

Theme Issue Article

Single-chain antibodies as diagnostic tools and therapeutic agents

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Summary

Over three decades after the generation of the first mouse monoclonal antibodies by Kohler and Milstein, recombinant antibodies are the fastest growing class of therapeutic proteins. Furthermore, antibodies are key detection reagents in research and diagnostics. Technology improvements have provided several approaches to manufacturing human antibodies with high affinity for biologically relevant targets. Approximately 300 development programs for therapeutic antibodies have been reported in industrial and academic laboratories, and this clearly demonstrates the expectations towards antibody technology.

Keywords

Antibody fragments, imaging, thrombosis, inflammation, phage-display

Antibody fragments are a subclass with growing clinical importance. This review focuses on single-chain antibodies as one of the smallest possible format for recombinant antibodies and their use as diagnostic tools and therapeutic agents. We describe the structure, selection and production of single-chain antibodies. Furthermore, we review current applications of antibody fragments focusing on thrombus targeting using fibrin- and platelet-specific single-chain antibodies as well as describing novel non-invasive imaging approaches for the diagnosis of thrombosis and inflammation.

Thromb Haemost 2009; 101: 1012–1019

Structure, selection and production of single-chain antibodies

With the advent of hybridoma technology, it was possible to produce monoclonal antibodies of defined antigen specificity (1). A major breakthrough in the field of antibody engineering was the generation of antibody fragments as recombinant proteins in the periplasmic space of *Escherichia coli* (2). The genes encoding the variable regions of monoclonal antibodies were cloned into *E. coli* expression vectors in order to produce antibody fragments, which preserve the binding specificity of the parental hybridoma antibodies (3). These generated single-chain antibodies (scFvs) are a minimal form of functional antibodies (Fig. 1). The scFv format demonstrates several advantages compared with mouse-derived antibodies or their fragments; the ease of cloning in bacteria offers the opportunity of genetic engineering, such as further optimisation and the construction of fusion molecules without loss of binding function. Single-chain antibodies are expected to be only minimally immunogenic, especially when gen-

erated from human libraries. Their small size facilitates tissue penetration, but also results in a rapid clearance from circulation (4).

Most of the growing number of antibodies in clinical trials are derived from phage-display technology (5) or transgenic mice that express human immunoglobulin genes (6). The binding affinity of human antibodies from transgenic mice is often high, reflecting affinity maturation *in vivo*. Using large natural and synthetic libraries, specific high-affinity antibodies can be selected by linking antigen binding to genotype, thereby allowing simultaneous recovery of the gene encoding the selected antibody. Despite the availability of various alternative methods (7) such as bacterial surface display, ribosomal display, puromycin display or yeast surface display, phage display has become the most widely used method. The technique for isolating specific antibodies by their binding activity *in vitro* is called “panning”. The antigen is immobilised on a solid surface, such as nitrocellulose, magnetic beads, a column matrix, plastic surfaces, microtiter plates or even presented on the cell surface (8). It is also possible to select scFv *in vivo* using phage display as de-

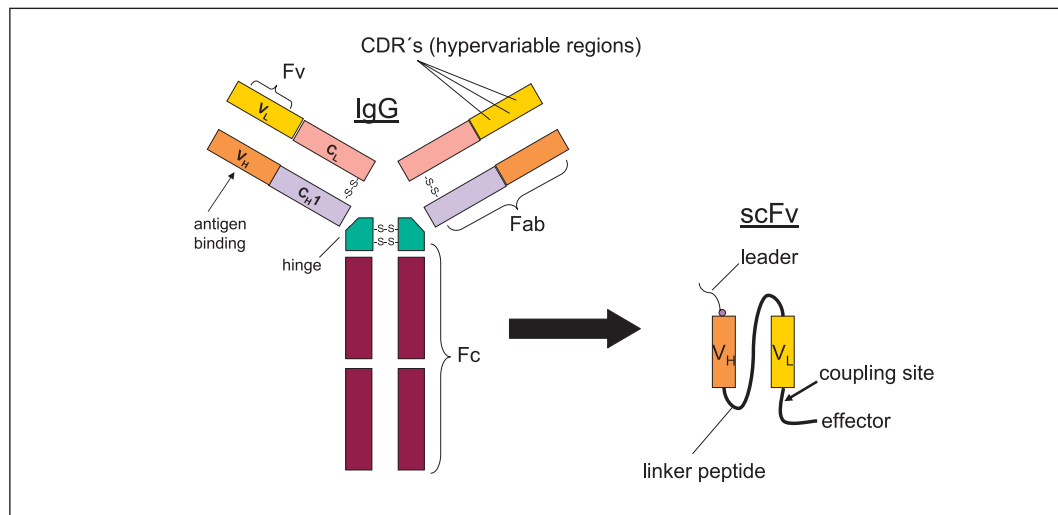
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Received: December 15, 2008
Accepted: February 13, 2009

