	yes	I & II
5-1	t-PA/PAI-1	IgG1	yes	III
5-2	t-PA/PAI-1	IgG1	yes	IV
R-2	t-PA/PAI-1	IgG1	yes	VI

Designated mAb code, antigen used for immunisation, heavy-chain isotype, reactivity with t-PA/PAI-1 complex, and designated epitope cluster (summary of Table 2). NA = not assigned to a group.

Table 2. Epitope competition ELISA

solid phase mAb	fluid phase mAb (biotin labelled)										
	7-8-4	10-1-3	2-1-3	15-4-2	1-1	1-3-1	5-1	19-1-3	5-2	12-5-3	R2
			I			II		III	IV	V	VI
7-8-4	C	C	C	C	C	-	-	-	-	-	-
10-1-3	C	C	C	C	C	-	-	-	-	-	-
2-1-3	C	C	C	C	C	-	-	-	-	-	-
15-4-2	C	C	C	C	C	-	-	-	-	-	-
1-1	C	C	C	C	C	C	-	-	-	-	-
1-3-1	-	-	-	-	C	C	-	-	-	-	-
5-1	-	-	-	-	-	-	C	C	-	-	-
19-1-3	-	-	-	-	-	-	C	C	-	-	-
5-2	-	-	-	-	-	-	-	-	C	-	-
12-5-3	-	-	-	-	-	-	-	-	-	C	-
R2	-	-	-	-	-	-	-	-	-	-	C
epitope cluster			I			II		III	IV	V	VI

Reactivity of biotin-labeled mAb in fluid-phase (horizontal) with t-PA bound to solid-phase mAb (vertical). C = competition, - = no competition. Roman numerals indicate designated epitope cluster (summarised in Table 1).

interfere with the binding of any other mAb to t-PA and apparently reacted with discrete epitopes on t-PA.

The reaction was judged competitive when no respons, or a repons of < 3 times the background at the highest t-PA concentration incubated (100 ng/ml), was measured. In those cases where no competition occurred, a clear dose-respons was obtained with 3 or more of the 5 t-PA concentrations incubated. The two IgM mAb 4-5-1 and 18-1 were not included in this test, since several attempts to obtain a biotinylated conjugate of these mAb failed.

Effect on PAI-1 binding to t-PA

All but one (12-5-3) of the mAb reacted both with free t-PA and t-PA in complex with PAI-1 (Table 1). The binding of PAI-1 to t-PA, as assessed in the ELISA, was not affected by the other mAb, since a dose-dependent binding of PAI-1 to the mAb-bound t-PA could be detected (Fig. 1). However, mAb 12-5-3 apparently prevented the t-PA/PAI-1 complex formation, since no binding of PAI-1 to the mAb-bound t-PA could be detected, while binding of t-PA to the mAb remained unchanged (Fig. 1). Furthermore, the reactivity of 12-5-3 with t-PA decreased in a dose-dependent fashion with increasing PAI-1 concentrations, when t-PA was allowed to react first with PAI-1 in solution before being added to the mAb-coated wells (Fig. 2). We conclude that 12-5-3 and PAI-1 are mutually exclusive for binding to t-PA.

Effect on enzymatic activity

The graphical representation of recovered against calculated t-PA activity bound to the solid phase mAb, clearly shows the effect of the mAb on the enzymatic activity of t-PA (Fig. 3a and 3b). Only 3 of the tested mAb (1-3-1, 2-1-3 and 4-5-1) had no apparent effect on the plasminogen activation by t-PA, since all the activity of the mAb-bound t-PA was recovered (Table 3). The other mAb inhibited the plasminogen activation by t-PA to varying extents. The amidolytic activity of t-PA, however, is less affected by most mAb, since a major fraction of t-PA activity was recovered. Some mAb inhibited the plasminogen activation by t-PA, but not the amidolytic activity of t-PA. Only 3 mAb completely inhibited both enzymatic activities of t-PA (7-8-4, 10-1-3 and 12-5-3).

Data on mAb 18-1 were omitted, since no significant binding of t-PA activity to the solid-phase mAb could be detected when t-PA was incubated at concentrations low enough to exclude aspecific binding, as determined using an adsorbed negative control antibody.

Control ELISA experiments showed that all t-PA remained bound to the solid phase mAb after incubation with the appropriate substrate mixture. In the case of a decreased recovery of t-PA activity bound to the solid-phase mAb, full binding of t-PA antigen was confirmed (results not shown).

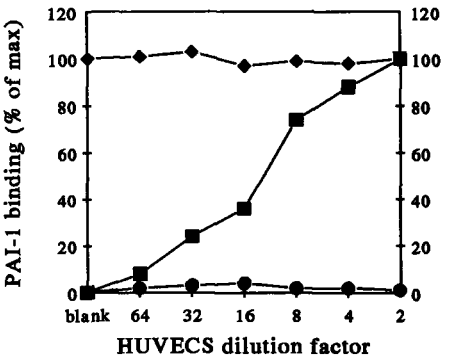


Figure 1. Influence of the anti-t-PA mAb on the interaction between t-PA and PAI-1; (■) binding of PAI-1 to t-PA bound to 7-8-4 (similar results obtained with other mAb but 12-5-3); (●) binding of PAI-1 to t-PA bound to 12-5-3; (♦) control for t-PA antigen on 12-5-3 after incubation with PAI-1 (other mAb showed a similar response). Abscissa: PAI-1 concentration; endotoxin-stimulated HUVECS was used as the source for active PAI-1. Ordinate: percentage of maximum binding.

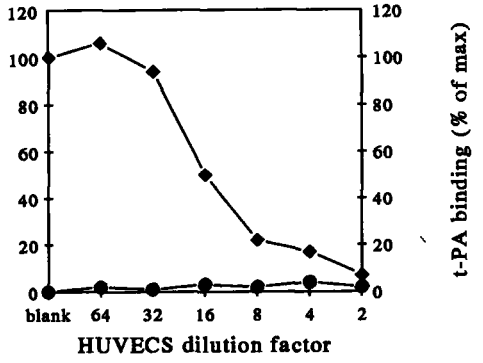
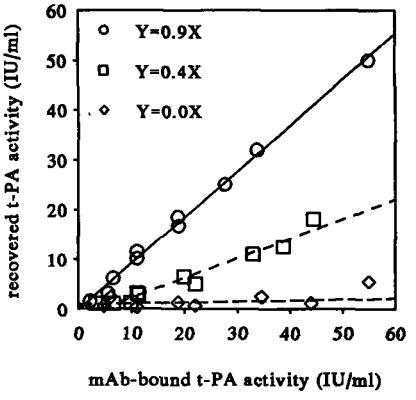


Figure 2. Binding of (♦) t-PA antigen and (●) PAI-1 antigen to solid phase 12-5-3 after pre-incubation of t-PA in a serial dilution of PAI-1. Abscissa: PAI-1 concentration; endotoxin-stimulated HUVECS was used as the source for active PAI-1. Ordinate: percentage of maximum binding.



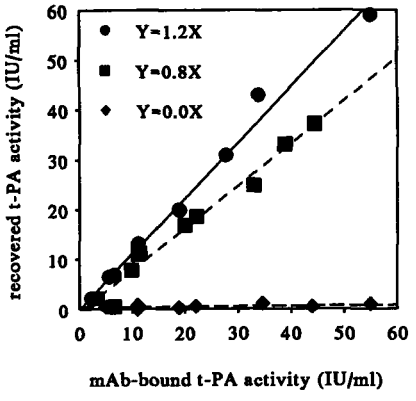


Figure 3. Study of the effect of the mAb on t-PA activity as determined using the activity recovery assay. Recovered t-PA activity was assessed (○ □ ◇) by plasminogen activation or (● ■ ♦) amidolytic activity. Results are shown of 3 representative mAb (complete data in Table 3). Recovered t-PA activity of (○ ●) mAb 1-3-1, (□ ■) mAb 1-1 and (◇ ♦) mAb 7-8-4. Abscissa: calculated t-PA activity bound to the solid phase mAb. Ordinate: recovered t-PA activity bound to the solid phase mAb. Insert: equations of the lines calculated by linear regression of data, used to determine the recovered fraction of mAb-bound t-PA activity.

Table 3. Influence of anti-t-PA mAb on some biological properties of t-PA

mAb-code	recovered mAb-bound t-PA activity as a fraction of calculated activity		relative capacity of t-PA to bind to fibrin (% of max)	
	PLG	AMIDOL	- 6-AHA	+ 6-AHA
7-8-4	0.0	0.0	28.8	00.0
10-1-3	0.1	0.1	71.1	13.4
12-5-3	0.1	0.0	99.1	92.4
1-3-1	0.9	1.2	90.1	97.7
2-1-3	0.8	1.0	91.6	69.8
4-5-1	1.2	0.9	99.5	91.8
15-4-2	0.3	1.2	79.8	50.1
19-1-3	0.2	0.7	48.2	13.9
18-1	ND	ND	98.5	92.6
1-1	0.4	0.8	60.7	54.7
5-1	0.3	0.6	38.4	00.0
5-2	0.0	0.9	80.9	60.2
R2	0.4	1.1	77.9	31.0

Recovered t-PA activity after binding to solid phase mAb as a fraction of calculated activity; assessed for (PLG) plasminogen activation by t-PA and (AMIDOL) amidolytic activity of t-PA (see also Fig. 3), and relative fibrin-binding capacity of t-PA after correction for blank values in (- 6-AHA) the absence (i.e. F- and K2-domain mediated binding) or (+ 6-AHA) presence of 0.1 M 6-AHA (i.e. F-domain mediated binding alone). ND = not determined. Means of at least 2 experiments, results did not vary more than 10%.

Effect on fibrin binding of t-PA

The effect of the mAb on the relative fibrin-binding capacity of t-PA, was determined and expressed as a percentage of the maximal fibrin-bound t-PA under the different test-conditions (i.e in the absence or presence of 0.1 M 6-AHA).

Most mAb interfered, to varying degrees, with the fibrin binding of t-PA, since the relative fibrin-binding capacity of t-PA was reduced (Table 3). If fibrin binding was affected, this also occurred in the presence of 6-AHA, indicating that these mAb interfered mostly with the F-domain mediated fibrin binding of t-PA. With 4 mAb (7-8-4, 10-1-3, 19-1-3 and 5-1) the F-domain mediated fibrin binding was inhibited dramatically.

Control ELISA experiments showed that with one mAb (1-3-1) a 20 minute incubation with 0.1 M 6-AHA caused an almost complete dissociation of the mAb/

t-PA complex, since bound t-PA antigen levels dropped by more than 90%. With all other mAb the mAb/t-PA complex remained intact.

Effect on liver cell association

As can be seen in Table 4, one mAb (1-3-1) specifically inhibited the liver endothelial cell mediated uptake of t-PA by 62%, with little effect on the association with liver parencymal cells (20%). In contrast, another mAb (7-8-4) specifically inhibited the liver parenchymal cell mediated uptake of t-PA by 75%, with significantly less effect on the association with liver endothelial cells (39%). These data suggest at least two cell types are involved in the clearance of t-PA by the liver, one mediated by a receptor on the liver endothelial cell, the other by a different receptor on the liver parenchymal cell, each distinctively inhibited by another mAb (i.e. 1-3-1 and 7-8-4, respectively). Furthermore, anti-t-PA mAb (1-3-1 and 7-8-4) can specifically modify the association of t-PA with liver cells *in vitro*, by binding to or near the site involved in receptor-binding or by inducing a conformational change in t-PA. The other mAb in this study did not affect the association of t-PA with liver cells.

Table 4. Effect of mAb on the association of t-PA with liver cells

^{125}I-t-PA added with	cell type	
	endothelial	parenchymal
buffer	100	100
1-3-1	37	80
7-8-4	61	25
t-PA	21	37

Percentage of t-PA associated with liver endothelial and liver parenchymal cells after pre-incubation of ^{125}I-t-PA with buffer (control), mAb 1-3-1, mAb 7-8-4 or excess unlabeled t-PA. Means of two experiments.

DISCUSSION

The aim of the present study was to find mAb which inhibit or eliminate characteristics of t-PA which are considered as negative for the use of t-PA as a thrombolytic agent, i.e. the rapid neutralisation of t-PA activity in the circulation through inhibition by PAI-1 and the rapid hepatic clearance. As shown in the results section, we succeeded in finding an antibody which prevents the binding of PAI-1 to t-PA (12-5-3), and two mAb that specifically inhibit the uptake of liver endothelial cells (1-3-1) or liver parenchymal cells (7-8-4). Apart from these, we obtained other mAb which appear to be valuable tools for structure-function analysis of t-PA and several other applications.

In our studies which investigated the effect of the mAb on the enzymatic activity of t-PA, a novel activity recovery assay was used. The design of the assay was such that all activity measured was from t-PA in complex with (the solid-phase) mAb. Thus differences in inhibitory effects between mAb are not related to different dissociation constants of the mAb with t-PA. With this assay, we found that with some mAb (1-3-1, 2-1-3 and 4-5-1) all t-PA activity could be recovered after binding of t-PA to the solid phase mAb. Some mAb (15-4-1, 19-1-3, 1-1, 5-1, 5-2 and R2) only inhibited the plasminogen activation by t-PA, but not the amidolytic activity of t-PA. This suggests that they either affect the interaction between t-PA and its substrate plasminogen, possibly by steric hindrance, or inhibit the binding of the K2-domain of t-PA to the rate-enhancing fragments FCB2 and FCB5, present in the CNBr digest of fibrinogen which was used as a stimulator in the plasminogen activation assay [1,2,35,39]. Additionally, these mAb also interfere with the F-domain mediated fibrin binding of t-PA, suggesting that some of these mAb may have their epitope on the F-domain. This could indicate that in native t-PA, the F-domain is folded either near the K2-domain of t-PA (effecting a steric hindrance by the mAb on the interaction of t-PA with the stimulatory fibrin(ogen) fragments) and/or folded near the P-domain of t-PA (effecting a steric hindrance by the mAb on the interaction of t-PA with plasminogen). Recently, Novokhatny et al. presented data on the thermostability of t-PA, suggesting that the F-domain and/or EGF-domain closely interact with and stabilise the P-domain [40]. Another possibility is that the mAb induce an unfavourable conformational change in t-PA.

It was difficult to categorize the epitopes on t-PA for the different mAb. Results from different assays were sometimes inconsistent with, or even apparently contradictory to, each other. This is best illustrated by the mAb from cluster 1. All inhibit the binding to t-PA of the other mAb in this group, as was demonstrated in the competition ELISA, yet they had different effects on the enzymatic activity of t-PA; mAb 7-8-4 and 10-1-3 inhibited both plasminogen activation and amidolytic activity; mAb 15-4-2 and 1-1 inhibited only the plasminogen activation but not the amidolytic activity; and mAb 2-1-3

had no apparent effect on either of the enzymatic activities.

The problems in identifying the epitopes on t-PA are further illustrated by mAb 7-8-4 and 10-1-3. Both strongly inhibit the F-domain mediated fibrin binding of t-PA, suggesting an epitope on the F-domain, but also dramatically inhibit the amidolytic activity, which strongly suggests an epitope in or near the active site of t-PA localised on the P-domain. One explanation may be that 7-8-4 and 10-1-3 bind to, or near, the active site of t-PA and cause steric hindrance for the fibrin binding of the F-domain, folded near to the P-domain in intact t-PA as suggested above. If this is correct, mAb 12-5-3, which also seems to have its epitope in the active site of t-PA, must recognise a different epitope, since it has no apparent effect on the binding of t-PA to fibrin and the association of t-PA with liver cells. This corresponds with the results obtained in the competition ELISA, showing that 12-5-3 did not compete with 7-8-4 and 10-1-3.

We must conclude that our attempts to map the epitopes of the mAb on t-PA, on the basis of the effect of the mAb on biological functions of t-PA, did not yield clear-cut results. Other groups reported similar, apparently conflicting results [41,42]. These and our results may be explained by a more complex organisation, and perhaps cooperative actions, of the different domains in t-PA.

Mapping the epitopes of the mAb using domain-deletion mutants of t-PA, as described by Zonneveld et al. [43], could prove to be inconclusive, as has also been suggested by Pannekoek et al. [5], since different t-PA mutants may react differently with the same mAb, whether or not the relevant antigenic sites are present. This is probably because a domain-deletion in t-PA has extensive effects on the conformation of other domains or may disrupt the correct orientation of distinct domains in the t-PA molecule.

The results obtained with mAb 12-5-3 indicate that this mAb only reacts with free t-PA. Furthermore, 12-5-3 prevents the binding of PAI-1 to mAb-bound t-PA. In addition to the results obtained in the epitope competition ELISA, this is another indication that 12-5-3 reacts with a different epitope than 7-8-4 and 10-1-3. Combined with the dramatic inhibitory effect of 12-5-3 on the enzymatic activity of t-PA, we conclude that 12-5-3 probably has its epitope in the active-site of t-PA.

The specificity of 12-5-3 for free t-PA was successfully used to remove excess free t-PA from endotoxin-stimulated endothelial cell culture supernatant containing t-PA/PAI-1 complex, to purify t-PA/PAI-1 for immunisation. The features of 12-5-3 also make it a good candidate for use in an ELISA to determine free (active) t-PA. This application is now under investigation.

The dissociation of the 1-3-1/t-PA immunecomplex, caused by 6-AHA, was a very interesting finding. One likely explanation for this is that 6-AHA and 1-3-1 compete for the same site on t-PA, possibly the lysine binding site in the K2-domain [7,34,35]. However, a CNBr digest of fibrinogen, in which FCB2 [34], and probably FCB5 [44], also bind to the K2-domain of t-PA, did not cause the disruption of the 1-3-1/t-PA

immunecomplex. Furthermore, 1-3-1 had no influence on the fibrin binding of t-PA in the absence of 6-AHA, presumed to be mediated at least partly by the K2-domain of t-PA. These results indicate that the contribution of the K2-domain to the fibrin binding of t-PA may not be mediated entirely by the high-affinity lysine binding site in this kringle, but may involve (an)other, yet unidentified part(s) of the K2-domain, perhaps in combination with other domains of t-PA. It is not inconceivable that 6-AHA induces an unfavourable conformational change either in t-PA, destroying the epitope for 1-3-1, or (less likely) in the mAb, rendering the antigen binding site incapable of reacting with t-PA.

This feature of 1-3-1 enabled us to purify t-PA/PAI-1 complex from endotoxin-stimulated endothelial cell culture supernatant (results not shown). After binding of t-PA (t-PA/PAI-1 complex) to 1-3-1, it could be eluted under mild conditions simply by adding 0.1 M 6-AHA to the buffer, without the need for chaotropic agents or extreme pH changes. This particular feature of 1-3-1 is promising for the large scale one-step purification of melanoma or recombinant t-PA from culture medium. This application is now under investigation.

As indicated in the introduction, we are in the process of producing bispecific mAb, with a view to improving the efficacy and specificity of t-PA in thrombolytic therapy. Such a bispecific mAb will combine the antigen binding sites, i.e. the Fab fragments, of an anti-fibrin mAb and an anti-t-PA mAb. Binding of t-PA to such a bispecific mAb enhances the affinity of t-PA for fibrin. However, because t-PA is bound to the bispecific mAb via the anti-t-PA Fab, the binding is predefined and uniform, and the effect of binding to t-PA can be well-characterised. By selecting an anti-t-PA mAb with special characteristics, we can modulate some adverse property of t-PA that renders it less suitable as a thrombolytic agent, such as the fast clearance of t-PA in the liver and/or the inhibition of t-PA by PAI-1. Potential candidates for use in a bispecific mAb would be mAb 1-3-1, 7-8-4 and 12-5-3, since they have a special effect on t-PA, i.e. 1-3-1 and 7-8-4 may reduce the clearance rate of t-PA as suggested by the effect of these mAb on the liver cell association of t-PA, and 12-5-3 prevents the binding of t-PA to PAI-1. However, mAb 7-8-4 and 12-5-3 also inhibit the plasminogen activation by t-PA, as determined by the activity recovery assay, which seems to make them less suitable for use in bispecific mAb. Nevertheless, it is possible that enough t-PA can dissociate from the bispecific mAb upon binding of the bispecific mAb/t-PA complex to the thrombus, with its high local concentration of fibrin for which t-PA also has some affinity. When the effect of mAb 7-8-4 and 12-5-3 on the t-PA activity in the fibrin plate assay was measured, we could indeed detect some t-PA activity despite the presence of the mAb (results not shown). Preliminary results show that, using mAb obtained in this study (1-3-1 and 12-5-3), we are indeed capable of preparing bispecific mAb that effectively accumulate t-PA antigen and/or activity on a fibrin surface [45,46].

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CHAPTER 4

ONE-STEP PURIFICATION OF TISSUE-TYPE PLASMINOGEN ACTIVATOR USING AFFINITY CHROMATOGRAPHY WITH A SPECIAL MONOCLONAL ANTIBODY UNDER MILD CONDITIONS

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SUMMARY

We have previously isolated a monoclonal antibody, designated as 1-3-1, specific for tissue-type plasminogen activator (t-PA). We have shown that t-PA dissociates from 1-3-1 in the presence of the lysine analogue 6-aminohexanoic acid (6-AHA). Here we describe a method for the one-step immunoaffinity purification of t-PA from conditioned melanoma cell medium, using 1-3-1 immobilised on Sepharose under mild elution conditions, favourable for t-PA.

The yield of t-PA (antigen or total protein) from a 1-3-1-Sepharose column, when eluted using a buffer supplemented with 0.2 M 6-AHA at neutral pH, was as effective as other buffers that involve a stronge pH-change, i.e. pH 2-3. However, the enzymatic activity of the t-PA purified with 6-AHA was 25 to 30% higher, as compared with t-PA eluted using a pH-change. This resulted in a markedly higher specific activity of t-PA purified with 0.2 M 6-AHA, as compared with t-PA purified using a strong pH-change.

The purity of t-PA, purified using the present method, was very high, as determined by gel electrophoresis. An additional advantage of the present procedure is that the mild elution conditions prolong the column-life.

INTRODUCTION

The fibrinolytic system, as the counterpart of the coagulation system, is responsible for the breakdown of the fibrin matrix of a blood clot. The end product of the fibrinolytic system is the serine protease plasmin, which degrades the insoluble fibrin to soluble fibrin degradation products [1]. Plasmin does not occur as such in the circulation, but as an inactive zymogen called plasminogen. The conversion of plasminogen into plasmin is mediated by so-called plasminogen activators. It has been shown that tissue-type plasminogen activator (t-PA) is a relatively fibrin-specific plasminogen activator, since it has an affinity for fibrin [2,3], and fibrin has a rate-enhancing effect on the t-PA mediated plasminogen activation [4,5,6]. Since these are favourable properties for thrombolysis, t-PA has been advocated as the agent of choice for thrombolytic therapy [7,8]. However, the recommended dosages of t-PA for an effective therapy are relatively high and range from 50 to 100 mg per patient.