Prepublished online: April 3, 2009
doi:10.1160/TH08-12-0816

Figure 1: Structure of single-chain antibodies.

Single-chain antibodies are a minimal form of functional antibodies, consisting of the variable regions of only the heavy and light chain of a full IgG antibody, connected by a small linker peptide. Effector molecules can be fused to the scFv-coupling site by standard molecular biology techniques.



scribed recently (9, 10). The antibody phages are incubated with the immobilised antigen, followed by washing to remove excess non-binding antibody phages. The bound phages can subsequently be eluted and reamplified by infection of *E. coli* for the next selection round. Usually two to six panning rounds are necessary to select specifically binding antibody phages. After their isolation some antibody fragments have a sufficiently high binding affinity or biological potency for therapeutic applications.

Production of antibodies for preclinical and clinical trials has been evaluated in numerous expression systems, but bacteria are favoured for small, non-glycosylated Fab and scFv fragments as they can be expressed at low cost and are easily amenable to commercial scale up, usually with terminal polypeptides for affinity purification (11). Several antibody fragments have been produced at gram-per-litre titres by *E. coli* fermentation, a feat that has only recently been approached for IgG molecules in this host (12).

Single-chain antibodies as diagnostic agents

Immunosensors

Recombinant antibodies provide an emerging strategy in the development of new diagnostic agents. In particular, scFvs can be isolated and expressed in bacterial systems that also allow their manipulation *in vitro* at the gene level to introduce linker groups for immobilisation and coupling to nanoparticles. For example, the introduction of a cysteine residue at the C terminus of an scFv construct allows covalent attachment to gold-coated sensor interfaces in directed orientation (13). An array of simultaneous detection measurements revealed specific antigen binding in this system, which was proportional to the antigen concentration. Shen et al. (14, 15) used an additional cysteine or two additional histidines within the linker peptide of the scFv to immobilise the antibody to a gold piezoelectrode surface. All scFvs were correctly oriented, retained antigen-binding activity, and coupled at high surface concentration. These results suggest that the location on the linker sequence, in which the amino acids were incorporated, was well tolerated and did not interfere with scFv

binding activity. Recently, the same group could further improve this approach by generating a polycationic arginine peptide linker absorbed to anionic charged template surfaces (16). These immunosensors have potential applications as highly sensitive detection assays.

Single-chain antibodies in magnetic resonance and near infrared imaging

Atherosclerosis is an inflammatory disease leading to plaque formation and expression of proinflammatory proteins, extracellular matrix proteins and proteases. Imaging of plaque-associated proteins appears to be interesting especially when their expression can be related to local inflammation or characteristics of plaque instability. It has been shown that Extra domain B-containing (ED-B) fibronectin is a potential target for plaque imaging. Dietrich et al. (17) used a novel anti-ED-B scFv conjugated for near infrared fluorescence imaging (NIRF). They examined the association of ED-B with the presence of macrophages in a murine model of atherosclerosis. Expression of ED-B was observed in plaque areas in apolipoprotein E-deficient (apoE^{-/-}) mice, which increased with age and plaque load. Plaque lesion ED-B was expressed by smooth muscle cell and was closely associated to macrophage infiltrates indicating plaque instability.

Platelets are the key to thrombus formation and play a role in the development of atherosclerosis (18, 19). They are lining rupture-prone atherosclerotic plaques and could therefore facilitate detection of platelet-mediated pathology in atherothrombotic disease. The platelet integrin GPIIb/IIIa mediates the final common pathway of platelet aggregation and is the key to thrombus formation on ruptured atherosclerotic plaques. We have recently developed a single-chain antibody that recognises ligand-induced binding sites (LIBS) of GPIIb/IIIa that become exposed only upon receptor activation (20). The LIBS-epitope is located outside the fibrinogen binding area and therefore yields high specificity for the activated GPIIb/IIIa receptor. Consequently, it can also be targeted even in the presence of fibrinogen or GPIIb/IIIa receptor antagonists.

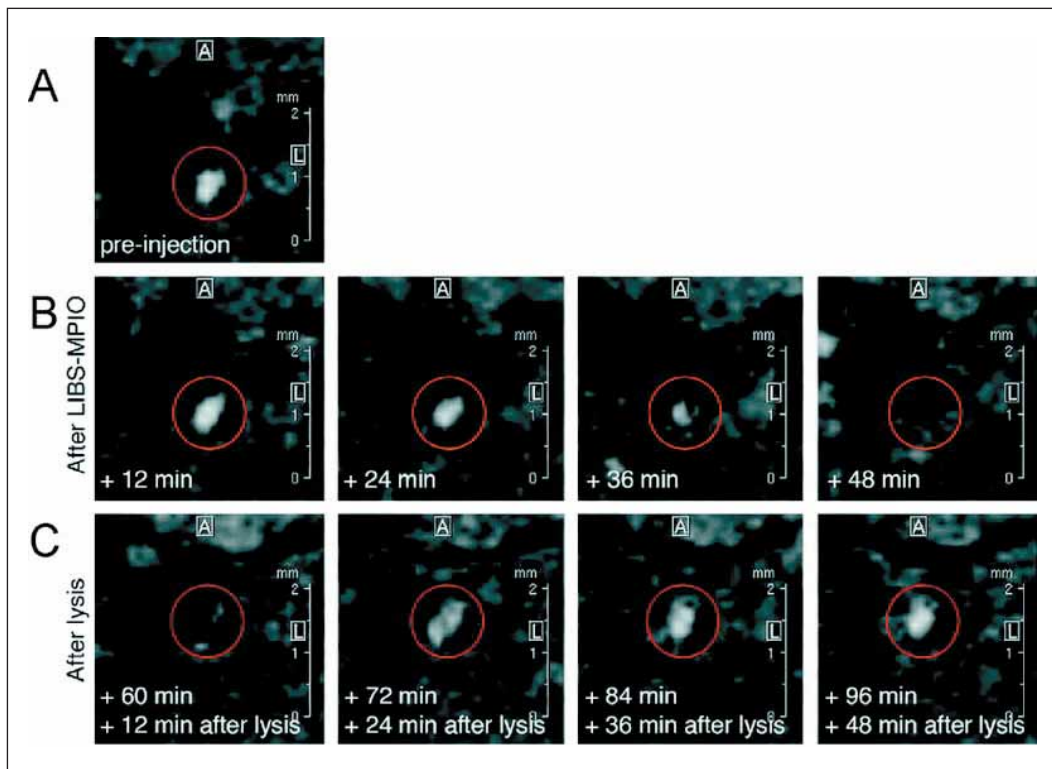


Figure 2: In vivo MRI after carotid artery injury and subsequent treatment with intravenous mouse urokinase. Enlarged transverse sections of the injured right carotid artery. A) The vessel before injection, with a marked and progressive diminution in signal after LIBS MPIO injection between 12 and 48 minutes. B) Thrombolysis was performed after the 48-minute scan. Images obtained after intravenous application of urokinase show the re-appearance of the vessel lumen signal over time indicated by (C).