For the large-scale production of t-PA, three methods are generally used, namely large-scale culture of human melanoma cells [9,10], of Chinese hamster ovary cells [11,12] or of *Escherichia coli* [13,14]. The latter two cell types have been transfected with the cDNA sequence for human t-PA. Though derived from different sources, and varying somewhat in molecular structure, all varieties of t-PA produced sofar are potent

plasminogen activators, with comparable enzymatic activities and (short) plasma half-lives [8,10,11,14,15].

The purification of t-PA from the cell-conditioned medium is usually achieved by laborious chromatographic procedures, involving zinc-chelate, Concanavalin A-Sepharose, and arginine- or lysine-Sepharose [9,10,17,18]. In more recently-developed methods, t-PA is purified with the aid of an immobilised monoclonal antibody (MoAb) specific for t-PA [19,20]. The latter method has the advantage of a highly t-PA specific solid-phase, requiring only one purification step. This may improve purity and yield, be less time-consuming, and decrease the risk for toxic products leaking from the resin (e.g. Concanavalin A). However, a chaotropic agent or an extreme pH-change is usually necessary to elute the t-PA from the MoAb, which is likely to result in a (partial) denaturation of t-PA and the immobilised MoAb. This may decrease the yield of enzymatically-active t-PA and diminish column-life.

Earlier, we isolated and functionally characterized a special MoAb (1-3-1) that binds specifically to t-PA [21]. In the presence of 0.1 M 6-aminohexanoic acid (6-AHA) t-PA was shown to dissociate from the MoAb, since 6-AHA and the MoAb compete for the same (lysine) binding site on t-PA, or since 6-AHA induces an unfavourable, reversible, conformational change in the t-PA molecule. This prompted us to investigate the possibility of purifying t-PA by using immobilised 1-3-1 and eluting t-PA under mild conditions with 6-AHA containing buffers at a neutral pH. These mild conditions may not only preserve the enzymatic activity of t-PA, but may also prolong the life-time of the column. The results of that study are reported here.

MATERIALS AND METHODS

Source of t-PA

Human melanoma (Bowes) cell conditioned serum free medium was kindly provided by Dr. J.H. Verheijen of this institute. The t-PA in this medium is predominantly one-chain t-PA.

Standard purified t-PA, derived from melanoma cells and purified according to Rijken and Collen (9) as modified by Kluft et al. (10), was kindly provided by Dr. J.H. Verheijen of this institute. The preparation contains 75% one-chain t-PA and 25% two-chain t-PA, and has a specific activity of about 500 IU/μg.

Antibodies

The t-PA specific monoclonal antibodies (MoAb), designated as 1-3-1, 7-8-4 and 10-1-3, were isolated and characterized as described elsewhere [21]. The MoAb were purified from ascites on protein A-Sepharose according to Ey et al. [22]. The MoAb 1-3-1, which

dissociates from t-PA in the presence of 6-AHA, was immobilised to activated CH-Sepharose 4B (10 mg protein to 6 ml gel) according to the manufacturer's recommendations (Pharmacia, Uppsala, Sweden). Columns were prepared as described below. The two other MoAb, 7-8-4 and 10-1-3, were used as catching antibodies in the ELISA to determine t-PA antigen levels as described below.

The polyclonal antibodies from a goat anti t-PA (GatPA) antiserum, obtained from Dr. J.H. Verheijen of this institute, were purified using protein G-Sepharose according to the manufacturer's instructions (Pharmacia, Uppsala, Sweden). The purified antibodies were labelled with horseradish peroxidase (HRP) using N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) according to the manufacturer's directions (Pharmacia, Uppsala, Sweden). The GatPA/HRP conjugate was used as tagging antibody in the ELISA described below.

t-PA antigen assay

t-PA antigen was assessed using an ELISA developed by us. Briefly, a mixture of MoAb 7-8-4 (2 μg/ml) and 10-1-3 (8 μg/ml) in 0.04 M Tris, pH 7.4 was adsorbed overnight at 4°C to 96-well polystyrene microtitre plates (Greiner, Frickenhausen, Germany). Prior to use, the plates were washed three times with 0.01 M phosphate, 0.15 M NaCl, pH 7.4 containing 0.1% (v/v) Tween 20 (PBST). Samples were serially diluted three-fold in PBST, added to the wells and incubated for two hours at room temperature. Non-bound material was washed away and GatPA/HRP, diluted in PBST supplemented with 0.1% (w/v) bovine serum albumin (BSA), was added. After two hours at room temperature, non-bound conjugate was washed away and the chromogenic peroxidase substrate mixture 3,3',5,5'-tetramethyl benzidine and H_2O_2 (TMB/H_2O_2) was added [23]. The reaction was stopped by addition of H_2SO_4 and the absorption was read at 450 nm. A calibration curve of standard purified t-PA was run simultaneously.

t-PA activity assay

The enzymatic activity of t-PA towards plasminogen was determined essentially as described by Verheijen et al. [24]. In brief, samples were serially diluted three-fold in 0.1 M Tris containing 0.1% (v/v) Tween 80, pH 8.0, in the presence of plasminogen and CNBr digested fibrinogen (stimulator). The t-PA mediated plasmin formation was determined with a plasmin specific chromogenic substrate. A calibration curve of standard purified t-PA was run simultaneously.

Total protein assay

The total protein content in the samples was determined essentially as described by Bensadoun & Weinstein [25]. In brief, to remove substances that may interfere in the protein assay and, at the same time, to concentrate the sample, proteins were

precipitated with 10% (w/v) trichloroacetic acid (TCA) in the presence of 0.02% (w/v) sodium deoxycholate. After centrifugation, the pellet was dissolved in a four-fold smaller volume, as compared with the initial sample size, of de-ionized water containing 1% (w/v) sodium dodecylsulphate. Protein was determined using the Micro BCA protein assay according to the manufacturer's recommendations using BSA as calibrator (Pierce, Rockford, Il., U.S.A.). Protein values were corrected for losses, expected at the used low protein concentrations. The correction factor was determined by assessing protein recovery in the pellet after TCA precipitation of standard purified t-PA, at various low concentrations.

Gel electrophoresis

To check the purity of the t-PA obtained by immunoaffinity purification with the present method, samples were subjected to sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE), under non-reducing conditions, according to Laemmli [26]. The gel was silverstained using Gelcode according to the manufacturer's instructions (Pierce, Rockford, Il, U.S.A.).

Affinity chromatography

Several identical columns were prepared by pouring one ml of 1-3-1-Sepharose into adapted 5 ml syringes. Before sample application, the columns were equilibrated with buffer: 0.1 M Tris, 0.15 M NaCl, in the absence or presence of 0.1% (v/v) Tween 80, pH 7.5 (TBS and TBST, respectively). Thirty ml of human melanoma cell conditioned medium (in the absence or presence of 0.1% (v/v) Tween 80) was applied to such a column and non-bound material was washed away using TBS or TBST as indicated. Subsequently, elution buffer was applied and 1 ml fractions were collected. The fractions were assessed for the presence of t-PA antigen; and the t-PA containing fractions (usually the first three) were pooled and used for further analysis.

Buffers used for elution were TBS or TBST, both supplemented with varying concentrations (see below) of 6-AHA; 0.1 M Glycine, pH 2.5; and 1.0 M acetic acid, pH 3.0. When appropriate, the fractions were immediately neutralized with 1.0 M Tris, pH 8.5. After elution, the 1-3-1-Sepharose was collected from the syringes, equilibrated with TBS supplemented with 0.05% (w/v) NaN_3 and stored at 4 °C until further use.

The original cell-conditioned medium (defined as 100% or control), non-bound material and the pooled fractions were assessed for t-PA antigen and/or t-PA activity and/or total protein content as described above. Since 6-AHA was shown to have some inhibitory effect in the ELISA, the original cell-conditioned medium used as control and the standard purified t-PA used as calibrator were supplemented, when appropriate, with 6-AHA to the same concentration used for elution to correct for this effect.

RESULTS

Binding and yield; effect of Tween 80

Initial experiments with TBS as equilibration, wash and (supplemented with 0.1 M 6-AHA) elution buffer, showed that binding of t-PA to the 1-3-1-Sepharose was low (34% t-PA not bound), as was the yield of bound t-PA after elution (50% of bound t-PA). However, the addition of 0.1% (v/v) Tween 80 to both TBS and the cell- conditioned medium, improved the binding of t-PA to 1-3-1-Sepharose (all t-PA bound) as well as the yield after elution with 0.1 M 6-AHA (73% of bound t-PA). In further experiments 0.1% (v/v) Tween 80 was added to both the TBS and the sample.

Yield; effect of the 6-AHA concentration

The above results indicate that elution of t-PA from 1-3-1-Sepharose was not optimal (73%) with 0.1 M 6-AHA in TBST. Therefore, the effect of the 6-AHA concentration in the elution buffer was further examined. To this end, TBST was supplemented with 0.05, 0.1, 0.2, 0.3 and 0.4 M 6-AHA. We found that increasing 6-AHA concentrations had a marked effect on the yield of t-PA up to 89% at 0.2 M (Table 1). Increasing the 6-AHA concentration above 0.2 M, hardly further increased the yield of t-PA. In further experiments t-PA was eluted with TBST containing 0.2 M 6-AHA.

Table 1. Yield of t-PA antigen from 1-3-1-Sepharose upon elution with TBST supplemented with various 6-AHA concentrations.

[6-AHA] M	yield (% of bound)
0.00	0
0.05	48
0.10	69
0.20	89
0.30	91
0.40	94

Yield and purity; comparison with other elution buffers

To determine the total protein in the various samples, we first determined the efficiency of TCA precipitation for t-PA at low concentrations. To this end, the protein recovery

of standard purified t-PA at 1.0, 2.0 and 4.0 μg/ml in TBST after TCA precipitation was determined. In the pellets 34 ± 16%, 39 ± 8% and 54 ± 7% t-PA, respectively, was recovered (n = 5). Therefore, a correction factor of 2.2 was used for measured total protein levels.

The yield of t-PA (total protein and t-PA antigen) from 1-3-1-Sepharose with TBST containing 0.2 M 6-AHA, was compared with two other, frequently-used elution procedures; i.e. 0.1 M Glycine, pH 2.5; or 1.0 M acetic acid, pH 3.0. The yield of total protein or of t-PA antigen from the columns was virtually identical for all three elution buffers (Table 2). Apparently, TBST containing 0.2 M 6-AHA elutes t-PA as efficiently as methods that involve an extreme pH-change.

On multiple occasions, a single band was observed with all samples after SDS-PAGE at the position of t-PA, indicating that the present method yields a pure product.

Table 2. Purification of t-PA using 1-3-1-Sepharose in combination with three different elution buffers. Tested samples were: cell conditioned medium; non-bound material; fractions eluted with (E1) TBST containing 0.2 M 6-AHA, pH 7.5; (E2) 0.1 M Glycine, pH 2.5; and (E3) 1.0 M acetic acid, pH 3.0. The samples were tested for; total protein (corrected for partial protein recovery after TCA precipitation); t-PA antigen; and t-PA activity. From these data are derived: yield of t-PA antigen; yield of t-PA activity; specific activity as calculated on the basis of total protein; and specific activity as calculated on the basis of t-PA antigen. Means of two experiments.

sample	volume	total protein (corrected)	t-PA antigen	t-PA activity	yield (antigen)	yield (activity)	specific activity (protein)	specific activity (antigen)
	ml	μg/ml	μg/ml	IU/ml	%	%	IU/μg	IU/μg
medium	30	4.48	0.20	116	100	100	26	591
non-bound	34	4.64	0.01	<0.05			<1	<4
E1	3	1.71	1.86	1045	95.1	91.6	611	561
E2	3	1.75	1.72	783	87.6	67.5	447	456
E3	3	1.64	1.80	745	91.8	64.2	455	414

Specific activity; effect of elution buffer

The pooled fractions of t-PA obtained after elution of the columns with either TBST + 0.2 M 6-AHA, 0.1 M Glycine pH 2.5 or 1.0 M acetic acid pH 3.0 were also tested for t-PA activity levels. As mentioned earlier, the fractions contained comparable protein and

t-PA antigen levels. However, there was a marked difference between the fractions in the enzymatic activity of t-PA towards plasminogen. Both the samples purified using 0.1 M Glycine and 1.0 M acetic acid contained notably lower t-PA activity levels, as compared with the sample purified using TBST containing 0.2 M 6-AHA (Table 2). Approximately 25% of t-PA activity was apparently lost upon elution using a pH-change, as compared with TBST containing 0.2 M 6-AHA. The decreased enzymatic activity of the samples purified by a procedure involving an extreme pH-change, resulted in a markedly decreased specific activity (IU/μg) of t-PA, both when calculated on the basis of total protein and when calculated on the basis of t-PA antigen, as compared with the sample eluted by TBST containing 0.2 M 6-AHA (Table 2). The extreme pH-change presumably affects the enzymatic activity of t-PA.

The susceptibility of t-PA to a low pH was confirmed by measuring the t-PA activity of the cell-conditioned medium, brought to pH 2.5 for 2 hours and then neutralized. This treatment decreased the enzymatic activity to 37 IU/ml, which was approximately three-fold lower than the original conditioned medium (116 IU/ml).

DISCUSSION

We describe a method for purification of t-PA from human melanoma (Bowes) cell-conditioned medium, using an immobilized special monoclonal antibody (1-3-1), under mild elution conditions. The MoAb/t-PA complex dissociates under the influence of 6-AHA, either by competition between 6-AHA and the MoAb for the same (lysine) binding site on t-PA; or by induction of an unfavourable conformational change in t-PA; or, less likely, the MoAb [21]. We investigated whether the use of 6-AHA for elution was beneficial for the enzymatic activity of t-PA, since no chaotropic agents or extreme pH-changes are necessary for t-PA elution.

We found that the yield of t-PA from 1-3-1-Sepharose was improved by the addition of Tween 80 to both the sample and the buffer, probably as a result of the prevention of non-specific binding of t-PA to the column material, as reported by others [9,10,27]. Additionally, Tween 80 improved the specific binding of t-PA to 1-3-1, since binding of t-PA to 1-3-1-Sepharose increased after Tween 80 was added. This might be due to the possible unmasking of epitopes on t-PA which are partially cryptic to the MoAb.

Elution of t-PA protein from the immobilized MoAb with 0.2 M 6-AHA was as efficient as other, more standard procedures for MoAb/antigen dissociation by means of an extreme pH-change, since the yield of t-PA (total protein or antigen) from the 1-3-1-Sepharose collumn was virtually the same for all three elution buffers. Moreover, the elution of t-PA from 1-3-1-Sepharose with 0.2 M 6-AHA was apparently beneficial for t-PA, since the enzymatic activity of the t-PA in the eluted fractions was notably higher,

as compared with t-PA eluted by an extreme pH-change. The latter is possibly caused by (partial) denaturation of the t-PA molecule. The susceptibility of t-PA to low pH was confirmed by the strongly decreased enzymatic activity of a sample subjected to pH 2.5 for two hours. These data are contradictory to the reported high stability of t-PA in a pH-range from 2 to 5 [28], but confirm earlier observations by Rijken [29].

In the purified samples the amounts for total protein and t-PA antigen were virtually equal, suggesting that all protein in the eluted fractions is t-PA. This was confirmed by SDS-PAGE in which a single band was obtained, indicating that one-step purification of t-PA with 1-3-1-Sepharose yields a highly pure product. However, there was a markedly higher t-PA activity in the sample purified using 6-AHA, as compared with the samples purified using a pH-change. This suggests that part of the t-PA purified with the latter methods is inactive. This is reflected in the notably decreased specific activity of t-PA in these samples, as compared with the sample purified using 6-AHA. The specific activity of t-PA purified with 6-AHA, is in concordance with (even somewhat higher than) the specific activity of the presently used standard purified t-PA (i.e. 500.000 IU/mg). This illustrates that the present method for t-PA purification, using 1-3-1-Sepharose and TBST containing 0.2 M 6-AHA as elution buffer, is perhaps even superior over other methods.

Additionally, it has been reported that arginine, lysine and their derivatives and analogues (such as 6-AHA) improve t-PA solubility at high t-PA concentrations [30]. In our experiments we never reached t-PA concentrations high enough to verify this aspect. However, if the present method is applied to the large-scale purification of t-PA, with a high-capacity column and with larger volumes of cell-conditioned medium, this aspect could further add to the positive properties of the method described here, since 6-AHA is already present in the elution buffer.

The possibility of avoiding chaotropic agents or extreme pH-changes may also be beneficial to the immobilized MoAb. The column-life of the 1-3-1-Sepharose was good, since several runs (approximately 20) were performed without any indications of decreased capacity or yield. However, this number of runs is too low on which to draw definite conclusions.

One-step affinity chromatography for the purification of t-PA using MoAb 1-3-1 under mild elution conditions, was shown to be favourable for t-PA and is probably also beneficial for the immobilized MoAb. The high specific activity of the purified t-PA, combined with the other advantages of using 6-AHA in the eluent (e.g. increased solubility) further add to the potential of the present procedure for the large-scale purification of t-PA from conditioned culture medium.

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CHAPTER 5

A ONE-STEP ENZYME IMMUNOASSAY FOR THE DETERMINATION OF TOTAL TISSUE-TYPE PLASMINOGEN ACTIVATOR (t-PA) ANTIGEN IN PLASMA

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ABSTRACT

A reproducible and sensitive one-step enzyme immunoassay (EIA) was developed to determine total tissue-type plasminogen activator (t-PA) antigen in plasma. The EIA comprises of two monoclonal catching antibodies and a polyclonal (goat) tagging antibody conjugated with horse-radish peroxidase. There is an equal reactivity towards the several physiological t-PA forms, i.e. single-chain t-PA, two-chain t-PA and t-PA in complex with its naturally occurring inhibitor plasminogen activator inhibitor-type 1 (t-PA/PAI-1 complex). Additionally, the EIA does not discriminate between human melanoma t-PA and recombinant t-PA (Activase). The assay has a lower detection limit of approximately 0.5 ng t-PA per ml plasma, with a time-to-result of only 3.5 hours.

INTRODUCTION

In a properly functioning haemostatic balance, there will be an equilibrium between the coagulation system and the fibrinolytic system. Activation of the coagulation system yields active thrombin, which converts the soluble plasma protein fibrinogen via a multi-step process to insoluble crosslinked fibrin, the protein matrix of a blood clot. Activation of fibrinolysis yields active plasmin, which degrades fibrin to soluble fibrin degradation products, and thereby disintegrates the clot (for review see (1)). Tissue-type plasminogen activator (t-PA) plays a pivotal role in the fibrinolytic system since it activates plasminogen to plasmin. This process is highly accelerated in the presence of fibrin, which therefore acts both as a substrate and as a cofactor of the fibrinolytic system. Disturbances of the haemostatic balance may, amongst others, be caused by an over-active fibrinolysis (which causes bleeding) or by a defective fibrinolysis (which causes thrombosis). t-PA is released into the blood by endothelial cells. This release can be strongly increased by stimuli such as exercise, stressfull conditions, venous occlusion and by the vasopressin analogue DDAVP (2). In thrombotic patients the ability of the vessel wall to respond to such stimuli is often found to be decreased (3).

Tissue-type plasminogen occurs in two forms i.e. a single-chain (sc-t-PA) and a two-chain (tc-t-PA) form. Both forms are active, and sc-t-PA can be converted to tc-t-PA by plasmin. Both forms are inactivated by naturally occurring protease inhibitors. The most important t-PA inhibitor is plasminogen activator inhibitor type one, PAI-1 (4,5). Thus, t-PA activity is not stable in blood and in plasma, and this may be the reason for the problems encountered in the assessment of t-PA activity in patient samples. Assays which determine total t-PA antigen circumvent these problems and have been shown to be clinically usefull (3,6).

Several assays for the quantification of t-PA antigen have been described. These include immunoradiometric assays (7-9), radioimmunoassays (10,11) and enzyme immunoassays (EIAs) based on only polyclonal antibodies (12-15), on combinations of polyclonal and monoclonal antibodies (16,17) and on two or more monoclonal antibodies (18-21).

Here we describe a novel one-step sensitive enzyme immunoassay, based on a mixture of two anti-t-PA monoclonal antibodies as the solid phase antibodies and a horse-radish peroxidase (HRP) conjugated IgG fraction of a polyclonal antiserum against t-PA as the tagging antibody.*

MATERIALS AND METHODS

Microtiter plates (Immulon, Dynatech) were purchased from Greiner, Alphen a/d Rijn, the Netherlands; 3,3',5,5'-tetramethylbenzidin (TMB) from Fluka (Buchs, Switzerland); N-succinimidyl-3-(2-pyridyl-dithio)propionate (SPDP), protein A Sepharose, protein G Sepharose, Sephacryl S-200 and CNBr activated Sepharose 4B from Pharmacia (Uppsala, Sweden); dimethylsulfoxide (DMSO) was bought from Baker Chemical Company (Philipsburg, USA). Casein was bought from Merck (Amsterdam, The Netherlands); horse-radish peroxidase (HRP, grade II) from Boehringer (Mannheim, FRG); plasmin from KabiVitrum (Stockholm, Sweden); and aprotinin (Trasylol) from Bayer (Leverkusen, Germany). t-PA (specific activity 500,000 IU/mg) was a gift from Dr. J. Verheijen of this institute and had been purified from Bowes melanoma cells according to Rijken et al. (22) as modified by Kluft et al. (23). Recombinant one-chain t-PA (r-t-PA; Activase) was from Genentech (San Francisco, USA).

Phosphate-buffered saline/Tween (PBST) was prepared by dissolving 1.4 g Na_2HPO_4, 0.215 g KH_2PO_4, 8.75 g NaCl and 0.5 ml Tween 20 in 1 liter of distilled water. TMB/H_2O_2 substrate solution was prepared as follows: 100 μl of a 42 mM solution of TMB in DMSO was added to 10 ml of a 0.1 M sodium acetate/citric acid buffer, pH 6.0 under constant agitation. Just before use 1.5 μl H_2O_2 (30% w/v) was added.
PBST/EDTA buffer was prepared by adding EDTA to PBST to a final concentration of 0.01 M.

CPBS buffer was prepared by adding a 0.01 M NaH_2PO_4 solution containing 0.15 M NaCl and 0.01 M sodium citrate to a 0.01 M Na_2HPO_4 solution containing 0.15 M NaCl and 0.01 M sodium citrate until the pH was 7.4.

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*Footnote: The assay will be available soon from Organon Teknika, Veedijk 58, 2300 Turnhout, Belgium.

Antibodies

Monoclonal antibodies (MoAb) t-PA7-8-4 and t-PA10-1-3 (from here on identified as OT.07 and OT.10, respectively) were selected from a panel of MoAbs which will be described in detail elsewhere (24). MoAbs OT.07 and OT.10 were chosen, since they were equally reactive, in purified systems, with sc-t-PA, tc-t-PA and t-PA/PAI-1 complexes. MoAbs OT.07 and OT.10 are both of the IgG_1 subclass, and have closely related epitopes. They were purified from ascites fluid by chromatography on protein A Sepharose according to Ey et al. (25).

Polyclonal antibodies against t-PA (G-α-t-PA) were elicited in goats, and purified on protein G Sepharose according to the manufacturers instructions. Elution was done with 0.1 M glycin, pH 2.5 containing 0.01% (w/v) sodium azide; followed by immediate neutralization using 2.0 M Tris added to the collection tubes.