Recent progress in molecular magnetic resonance imaging (MRI) provides the opportunity to image cells and cellular receptors using iron oxide particles, which provide a potent negative contrast in T2*-weighted MRI. Recent studies have used microparticles of iron oxide (MPIOs) for this purpose (21–23). These MPIOs convey a payload of iron that is many orders of magnitude greater compared to ultrasmall particles of iron oxide. They cause local magnetic field inhomogeneity and therefore MRI signal distortions within an area up to 50 times of the size of the particle depending on the MRI imaging method. We conjugated MPIOs to anti-LIBS-scFv's via the poly-his tag at the C-terminus. In a well-established mouse model of carotid injury, we induced non-occlusive mural thrombi with 6% ferric chloride and imaged thrombosis and thrombolysis at 9.4 tesla by MRI (24). A significant signal void in the carotid artery was observed with LIBS MPIOs, corresponding to MPIO accumulation. After thrombolysis, the signal void subsided, indicating successful thrombolysis (Fig. 2). On histology, the MPIO-content of the thrombus, as well as thrombus size, correlated significantly with LIBS MPIO-induced signal void. In another study, we were able to image platelet adhesion at the site of injury on a 11.7-tesla MRI scanner *ex vivo*, following guidewire injury to mouse femoral artery (25). LIBS-MPIO binding, as quantified by MRI, was four-fold higher with anti-LIBS-MPIO in comparison to control MPIO. We could also validate LIBS-MPIOs at a lower and clinically more relevant magnetic field strength on a 3 Tesla clinical MRI-scanner (26). Ex-vivo binding to human platelet-rich clots as well as significant binding of LIBS-MPIO to a platelet matrix under venous/arterial flow conditions *in vitro* was observed.

Platelets also play an important role in acute inflammation, interacting with leukocytes, and endothelial cells. Cerebral malaria is associated with elevated levels of cytokines in the brain and adherence of platelets to the microvasculature. Using the LIBS-MPIO contrast agent in a mouse model of cerebral malaria, we could detect activated platelets in brains of malaria-infected mice six days after inoculation with *Plasmodium berghei* (27). Importantly, this was at a time when the pathology was otherwise undetectable by conventional MRI. Temporal studies revealed that the level of MPIO-induced contrast was proportional to the number of platelets bound.

These studies highlight the potential of platelet targeted LIBS-MPIO contrast agents for diagnostic, mechanistic, and therapeutic studies *in vitro* and *in vivo* in inflammation and thrombotic events. Further studies will have to prove if the identification of vulnerable, rupture-prone atherosclerotic plaques is possible via non-invasive MRI.

Single-chain antibodies as therapeutic agents

Inhibition of inflammation and complement system suppression

The complement system contributes significantly to the pathogenesis of numerous acute and chronic diseases. Inflammation is a cornerstone of the post-myocardial infarction. Also, during a heart bypass procedure, the „complement activation“ causes an inflammatory response that can lead to side effects such as chest pain, heart attack, stroke, heart failure, or death. Pexelizumab (Alexion Pharmaceuticals), a recombinant humanised scFv to C5, was the first scFv to enter clinical trial (28–30). Pexelizumab

is designed to inhibit complement-mediated tissue damage associated with reperfusion injury and inflammation that occurs during open heart surgery. In clinical trials it appears to reduce cardiac enzyme release and possibly mortality in patients undergoing coronary artery bypass graft surgery and also in the treatment of acute myocardial infarction (31, 32). Pexelizumab can also be used as adjunctive therapy to fibrinolysis and primary percutaneous coronary intervention.

Selectins are a family of adhesion receptors, which mediate rolling of leukocytes on the luminal surfaces of the vascular endothelium. Selectin-mediated rolling is a particularly attractive target for therapeutic intervention because it occurs early in the inflammatory cascade before firm adhesion and extravasation into the surrounding tissue. Blocking selectin binding may reduce tissue damage by both decreasing the number of leukocytes that enter the surrounding tissue and reducing the quantity of proinflammatory cytokines that are released. Blocking scFv have been described against two prominent members of the selectin family: E-selectin (33) and P-selectin (34). Anti-E-selectin antibodies have been effective for the therapy of septic shock, where signs of shock resolution were registered after treatment (35). Blocking P-selectin has been shown to inhibited neutrophil recruitment into chemically inflamed mouse peritoneum after stimulation (36).

Another important adhesion molecules involved in inflammation is the leukocyte integrin Mac-1. Upon leukocyte activation, Mac-1 undergoes a conformational change exposing interaction sites for multiple ligands. We recently generated scFvs directed against activation-specific Mac-1 ligand-binding sites using human scFv phage libraries (37). Potential therapeutic use was tested in adhesion assays under static and flow conditions demonstrating the selective blockade of activated monocytes only. To demonstrate the feasibility of activation-specific anti-Mac-1 scFvs as diagnostic tools, we investigated patients with severe sepsis. Compared to a matched control group without clinical or laboratory signs of inflammation, patients with sepsis demonstrated a significantly increased binding of MAN-1 to peripheral blood monocytes. Thus, anti-Mac-1 scFv's also represent a diagnostic tool for the detection of monocyte activation in sepsis.

Fibrin-targeted anticoagulants

Fibrin is an obvious target to concentrate antithrombotic or fibrinolytic agents at the clot. Sufficient amounts of fibrin are present even in platelet-rich thrombi. Fibrin targeting can be achieved with the monoclonal antibody 59D8, which selectively binds to the amino-terminus of the fibrin beta-chain that becomes exposed after cleavage of fibrinopeptide B by thrombin (38). Because exposure of this epitope is an early event in the conversion of fibrinogen to fibrin, it is likely that 59D8 accumulates at sites of high thrombin activity, such as a developing arterial clot. Other antibodies such as MA-15C5, directed against the fragment D-dimer of cross-linked human fibrin, have also been used successfully to target clots (39), but these are predominately directed against structures of already cross-linked and thus older clots. Another scFv antibody was developed directed against human fibrin clots by using a fully human phage display library (40). The scFv could specifically

recognise human fibrin clots and indicate no binding ability with human fibrinogen shown by ELISA.

Based on the monoclonal antibody 59D8, we generated a fibrin-specific scFv and constructed three different anti-thrombotic fusion proteins. We fused hirudin to the generated fibrin-specific scFv of 59D8 using recombinant techniques to target a developing clot (41). Hirudin can inhibit thrombin that is bound to the clot or to soluble fibrin degradation products (42) and, in contrast to heparin, has no natural inhibitors. Furthermore, hirudin can displace thrombin from platelet thrombin receptors (43). Because hirudin needs a free amino- as well as a free carboxyl-terminus for antithrombin activity, a direct fusion at the terminus of hirudin was expected to result in a functional loss. Therefore, a factor Xa (FXa) cleavage site was introduced between the scFv_{59D8} and hirudin. Without an activated coagulation system, the fusion protein would be inert. However, as a clot develops, the combination of fibrin targeting and dependence on cleavage by FXa could result in an effective thrombin inhibition at the clot without systemic anticoagulation. The generated fusion protein inhibited appositional growth of whole blood clots *in vitro* more efficiently than native hirudin.

Encouraged by the promising results obtained with the targeted thrombin inhibitor scFv_{59D8}-Xa-hirudin, we designed a targeted direct FXa inhibitor, which promises to enhance local anticoagulative potency at the clot and reduce systemic anticoagulation, which potentially results in less bleeding complications (44).

Efficient blocking of FXa can be achieved with the serine protease inhibitor tick anticoagulant peptide (TAP), originally isolated from *Ornithodoros moubata* (45). The recombinant form of TAP has been shown to have a strong antithrombotic effect in models of experimental arterial and venous thrombosis (46) mediated through a specific and potent direct inhibition of fXa independent of other plasma cofactors. In contrast to the scFv_{59D8}-Xa-hirudin, we could show that no FXa cleavage site was necessary and the recombinant fusion did not destroy the function of the fusion partners. This finding introduces TAP as a potent partner for fusion molecules. In human whole blood clots, scFv_{59D8}-TAP revealed anticoagulative properties at concentrations where non-targeted TAP did not reveal anticoagulative activity at all.