Conjugation of G-α-t-PA antibodies

Purified G-α-t-PA antibodies were conjugated with HRP essentially as described in the Pharmacia booklet on SPDP, based on the method of Carlsson (26). The conjugate was purified from contaminating free HRP by passing the conjugation mixture over a Sephacryl S-200 column (150 x 1.2 cm) run in 0.3 M NaCl. The conjugate-containing fractions were pooled and kept frozen at -80°C. The conjugate (G-α-t-PA/HRP) was diluted with PBST/EDTA containing 0.1% (w/v) of casein to the proper concentration prior to use.

Coating of microtiter plates

Aliquots of 135 μl of a mixture of MoAb OT.07 (2 μg/ml) and OT.10 (8 μg/ml) in 0.04 M Tris, pH 7.4 to give a total MoAb concentration of 10 μg/ml were pipetted into the wells of microtiter plates. After incubation overnight at 4°C, the plates were emptied and washed with 0.04 M Tris, pH 7.4.

Conversion of sc-t-PA to tc-t-PA

sc-t-PA, 3.9 μg/ml in 0.1 M Tris, 0.1% Tween 80, pH 7.6, was incubated with plasmin (final concentration 0.1 CU/ml) or buffer (control) for 15 minutes at 37°C. Then aprotinin was added to a final concentration of 3520 KIU/ml. Conversion was determined as described by Verheijen et al. (27).

Calibration material

Purified G-α-t-PA antibodies were immobilized to CNBr-activated Sepharose 4B according to the manufacturers instructions. The column was equilibrated with CPBS buffer. Two ml portion of pooled normal plasma was applied to this column at a rate of 1 ml/hr; fractions of 100 μl were collected. The fractions with the highest absorbance at

280 nm (and free of t-PA antigen as assessed by the EIA procedure, described below) were pooled and designated as t-PA depleted plasma. To the t-PA depleted plasma, pure sc-t-PA was added to give a final concentration of 20 ng/ml. This depleted and reconstituted plasma is the calibrator for the EIA.

EIA procedure

A five-fold dilution of the calibrator is made in PBST/EDTA. Subsequently also 10-, 20-, 40-, and 80-fold dilutions are made by serial two-fold dilution in PBST/EDTA. Test samples are routinely diluted 5-fold in PBST/EDTA, but a 2.5-fold dilution is acceptable for samples with a (suspected) low t-PA antigen level. The blank is PBST/EDTA. Aliquots of 50 μl of diluted G-α-t-PA/HRP are pipetted into the wells of the MoAb OT.07/OT.10 coated wells of the microtiter plates. Then 50 μl aliquots of the serially diluted calibrator, the blank and the diluted samples are added. Thus, the final dilutions of the calibrator are 10-, 20-, 40-, 80- and 160-fold, and those of the samples 10-fold (5-fold).

After incubation of the plates for 3 hours at room temperature in the dark, the wells are washed 4 times with PBST and tapped dry. Aliquots of 100 μl TMB/H_2O_2 substrate are then added to the wells and the plates are incubated for 30 minutes at room temperature. A blue colour develops, which turns to yellow when the reaction is stopped by addition of a 100 μl aliquot of 1 M H_2SO_4 to each well. The absorbance at 450 nm is read using a multichannel spectrophotometer (Multiskan, Flow Laboratories Ltd., Ayrshire, Scotland).

A calibration curve is made by plotting the absorbances at 450 nm against the final t-PA concentrations in the calibrator (i.e. 2, 1, 0.5, 0.25 and 0.125 ng/ml). The t-PA concentrations in the samples are read from this curve.

RESULTS AND DISCUSSION

The MoAbs used in this EIA were selected from a panel of MoAbs prepared against t-PA. The MoAbs OT.07 and OT.10 were chosen since they appeared to fullfil an important specificity requirement for the EIA which we planned to develop i.e. an equal reactivity with sc-t-PA, tc-t-PA and t-PA/PAI complexes. As will be described in detail elsewhere (24), the MoAbs OT.07 and OT.10 have similar specificities towards t-PA. In competition assays they are even mutually exclusive. Therefore it was somewhat surprising to find that a combination of OT.07 and OT.10 in the solid phase of our EIA yielded higher responses than each separately, i.e. plates coated with 10 μg/ml of either OT.07 or OT.10 showed a lower response than plates coated with a mixture of the two, containing a total concentration of 10 μg MoAb/ml. Optimum results were obtained with

a mixture of 8 μg OT.10/ml and 2 μg OT.07/ml (total concentration of 10 μg MoAb/ml) (Fig. 1).

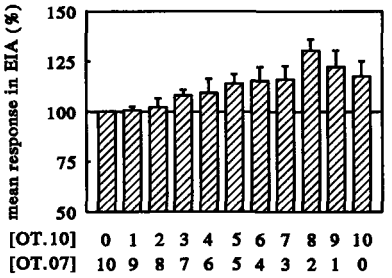

Figure 1. Mean response of 4 different concentrations of sc-t-PA in the EIA coated with OT.07 and OT.10 at different ratios. The response on the plates coated with OT.07 alone was defined as 100%.

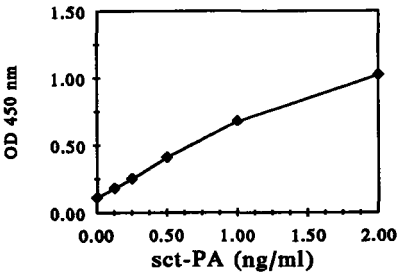

Figure 2. Dose-response curve for the present t-PA antigen assay.

Others have found that the use of EDTA in EIAs for t-PA increases the response (7,10,13). This phenomenon has been ascribed to unmasking of t-PA, which may be partially cryptic to antibodies in plasma (8). We made the same observation, and therefore 10 mM EDTA was added to all incubation buffers used in our EIA.

In order to keep the matrix of the calibrator and that of the samples comparable, we preferred to use plasma to which pure sc-t-PA was added, as a calibrator. Since, however, normal plasma already contains an unknown level of t-PA antigen, it was necessary to remove all t-PA antigen first by immunoadsorption on a column of immobilized G-α-t-PA antibodies, and then adding a known amount of purified t-PA.

A typical dose-response curve for the t-PA EIA is shown in Fig. 2.
As can be seen concentrations down to 0.1 ng t-PA/ml in the test are readily measurable.

The specificity of the EIA for t-PA and its various forms was assessed. First, sc-t-PA or tc-t-PA, both in aprotinin-containing buffer to prevent plasmin-mediated conversion of sc-t-PA, were added to normal plasma and tested in the present EIA. The dose-response curves are not significantly different (Fig. 3), indicating equal reactivity towards sc-t-PA and tc-t-PA. The higher response of the two t-PA-spiked samples, as compared with the calibrator, is due to the t-PA antigen level present in normal plasma. In another experiment, two concentrations (4.55 and 45.5 ng/ml) of each of sc-t-PA, tc-t-PA or r-t-PA were added to a plasma (depleted from t-PA by immunoadsorption) with a low concentration of PAI-1 (approximately 2 IU/ml; 1 IU PAI-1 is capable to inactivate 2 ng t-PA); a plasma with a normal PAI-1 concentration (approximately 7 IU/ml); and a plasma with a high PAI-1

concentration (approximately 13 IU/ml), and incubated for 16 hours at 37°C. Thus the ratio of free t-PA over t-PA/PAI-1 complex varies between all samples. The t-PA concentrations were measured against the calibrator in the EIA and recoveries of the two different concentrations of the various forms of t-PA in the three plasma's was determined. In all cases virtually all t-PA was recovered (Fig. 4) showing that r-t-PA, tc-t-PA, sc-t-PA and t-PA/ PAI-1 (and possibly other t-PA/inhibitor complexes) are detected equally well.

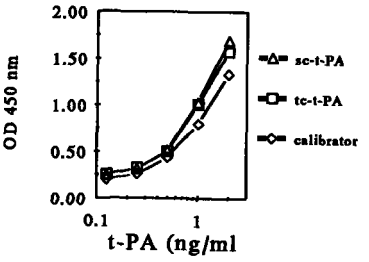

Figure 3. Dose-response curves for calibrator, sc-t-PA and tc-t-PA in the present EIA.

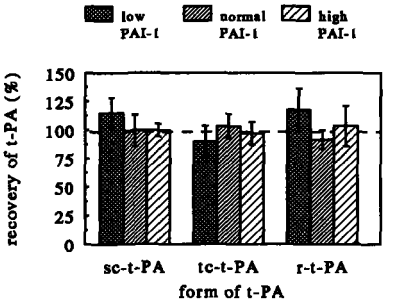

Figure 4. Recovery of various forms of t-PA in plasma containing low, normal and high PAI-1 levels.

In a panel of 78 plasma samples with a range of t-PA concentrations the correlation between the values found with our EIA and that from Biopool (IMULYSE tPA) was assessed. The results are shown in Fig. 5. The correlation between the two assays is good i.e. the R-value is 0.73 over all samples. There is an approximately 30% bias towards higher values in the Biopool assay, which may be due to different calibrators used.

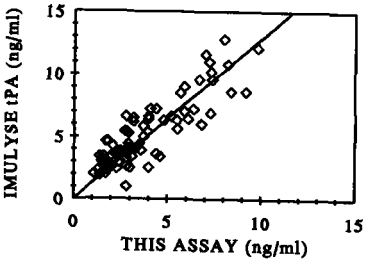

Figure 5. Correlation between plasma samples measured for t-PA antigen using this assay and the IMULYSE tPA assay. The line obtained after linear regression has as R-value 0.73 and follows the equation Y = 1.29 X.

We also assessed the within- and between-run variability of the assay.

The within-run variability was determined at two different calibrator concentrations i.e. 1 ng/ml and 0.5 ng/ml in six-fold in the test, on four different days. The CV values, as determined on the absorbance at 450 nm, ranged from 2.8% to 7.5% and 3.4% to 9.4% for 1 ng/ml and 0.5 ng/ml, respectively.

The between-run variability was determined from 16 independent dose-response curves of the calibrator (like the one shown in Fig. 2). The CV values were 5.4%, 4.5%, 6.8%, 6.1%, 7.3% and 4.3% for the 5-, 10-, 20-, 40-, and 80-fold diluted calibrator and the blank, respectively.

In conclusion: we have developed a highly reproducible one-step EIA with a time-to-result of only 3.5 hours, to determine total t-PA antigen in plasma. The lower detection limit of the assay is in the order of 0.5 ng t-PA/ml plasma.

No dilution curves using normal goat serum as in the IMULYSE tPA assay (28) need to be run. The only other one-step EIA similar to that described here is to our knowledge that described by Kaizu et al. (18). The latter is based on plates coated with a monoclonal antibody and another monoclonal antibody against t-PA conjugated with HRP as the tagging antibody. That EIA, however is about two-fold more reactive with tc-t-PA than with sc-t-PA, it is somewhat less sensitive than our EIA (i.e. the lower detection limit is 1 ng/ml) and it takes 4.5 hours.

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CHAPTER 6

ENHANCED BINDING OF t-PA TO FIBRIN USING BISPECIFIC MONOCLONAL ANTIBODIES

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ABSTRACT

Using somatic cell fusion, we produced a bispecific monoclonal antibody with affinity for both fibrin and t-PA. This bispecific MoAb was made with a view to enhance the efficacy of t-PA in thrombolytic therapy, since the bispecific MoAb increases the binding affinity of t-PA for fibrin, and may simultaneously decrease the clearance rate of t-PA *in vivo*. *In vitro* tests show, that in the presence of the bispecific MoAb, a ten- to twentyfold lower concentration of t-PA was needed to obtain the same amount of t-PA binding to fibrin coated wells, as with t-PA alone. The second possible effect of the bispecific MoAb, i.e. the increased half-life of t-PA *in vivo*, is under investigation.

INTRODUCTION

Tissue-type Plasminogen Activator (t-PA) is frequently used in thrombolytic therapy [1]. It is, however, rapidly neutralised by clearance in the liver [2] and/or by a fast-acting t-PA inhibitor (PAI-1) [3]. Therefore high dosages of t-PA have to be administered to obtain efficient reperfusion of the occluded blood vessel. Combined with the limited thrombus (i.e. fibrin) specificity of t-PA, the high dosages of t-PA can give rise to adverse side effects.

One of the side effects is the systemic activation of free plasminogen. The plasminogen in plasma is converted to plasmin, resulting in a rapid decline in the amount of circulating α2-anti-plasmin, a major down regulator of plasmin activity. Plasmin is a serine protease with a broad substrate specificity, and free circulating plasmin will, amongst others, degrade coagulation factors such as fibrinogen (coagulation factor I) and factors V and VIII [1 & 4]. It is important that plasminogen activation takes place only at the thrombus. Furthermore, there are reports that plasmin also has an effect on platelet aggregation [5] and complement activation [6]. All this may contribute to a higher risk for complications during thrombolytic therapy.

Reducing the required effective dose of t-PA, by increasing its fibrin affinity and specificity and/or by decreasing its clearance rate, could alleviate these t-PA induced systemic lytic effects [7, 8, 9, 10 & 11].

Several investigators reported on the use of bispecific monoclonal antibodies (MoAbs) for the site-specific delivery of drugs (i.e. drugs targeting) [12, 13 & 14]. Bispecific MoAbs combine the antigen-binding sites of two different MoAbs; e.g. one for the target, the other for the drug (Fig. 1) [15]. Thus the binding of the drug to its carrier (the bispecific MoAb) is predefined and uniform. We aim to use such bispecific MoAbs to target t-PA to fibrin (i.e. the thrombus) in an attempt to enhance the efficacy of t-PA in thrombolytic therapy.

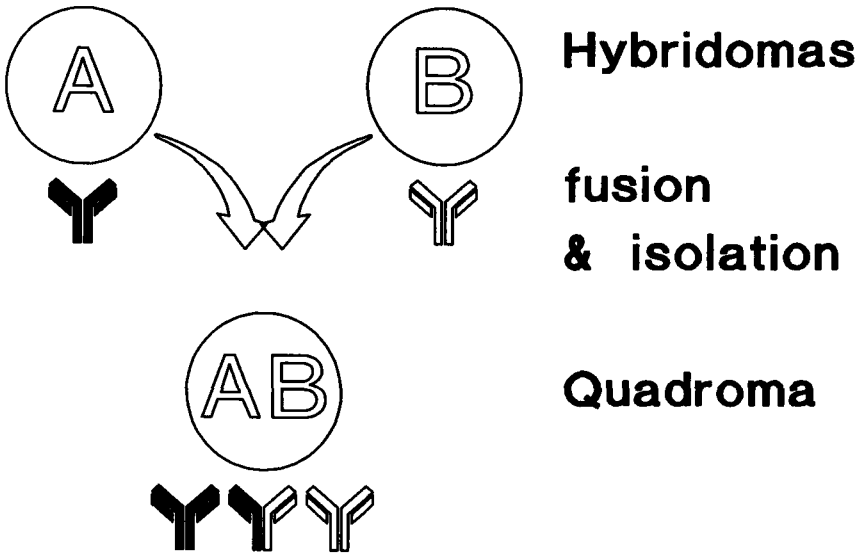

Figure 1. Outline for the production of bispecific MoAb producing cell lines (quadromas).

We produced a special bispecific MoAb by fusion of two selected hybridoma cell lines; the anti-fibrin producing cell line "Y22" [16], with an anti-t-PA producing cell line "tPA 1-3-1" with special properties [17]. Binding t-PA to this bispecific MoAb increases, as we will show, the affinity of t-PA for fibrin (i.e. its target), and may simultaneously reduce the clearance rate of t-PA.

MATERIALS AND METHODS

Hybridoma cell lines

- Y22-HATs; a hybridoma which produces MoAbs specific for fibrin, the major constituent of blood clots [16]. The Y22 hybridoma cell line was back-selected for the sensitivity to hypoxantine, aminopterin and thymidine (HATs) by selection for mutants resistant to the toxic purine analogue 6-thioguanine [18].
- tPA 1-3-1 and tPA 7-8-4; hybridomas which produce MoAbs specific for t-PA [17]. The tPA 1-3-1 cell line produces MoAb's against tPA that quench the receptor mediated uptake of tPA by liver endothelial cells and, to a lesser extent, the receptor mediated uptake by liver parenchymal cells (with 60% and 20% respectively) [19]. Plasminogen activation remains unaffected. The tPA 7-8-4 produces MoAbs that bind to a different epitope on t-PA than tPA 1-3-1 [17].

- Cell culture (in Dulbecco's modified Eagle's medium with 10% fetal calf serum and the standard additives (DMEM+FCS)), and ascites production were done as described [20].

Fibrin coated magnetic beads

For the preparation of fibrin coated magnetic beads, tosyl activated Dynobeads (Dynal A.S., Norway) were incubated with fibrinogen (150 μg/ml) according to the manufacturers instructions. Covalently bound fibrinogen was converted to fibrin by incubation with 1 NIH unit thrombin/ml 0.15M NaCl, 30 minutes at 37°C.

Cell fusion and quadroma isolation

Y22-HATs cells and tPA 1-3-1 cells were mixed (5 x 10^6 of each) and fused in PEG 1500 using standard procedures [21]. After fusion, the cells were diluted in 30 ml of a selection medium (HAT containing DMEM+FCS) and divided over two cell-culture flasks. In this selection medium, non-fused Y22-HATs cells are killed. The remaining cells are non-fused tPA 1-3-1 cells and quadromas. After 72 hours, the cells were collected, washed twice with ice cold PBS, and pelleted in the presence of fibrin coated magnetic beads, and kept on ice for 60 minutes. Anti-fibrin producing cells (i.e. quadromas) bind to these magnetic beads and form rozets. These can be separated from non-rozetted cells using a magnet. The rozetted cell fraction was plated in two 96-well plates and screened after 10 to 14 days for the production of active MoAbs using three different EIAs (see below).

EIAs

I The screening for the presence of anti-fibrin activity was done using the following procedure. Microtitre plates were coated with 10 μg fibrinogen/ml phosphate buffered saline (PBS) pH 8.0. After overnight incubation at 4°C, the plates were washed and the adsorbed fibrinogen was converted to fibrin by incubation with 1 NIH unit thrombin/ml 0.15 M NaCl for 30 minutes at 37°C. After washing the plates, diluted cell culture medium was added to the wells and incubated. Subsequently the plates were washed and bound MoAbs were quantified by incubation with horseradish peroxidase-conjugated goat-anti-mouse-Ig (GAMPO), and conversion of the peroxidase substrate TMB/H_2O_2.

II The screening for the presence of anti-t-PA activity was carried out as follows. Microtitre plates were coated with 250 ng t-PA/ml 0.05 M TRIS pH 9.4. After overnight incubation at 4^0C, the plates were washed and diluted cell culture medium was added to the wells and incubated. Subsequently the plates were washed and bound MoAbs were quantified as described above.

III The screening for the presence of bispecific activity was carried out as follows. Fibrin plates were prepared as described above, and the cell culture fluid was diluted, and

incubated in the fibrin coated plates. After washing, t-PA (100 ng/ml) was added. After incubation, the plates were washed, and bound t-PA was quantified by incubation with horseradish peroxidase-conjugated anti-t-PA 7-8-4 (7-8-4-PO), and conversion of TMB/H_2O_2.

In all three EIAs PBS pH 7.4 + 0.1% Tween 20 (PBST) was used as washing and dilution buffer. All incubations were carried out for 45 minutes at room temperature. The conjugates were diluted in PBST + 0.1% bovine serum albumin.

Purification of bispecific MoAbs

The MoAbs, produced by the quadromas, were first purified from ascites on Protein A-Sepharose [22]. Purified IgG (IgG-MIX) was, after neutralisation to pH 7.4, passed over a column of fibrin immobilised to Sepharose. Non-bound material was collected for further analysis (WASH I). Bound IgG was eluted with 0.1 M Glycine pH 2.5, followed by immediate neutralisation of the fractions with a few drops of 2 M TRIS pH 8.9. This material (ELUTE I) was passed over a column of t-PA immobilised to Sepharose. Non-bound IgG was collected (WASH II) and bound IgG was eluted as described above (ELUTE II). The IgG-MIX, WASH I and II, and ELUTE I and II fractions were analyzed for antigen specificity using the three EIAs described above.

Effect of bispecific MoAbs on the binding of t-PA to fibrin, using different quantification techniques

A t-PA solution was made (final concentration as required per experiment) in 0.1 M TRIS + 0.1% Tween 80, pH 8.0 (TTB) in the presence or absence of an equimolar amount of purified bispecific antibodies, and pre-incubated for 30 minutes at room temperature.

Fibrin plates were prepared as described above, and the pre-incubated t-PA solutions were incubated in serial dilutions in TTB on these plates for 2 hours. Non-bound material was removed by washing with TTB, and the amount of bound t-PA was determined using different methods:

- Bound t-PA protein was determined using tPA 7-8-4-PO or a polyclonal anti t-PA conjugate (GatPA-PO), both diluted in TTB + 0.1% BSA, followed by the conversion of TMB/H_2O_2 as in the EIA described above.
- Bound, active t-PA values were determined by adding either the t-PA specific chromogenic substrate S2288 in TTB (amidolytic activity), or by adding plasminogen and the plasmin chromogenic substrate S2251 in TTB (plasminogen activation) [23]. A calibration curve of t-PA activity was used to assess the amount of bound t-PA. For amidolytic activity, the increase in A_{405} per hour, and for plasminogen activation, the increase in A_{405} per hour squared, was calculated.

Isolation of quadromas

After fusion and the two selection procedures, most of the rozetted cells produced MoAbs with only anti-tPA activity. Only three clones (out of 6 fusion experiments) were found that produced MoAbs with bispecific activity, i.e. the overall frequency is less than 1 quadroma per 10^7 parent cells. One clone, designated as Q6-3, was selected for its relatively high titre in the EIA for bispecific activity, expanded and recloned. All subclones produced bispecific antibodies, indicating the genetic stability of the quadromas. One subclone, designated as Q6-3-8, was further expanded and ascites was produced.

Purification of bispecific MoAbs

The quadroma cells produce, besides the desired bispecific MoAb, several functionally different types of MoAb's. This is explained by the random heavy-light and heavy-heavy chain association during IgG assembly in the cell. Functional bispecific MoAbs were purified by sequential immuno-affinity chromatography on immobilised fibrin and immobilised t-PA. The ELUTE II fraction contains MoAbs which bind to both t-PA and fibrin (Figs. 2a and 2b). This fraction consists of 10-15% of the total IgG present in the ascites produced by Q6-3-8. The other fractions (WASH I and WASH II) contain material with a binding capacity for only one antigen, or are still contaminated with such material (IgG-MIX and ELUTE I).

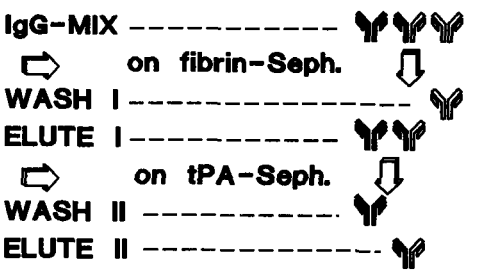

Figure 2a. Schematic representation of the purification of bispecific MoAbs.

	t-PA	fibrin	bispecific
IgG-MIX	+	+	+
WASH I	+	-	-
ELUTE I	+	+	+
WASH II	-	+	-
ELUTE II	+	+	+

Figure 2b. Reactivity of the fractions obtained after sequential immuno-affinity chromatography of the bispecific MoAbs.

Effect of bispecific MoAbs on the binding of t-PA to fibrin

The effect of the bispecific MoAbs on the affinity of t-PA for fibrin was measured using different quantification techniques. In the presence of purified Q6-3-8 MoAbs, a

10 to 20 fold lower concentration of t-PA was needed to obtain the same amount of binding to a fibrin coating, as compared with free t-PA. This was not only the case for t-PA antigen (Figs. 3a and 3b), but also for t-PA activity (Figs. 4 and 5). The difference in the amount of incubated t-PA, needed for measurable values, is a result of the different sensitivities of the used quantification techniques.

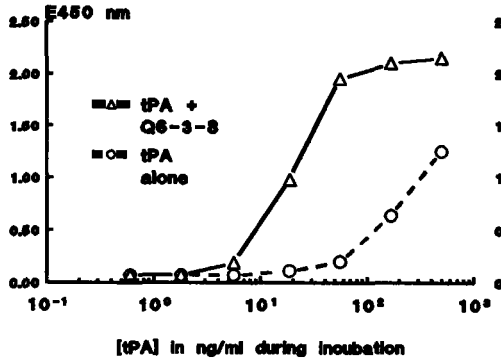

Figure 3a. Effect of bispecific MoAb Q6-3-8 on the binding of t-PA antigen to a fibrin coating, assessed using tPA7-8-4-PO.

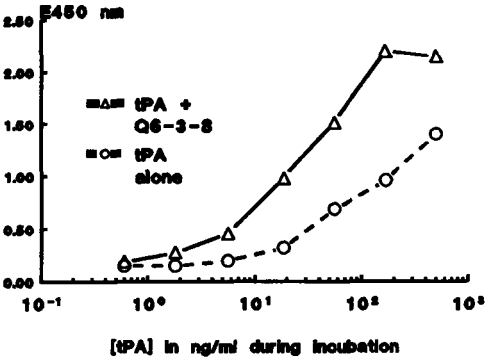

Figure 3b. As Fig. 3a, assessed using GatPA-PO.

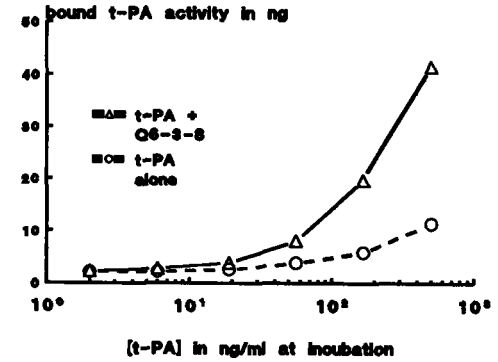

Figure 4. Effect of bispecific MoAb Q6-3-8 on the binding of t-PA activity to a fibrin coating, assessed for amidolytic activity.

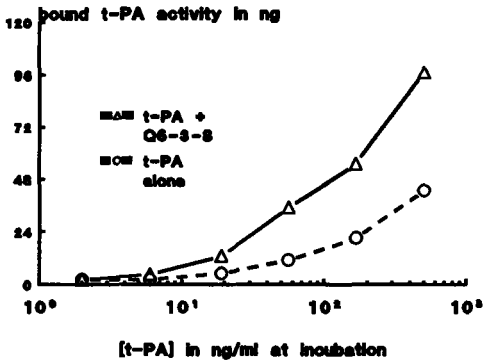

Figure 5. Effect of bispecific MoAb Q6-3-8 on the binding of t-PA activity to a fibrin coating, assessed for plasminogen activation.

DISCUSSION

After fusion of two hybridoma cell lines, quadromas were isolated for HAT resistance and the ability to bind to immobilised fibrin. The first selection procedure is fail-safe since all Y22-HATs parent cell die in the selective HAT medium within 2 days. Control experiments, with non-fused "Y22-HATs" and "tPA 1-3-1" hybridomas that did not undergo HAT selection, showed that selection for the presence of membrane bound anti-fibrin activity was not very effective. The occurrence of "false-positive" cells (i.e. anti-t-PA producing cells in the rozetted fraction), and "false-negative" cells (i.e. anti-fibrin producing cells in the non-rozetted fraction), was very high (results not shown). This could explain the observed high occurrence of monospecific (anti-t-PA) MoAb producing cells, and indicates the importance of a good post-fusion selection method.

Several authors speculate on the preferential pairing of homologous light and heavy chains [15, 24 & 25]. The synthesised heavy and light chains combine for the assembly into mature (4-chain) immunoglobulins. This gives, theoretically, 10 different types of IgG. If chain assembly occurs at random (scrambled), 12% will be bispecific. The yield of functional bispecific MoAbs we find, 10 - 15% (in ELUTE II) from the sum of IgG produced by the quadroma cells, concurs with this theoretical value. Provided that all four heavy and light chain genes in the Q6-3-8 cells are expressed with an approximately equal efficiency, this indicates that there is no preferential pairing of light chains with their homologous heavy chains in our cells.

We found that the bispecific MoAbs enhanced the fibrin affinity of t-PA ten- to twentyfold. It is important to note that our system of measuring the effect of the bispecific MoAbs on the binding of t-PA to fibrin, is not physiological. The fibrin coating can best be described as a two dimensional monolayer of fibrin molecules. The three dimensional configuration, and perhaps different properties of fibrin in a blood clot, could modify the results. Further experiments, using more physiological models, should answer the question whether the observed enhanced fibrin affinity of tPA inferred by Q6-3-8 also occurs in a blood clot system.

The experiments done so far, only measure the contribution of the bispecific MoAb to an enhanced fibrin affinity *in vitro*. The effect *in vivo* may be augmented by the possible lower clearance rate of t-PA when it is bound to this particular bispecific MoAb, since the tPA 1-3-1 moiety decreases the receptor mediated uptake of liver cells. A recent report on *in vivo* clot lysis experiments with t-PA deletion mutants, that have a lower clearance rate (but also a lower fibrin affinity), show that they improve reperfusion rates and diminish re-occlusion, with a reduced consumption of coagulation factors [9].

We are now in the process of isolating other quadroma's, using a more efficient procedure for quadroma selection, from MoAbs with specific effects on t-PA and fibrin, to compare the efficacy of different types of bispecific MoAbs in clot lysis [26].

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CHAPTER 7

ENHANCED TRANSFECTION OF A BACTERIAL PLASMID INTO HYBRIDOMA CELLS BY ELECTROPORATION; APPLICATION FOR THE SELECTION OF HYBRID HYBRIDOMA (QUADROMA) CELL LINES

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ABSTRACT

A procedure was investigated to transduce a bacterial plasmid containing a specific drug resistance marker (pSV2-neo), into a hybridoma cell line using electroporation. The effect of several buffers and the form of plasmid DNA (circular or linearized) on the stable transfection frequency were examined.

When complete cell culture medium (DMEM) was used as electroporation buffer, we observed a two-fold increase in post-pulse viability and a ten- to thirty-fold increase in the transfection frequency of pSV2-neo, as compared with HEPES buffered 0.15 M sodium chloride. Supplementing DMEM with fetal bovine serum (DMEM+FBS) had some beneficial effect on post-pulse viability of the cells after electroporation, but did not markedly increase stable transfection frequency as compared with DMEM alone.

Furthermore, with DMEM+FBS, the intact plasmid was transfected as effectively as linearized PSV2-neo. However, when using HEPES buffered saline, the transfection frequency of pSV2-neo increased two-fold after linearization as compared with intact plasmid.

The drug resistance was used successfully as a marker for the selection of hybrid hybridoma (quadroma) cell lines after fusing two different hybridoma cell lines, producing anti-fibrin and anti-plasminogen activator antibodies respectively. The quadroma cells produced bispecific antibodies that are capable of accumulating plasminogen activator on a fibrin surface.

INTRODUCTION

In our studies to improve the efficacy of tissue-type plasminogen activator (t-PA) as a thrombolytic agent, we investigated the possibility of using a thrombus (i.e. fibrin) specific monoclonal antibody (MAb) as a means for the site-specific delivery of t-PA at the occluding blood clot (drugs targeting) [1, 2, 3, 4]. For this purpose, a stable association of drug (i.e. t-PA) and carrier (i.e. anti-fibrin MAb) is required. Several investigators have reported on the use of bispecific monoclonal antibodies (bs-MAb) for the targeting of drugs [5, 6, 7]. Bispecific antibodies combine the antigen-binding sites (Fab'-fragments) of two different antibodies; one specific for the target (e.g. fibrin), the other for the drug (e.g. t-PA).

Molecules with bispecific properties can be prepared by cross-linking two intact antibodies [8]. Smaller, more adequate molecules for *in vivo* purposes, can be prepared by the chemical conjugation of (separately prepared) Fab'-fragments from two different MAb [9, 10]. However, the disadvantages of this method are potential loss of activity and batch to batch variation. To avoid these disadvantages, we have chosen the method of

somatic cell hybridization, that involves the fusion of two hybridoma cell lines, each secreting a MAb of the desired specificity. This will result in a cell type, called quadroma, that produces MAb with bispecific properties [11, 12]. bs-MAb can thus be produced in large quantities of constant quality. Our aim is to prepare a bs-MAb, reactive with both fibrin and t-PA, and investigate its effect on the efficacy of t-PA in clot lysis.

Obviously, the post-fusion selection procedure, that isolates the desired quadroma cells from the mass of non-fused (or incorrectly fused) cells, is crucial. To date, three methods are frequently used. In one method each of the two cell lines is first labelled with a different fluorescent dye, followed by a post-fusion selection for double-stained cells using a fluorescent-activated cell sorter (FACS) [13, 14]. A second procedure uses irreversible biochemical inhibitors [12, 15]. In this method, each cell line is treated with a lethal dose of a complementary agent (i.e. destroying different parts of the cell metabolism). Fused cells can survive since they are metabolically complete. A third method involves two genetically controlled drug resistance markers in the hybridoma cells. Quadroma cells are subsequently selected by culture in a selective medium containing both drugs [16, 17, 18]. This method appeared the most attractive.

We searched for a method that could transduce a specific drug resistance to hybridoma cells with a relatively high frequency. Recent publications have described the transfection of bacterium derived plasmid deoxyribonucleic acid (DNA), containing a marker-gene, into mammalian cells by electroporation. Stable transfection of the plasmid into a cell (i.e. integration in the eukaryotic genome and expression of the coded gene) will permanently confer the drug resistance to this cell [19, 20]. The mechanisms of DNA transfer into the genome during electroporation are still poorly understood [21]. Others have found that the electroporation buffer in which the cells are pulsed and the form of the plasmid DNA (circular or linearized) are crucial [17, 22, 23, 24, 25, 26, 27, 28, 29, 30].

We, therefore, investigated what conditions are favorable for the stable transfection of hybridoma cells using electroporation. We then applied the transduced drug resistance marker for post-fusion quadroma isolation. The quadroma cell lines were prepared by fusing two different hybridoma cell lines, producing anti-fibrin and anti-plasminogen activator antibodies respectively. The quadroma cells were screened for their capacity to produce bs-MAb, capable of accumulating plasminogen activator on a fibrin surface.

MATERIALS AND METHODS

Cell Culture

Hybridoma cells were cultured at 37°C and 98% humidity under 7.5% CO_2, in

Dulbecco's modified Eagle medium (Gibco, Paisley, Scotland) containing D-Glucose (4.5 mg/ml), penicillin (100 IU/ml), streptomycin (100 μg/ml), 2-mercaptoethanol (50 μM), pyruvate (100 μg/ml) and glutamine (2 mM), supplemented with 10% (v/v) fetal bovine serum (DMEM+FBS).

Hybridoma cell lines and monoclonal antibodies

Y22/HAT^S; a hybridoma cell line producing (IgG1) MAb specific for fibrin, the major constituent of blood clots [31]. The Y22 hybridoma cell line was back-selected for sensitivity to hypoxantine, aminopterin and thymidine (HAT^S) by selection for mutants resistant to the toxic purine analogue 6-thioguanine [32]. To this end, Y22 cells were cultured for three weeks in (hypoxantine/thymidine free) DMEM+FBS supplemented with 0.1 mM 6-thioguanine [16, 33]. Surviving cells were cloned and expanded in the same medium. The cell lines were checked for sensitivity to HAT in DMEM+FBS supplemented with 100 μM hypoxanthine, 0.4 μM aminopterin and 16 μM thymidine.

tPA12-5-3; a hybridoma cell line producing (IgG2a) MAb specific for tissue-type plasminogen activator (t-PA) [34]. The tPA12-5-3 MAb only reacts with free t-PA, i.e. not in complex with its naturally occurring inhibitor plasminogen activator inhibitor type-1 (PAI-1) [34, 35].

tPA7-8-4; a hybridoma cell line producing (IgG1) MAb specific for t-PA, recognizing another epitope on t-PA than tPA12-5-3 [34]. This MAb was purified on Protein A-Sepharose (LKB-Pharmacia, Uppsala, Sweden) according to Ey et al. [36] and labelled with horseradish peroxidase (HRP) using N-succinimidyl-2-pyridyldithiopropionate (SPDP) according to the manufacturer's instructions (Pharmacia, Uppsala, Sweden). The tPA7-8-4/HRP conjugate was used for the assessment of bound t-PA antigen in the ELISA system described below.

Plasmid-DNA

The pSV2-neo plasmid [20] was produced in *E. coli*; isolated by double CsCl-gradient banding, and concentrated by ethanol precipitation, using standard procedures [37]. Expression of the aminoglycoside phosphotransferase gene, coded on this plasmid, in eukaryotic cells makes them resistant to the neomycin analog G418 (NEO^R). Plasmid linearization was performed by digestion with the restriction enzyme Pvu I [30] according to the manufacturer's specifications (Boehringer Mannheim, Mannheim, Germany).

Electroporation

Hybridoma cells were washed with and concentrated in the electroporation buffer under investigation to 5 x 10^6 cells/ml. One ml of the cell suspension was then pulsed once at room temperature [28] using a Bio-Rad Gene Pulser with capacitance extender (Bio-Rad,

Richmond, CA, USA). The electric discharge, generated by this apparatus, has the form of an exponentially decaying pulse. The pulse-length (τ), i.e. the time needed for the field strength to decay to 37% of its peak voltage, is registered in milliseconds.

After pulsing, the cells were allowed to rest for 10 minutes at room temperature before dilution into 6 ml of cell culture medium. Post-pulse viability of the cells was determined 24 hours after pulsing, by use of the trypan blue exclusion method, and expressed as a percentage of identically treated, but non-pulsed control cells.

Cell viability

Initial experiments were performed to study the effect of different electroporation buffers on the viability of the Y22/HAT^S cells exposed to different field strengths and capacitances. The strongest electric discharge to which the cells could be exposed with relatively little cell death was determined, since these conditions were found to correlate with the highest transfection frequency [23, 28]. Therefore, post-pulse viability was determined after pulsing Y22/HAT^S cells, as described above, with different field strengths and capacitances using three different electroporation buffers. Tested buffers were: 0.05 M HEPES, 0.15 M NaCl, pH 7.4 (HBS); complete cell culture medium (DMEM); and complete cell culture medium supplemented with FBS (DMEM+FBS). No DNA was added to the cells in these experiments.

Transfection

For transfection studies, five μg of plasmid DNA were added to one ml of Y22/HAT^S cell suspension prior to electroporation. The suspension was then pulsed and post-pulse viability determined as described above. The cells were subsequently washed with culture medium and seeded in two 96-well microtitre plates (Costar, Cambridge, MA, USA). Selection for stable transfected cells was not commenced until 48 hours after pulsing, by the addition of G418-sulphate (Sigma, St.Louis, MO, USA) to a final concentration of 2 mg/ml. The number of wells with growing Y22/HAT^S/NEO^R clones was scored after 10 to 14 days, and the production of anti-fibrin MAb was determined using an ELISA (see below).

Cell fusion

tPA12-5-3 cells and Y22/HAT^S/NEO^R (see above) cells, 10^7 each, were mixed and washed with DMEM. Next, they were fused using 50% (w/v) polyethylene glycol 1500 (Boehringer, Mannheim, Germany) according to standard procedures [34, 38]. After fusion, the cells were seeded in two 96-well plates in a double selection medium; DMEM+FBS supplemented with G418 (2 mg/ml) and HAT (100 μM hypoxanthine, 0.4 μM aminopterin and 16 μM thymidine). As a control, a sample of non-fused cells was grown in this double selection medium in separate wells. Selection medium was renewed

after 2, 5 and 10 days. The number of growing clones was scored after 2 weeks, and the culture medium was screened for the presence of bs-MAb in a double antigen ELISA (see below).

ELISA

I) To demonstrate the presence of anti-fibrin antibodies in the supernatant of Y22(/HAT^S/NEO^R) cells, the following procedure was followed. Fibrinogen (Kabi-Vitrum, Mölndal, Sweden) was adsorbed to microtitre plates (Greiner, Frickenhausen, Germany) at 10 μg/ml in 0.01 M Phosphate, 0.15 M NaCl, pH 7.4 (PBS) by incubation overnight at 4°C. Prior to use, the plates were washed three times with PBS containing 0.1% (v/v) Tween 20 (PBST), and the solid phase fibrinogen was converted to fibrin by incubation with 10 NIH units thrombin/ml in 0.15 M NaCl for 30 minutes at 37°C. The plates were then washed with PBST, and Y22(/HAT^S/NEO^R) supernatant, three-fold diluted in PBST, was added. After incubation for 1 hour at room temperature, the plates were washed with PBST and bound MAb was quantified by adding peroxidase-labelled polyclonal goat-anti-mouse-Ig (Nordic, Tilburg, the Netherlands) diluted in PBST containing 0.1% (w/v) bovine serum albumin (BSA), and the subsequent conversion of the chromogenic peroxidase substrate 3,3',5,5'-tetramethyl benzidine in the presence of H_2O_2 (TMB/H_2O_2) [39]. The reaction was stopped by the addition of an equal volume of 2N H_2SO_4, and the absorbance was read at 450 nm.

II) To assess the presence of bs-MAb, reactive with both fibrin and t-PA, a double antigen ELISA was developed. Fibrin coated microtitre plates were prepared as described above. The supernatant of quadroma cells was diluted three-fold in PBST, and added to the wells. After a 1 hour incubation at room temperature, the plates were washed three-times with PBST and t-PA was added at 100 ng/ml in PBST containing 0.1% (w/v) BSA. The plates were then incubated for another hour at room temperature. Non-bound t-PA was removed by washing with PBST and bound t-PA antigen was assessed using tPA7-8-4/HRP, diluted in PBST containing 0.1% (w/v) BSA, and the subsequent conversion of TMB/H_2O_2.

RESULTS

Cell viability

With all three buffers, a rapid decline in cell viability of Y22/HAT^S cells was observed at field strengths above 375 V/cm with the capacitance set at 960 μF (Table 1). At lower capacitances, no cell death was observed. Both with DMEM and DMEM+FBS as electroporation buffers, the post-pulse viability of the Y22/HAT^S cells was markedly

increased, as compared with HBS (Table 1). The presence of serum in DMEM is apparently favorable to cell viability after pulsing, since a slightly higher post-pulse viability is observed when using DMEM+FBS instead of DMEM (Table 1). We also observed a slightly increased pulse-length in DMEM and DMEM+FBS, as compared with HBS. On the basis of these results, as shown in Table 1, we decided to pulse the cells in further transfection experiments with the capacitance set at 960 μF, varying the field strength around 500 V/cm.

Table 1. Effect of electroporation buffer and electric field strength, at a capacity of 960 μF, on pulse-length and post-pulse viability

	Electroporation buffer					
	HBS		DMEM		DMEM + FCS	
V/cm	τ	ppv	τ	ppv	τ	ppv
250	13.8	87	14.8	92	14.2	87
375	12.5	72	14.5	80	14.3	88
500	11.1	24	13.1	54	13.8	68
625	10.6	12	12.6	39	12.6	43
750	9.9	0	11.5	3	12.2	7
875	8.3	0	9.5	0	9.9	0

V/cm = electric field strength; τ = pulse length (ms); ppv = post-pulse viability (% of control). Means of two experiments.

Transfection of pSV2-neo

Upon pulsing the Y22/HAT^S cells in the presence of 5 μg intact pSV2-neo, varying the field strength at 960 μF, we observed for all three buffers a clear effect of the field strength on stable transfection. However, a significantly higher number of growing NEO^R clones was found with DMEM and DMEM+FBS as electroporation buffer, as compared with HBS (Fig. 1). These results indicate, that with DMEM(+FBS) about a ten- to thirty-fold higher stable transfection frequency is achieved than with HBS.

The effect of plasmid linearization was studied comparing HBS and DMEM+FBS as buffers at 500 V/cm and 960 μF. Using DMEM+FBS, the intact plasmid was transfected almost as effectively as linearized PSV2-neo, since the numbers of NEO^R clones were about the same (Table 2). Using HBS, the stable transfection frequency

increased approximately two-fold with linearized pSV2-neo, as compared with the intact plasmid.

The presence of DNA did not affect pulse-length or cell viability (not shown), since these values were virtually the same as those observed in earlier experiments performed in the absence of DNA (Table 1).

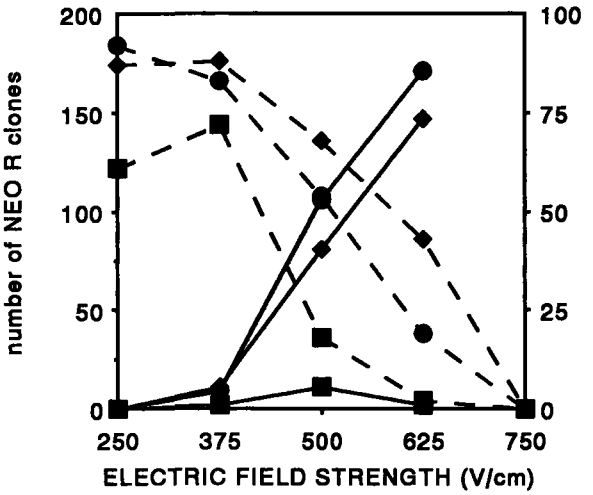

Figure 1. Effect of field strength, at 960 μF in the presence of 5 μg intact plasmid, on the stable transfection frequency (—) and post-pulse viability (---), using either HBS (■), DMEM (●) or DMEM + FCS (♦) as electroporation buffer.

Table 2. Effect of the form of DNA on the stable transfection of pSV2-neo (at 500 V/cm and 960 μF) with HBS or DMEM + FCS

		Electroporation buffer	
		HBS	DMEM + FCS
plasmid DNA		NEO^R	NEO^R
circular	exp. 1	4	124
	exp. 2	3	86
linearized	exp. 1	16	101
	exp. 2	8	116

NEO^R = number of wells with C418 resistant clones out of 192 wells initially seeded.