Targeted recombinant plasminogen activators

Fibrinolysis has been proven to be very beneficial in patients with acute myocardial infarction (47). The single-chain urokinase-type plasminogen activator (scuPA) is a glycoprotein of 54 kDa. Cleavage by plasmin or kallikrein results in a two-chain derivate (tcuPA) connected by a disulfide bond with a much higher plasminogen activation rate than scuPA. Thus, scuPA generates plasmin activity at the site of a clot, which then potentiates plasminogen activation by converting scuPA to the highly active form tcuPA. However, limitations in thrombolytic efficacy have caused an ongoing search for improved thrombolytic drugs. Using an scFv directed against the fragment D-dimer of cross-linked human fibrin, scuPA has been successfully targeted to clots (39) and tested in different venous thrombosis models *in vivo* (48). We used the fibrin-specific scFv 59D8 to target scuPA to a thrombus in order to enhance thrombolytic potency and clot

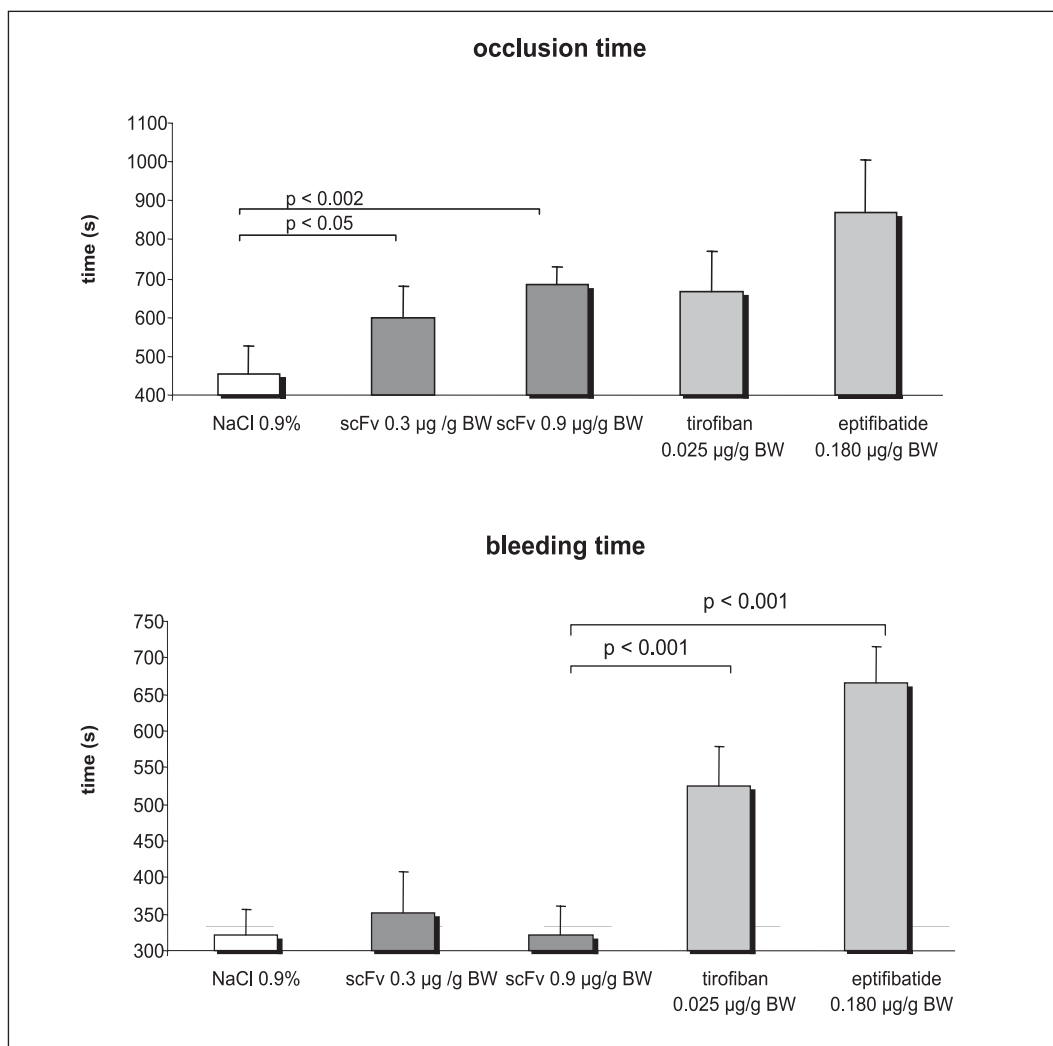


Figure 3: Efficient anti-thrombotic effects without prolongation of bleeding times by activation-specific inhibition of GPIIb/IIIa in mice. Thrombus development was induced by ferric chloride and monitored with a nano-flow probe. Tail transection revealed a prolongation of bleeding time for tirofiban and eptifibatide but not for the activation-specific anti-GPIIb/IIIa antibody (BW = body weight).

specificity (49). In human plasma clots, thrombolysis by scFv_{59D8}-scuPA^{LMW} is significantly faster and more potent compared with the clinically used urokinase.

Recent work was published about a chimeric protein consisting of the C-terminal sequence of hirudin, scu-PA and an RGD sequence derived from the leech protein decorsin also targeting platelets (50). The constructed protein has plasminogen-dependent fibrinolytic activity, platelet aggregation inhibitory activity and antithrombin activity. A thrombus-targeting chimera composed of annexin B1, scuPA and a fibrin-adherent peptide was also described. An in-vitro test showed that the chimera was a thrombolytic agent with activated-platelet membrane and fibrin binding activity. Compared to scuPA, the chimera had less reperfusion time, higher reperfusion ratio, and less bleeding effects on coronary thrombolysis in a dog model (51).