MAb production and genetic stability

We tested 124 G418 resistant Y22/HAT^S clones, isolated after transfection using DMEM+FBS as electroporation buffer for MAb production in the ELISA. We found that only one clone did not produce functional anti-fibrin MAb. Furthermore, the transformed cells were resistant to concentrations of G418 up to 10 mg/ml, illustrating a high level of aminoglycoside phosphotransferase expression. Non-transformed Y22/HAT^S cells died in medium with G418 at concentrations as low as 0.5 mg/ml within 4 days.

To test the genetic stability of the transformed cells, two arbitrarily selected Y22/HAT^S/NEO^R clones were cultured in medium without G418 for several weeks. Subsequently, these cell lines were cloned (seeded at 1 cell/well) in culture medium with or without 2 mg/ml G418 added. No difference was observed in the number of growing sub-clones, indicating that all cells had retained their drug resistance marker.

Quadroma isolation and bs-MAb production

One of the Y22/HAT^S/NEO^R hybridoma cell lines was selected for further fusion experiments. Cells from this line were fused with cells from the tPA12-5-3 cell line, which are 'wildtype' hybridomas, i.e. HAT^R and NEO^S. After selection in G418 and HAT containing medium, clones were observed in all wells. Non-fused control cells died in this selection medium within 4-5 days. bs-MAb were detected (using the double antigen ELISA) in the culture medium of all clones. Two clones, designated as Q8-1 and Q8-2, were picked for the high response of their supernatant in the double antigen ELISA (Fig. 2); and were expanded and recloned. All sub-clones produced bs-MAb, indicating the genetic stability of the isolated quadroma cell lines.

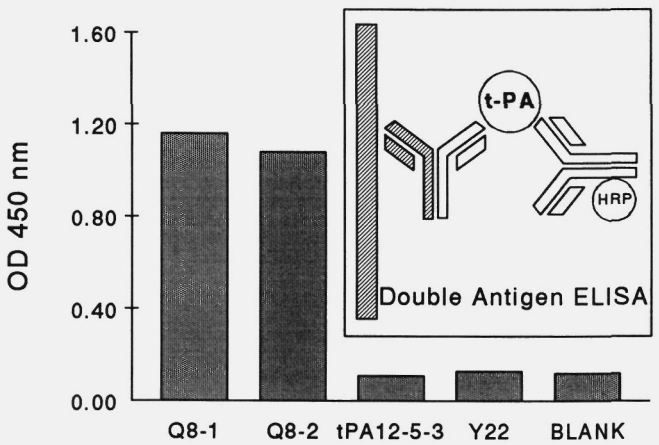

Figure 2. Response of diluted cell culture medium from parental hybridomas (Y22 and tPA12-5-3) and quadromas (Q8-1 and Q8-2) derived thereof in double antigen ELISA. Insert: Schematic representation of the double antigen ELISA, comprising of (from left to right): solid-phase fibrin (antigen 1); bispecific MAb; t-PA (antigen 2); tPA7-8-4/HRP conjugate.

DISCUSSION

Here we report on a method with an improved efficiency for the transfection of plasmid DNA, containing a drug resistance marker gene, into hybridoma cells using electroporation. Both HBS, generally used as electroporation buffer by most researchers [22, 23, 24, 27, 28, 29, 30] and DMEM(+FBS) are isotonic low-resistance buffers. However, the use of DMEM(+FBS) instead of HBS dramatically increases the yield of the stable transfected cells. Additionally, we showed that the NEO^R selection-marker, in combination with HAT sensitivity, can be used successfully for the isolation of bispecific antibody-producing quadroma cells. Furthermore, when using DMEM(+FBS) as electroporation buffer, plasmid linearization is apparently no longer required, whereas linearization is beneficial for stable transfection with HBS [22, 23, 24, 30, this study].

The mechanism which yields the observed increase in the stable transfection frequency when using DMEM(+FBS) instead of HBS as electroporation buffer is not known. It may be due to an effect on cell permeability, genomic insertion ratio, protection of DNA against enzymatic digestion, or perhaps some other mechanism. There is as yet no straightforward model on the mechanism(s) involved by which DNA enters the cell during electroporation; this may occur by passive diffusion of DNA through the pores present in the cell membrane after pulsing; active electrophoresis of the highly negatively charged DNA, mediated by the electric field; and pinocytosis of suspended or membrane bound DNA, induced by the electric discharge [21]. Furthermore, distinction should be made between methods that assess transfection either at the level of transient expression, or for stable transfection frequency [22, 23, 25]. The latter involving the more complex integration (by recombination) and expression of the plasmid in the eukaryotic genome.

The strong increase in stable transfection frequency when using DMEM(+FBS) instead of HBS as electroporation buffer was unexpected. Earlier studies on DNA transfection in mammalian cells using electroporation describe that divalent cations such as Ca^{2+} and Mg^{2+} (present in DMEM at 1.8 mM and 0.81 mM respectively) are to be avoided because of inhibitory effects, caused by binding of DNA to the cell membrane [21, 22, 26, 27]. Our results, however, indicate that with DMEM(+FBS), not only the post-pulse survival of the cells is increased (about two-fold), but also the genomic integration and expression of the plasmid genes (about ten- to thirty-fold).

Our results do not agree with those reported earlier by Knutson and Yee [40], who did not observe an increased stable transfection frequency when pulsing several mammalian (not hybridoma) cell lines in DMEM+FBS, as compared with HBS. With some cell lines, in agreement with our results, they did observe an increased post-pulse viability with DMEM+FBS, as compared with HBS.

Our results are in line with the observations of Bahnson and Boggs [41], who recently reported on the beneficial effect of FBS on the transfection frequency when added to HBS during or immediately after pulsing. We did not observe such an increase in the transfection frequency upon supplementing DMEM with FBS, maybe because transfection frequencies were already optimal. An other explanation for their results might be that when supplementing HBS with serum, divalent cations (and other elements) are also added to the HBS besides proteins. The Ca^{2+} and Mg^{2+} ions (present in serum at approximately 2.5 mM and 0.85 mM respectively) might contribute to the observed increased transfection frequency, in concordance with our results when HBS is substituted for DMEM.

The use of two drug resistance selection-markers for post-fusion quadroma isolation has several advantages over other methods. In other methods, the two hybridoma cell lines to be fused are either incubated with different fluorescent dyes and sorted on a FACS [13, 14], or they are pre-incubated with a toxic dose of complementary irreversible biochemical inhibitors [12, 18]. Both methods involve a pre-treatment of the hybridoma cell lines, that has to be repeated for every cell fusion. In contrast, drug resistance markers, once introduced in (one of) the fusion partners, allow a simple selection in a selective culture medium. Moreover, this procedure selects for functionally successful synkarions (fused nuclei). The other selection procedures only select for heterokaryons (fused membranes), increasing the number of isolated clones not actually producing bs-MAb. This is best illustrated by the fact that all the quadroma cells isolated using our double drug-resistance method, produced bs-MAb, whereas others [13, 14] reported that only a small percentage of double fluorescent cells, isolated after fusion using the double fluorescent dye/FACS procedure, actually produced bs-MAb.

We chose for a post-fusion selection based upon a double mutation in one cell line. First a recessive selection-marker was applied to the Y22 cell line, i.e. sensitivity to HAT. We then applied, by electroporation, a dominant selection marker, the NEO^R gene, inducing a resistance to G418. This is practical for our purpose, since we plan to use the (anti-fibrin) Y22 cell line as primary fusion partner with a range of hybridoma cell lines that produce MAb with different specificities for t-PA and other plasminogen activators.

As shown by our results, transfection of hybridoma cells is an efficient method for introducing drug resistance markers. This in contrast to the low success rate and time-consuming isolation of spontaneous ouabain or actinomycin D resistant mutants. Furthermore, the mechanisms (i.e. responsible genes) of such types of drug resistances are often not known. As we have shown, these problems can be overcome using transfection with well defined, bacterial plasmid constructs. It is possible to transfect other dominant drug resistance selection-markers to the cells using additional plasmids. This could be significant when selection for HAT^S mutants is arduous [18], or when the co-transfection of two genes is required [24, 25].

Transfection of a bacterial plasmid by electroporation, to transduce a drug resistance marker to the hybridoma cell line, is preferable over the method using a retroviral shuttle system [18], based upon modified retroviruses not capable of autonomous reproduction [42]. The reported stable transfection frequency, obtained with the latter method is very high, making the system ideal for e.g. *in vitro* gene-regulation studies. There is, however, a risk for infection with an amphotropic replication competent (helper) retrovirus, which can have serious consequences when large scale production of bs-MAb, or especially when *in vivo* application in humans is desired.

Our preliminary results with bs-MAb, that are reactive with both fibrin and a plasminogen activator (as produced by the "quadroma-technology"), showed that targeting of plasminogen activators to fibrin can enhance the fibrin-specificity and clot-lysis capacity of such plasminogen activators dramatically [43, 44].

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CHAPTER 8

BISPECIFIC MONOCLONAL ANTIBODIES CAN INCREASE THE FIBRIN-SPECIFIC FIBRINOLYTIC ACTIVITY OF PLASMINOGEN ACTIVATORS

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SUMMARY

With a view to enhancing the efficacy of tissue-type plasminogen activator (t-PA) or urokinase-type plasminogen activator (u-PA) in thrombolytic therapy, bispecific monoclonal antibodies (bs-MoAb) were produced which target t-PA or u-PA, respectively, to a fibrin surface. The bs-MoAb, reactive with both fibrin and t-PA or fibrin and u-PA, were produced by fusing two appropriate hybridoma cell lines. The hybrid hybridoma (quadroma) cell produces, amongst others, antibodies with bispecific properties. By a rational choice of parental hybridomas for bs-MoAb production, we aimed at the simultaneous modification of some of the adverse properties of t-PA and u-PA *in vivo*, i.e. their limited fibrin specificity and their short plasma half-life by rapid hepatic clearance; and/or inhibition by plasma inhibitors such as PAI-1.

In the present study, we have investigated the effect of such bs-MoAb on the fibrin-specific fibrinolytic activity of t-PA, two-chain (tc) u-PA and single-chain (sc) u-PA. To this end a novel *in vitro* assay was employed that measures plasmin-mediated degradation of a fibrin gel, rather than enzymatic activity towards soluble chromogenic substrates.

All bs-MoAb were able to accumulate t-PA or u-PA antigen, respectively, onto adsorbed fibrin monomer. Using the novel *in vitro* assay, we found that one bs-MoAb reactive against fibrin and t-PA and designated as Q6-3-8, enhanced the fibrin-specific fibrinolytic activity of t-PA 10- to 20-fold. Another bs-MoAb reactive against fibrin and t-PA and designated as Q8-1-2, however, had no effect on the fibrin-specific fibrinolytic activity of t-PA. The bs-MoAb designated as QUK52, reactive against fibrin and u-PA, conferred to tcu-PA and especially scu-PA a very high fibrin-specific fibrinolytic activity, a property which the two u-PA forms do not have without the bs-MoAb. In these *in vitro* experiments, only an increased fibrin-specificity is expressed. On the basis of the specificities of the anti-t-PA and anti-u-PA moieties of the bs-MoAb, additional effects are expected *in vivo*, since these MoAb may (specifically) modulate hepatic clearance and/or PAI-1 inhibition.

INTRODUCTION

The conversion of the zymogen plasminogen into the active enzyme plasmin, mediated by so-called plasminogen activators, is the essential step in thrombolytic therapy. Plasmin, in turn, degrades the fibrin matrix of a thrombus to soluble fibrin degradation products [1]. It is considered crucial that the activation of plasminogen is restricted to the desired site of action, i.e. the thrombus, since plasmin in the circulation will readily degrade several other essential plasma proteins, increasing the risk for bleeding complications.

In recent years, many reports have been published on the use of tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA) as thrombolytic agents [2-5]. Both t-PA and u-PA appear in two forms, i.e. as single-chain or two-chain molecules. In the case of t-PA, both the single- and two-chain form are equally reactive towards plasminogen in the presence of fibrin, and are susceptible to inhibition by plasminogen activator inhibitor type-1 (PAI-1) [6]. In the case of u-PA, the two-chain form (tcu-PA) has a high enzymatic activity and is susceptible to PAI-1 inhibition, whereas the single-chain form (scu-PA) has, supposedly, no enzymatic activity and does not interact with PAI-1 [7-10]. t-PA is a relatively fibrin-specific plasminogen activator, since it has some affinity for fibrin [11-13], and since fibrin greatly enhances the t-PA-mediated plasminogen activation [13-15]. Although scu-PA has little enzymatic activity *in vitro*, it has been suggested that it can activate fibrin-bound plasminogen [9,16,17]. However, it is generally believed that the major thrombolytic activity of scu-PA is mediated by the conversion to the enzymatically more active two-chain form, e.g. by plasmin (present, in low concentrations, at the thrombus) [8,10,18].

The use of t-PA and u-PA as thrombolytic agents, is hampered by some of their adverse characteristics *in vivo*. Both are cleared very fast from the circulation, and can be inhibited by PAI-1 (with the exception of scu-PA) [19-21]. Therefore, large doses of the agents, over long infusion periods, are required for effective thrombolytic therapy. Combined with the limited fibrin-specificity of exogenously administered t-PA and especially u-PA, these high doses may lead to systemic plasminogen activation.

Reported strategies for improving the efficacy of these agents in thrombolytic therapy, have been aimed at either increasing their affinity for fibrin, or at decreasing their hepatic clearance rates, by the generation of mutant plasminogen activators with modified selected structural domains, supposedly involved in fibrin binding or hepatic clearance [22-28]. Such mutations, however, often also affected the enzymatic activity and/or fibrin affinity, even though the mutations were made in regions of the molecule not considered to play a role in these functions [29,30].

We aimed at the simultaneous modification of some of the negative properties of t-PA or u-PA *in vivo*, i.e. their limited fibrin-specificity and their short plasma half-life, by targeting them to a thrombus using special bispecific monoclonal antibodies (bs-MoAb). A bs-MoAb combines the antigen binding domains of two different monoclonal antibodies (MoAb). We produced such bs-MoAb with one binding domain directed against fibrin, the other directed against the thrombolytic agent (i.e. t-PA or u-PA). As well as increasing the affinity of the agent for fibrin, some adverse property of the agent (e.g. rapid hepatic clearance and/or PAI-1 binding), may be modulated simultaneously by a rational choice of MoAb for bs-MoAb production. The selected MoAb could bind to a specific site on t-PA or u-PA, thereby affecting the interaction with PAI-1, or binding to the liver cell receptor(s) involved in clearance.

Earlier, we and others have shown that bs-MoAb can successfully be produced by fusing two appropriate hybridoma cells to form a hybrid hybridoma or quadroma cell [31-35]. These quadroma cells produce various antibodies including those with bispecific properties. In order to test the effect of bs-MoAb on the fibrin-specificity of t-PA and u-PA, a novel *in vitro* assay system was developed, by which binding of plasminogen activator to a fibrin gel is assessed by measurement of the plasmin-mediated degradation of this fibrin (fibrinolytic activity) [36]. Here we report on the first phase of our studies, i.e. the production and characterization *in vitro* of such bs-MoAb to assess their potential in antibody-targeted thrombolytic therapy *in vivo*. These bs-MoAb may simultaneously increase the fibrin-specificity and plasma half-life of t-PA, tcu-PA or scu-PA, and thus greatly improve their potency in thrombolytic therapy. Preliminary results of this study have been published elsewhere [37].

MATERIALS AND METHODS

Thrombolytic agents

Purified t-PA, from human melanoma (Bowes) cells, was kindly provided by Dr J.H. Verheijen of our institute. Purified tcu-PA (Ukidan®), from the urine of healthy males, was obtained from Serono (Pharma-import, Haarlem, the Netherlands). Purified scu-PA, from human embryonic kidney cells, was kindly provided by Dr. J. Henkin of Abbott Laboratories (Abbott Park, Il., U.S.A.).

Other proteins

Bovine thrombin was obtained from Leo Pharmaceuticals (Ballerup, Denmark); horseradish peroxidase grade-I (HRP) from Boehringer Mannheim (Mannheim, Germany); fibrinogen from KabiVitrum (Stockholm, Sweden); and bovine serum albumin (BSA) from Organon Teknika (Turnhout, Belgium). Purified Glu-plasminogen was kindly provided by Dr D.W. Traas of our institute.

Antibodies

Y22: A MoAb (IgG1) specific for fibrin and directed against a conformation-dependent epitope in the D-domain of fibrin [38]. The MoAb's specificity for fibrin has been demonstrated *in vivo* by imaging experiments in rabbits and rats. Additionally, Y22 cross-reacts between human, rat and rabbit fibrin, a property useful for studies *in vivo*, since autologous fibrin can be used.

1-3-1: A MoAb (IgG1) specific for t-PA [39]. The MoAb does not affect the enzymatic activity of t-PA, but it specifically inhibits the receptor-mediated uptake of t-PA by liver endothelial cells.

12-5-3: A MoAb (IgG2a) specific for t-PA [39]. The MoAb reacts exclusively with free (non-PAI-1 bound) t-PA and prevents the binding of PAI-1 to t-PA. In an *in vitro* assay system, the MoAb was shown to inhibit the enzymatic activity of t-PA.

UK50.2: A MoAb (IgG2b) specific for u-PA [40]. It reacts equally well with tcu-PA and with scu-PA, and has its epitope on the amino-terminal fragment of u-PA. The MoAb does not affect u-PA-mediated plasminogen activation.

Polyclonal antibodies: Goat antiserum against t-PA (GatPA) or u-PA (GauPA) were donated by Dr. J.H. Verheijen and Dr. G. Dooijewaard of our institute, respectively. The antibodies were purified from the serum by affinity chromatography using Protein G-Sepharose according to the manufacturer's recommendations (Pharmacia, Uppsala, Sweden). The purified immunoglobulins (IgG) were labeled with horseradish peroxidase (HRP) using N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) according to the manufacturer's directions (Pharmacia, Uppsala, Sweden). The GatPA/HRP and GauPA/HRP conjugates were used in the double antigen ELISA described below.

Bispecific monoclonal antibodies

Q6-3-8: Bispecific monoclonal antibodies, which combine the special properties of the MoAb Y22 and 1-3-1, were produced using a method described in detail by us in an earlier publication [31]. The bs-MoAb, designated as Q6-3-8, was expected to be specific for t-PA and fibrin, to inhibit liver endothelial cell-mediated uptake of t-PA, and not to affect the enzymatic activity of t-PA.

Q8-1-2: Bispecific monoclonal antibodies, which combine the special properties of the MoAb Y22 and 12-5-3, were produced using an improved method described by us earlier [32]. The bs-MoAb, designated as Q8-1-2, was expected to be specific for fibrin and free t-PA, and to prevent binding of PAI-1 to t-PA.

QUK52: Bispecific monoclonal antibodies, which combine the special properties of the MoAb Y22 and UK50.2, were isolated as described before [32]. The bs-MoAb, designated as QUK52, was expected to be specific for u-PA and fibrin, and able to target both active tcu-PA and (PAI-1 resistant) scu-PA, while having no effect on the enzymatic activity of u-PA.

Production and purification of bs-MoAb

The bs-MoAb were produced as ascites by intraperitoneal inoculation of 3 x 10^6 quadroma cells in pristane primed, female BALB/c mice. Antibodies were isolated from the ascites using Protein A-Sepharose according to Ey et al. [41].

To separate functionally active bispecific MoAb from co-produced monospecific and non-functional MoAb, the protein A-Sepharose isolated proteins were subjected to double affinity chromatography, essentially as described earlier [31]. In brief, the MoAb were first applied to a column of fibrin-Sepharose. Bound material was eluted,

immediately neutralised and applied to a second column of either t-PA-Sepharose (for Q6-3-8 and Q8-1-2) or u-PA-Sepharose (for QUK52). Material which also bound to the second column was eluted and immediately neutralized. Samples from each chromatography step were tested in the double antigen ELISA for bs-MoAb (see below). Protein levels were determined by absorption at 280 nm, assuming an A(1%,1cm) of 14.5.

Double antigen ELISA

The ability of the bs-MoAb to accumulate t-PA or u-PA *antigen* onto a fibrin surface, was determined using a double antigen ELISA. Fibrinogen was adsorbed to microtitre plates (Greiner, Frickenhausen, Germany) at 10 μg/ml in 0.01 M Phosphate, 0.15 M NaCl, pH 7.4 (PBS) by incubation overnight at 4 °C. Prior to use, the plates were washed three-times with PBS containing 0.1% (v/v) Tween 20 (PBST), and the adsorbed fibrinogen was converted into fibrin by incubation with 10 NIH/ml thrombin in 0.15 M NaCl for 30 minutes at 37 °C.

The plates were then washed with PBST, and purified bs-MoAb, 0.25 μg/ml in PBST, or buffer (control) was added to the wells. After a one-hour incubation at room temperature, the plates were washed with PBST, and t-PA (for Q6-3-8 and Q8-1-2) or tcu-PA (for QUK52) was incubated at 100 ng/ml in PBST containing 0.1% (w/v) bovine serum albumin (BSA) for another hour at room temperature. Non-bound t-PA or tcu-PA was removed by washing with PBST and GatPA/HRP or GauPA/HRP, respectively, diluted in PBST containing 0.1% (w/v) BSA was added. Non-bound conjugate was washed away after one hour, and a mixture of the chromogenic peroxidase substrate 3,3',5,5'-tetramethyl benzidine and H_2O_2 (TMB/H_2O_2) was added [42]. The reaction was stopped by the addition of 2N H_2SO_4, and the absorption was measured at 450 nm in a multichannel spectrophotometer (Organon Teknika, Turnhout, Belgium).

Enzymatic activity

Additionally, the potential of the bs-MoAb to accumulate t-PA or u-PA *activity* onto a fibrin surface was assessed. To this end, 96-well plates with adsorbed fibrin were prepared as described above. Purified bs-MoAb, 0.25 μg/ml in 0.1 M Tris, 0.05% Tween 80, pH 8.0 (TTB), or buffer (control) was added to the wells of the plate. After a one-hour incubation the plates were washed with TTB, and 10 IU/ml t-PA (for Q6-3-8 and Q8-1-2) or 50 U/ml tcu-PA (for QUK52) in TTB containing 1.0% (w/v) BSA was added. After a one-hour incubation at room temperature, non-bound plasminogen activator was washed away and plasminogen (0.11 μM) and the chromogenic plasmin substrate S2251 (0.3 mM) (KabiVitrum, Stockholm, Sweden) in TTB were added. When indicated, the latter solution was supplemented with 80 μg/ml of CNBr digested fibrinogen (stimulator) [43]. At timed intervals, the absorption at 405 nm was determined. The ($\Delta OD/t^2$) x 1000,

was calculated and used as a measure for t-PA or u-PA activity.

Fibrinolytic activity; one-step assay

To determine the effect of the bs-MoAb on the fibrinolytic activity of t-PA and u-PA, the novel enzyme linked fibrinolytic assay (ELFA) was employed (Elcatech, Winston-Salem, N.C., U.S.A.). The assay consists of a solid-phase peroxidase-labeled fibrin *gel*, prepared by *thrombin-mediated polymerization* of a *solution* of HRP-labeled fibrinogen in the wells of a 96-well microtitre plate [36]. After addition of plasminogen activator and plasminogen, the fibrinolytic activity can be determined by assessing peroxidase levels released in the fluid-phase, which result from labeled fibrin degradation products.

In the original manufacturer's assay, used to determine overall fibrinolytic activity, plasminogen and plasminogen activator are incubated simultaneously (one-step version). For our purposes, t-PA, tcu-PA or scu-PA, in the absence or presence of an equimolar amount of the appropriate bs-MoAb, was serially diluted four-fold in 0.15 M Tris, 2 mM EDTA, 0.15 M NaCl, 0.1% (v/v) Tween 80, pH 7.5 (TEST buffer) supplemented with 0.11 μM plasminogen, and added to the wells of an ELFA plate. After a two-hour incubation at room temperature, a sample of the supernatant was taken in duplicate, and transferred to a non-treated 96-well plate. Peroxidase levels in these samples were determined as in the ELISA (see above).

Fibrin-specific fibrinolytic activity; two-step assay

To determine exclusively fibrin-specific fibrinolytic activity and not fluid-phase plasminogen activation, a two-step version of the ELFA was developed. To this end, t-PA, tcu-PA or scu-PA, in the absence or presence of an equimolar amount of the appropriate bs-MoAb, was serially diluted four-fold in TEST buffer containing 1.0% (w/v) BSA and added to the wells of an ELFA plate. After a two-hour incubation, non-bound material was washed away with TEST buffer. Then 0.11 μM plasminogen in TEST buffer, was added to determine fibrin-bound fibrinolytic activity. After another two-hour incubation, a sample of the supernatant was taken in duplicate, and peroxidase levels were assessed as described above.

RESULTS

Production and purification of bs-MoAb

The bs-MoAb were purified by double affinity chromatography. As shown in Table 1, a major portion of the protein A-Sepharose purified IgG (defined as 100%) bound to the fibrin-Sepharose column. This portion contained bispecific activity, as determined in the double antigen ELISA, whereas the non-bound material did not. When the bound IgG

were eluted from the fibrin-Sepharose column and applied to a second column with either t-PA-Sepharose (in the case of Q6-3-8 and Q8-1-2) or u-PA-Sepharose (in the case of QUK52), part of the IgG again bound. This second fraction, therefore, contains the purified bispecific MoAb, since these antibodies are able to bind to both immobilized fibrin and to immobilized t-PA or u-PA, as also demonstrated in the double antigen ELISA (see below), whereas the non-bound material did not. From the IgG initially purified on protein A-Sepharose from the ascites, approximately 15% was functionally bispecific. The non-bound material washed from the columns consists of monospecific (mono- or bivalent) and/or non-functional MoAb.

Table 1. Purification of bs-MoAb by double affinity chromatography. The eluted material from protein A-Sepharose was applied to a column of fibrin-Sepharose. Bound material was eluted and applied to a second column of either t-PA-Sepharose, or u-PA-Sepharose. Bound material was eluted. The protein content in the eluted fractions was determined spectrophotometrically, and the average yield of IgG in the eluted fractions estimated (N=3). ND = not done.

Column	Q6-3-8 bound (%)	Q8-1-2 bound (%)	QUK52 bound (%)
protein A-Sepharose	100	100	100
fibrin-Sepharose	50	60	35
t-PA-Sepharose	16	14	ND
u-PA-Sepharose	ND	ND	17

As assessed in the double antigen ELISA, all the purified bs-MoAb were able to accumulate t-PA (for Q6-3-8 and Q8-1-2) or u-PA (for QUK52) antigen onto the adsorbed fibrin (Fig. 1). Control experiments with a 1:1 mixture of the corresponding parental MoAb gave no response in the ELISA. Furthermore, the bs-MoAb were shown to accumulate only the expected plasminogen activator, i.e. Q6-3-8 and Q8-1-2 accumulated t-PA but not u-PA, whereas QUK52 accumulated u-PA but not t-PA onto the adsorbed fibrin.

Effect of bs-MoAb on the enzymatic activity of t-PA and u-PA

The results of the double antigen ELISA, show that the bs-MoAb are able to accumulate t-PA or u-PA antigen onto a fibrin surface. Therefore, we tested whether the accumulation of t-PA or u-PA *antigen*, coincided with an accumulation of *enzymatic activity* towards plasminogen. Both with Q6-3-8 (Fig. 2a) and QUK52 (Fig. 2b), we

observed that the accumulated t-PA or u-PA, respectively, is active. In the presence of bs-MoAb significantly more plasmin is formed, as compared without bs-MoAb (i.e. the control). With Q8-1-2 (Fig. 2a), hardly any t-PA activity is found. Since Q8-1-2 is able to accumulate t-PA antigen onto fibrin (see above), this is probably due to the inhibition of t-PA by the 12-5-3 moiety in the bs-MoAb.

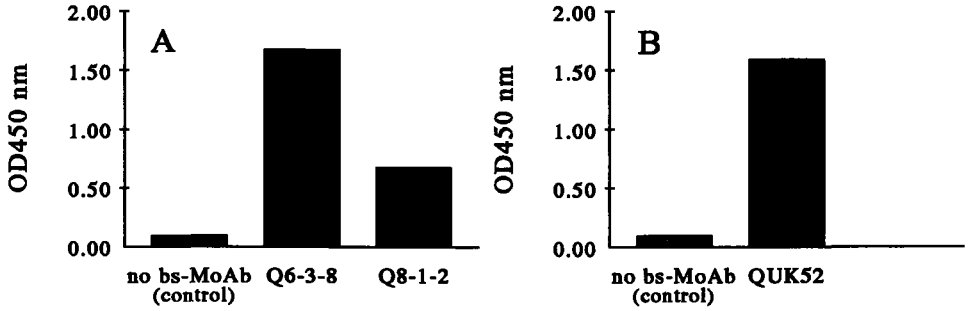

Figure 1. Ability of bs-MoAb to accumulate t-PA or u-PA antigen on fibrin.
Fig. 1A: Ability of Q6-3-8 and Q8-1-2 to accumulate t-PA antigen on adsorbed fibrin (details in text).
Fig. 1B: Ability of QUK52 to accumulate u-PA antigen on adsorbed fibrin (details in text).

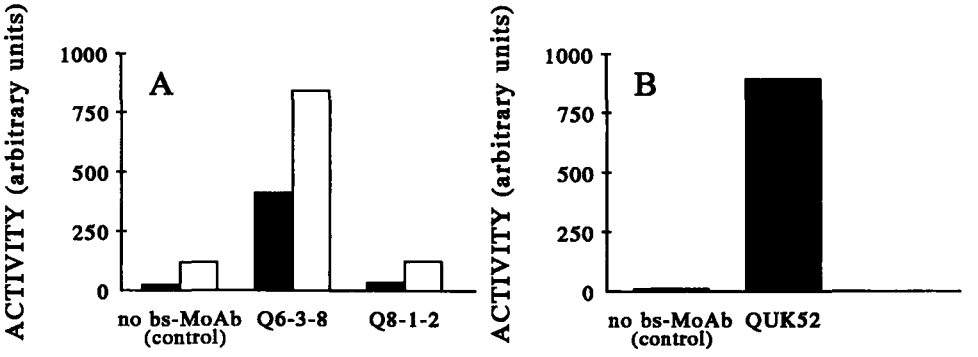

Figure 2. Ability of bs-MoAb to accumulate t-PA or u-PA enzymatic activity on fibrin.
Fig. 2A: Ability of purified Q6-3-8 and Q8-1-2 to accumulate t-PA enzymatic activity on adsorbed fibrin (details in text). Activity was determined in the (■) absence or (□) presence of CNBr digested fibrinogen (stimulator).
Fig. 2B: The ability of purified QUK52 to accumulate u-PA enzymatic activity on adsorbed fibrin (details in text).

When the assay is performed in the presence of a CNBr digest of fibrinogen (stimulator), a strong increase in the enzymatic activity of the immobilised t-PA is measured, as compared with in the absence of stimulator (Fig. 2a). This effect was observed both with t-PA bound to adsorbed fibrin directly (i.e. the control), and with t-

PA bound to adsorbed fibrin via Q6-3-8. This indicates that the interaction between the immobilized t-PA and plasminogen, i.e. the formation of the cyclic ternary-complex between fibrin, t-PA and plasminogen, on the adsorbed fibrin monolayer may not be optimal. With Q8-1-2, the presence of stimulator only increased blank values, but no accumulation of t-PA activity was found (Fig. 2a). The presence of stimulator had no effect on the activity of u-PA, when accumulation of u-PA was assessed with the bs-MoAb QUK52.

Fibrinolytic activity; one-step assay

A novel assay (ELFA) was used to study the effect of the bs-MoAb on the targeting of t-PA and u-PA to fibrin. Rather than measuring antigen, or enzymatic activity of the immobilized plasminogen activator towards soluble (chromogenic) substrates, this assay actually measures plasmin-mediated degradation of a true fibrin gel. The effect of the bs-MoAb on the fibrinolytic activity of t-PA and u-PA was tested using the one-step version of the assay.

We found that QUK52 enhanced the fibrinolytic activity of tcu-PA and especially scu-PA, since a notably lower concentration of tcu-PA or scu-PA was needed in the presence of QUK52 to obtain similar fibrin degradation, as compared without bs-MoAb (Fig. 3). Apparently, QUK52 accumulates u-PA on fibrin, which results in an increased fibrin-localised plasmin formation.

Neither the bs-MoAb Q6-3-8, nor Q8-1-2, had an effect on the fibrinolytic activity of t-PA in this one-step version of the assay (Fig. 4), despite the ability of these bs-MoAb to accumulate t-PA onto a fibrin surface (Fig. 1).

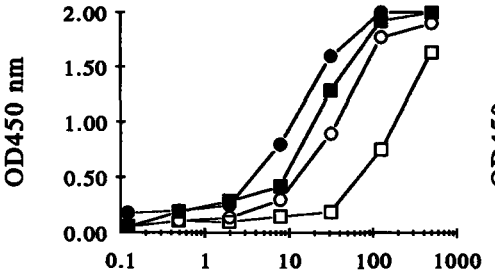

Figure 3. Effect of bs-MoAb on the fibrinolytic activity of u-PA.

Fibrinolytic activity of (●,○) tcu-PA and (■,□) scu-PA in the (○,□) absence or (●,■) presence of QUK52, as determined in the one-step ELFA (details in text). Abscissa: concentration of u-PA (U/ml); ordinate: OD450 nm as a measure of fibrinolytic activity.

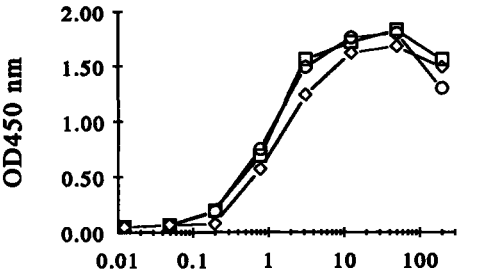

Figure 4. Effect of bs-MoAb on the fibrinolytic activity of t-PA.

Fibrinolytic activity of t-PA in the presence of bs-MoAb (□) Q6-3-8 and (◇) Q8-1-2 or (○) absence of bs-MoAb, as determined in the one-step ELFA (details in text). Abscissa: concentration of t-PA (IU/ml); ordinate: OD450 nm as a measure of fibrinolytic activity.

Fibrin-specific fibrinolytic activity; two-step assay

To exclusively determine fibrin-associated fibrinolytic activity, and minimize the contribution of plasminogen activated in the fluid-phase, a two-step version of the ELFA was developed. First the plasminogen activator was allowed to bind to the fibrin matrix, and the non-bound material was then washed away and plasminogen was added.

We first examined the effect of the wash-step on the t-PA- and u-PA-mediated plasminogen activation, in the absence of bs-MoAb. The wash step had only a small effect on the fibrinolytic activity of t-PA, indicating that most t-PA-mediated plasminogen activation is fibrin-specific, in accordance with its predicted behaviour (Fig. 5a). With tcu-PA, the fibrinolytic activity virtually disappeared after washing. This was anticipated, since u-PA has no affinity for fibrin (Fig. 5b). These results illustrate that the two-step version of the ELFA can be used to discriminate between fibrin-specific and non-specific plasminogen activation.

Furthermore, CNBr digested fibrinogen (stimulator), added to plasminogen in the final step of the two-step version assay, did not further enhance the fibrinolytic activity of the immobilized t-PA (not shown). This indicates that on the fibrin gel as used in the ELFA, t-PA and plasminogen can apparently interact optimally, in contrast with on the adsorbed fibrin monolayer (see above).

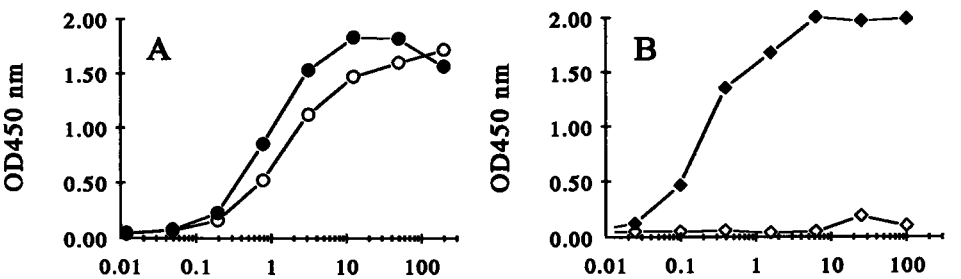

Figure 5. Effect of the assay-format on the fibrinolytic activity of t-PA and u-PA.
Fibrinolytic activity of A: (●,○) t-PA and B: (♦,◇) tcu-PA, as determined by the (○,◇) two-step version or (●,♦) one-step version of the ELFA (details in text). Abscissa: concentration of t-PA (IU/ml) or tcu-PA (U/ml); ordinate: OD450 nm as a measure of fibrinolytic activity.

Fibrin-specific fibrinolytic activity; effect of bs-MoAb

Using the two-step ELFA, we investigated the effect of the bs-MoAb on the fibrin-specific fibrinolytic activity of t-PA, tcu-PA or scu-PA. In this assay Q6-3-8 induced a marked increase in the fibrin-specific activity of t-PA. A 10- to 20- fold lower concentration of t-PA was needed in the presence of Q6-3-8 to obtain a similar extent

of fibrin degradation, as compared with in the absence of Q6-3-8 (Fig. 6). With Q8-1-2 the results were less pronounced; Q8-1-2 did not increase, but neither inhibited, the fibrin-specific fibrinolytic activity of t-PA (Fig. 6).

With QUK52, the effects were very strong: in the presence of QUK52, a high fibrin-specific fibrinolytic activity was conferred to both tcu-PA and scu-PA, a property they do not exhibit in the absence of QUK52 (Fig. 7).

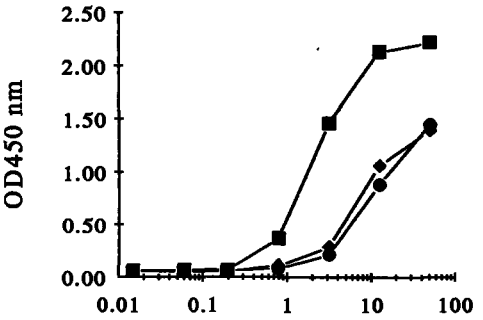

Figure 6. Effect of bs-MoAb on the fibrin-specific fibrinolytic activity of t-PA.
Fibrin-specific fibrinolytic activity of t-PA in the presence of (■) Q6-3-8 and (♦) Q8-1-2 or (●) absence of bs-MoAb, as determined in the two-step ELFA (details in text). Abscissa: concentration of t-PA (IU/ml); ordinate: OD450 nm as a measure of fibrinolytic activity.

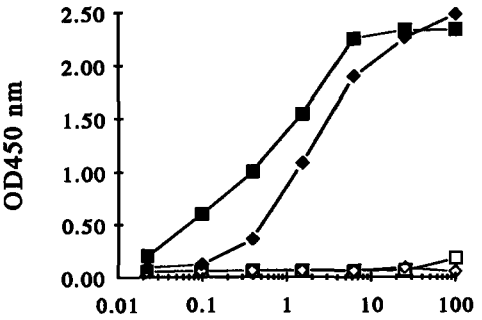

Figure 7. Effect of bs-MoAb on the fibrin-specific fibrinolytic activity of u-PA.
Fibrin-specific fibrinolytic activity of (■,□) tcu-PA and (♦,◇) scu-PA in the (□,◇) absence or (■,♦) presence of QUK52, as determined in the two-step ELFA (details in text). Abscissa: concentration of u-PA (U/ml); ordinate: OD450 nm as a measure of fibrinolytic activity.

DISCUSSION

Bispecific monoclonal antibodies are powerful tools for targeting plasminogen activators to the desired site of action [31-35,44,45]. Here we have demonstrated that the present bs-MoAb, reactive against both fibrin and t-PA, or fibrin and u-PA, can accumulate t-PA and u-PA, respectively, on a fibrin surface. Furthermore, the fibrin-specificity of the plasmin-mediated fibrin degradation, was increased. The bs-MoAb Q6-3-8 notably increased the fibrin-specific fibrinolytic activity of t-PA; and QUK52 conferred to tcu-PA and scu-PA a high-fibrin specificity, which is non-existent in the absence of QUK52.

We chose to produce bs-MoAb by somatic cell fusion, rather than by chemical cross-linking [44,45]. The major advantage to this method is that bs-MoAb can be produced both in unlimited quantities and of a consistent quality. There is no risk of the occurrence of batch-to-batch variation, loss of activity and the formation of tri-

and multimers. Furthermore, it has been suggested that chemically prepared bs-MoAb are more immunogenic [34].

Initial studies on the effect of bs-MoAb on the affinity of t-PA or u-PA for fibrin, suggest that adsorbed fibrin is not an optimal model for the study of increased fibrinolysis. This is best illustrated by the enhancing effect a CNBr digest of fibrinogen (stimulator) on the plasminogen activation of fibrin-bound t-PA. Apparently, the formation of the cyclic ternary complex, essential for optimal plasminogen activation by t-PA, is impeded as a result of the adsorption of fibrin in a monolayer. Moreover, plasmin activity is assessed by using a soluble chromogenic substrate, and not by its ability to lyse fibrin. We, therefore, searched for an improved model for assessing fibrin degradation by plasmin, generated by plasminogen activators bound to fibrin. The ELFA is an assay that apparently meets our requirements: it consists of a true fibrin gel; the fibrin gel binds t-PA but not u-PA; plasmin-mediated fibrin degradation is measured; and fibrin-bound t-PA is fully able to activate plasminogen, since the presence of stimulator no longer has an enhancing effect. Furthermore, it has a microtitre-plate format, facilitating the determination of many samples in duplicate requiring little material, and fibrin degradation is simply assessed by measuring fluid-phase peroxidase levels.

In the original one-step version of the ELFA, a clear effect of the bs-MoAb QUK52 was observed on the fibrinolytic activity of tcu-PA and scu-PA. Apparently, the targeting of u-PA to the fibrin gel by QUK52, increases the local concentration of u-PA, such that plasmin formation at or near the fibrin surface is favoured. The positive feedback of fibrin-associated plasmin on the subsequent activation of more scu-PA, may explain the stronger effect of QUK52 on scu-PA than on tcu-PA. Comparable results were reported for a similar bs-MoAb by Kurokawa et al. [35]. In the two-step version of the ELFA, the QUK52 conferred fibrin-specificity of u-PA is even more clearly demonstrated, since in the absence of QUK52 no fibrinolytic activity was detected, in contrast with the high fibrinolytic activity observed in the presence of QUK52. *In vivo*, targeting of especially scu-PA with QUK52, may prove to be beneficial, since scu-PA in the circulation is not very reactive towards free plasminogen and resistant to PAI-1 inhibition. Once on the clot, scu-PA will rapidly be converted to active tcu-PA, which can efficiently activate clot-associated plasminogen. It is not unlikely, that complexing u-PA with the bs-MoAb QUK52, may also alter its pharmacokinetic behaviour.

The ability of the bs-MoAb Q6-3-8 to increase t-PA levels at a fibrin surface, could only be detected in a two-step assay. The results with the ELFA strongly suggest that t-PA immobilised to fibrin via the bs-MoAb, is capable of associating with its appropriate binding site(s) on fibrin and of interacting with plasminogen. We can give no rational explanation why the increased fibrin-specificity of t-PA is not

observed in the one-step version of the assay. Preliminary studies with an *in vitro* clot-perfusion model mounted on a gamma-camera, using a system described earlier [46], showed that with Q6-3-8, radioactively-labeled t-PA accumulated much faster on a fibrin clot than it did in the absence of Q6-3-8. Apparently a static model (e.g. one-step ELFA) does not bring to expression an increased fibrin-specificity as well as a dynamic system (e.g. two-step ELFA or clot-perfusion model). These findings are comparable with the results described by Nelles et al., who reported similar results with a t-PA/scu-PA chimeric protein [47]. It is likely, that *in vivo* Q6-3-8, besides increasing the fibrin-specificity of t-PA, will increase the plasma half-life of t-PA, since the 1-3-1 moiety in the bs-MoAb specifically inhibits the receptor-mediated uptake of t-PA by liver endothelial cells [39].

With Q8-1-2, no increase in fibrin-specific fibrinolytic activity of t-PA was observed in the presence of the bs-MoAb. To our surprise, however, we observed some activity in the ELFA when t-PA was targeted to fibrin with the bs-MoAb Q8-1-2. Since the 12-5-3 moiety in Q8-1-2 inhibits the enzymatic activity of t-PA *in vitro* [39], the absence of inhibition in the ELFA suggests that in the presence of an abundance of fibrin enough t-PA dissociates from the bs-MoAb to induce plasminogen activation. *In vivo* this may prove to be beneficial: while in circulation, Q8-1-2 bound t-PA is inactive towards plasminogen and protected from PAI-1 binding; once immobilised to the clot, active t-PA is (slowly) released from the bs-MoAb to induce clot-lysis. In addition, it is not unlikely that complexing of t-PA with Q8-1-2, may also modify its pharmacokinetic behaviour.

From the strategies that aim to enhance the efficacy of t-PA and u-PA as thrombolytic agents, the best results so far obtained were with mutants of t-PA that have a reduced hepatic clearance [22,23,28,48]. However, these mutations often simultaneously caused a reduced fibrin affinity and enzymatic activity, even though the mutations were made in domains that supposedly do not play a role in these functions. Attempts to produce chimeric proteins, with the fibrin binding structures of plasminogen, t-PA or monoclonal antibodies, linked to the proteolytic domains of u-PA or t-PA, also proved to be less effective than anticipated [47,49-51]. Several explanations can be given for this. The correct folding of the functional domains in these chimeric molecules, or t-PA and u-PA mutants, may be disrupted, due to the interfering presence of 'foreign' protein material, or to the absence of autologous structural domains. Furthermore, the correct steric assembly (mutual orientation) of the active site of t-PA and its substrate plasminogen on the fibrin surface may be disrupted [47,52]. Antibody-targeted thrombolytic therapy using bispecific monoclonal antibodies, avoids these disadvantages. Because of the flexible nature of the antibody molecule, t-PA may have more freedom to associate with its binding site(s) on fibrin which would allow for optimal plasminogen activation. Additionally, the wildtype

configuration of the plasminogen activator is left intact, and the effect of the bs-MoAb on the biological properties (i.e. enzymatic activity, hepatic clearance, PAI-1 inhibition and fibrin binding) of the plasminogen activator can be well characterized.

Only a few reports have so far appeared on the use of bs-MoAb produced by 'quadroma technology', for the targeting of t-PA or u-PA to fibrin [31-35]. In three studies an increased thrombolytic potency *in vivo* is also shown [33-35]. In the first two, it was not made clear, however, whether this is due to the enhanced fibrin-affinity or to a prolonged plasma half-life caused by the complexing of t-PA with the bs-MoAb, since t-PA clearance was not determined. The latter study showed that the major effect of their bs-MoAb on the increased thrombolytic potency of scu-PA, was due to an altered clearance rate. The contribution of the bs-MoAb enhanced fibrin-specificity to the increased thrombolytic potency of scu-PA was marginal. This could be explained by the limited fibrin-specificity of the anti-fibrin which Kurokawa et al. used for bs-MoAb production, since it strongly cross-reacts with fibrinogen.

A reduced inhibition of t-PA or tcu-PA by PAI-1 is generally considered of less importance, since in the current protocols of thrombolytic therapy the dosages of t-PA and u-PA are much higher than that of plasma PAI-1 [52]. When dosages can be reduced significantly, and given as a single bolus-injection, inhibition by PAI-1 may play a more important role. Hence, targeting scu-PA with QUK52, or t-PA with Q8-1-2, may prove to be effective.

In the tests *in vitro* presented here, only an increased affinity/specificity of the plasminogen activators for fibrin is expressed. *In vivo*, additional effects are expected which are related to a reduced clearance and/or inhibition by PAI-1 after complexing of the plasminogen activator to the bs-MoAb. On the basis of the specificities of the anti-t-PA and anti-u-PA moieties of our bs-MoAb, a reduced hepatic clearance of t-PA is expected for Q6-3-8; and a reduced PAI-1 inhibition for Q8-1-2. In addition these bs-MoAb probably alter the biodistribution of t-PA and u-PA. These aspects are currently under investigation.

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CHAPTER 9

GENERAL DISCUSSION AND SUMMARY

This thesis contains an investigation of various applications of monoclonal antibodies (MoAb) against components of the fibrinolytic system. The fibrinolytic system is characterized by intricate interactions between several proteins, such as (pro-)enzymes, activators, potentiators and inhibitors. Although the fibrinolytic system is generally considered as part of the haemostatic balance (the equilibrium between the two opposing processes of coagulation and fibrinolysis), components of the fibrinolytic system also play a role in several other physiological or pathological processes. Furthermore, components of the fibrinolytic system, i.e. tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA), are frequently used as agents for thrombolytic therapy. This latter application is studied intensively, since vascular diseases, such as myocardial infarctions, are a major cause of death in western society. Plasminogen activators are administered which activate the endogenous fibrinolytic system, rapidly degrading the fibrin matrix of the occluding blood clot which causes the infarction.

The success of t-PA and u-PA as thrombolytic agents is limited by some of their adverse properties *in vivo*, i.e. they have no, or only a limited, fibrin-specificity; they are cleared very fast from the circulation by the liver; and they may be inhibited by fast acting inhibitors such as PAI-1. A promising strategy for improving the efficacy of t-PA and u-PA as thrombolytic agents, is by targeting them to thrombi using bispecific MoAb (bs-MoAb) (chapter 2). In bs-MoAb, one antigen binding-site is directed against the target (here the fibrin in a thrombus), the other is directed against the agent (here t-PA or u-PA). In addition to increasing the fibrin-affinity, we aimed to decrease simultaneously some other adverse property of t-PA or u-PA *in vivo* mentioned above, by a rational choice of MoAb for bs-MoAb production. To this end, MoAb were selected that bind to or near the site involved in binding of t-PA to liver cell receptors or to PAI-1; and MoAb that bind to PAI-1 resistant scu-PA. Hybridoma cell lines, producing suitable anti-fibrin or anti-u-PA MoAb were available during the studies. Therefore, first a set of hybridoma cells producing suitable anti-t-PA MoAb had to be produced.

In chapter 3, the isolation and characterization of this set of MoAb against t-PA is described. Here, a novel assay is described, i.e. the activity-recovery assay, based on the measurement of t-PA activity when bound to immobilized MoAb. The assay allows for the more precise determination of the effect of the MoAb on the enzymatic activity of

t-PA. This is more reliable than other (fluid-phase) assays, since mutual differences between MoAb, such as purity, subclass and affinity for the antigen, are eliminated. Structure/function analysis with these MoAb on t-PA revealed some interesting aspects. Some MoAb, which apparently recognized the same epitope on the t-PA molecule, since they were mutually exclusive for binding to t-PA, had completely different effects on t-PA. Some MoAb gave seemingly conflicting results when their effect on biological properties of t-PA was studied. For instance, some MoAb inhibit the amidolytic activity of t-PA, which suggests that these MoAb have their epitope at or near the active site of t-PA. However, these MoAb also inhibit the finger-domain mediated binding of t-PA to fibrin. This indicates that epitope definition of the MoAb by studying the influence on t-PA functions is a complicated matter. Furthermore, it indicates that the structure of t-PA is relatively complex, since functional domains in the native molecule may interact in a different manner than anticipated on the basis of earlier results with domain-deletion mutants of t-PA.

On the basis of the results of chapter 3, several anti-t-PA MoAb were identified in having special characteristics: MoAb 1-3-1 and 7-8-4 specifically inhibit the receptor-mediated uptake of t-PA by liver endothelial cells and by liver parenchymal cells, respectively. These results confirmed the involvement of at least two distinct receptor-mediated clearances routes for t-PA by the liver. MoAb 12-5-3 was found to react exclusively with free t-PA, i.e. t-PA not in complex with the fast acting plasminogen activator inhibitor type-1 (PAI-1); and to prevent the binding of PAI-1 to t-PA. It presumably reacts at or near the active site of t-PA, since 12-5-3 also inhibits the enzymatic activity of t-PA. The specificity of 12-5-3 for free t-PA was applied to separate free t-PA from t-PA/PAI-1, in order to use the t-PA/PAI-1 complex as immunogen for MoAb production (chapter 3). Additionally, the specificity of 12-5-3 for free t-PA can be applied to study t-PA/inhibitor interactions, e.g. in a specific ELISA for free t-PA. Finally, MoAb 1-3-1 was found to dissociate from t-PA in the presence of the lysine analogue 6-amino hexanoic acid (6-AHA). Apparently, 1-3-1 and 6-AHA compete for the same (lysine) binding-site on t-PA, or 6-AHA induces a conformational change in t-PA. However, physiological ligands of t-PA, such as fibrin, did not affect the binding of t-PA to 1-3-1. The special properties of these MoAb were used later in this study for several applications (chapters 4 to 8).

One application investigated was the large-scale purification of t-PA from (melanoma) cell-conditioned medium by one-step immunoaffinity chromatography. The ability of 6-AHA to dissociate the 1-3-1/t-PA complex was used to elute bound t-PA from immobilized 1-3-1 under mild conditions, by the addition of 6-AHA to Tris/Tween pH 7.5 (chapter 4). This was shown to increase the yield of active t-PA. Frequently used methods to elute antigen from immobilized MoAb, e.g. by a strong pH-change, reduced the yield of t-PA activity, as illustrated by the 25% lower specific activity of t-PA eluted

from the immobilized MoAb with 0.1 M Glycine pH 2.5 and 1.0 M acetic acid pH 3.0. The possibility of avoiding chaotropic reagents or a strong pH-change will possibly also increase column-life. The specific activity of t-PA purified by elution with 6-AHA containing buffers, was also 10% higher than that of t-PA purified by the sequentive use of zinc-Sepharose, fibrin-Sepharose and gel-filtration. This illustrates the potential of the present method.

Some of the isolated MoAb against t-PA were also used to develop a sensitive one-step enzyme immunoassay to determine total t-PA antigen in plasma (chapter 5). The MoAb used, i.e. 7-8-4 and 10-1-3, react equally well with all major forms of t-PA (single-chain and two-chain t-PA; free t-PA and t-PA in complex with PAI-1). Thus, total t-PA antigen can be measured using a relatively simple assay. The immunoassay avoids the potentially disturbing effects of other plasma proteins, which may occur in functional assays.

As mentioned earlier, we aimed to produce special bs-MoAb that combine the distinct properties of two MoAb, one directed towards fibrin, the other towards t-PA or u-PA, respectivly, that may decrease several negative properties of these plasminogen activators simultaneously. Such a bs-MoAb was produced by fusion of the anti-fibrin producing cell line Y22 with the anti-t-PA producing cell line 1-3-1 (chapter 6). The resulting hybrid hybridoma (also called quadroma), designated as Q6-3-8, is expected to produce bs-MoAb which combine the special properties of both Y22 and 1-3-1, i.e. the bs-MoAb is specific for fibrin and t-PA; does not affect the enzymatic activity of t-PA; and inhibits the receptor-mediated uptake of t-PA by liver endothelial cells. During this study methods were developed to isolate, detect (double-antigen ELISA) and purify (double affinity chromatography) bs-MoAb which are able to accumulate t-PA antigen and/or activity on a fibrin surface. As anticipated, the bs-MoAb produced by Q6-3-8 were able to accumulate t-PA onto a fibrin surface and to increase the affinity of t-PA for fibrin; a 10- to 20-fold increase was found.

The crucial step in 'quadroma technology' is the isolation of the desired quadroma cell from the mass of unfused or incorrectly fused hybridoma cells. In chapter 6 a novel selection mechanism was used to isolate quadroma cells that appeared to be relatively inefficient. Part of the selection was based on the assumption that hybridoma cells still express functional membrane-bound antibody-like structures (surface Ig), as they do during B-cell development in the initial immune response. Most of the isolated cells did not produce bs-MoAb, but only monospecific MoAb with the specificity of anti-t-PA 1-3-1. Since we intended to isolate more combinations of MoAb, a more efficient method for quadroma isolation was developed, based entirely on drug-selection (chapter 7). For this, a double-mutant of the anti-fibrin MoAb producing hybridoma cell line (Y22) was isolated. First, cells deficient of the gene hypoxantine guanine phosphoribosyl transferase ($HGPRT^-$) were selected, which are sensitive to a combination of hypoxantine,

aminopterine and thymidine (HAT) in the culture medium. These cells were then made resistant to G-418, a neomycin analogue, by transfection with a bacterium derived plasmid construct. The plasmid contains a gene coding for neomycin aminoglycoside phosphotransferase, expression of which renders the cell resistant to G-418. The double-mutated Y22 hybridoma cell line (HAT sensitive and G-418 resistant) could then be used as fusion partner with any (wildtype) anti-t-PA or anti-u-PA producing hybridoma cell line (HAT resistant and G-418 sensitive), enabling us to rapidly isolate several different quadroma cell lines.

As mentioned above, the hybridoma cells were transfected with a plasmid construct. For the transfection of non-attached growing eucaryotic cells (such as hybridoma cells), electroporation, a procedure that involves a strong electrical discharge through a suspension of cells in the presence of plasmid DNA, is reported to be the method of choice. The buffer in which the cells were pulsed, the form of plasmid DNA, and the strength of the electric field were described to be crucial factors for successful transfection. Therefore, optimal conditions for the electroporation of hybridoma cells were investigated. Experiments showed that the use of serum containing culture medium as electroporation buffer was beneficial for the transfection of hybridoma cells, since about twice as many cells survived the electrical discharge; and the stable transfection frequency increased about 10- to 30-fold, as compared with standard electroporation buffers such as Hepes buffered saline (chapter 7). Moreover, using (serum-containing) culture medium instead of Hepes buffered saline, plasmid linearization was no longer required, since this did not further enhance the transfection frequency. The present method for enhanced transfection of eucaryotic cells may also be applicable for various other purposes besides the introduction of dominant drug-selection markers for quadroma isolation.

The selection procedure based entirely on drug-selection was first applied for the isolation of a quadroma cell line combining the properties of Y22 and 12-5-3, i.e. specificity for fibrin and for free t-PA, respectively. The procedure was very effective, since all isolated cells produced bs-MoAb (chapter 7). One cell line, designated as Q8-1-2, was selected for its relatively high response in the assay which measures bs-MoAb activity (accumulation of t-PA antigen onto a fibrin surface).

Using the improved method for quadroma isolation as developed in chapter 7, an additional bs-MoAb was produced, directed against both fibrin and u-PA. The bs-MoAb was prepared by fusion of Y22 with the anti-u-PA MoAb producing hybridoma UK50.2. The latter MoAb was partly characterized and shown to produce MoAb specific for u-PA which do not affect the enzymatic activity of u-PA towards plasminogen. Furthermore, UK50.2 reacts both with the active two-chain form of u-PA (tcu-PA) and with its precursor form single-chain u-PA (scu-PA). Scu-PA is, as a true pro-enzyme, enzymatically inactive, and resistant to inhibitors such as PAI-1. The quadroma cell line

isolated from this combination, designated as QUK52, produces bs-MoAb that confers to u-PA a high affinity for fibrin, a property u-PA does not have in the absence of the bs-MoAb.

During the studies on the effect of the bs-MoAb Q6-3-8, Q8-1-2 and QUK52 on the fibrinolytic potency of t-PA or u-PA, respectively, some shortcomings were observed in the *in vitro* model initially chosen. This model consists of a fibrin surface prepared by adsorbing fibrinogen to a microtitre plate and then converting the adsorbed fibrinogen to fibrin by thrombin. Binding of plasminogen activator to adsorbed fibrin was assessed by measurement of either t-PA or u-PA antigen or enzymatic activity. The enzymatic activity of immobilized plasminogen activator was measured using soluble (chromogenic) substrates. Apparently, the interaction of t-PA with plasminogen when immobilized to the adsorbed fibrin, either directly or via a bs-MoAb, was not optimal, since the addition of CNBr digested fibrinogen (stimulator) strongly increased the t-PA-mediated plasminogen activation (chapter 8). Presumably, the formation of the cyclic ternary complex between fibrin, t-PA and plasminogen, essential for efficient plasminogen activation, is impaired on the adsorbed fibrin. This is probably due to the absence of polymerization or incorrect polymerization of the adsorbed fibrin molecules, resulting in an improper assembly of the binding sites for t-PA and plasminogen. The introduction of the Enzyme Linked Fibrinolytic Assay (ELFA) compensated these shortcomings. The ELFA consists of a pre-formed fibrin gel, prepared by the thrombin-mediated polymerization of peroxidase-labeled fibrinogen in the wells of a microtitre plate. Fibrinolytic activity is assessed, after the addition of plasminogen activator and plasminogen, by determining fluid-phase peroxidase levels. The fibrin gel apparently allows for the correct binding of t-PA and plasminogen to their appropriate binding sites on fibrin, as shown after the addition of stimulator which did not further enhance the fibrinolytic activity of fibrin-bound t-PA (chapter 8). Furthermore, the assay is more physiological, since it measures plasmin-mediated degradation of pre-formed fibrin, instead of the degradation of soluble synthetic substrates.

To determine fibrin-specific fibrinolytic activity, a two-step variant of the ELFA was developed, which involves a wash-step to remove unbound plasminogen activator before plasminogen is added (chapter 8). With this assay, Q6-3-8 was shown to increase the fibrin-specific fibrinolytic activity of t-PA 10- to 20-fold. The bs-MoAb Q8-1-2 had no effect on the fibrin-specific fibrinolytic activity of t-PA. However, since the anti-t-PA moiety (i.e. 12-5-3) in the bs-MoAb Q8-1-2 inhibits the enzymatic activity of t-PA towards plasminogen, and since t-PA activity was detected, this suggests that in the ELFA some t-PA dissociates from the bs-MoAb and binds to the fibrin to induce plasminogen activation and fibrinolysis. The bs-MoAb QUK52 conferred a high fibrin-specific fibrinolytic activity to both tcu-PA and scu-PA, a property they do not exhibit in the absence of QUK52.

All experiments described in this thesis illustrate that the bs-MoAb induced increased fibrinolytic potency of t-PA or u-PA *in vitro*. In these assays only an increased targeting of the plasminogen activator to fibrin is expressed. *In vivo*, additional effects are expected on the basis of the specificities of the MoAb selected for bs-MoAb production, related to an altered behaviour of the plasminogen activator after complexing to the bs-MoAb. With Q6-3-8, the plasma half-life of t-PA is expected to increase, since the anti-t-PA moiety in the bs-MoAb (i.e. 1-3-1) specifically inhibits the receptor mediated uptake of t-PA by liver endothelial cells. The other two bs-MoAb, Q8-2-1 and QUK52, do not specifically inhibit the uptake of t-PA or u-PA, respectively, but an effect on their pharmacokinetic behaviour is not unlikely. The potential benefits of targeting t-PA with Q8-1-2 may be that while in circulation as a complex with Q8-1-2, t-PA is inactive towards plasminogen, since the anti-t-PA moiety in Q8-1-2 inhibits the enzymatic activity of t-PA, and at the same time t-PA is protected from inhibition by PAI-1. Once accumulated on the clot, as suggested by the results in the ELFA, enough t-PA may dissociate from the bs-MoAb to initiate clot lysis. For QUK52, the major application may be the targeting of especially scu-PA. While in circulation, scu-PA is inactive towards plasminogen and resistant to PAI-1 inhibition. The high affinity of scu-PA for fibrin conferred by QUK52, results in a fast accumulation of scu-PA at the clot surface, where scu-PA is rapidly converted to the active form tcu-PA, by clot-associated (formed) plasmin, to initiate clot lysis. Experiments *in vivo*, planned for the near future, will tell whether these predicted effects actually happen and whether antibody-targeted thrombolytic therapy using the present bs-MoAb increase the thrombolytic potency and thrombus specificity of t-PA and u-PA.

As mentioned in chapter 2, other strategies have been developed to increase the efficacy of t-PA or u-PA in thrombolytic therapy. Most often these strategies involve the production of mutant or chimaeric t-PA or u-PA molecules, or the chemical cross-linking of proteins. These strategies may be less successful when compared with the presently used bs-MoAb produced by somatic cell fusion. In many cases, mutants of t-PA or u-PA and chimaeric proteins will probably not have the correct folding and delicate structure of the native plasminogen activator. This may decrease enzymatic activity and/or hamper the correct assembly (mutual orientation) of the active site of the plasminogen activator and its substrate plasminogen at the fibrin surface. Chemical cross-linking will often lead to a decreased activity and heterogeneous preparations. Because of the flexible nature of the antibody molecule, and optimal preservation of the wildtype configuration of the plasminogen activator, the chance of an impeded plasminogen activation at the fibrin surface is less likely with the present bs-MoAb. Results with the bs-MoAb Q6-3-8 indicate that t-PA, immobilized to a fibrin gel via the bs-MoAb, is able to associate with its binding site(s) on fibrin and to effectively activate plasminogen.

Fibrin and aggregated platelets, both major components of thrombi, are commonly

used as targets in antibody-targeted thrombolytic therapy. In this approach, MoAb specific for fibrin or for activated GIIa/IIIb complex, respectively, are used for bs-MoAb production. However, these components also occur in the so-called haemostatic plugs. Thus, while a reduced systemic plasminogen activation may be achieved during thrombolytic therapy, due to the increased fibrin- or platelet-specificity of the plasminogen activators after complexing to the bs-MoAb, this may coincide with an increased degradation of the haemostatic plugs. As yet, there is no true thrombus-specific antigen known that can be used for thrombus-specific antibody-targeted thrombolytic therapy. However, a thrombus-specific antigen, or a specific combination of antigens, might be found. In that case, absolute thrombus-specificity in thrombolytic therapy would be within reach.

One of the advantages of our bs-MoAb, is the specificity of the anti-fibrin moiety Y22 used for bs-MoAb production. Earlier experiments have shown that the Y22 MoAb binding to freshly-formed clots, is faster than to aged clots. The matrix in a thrombus, and especially the occluding part of a thrombus in acute myocardial infarctions, consists mainly of recently-polymerized fibrin. This suggests our bs-MoAb would target t-PA or u-PA preferably to such thrombi instead of to the often more established haemostatic plugs.

Other applications for MoAb against t-PA and u-PA currently under investigation, are their use in immunohistochemistry to visualize t-PA and/or u-PA antigen in pathological tissue-samples. The absence or presence of t-PA and/or u-PA on these cells may give an indication for the malignancy/benignancy of these tissues (tumour cell markers), since t-PA and especially u-PA have been identified as playing an important role in tumour growth and metastasis. The presence of these antigens on the cell surface may correlate with (an increased risk for) these processes.

SAMENVATTING

In dit proefschrift worden de isolatie, karakterisering en de toepassingen van monoklonale antilichamen gericht tegen een aantal componenten van het fibrinolyse systeem beschreven. Het fibrinolyse systeem is hoofdzakelijk verantwoordelijk voor de afbraak van bloedstolsels, maar onderdelen van het fibrinolyse syteem spelen ook een rol bij andere fysiologische en pathologische processen. Daarnaast worden bepaalde onderdelen van het fibrinolyse systeem, namelijk weefsel-type plasminogeen aktivator (t-PA) en urokinase-type plasminogeen aktivator (u-PA) gebruikt voor trombolytische therapie. Voor het onderzoek naar de vaak complexe processen in het fibrinolyse systeem kunnen monoklonale antilichamen worden ingezet. Zo kunnen zij bijvoorbeeld worden gebruikt voor struktuur/funktie onderzoek of de zuivering van afzonderlijke componenten. Verder kunnen monoklonale antilichamen worden gebruikt voor het doelgericht transporteren van t-PA en u-PA naar een trombus (zie ook hoofdstuk 2).

Allereerst werd een set antistoffen tegen t-PA, een sleutel-enzym in het fibrinolyse systeem, geïsoleerd en gekarakteriseerd (hoofdstuk 3). Analyse van het effekt van deze antistoffen op t-PA leverden een aantal interessante resultaten:

* Zo bleken de antistoffen 1-3-1 en 7-8-4 de opname van t-PA via de receptoren op respektievelijk lever-endotheelcellen en lever-parenchymcellen specifiek te remmen. Dit bevestigt de aanwezigheid van minstens twee afzonderlijke routes voor de klaring van t-PA uit plasma.
* Een andere antistof, 12-5-3, bleek specifiek te reageren met vrij (niet-remmer-gebonden) t-PA. Kennelijk competeren 12-5-3 en PAI-1 voor de zelfde bindingsplaats op t-PA, omdat de één de binding van de ander uitsloot. Waarschijnlijk heeft 12-5-3 zijn epitoop in of bij het aktieve centrum van t-PA, omdat 12-5-3 ook de amidolytische aktiviteit van t-PA sterk remt.
* Tenslotte bleek 1-3-1 onder invloed van de lysine analoog 6-amino hexaanzuur (6-AHA) te dissocieren van t-PA. Kennelijk competeren 6-AHA en 1-3-1 voor de zelfde (lysine) bindingsplaats op t-PA; of 6-AHA induceert een voor 1-3-1 ongunstige conformatie verandering in t-PA.

Met behulp van deze (en andere) monoklonale antistoffen werden, gebaseerd op hun specifieke eigenschappen, enkele praktische toepassingen ontwikkeld.

Een onderzochte toepassing is de mogelijke grootschalige zuivering van t-PA uit geconditioneerd (melanoma) medium met behulp van immunoaffiniteits chromatografie onder milde condities (hoofdstuk 4). Aangetoond werd, dat wanneer t-PA van geïmmobiliseerde 1-3-1 werd geëlueerd bij neutrale pH met behulp van een buffer waaraan 6-AHA was toegevoegd, de opbrengst aan t-PA aktiviteit in de gezuiverde frakties veel hoger was (+ 30%), dan wanneer t-PA werd geëlueerd met behulp van een

meer gebruikelijke methode voor de verbreking van antistof/antigeen binding zoals een sterke pH-daling. De mogelijkheid een sterke pH verandering te vermijden, vermindert blijkbaar de denaturatie van het gezuiverde t-PA en waarschijnlijk ook van de geïmmobiliseerde antistof.

Een andere toepassing was de ontwikkeling van een antigeen bepaling voor totaal t-PA in plasma (hoofdstuk 5). De gebruikte anti-t-PA antistoffen bleken in gelijke mate te reageren met de meest voorkomende vormen van t-PA; een- en twee-ketenig t-PA; vrij t-PA en t-PA gebonden aan PAI-1. De concentratie van totaal t-PA in plasma is een mogelijke indicatie voor een verstoorde hemostatische balans, en/of het voorkomen van pathologische (trombotische) processen. Bovendien reageert de test in gelijke mate met t-PA van verschillende herkomst, zodat de test ook geschikt is voor het vervolgen van patiënten tijdens trombolytische therapie met bijvoorbeeld recombinant t-PA.

Zoals vermeld, worden t-PA en u-PA gebruikt voor trombolytische therapie. Het succes van deze stoffen is beperkt, hetgeen verklaard wordt door de beperkte fibrine specificiteit van t-PA en met name u-PA; de snelle klaring van t-PA en u-PA in de lever; en/of de remming van t-PA en u-PA door PAI-1. De efficiëntie van t-PA en u-PA in trombolytische therapie kan worden verbeterd door verhoging van hun fibrine specificiteit met behulp van zogenaamde bispecifieke antistoffen (hoofdstuk 2). Door een juiste selectie van monoclonale antistoffen voor de bereiding van dergelijke bispecifieke antistoffen, beoogden wij een gelijktijdige verbetering van meerdere negatieve eigenschappen van t-PA en u-PA te bereiken: de geringe fibrine specificiteit èn de snelle klaring in de lever en/of de remming door PAI-1.

Bispecifieke antistoffen worden geproduceerd door hybride hybridoma's, ook wel quadroma's. Een cruciale stap in deze techniek voor bispecifieke antistof produktie is, na fusie van de twee hybridomas's, de isolatie van de quadroma cel uit het mengsel van ongefuseerde en verkeerd gefuseerde hybridoma's. Uiteindelijk werd gekozen voor een selectie-systeem gebaseerd op dubbele cellulaire resistentie. Daartoe werd een anti-fibrine producerende hybridoma cellijn geselecteerd voor i) gevoeligheid voor de combinatie van hypoxantine, aminopterine en thymidine (HAT) en ii) resistentie tegen G-418, een neomycine analoog. Deze dubbel-gemuteerde cellijn kon zodoende gebruikt worden als universele fusiepartner voor iedere (wildtype) anti-t-PA of anti-u-PA producerende hybridoma (HAT resistent en G-418 gevoelig). Selectie van quadroma's na celfusie geschiedde eenvoudigweg door toevoeging van HAT en G-418 aan het kweekmedium.

Een aantrekkelijke methode om cellen resistent te maken tegen een bepaalde stof is door transfektie met een bacterieel plasmide, met daarop het gen dat codeert voor een bepaalde resistentie. Daartoe werd naar geschikte condities gezocht voor de transfektie van hybridoma's met behulp van elektroporatie (hoofdstuk 7). Aangetoond werd dat het gebruik van met serum gesuplementeerd kweekmedium, in plaats van de meer

gebruikelijke Hepes-gebufferde-zout-oplossing, als elektroporatiebuffer een gunstig effekt had op zowel de overleving van de cellen na de puls (2 tot 3 maal verhoogd), als op de stabiele transfektie frequentie (20 tot 30 maal verhoogd). De op deze wijze getransfekteerde resistentie werd succesvol toegepast voor de selectie van enkele quadroma cellen.

Uiteindelijk werden drie verschillende bispecifieke monoklonale antilichamen, reagerend met fibrine en t-PA of met fibrine en u-PA, geproduceerd, gezuiverd en getest. Deze bispecifieke antilichamen kombineren de speciale eigenschappen van de monoklonale antistoffen gebruikt voor bispecifieke antistof produktie: anti-fibrine Y22 in kombinatie met anti-t-PA 1-3-1; met anti-t-PA 12-5-3; of met anti-u-PA UK50.2.

- De eerste bispecifieke antistof (Y22 & 1-3-1), werd gecodeerd als Q6-3-8 en is specifiek voor fibrine en t-PA; heeft geen effekt op de enzymatische aktiviteit van t-PA; en zal *in vivo* waarschijnlijk de opname van t-PA door lever-endotheel cellen remmen (hoofdstuk 6). Van Q6-3-8 werd in een testsysteem *in vitro* aangetoond dat het de fibrinespecificiteit van de werking van t-PA 10- to 20-voudig verhoogde (hoofdstuk 8).
- Een tweede bispecifieke antistof (Y22 & 12-5-3), werd gecodeerd als Q8-1-2 en is specifiek voor fibrine en vrij t-PA; en voorkomt de binding van PAI-1 aan t-PA (hoofdstuk 7). In een testsysteem *in vitro* bleek Q8-1-2 de fibrine specificiteit voor de werking van t-PA niet te verhogen, ondanks de door Q8-1-2 verhoogde binding van t-PA aan fibrine (hoofdstuk 8). Dit is hoogstwaarschijnlijk het gevolg van het remmende effekt van het anti-t-PA gedeelte 12-5-3 van deze bispecifieke antistof op de enzymatische aktiviteit van t-PA. Omdat de t-PA aktiviteit niet volledig werd geremd, suggereert dit dat er kennelijk voldoende t-PA dissocieert van de bispecifieke antistof naar het fibrine oppervlak om daar plasminogeen te aktiveren.
- De derde bispecifieke antistof (Y22 & UK50.2), werd gecodeerd als QUK52 en is specifiek voor fibrine en u-PA; heeft geen effekt op de enzymatische aktiviteit van u-PA; en is in staat om zowel twee-ketenig u-PA als een-ketenig u-PA te binden (hoofdstuk 8). Deze bispecifieke antistof gaf aan de werking van u-PA een hoge fibrine specificiteit; een eigenschap die u-PA in het geheel niet heeft in afwezigheid van de QUK52 (hoofdstuk 8).

Met deze testen *in vitro* werd alleen het effekt van de bispecifieke antistoffen op de fibrine specificiteit gemeten. Door de speciale eigenschappen van de geselecteerde monoklonale antistoffen zullen bij gebruik *in vivo* waarschijnlijk additionele voordelen optreden:

* Met Q6-3-8 zal mogelijk de plasma halfwaardetijd van t-PA aanmerkelijk worden verlengd, want t-PA zal in complex met Q6-3-8 naar verwachting minder snel door lever-endotheel cellen worden opgenomen.

* Met Q8-1-2 zal waarschijnlijk de binding van PAI-1 aan t-PA worden verhinderd. Bovendien zal tijdens het transport van t-PA in complex met Q8-1-2 naar de trombus, t-PA niet in staat zijn vrij plasminogeen te aktiveren. Op de trombus kan, zoals gesuggereerd uit de testen *in vitro*, voldoende t-PA dissociëren van het bispecifieke antilichaam om fibrinolyse te induceren. Het is niet onwaarschijnlijk dat na binding van t-PA aan Q8-1-2 ook de plasma halfwaardetijd van t-PA zal worden verlengd.
* De grootste winst bij het gebruik van QUK52 zal liggen in het transporteren van met name een-ketenig u-PA naar de trombus. Tijdens het transport in complex met QUK52 is deze vorm van u-PA niet in staat vrij plasminogeen te aktiveren, en bovendien ongevoelig voor remmers als PAI-1. Het een-ketenig u-PA zal via QUK52 snel accumuleren op de trombus. Daar kan het worden omgezet in het aktieve twee-ketenig u-PA, om vervolgens plasminogeen in/aan de trombus te aktiveren. Het is niet onwaarschijnlijk dat na binding van u-PA aan QUK52 ook de plasma halfwaardetijd van u-PA zal worden verlengd.

Experimenten in proefdieren, die op korte termijn worden uitgevoerd, zullen moeten aantonen of de trombus specificiteit en de trombolytische potentie van t-PA en u-PA door deze speciale bispecifieke monoklonale antistoffen inderdaad wordt verhoogd.

EENVOUDIG GEZEGD

Monoklonale antistoffen zijn oorspronkelijk afkomstig uit het natuurlijke afweersysteem. Zij reageren zeer specifiek met bepaalde structuren op cellen of eiwitten die niet in ons lichaam thuishoren (antigenen). Deze specificiteit is de basis voor het gebruik van monoklonale antistoffen in talloze technieken in het medisch en biologisch onderzoek. Zo kunnen zij worden gebruikt voor het zuiveren van bepaalde eiwitten uit ingewikkelde mengsels (immunozuivering). Ook kan het effect van monoklonale antistoffen op een bepaalde werking van een eiwit opheldering geven over het verband tussen de structuur en de functie van dit eiwit (structuur/functie relaties). Verder kunnen monoklonale antistoffen worden gebruikt als zogenaamde 'toverkogels' (in het Engels 'Magic Bullets'); dat wil zeggen monoklonale antistoffen kunnen gebruikt worden om heel specifiek bepaalde stoffen te transporteren naar de plaats waar deze werkzaam dienen te zijn. Zodoende wordt het lichaam als geheel minder belast met hoge concentraties van deze actieve stoffen, die mogelijkerwijs schadelijk kunnen zijn voor andere delen van het organisme.

Het fibrinolyse systeem is verantwoordelijk voor de afbraak van bloedstolsels. Dit gebeurt via een ingewikkeld proces waarbij verschillende (voorlopers van) enzymen, aktivatoren, remmers en versnellers een rol spelen. Een eindprodukt van het fibrinolyse systeem is het aktieve enzym plasmine, dat wordt gevormd uit zijn inaktieve voorloper plasminogeen door zogenaamde plasminogeen aktivatoren. Voorbeelden van plasminogeen aktivatoren zijn weefsel-type plasminogeen aktivator (t-PA) en urokinase-type plasminogeen aktivator (u-PA). Het gevormde plasmine breekt het fibrine netwerk af, dat zorgt voor de stabiliteit van een bloedstolsel. Door gebruik te maken van (de specificiteit van) monoklonale antistoffen, is men in staat nauwkeuriger onderzoek te doen aan al die complexe processen.

Sommige onderdelen van het fibrinolyse systeem, de eerder genoemde plasminogeen activatoren, worden bovendien gebruikt voor de behandeling van patiënten met een ziekmakend bloedstolsel, een zogenaamde trombus. De trombus sluit een bloedvat af, zodat achterliggend weefsel verstoken blijft van voedsel en zuurstof. Door plasminogeen aktivatoren toe te dienen wordt het fibrinolyse systeem geaktiveerd zodat het fibrine in de trombus wordt afgebroken. Deze behandeling heet trombolytische therapie. Het nadeel bij het gebruik van de huidige plasminogeen aktivatoren is hun beperkte specificiteit voor fibrine; hun korte aanwezigheid in de bloedbaan door de snelle opname in de lever; en hun gevoeligheid voor natuurlijk voorkomende remmers. Als gevolg hiervan moet veel van het medicijn worden toegediend aan de patiënt, hetgeen resulteert in bijwerkingen zoals de aktivering van plasminogeen in de bloedbaan en niet uitsluitend ter plaatse van een trombus. Plasmine in de bloedbaan kan ook talloze andere

belangrijke bloedeiwitten afbreken, met allerlei nare gevolgen. Met behulp van monoklonale antistoffen kunnen deze plasminogeen aktivatoren heel specifiek worden getransporteerd naar de trombus, waardoor lagere doses nodig zijn en de kans op bijverschijnselen wordt verkleind.

In het kader van dit proefschrift werden enkele toepassingen van monoklonale antistoffen reagerend met verschillende onderdelen van het fibrinolyse systeem onderzocht. Daartoe werd eerst een verzameling van monoklonale antistoffen gemaakt en gekarakteriseerd die specifiek binden aan t-PA. Van deze verzameling bleek een aantal zeer speciale eigenschappen te hebben. Zo verminderden sommige monoklonale antistoffen de snelle opname van t-PA door levercellen. Een andere monoklonale antistof vehinderde de binding van t-PA aan een ander eiwit (PAI-1), dat de werking van t-PA remt. Van deze speciale eigenschappen werd gebruik gemaakt voor de ontwikkeling van enkele bijzondere toepassingen voor deze monoklonale antistoffen.

De eerste toepassing die wij onderzochten was het zuiveren van t-PA uit het medium (kweekvloeistof) van cellen die veel t-PA produceren. Dergelijke celkweken worden bij de bereiding van t-PA voor trombolytische therapie veel gebruikt. Met behulp van een van de monoklonale antistoffen uit de verzameling kon nu t-PA op een eenvoudige en snelle methode worden gezuiverd. Door een bijzondere eigenschap van deze monoklonale antistof, bleek de opbrengst aan t-PA hoger te zijn dan met standaard zuiveringsmethoden; het t-PA werd minder beschadigd en was dus beter werkzaam.

Andere monoklonale antistoffen uit de verzameling werden gebruikt om een relatief eenvoudige, maar zeer gevoelige test op te zetten om t-PA in bloed te bepalen. Dit kan van pas komen, omdat een afwijkende concentratie van t-PA in bloed een mogelijke indicatie is voor storingen in de stolselafbraak. De test kan ook worden gebruikt om tijdens trombolytische therapie met t-PA te meten of de toegediende hoeveelheden voldoende zijn.

Zoals reeds vermeld kunnen monoklonale antistoffen ook gebruikt worden als toverkogels voor het gericht transporteren van aktieve stoffen (zoals bijvoorbeeld plasminogeen aktivatoren) naar hun doel (zoals bijvoorbeeld een trombus). Deze toepassing werd uitgebreid onderzocht en verschillende nieuwe strategieën en methoden werden ontwikkeld om verbeterde toverkogels te maken. Daartoe werden zogenaamde bispecifieke monoklonale antistoffen ontwikkeld. Bispecifieke antistoffen zijn in staat om niet aan één, maar aan twee antigenen heel specifiek te binden. Een bispecifieke antistof bindt enerzijds aan bijvoorbeeld t-PA of u-PA, en anderzijds aan bijvoorbeeld fibrine, een hoofdbestandeel van de trombus. Zodoende krijgt het t-PA of u-PA na binding aan de bispecifieke antistof een hoge affiniteit voor (neiging tot binden aan) fibrine. Wij zochten nu naar speciale bispecifieke antistoffen waarbij de binding van t-PA of u-PA aan de bispecifieke antistoffen -naast een versterkte binding aan fibrine-een extra effect bewerkstelligde, bijvoorbeeld een verminderde opname van t-PA of u-PA door

levercellen of een verminderde binding van t-PA of u-PA aan remmers. Dergelijke bispecifieke antistoffen zullen dus niet alleen de binding van t-PA of u-PA aan fibrine verhogen en hun werking doelgerichter maken, maar gelijktijdig hun effektieve levensduur in de circulatie verlengen. Wij hopen dat daardoor lagere concentraties van t-PA of u-PA nodig zijn voor een effectieve therapie, zodat minder bijverschijnselen zullen optreden.

Monoklonale antistoffen worden geproduceerd door speciale cellen; de hybridoma's. Wanneer nu twee verschillende hybridoma cellen met elkaar worden versmolten (gefuseerd), ontstaat een nieuwe soort cel die hybride hybridoma of quadroma wordt genoemd. Deze quadroma zal nu monoklonale antistoffen produceren die de specifieke bindings-eigenschappen van de beide afzonderlijke monoklonale antistoffen, geproduceerd door de hybridoma's kombineert. Zo'n monoklonale antistof is dus bispecifiek. Door een juiste selectie van de te fuseren hybridoma's, kan men de speciale eigenschappen van de door hun geproduceerde monoklonale antistoffen in de gewenste kombinatie samenvoegen tot speciale bispecifieke antistoffen.

Om bispecifieke antistoffen te maken, moet men eerst in staat zijn een quadroma te isoleren. Een methode daarvoor is de twee te fuseren hybridoma's ieder ongevoelig (resistent) te maken voor een voor de andere hybridoma dodelijke stof. Na celfusie worden de cellen gekweekt in aanwezigheid van beide dodelijke stoffen. Alleen quadroma's kunnen dan groeien, want alleen zij hebben de resistenties van de beide gefuseerde hybridoma's overgeërfd. Om nu een hybridoma resistent te maken, werd een speciale techniek gebruikt. In deze techniek wordt een stukje erfelijk materiaal (DNA) van een bacterie, met daarop het gen voor een bepaalde resistentie, in de hybridoma cel gestopt (transfektie). Wij vonden een methode waarbij de transfektie van dit stukje bacterie DNA in de hybridoma cel veel efficiënter verloopt dan onder de gebruikelijke condities. Zo kunnen snel verschillende quadroma's worden gemaakt.

Uiteindelijk werden drie quadroma cellijnen geïsoleerd, die ieder een speciale bispecifieke antistof produceren. Deze bispecifieke antistoffen reageren met zowel fibrine als t-PA (gecodeerd als Q6-3-8 en Q8-1-2), of met fibrine en u-PA (gecodeerd als QUK52). Alle drie de bispecifieke antistoffen bleken in staat de binding van t-PA of u-PA moleculen op fibrine te verhogen. Twee van de drie bispecifieke antistoffen zorgden er bovendien voor dat de aktivering van plasminogeen door t-PA (met Q6-3-8) of door u-PA (met QUK52) effectiever verloopt op het fibrine oppervlak dan in oplosssing; de fibrinespecificiteit van de werking van t-PA en u-PA was aanmerkelijk verhoogd. De derde bispecifieke antistoffen (Q8-1-2) verhoogde niet de fibrinespecifieke werking van t-PA, ondanks de door deze bispecifieke antistoffen geïnduceerde verhoging van de binding van t-PA aan fibrine. Dit is het gevolg van een remmend effekt van deze bispecifieke antistof op de werking van t-PA.

Deze laboratorium testen laten alleen het effekt zien van de bispecifieke antistoffen

op de fibrinespecificiteit van de werking van t-PA en u-PA. Dat is maar één beoogd aspect van deze speciale bispecifieke antistoffen. In een levend organisme zullen mogelijk extra effekten een rol spelen, door de speciale eigenschappen van de monoklonale antistoffen die zijn geselecteerd voor bispecifieke antistof produktie. Zo zal de bispecifieke antistof Q6-3-8, naast een verhoging van de binding van t-PA aan fibrine, wellicht ook de verblijfsduur van het t-PA in de bloedbaan verlengen, want de gebruikte monoklonale anti-t-PA antistof in deze bispecifieke antistof remt de opname van t-PA door levercellen. Zodoende heeft t-PA langer de tijd zijn doel te bereiken. De bispecifieke antistof Q8-1-2 zal tijdens het transport van t-PA naar de trombus de remmende werking van PAI-1 op t-PA verhinderen, want dat is een eigenschap van de anti-t-PA monoklonale antistof die werd gebruikt voor deze bispecifieke antistof. Bovendien, zal tijdens het transport van het t-PA door de bispecifieke antistof Q8-1-2 naar de trombus, t-PA niet in staat zijn om plasminogeen in circulatie te aktiveren. Voor de bispecifieke antistof QUK52 ligt de grootste winst in het transporteren van de inaktieve voorloper van u-PA (pro-u-PA). Tijdens het transport door QUK52 van pro-u-PA naar de trombus, zal deze voorloper niet in staat zijn om plasminogeen in de circulatie te aktiveren. Bovendien, ook een eigenschap van voorlopers van enzymen, is het pro-u-PA ongevoelig voor remmers als PAI-1. Na de ophoping van het pro-u-PA door QUK52 op het fibrine van de trombus, zal aldaar het pro-u-PA snel worden omgezet tot aktief u-PA en vervolgens fibrine afbraak induceren.

Op grond van het totaal van deze effekten van de bispecifieke antistoffen op t-PA of u-PA, verwachten wij dat de effektieve doses van t-PA of u-PA drastisch kunnen worden verlaagd en de bijwerkingen van deze middelen, die ontstaan door aktivatie van plasminogeen in het bloed in plaats van op het stolsel, sterk worden verminderd. Dit waren maar een paar toepassingen voor deze monoklonale antistoffen. Ongetwijfeld zullen in de toekomst meer toepassingen met meer monoklonale antistoffen worden gevonden.

NAWOORD

Een proefschrift is niet af zonder een woord van erkentelijkheid aan de mensen die direct of indirect hun bijdrage hebben geleverd aan de totstandkoming ervan.

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CURRICULUM VITAE

De auteur van dit proefschrift werd op 19 september 1960 geboren in Vlaardingen. Het basis- en middelbaaronderwijs, genoten aan verschillende scholen verspreid over Nederland (Vlaardingen, Nijmegen, Leiden, Vlaardingen en Schiedam), werd medio 1979 succesvol afgerond met het VWO-B diploma. In hetzelfde jaar begon hij aan zijn studie Biologie aan de Rijksuniversiteit Leiden. In januari 1987 werd deze studie afgerond met het doctoraalexamen.

In het kader van de vervangende dienstplicht verbleef de auteur van februari 1987 tot september 1988 aan het Gaubius Instituut-TNO te Leiden als wetenschappelijk onderzoeker bij de sektie monoklonale antilichamen. In oktober 1988 werd hij aangesteld als Assistent in Opleiding aan de Rijksuniversiteit Leiden, gedetacheerd bij het Gaubius Instituut-TNO te Leiden, dat later overging in het IVVO-TNO Gaubius laboratorium te Leiden, alwaar onder begeleiding van Dr. W. Nieuwenhuizen de basis werd gelegd voor het werk zoals hier in dit proefschrift beschreven.

Vanaf februari 1992 is de auteur met steun van het Astma-fonds als post-doc in dienst bij het Academisch Ziekenhuis Leiden afdeling Longziekten, wederom gedetacheerd bij het IVVO-TNO Gaubius laboratorium te Leiden.

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*1 = Recipient of the ASH/Chugai-Upjohn student/resident award.
*2 = Recipient of the ISTH young investigators merit award.