The group of Vladimir Muzykantov elegantly used a scFv antibody against platelet-endothelial-cell adhesion molecule 1 (PECAM-1) for targeted thrombolysis. To prevent thrombus formation, they anchored scuPA to the vascular lumen (52–54) in the lung and the brain. In a model of pulmonary embolism and stroke the chimera protein was more effective than scuPA alone, thereby providing support for the concept of thromboprophylaxis

using recombinant scFv-fibrinolytic fusion proteins. To avoid premature activation and inactivation and to limit systemic toxicity, they also replaced the native plasmin activation site in scuPA with a thrombin activation site, activated by thrombin instead of plasmin (55). In mouse models of pulmonary thrombosis caused by thromboplastin and ischaemia-reperfusion, the targeted construct provided more potent thromboprophylaxis and greater lung protection than the plasmin-sensitive counterpart.

Most recently a heterodimer diabody that inhibits thrombin activatable fibrinolysis (TAFI) and plasminogen activator inhibitor-1 (PAI-1) simultaneously has been reported (56). Both play important roles in fibrinolysis by reducing plasmin generation. It was shown that the diabody revealed a similar affinity and inhibitory properties for TAFI and PAI-1 as that of the parental antibodies in vitro. These observations demonstrate that simultaneous inhibition of TAFI and PAI-1 results in faster lysis of the formed thrombus.

ScFvs against the platelet integrin GPIIb/IIIa

The platelet integrin GPIIb/IIIa is of pivotal importance for coagulation and thrombosis. Its conformation-unspecific blockade has been one of the major advances in antithrombotic therapy of

recent years. The chimeric mouse-human Fab-fragment abciximab, the cyclic peptide eptifibatid, and the synthetic peptidomimetic tirofiban have been successfully used in patients with acute myocardial infarction, unstable angina, or angioplasty (57). However, this current type of GPIIb/IIIa blockade is accompanied by several adverse effects. All of these problems are associated with the property of the inhibitors to bind to the GPIIb/IIIa receptor, irrespective of its activation state, and to act partially as ligand-mimetics.

We (58, 59) and others (60–62) have taken advantage of the existence of different conformational states of GPIIb/IIIa to develop an alternative, unique strategy that targets only activated platelets. As scFv phage display permits an *in-vitro* imitation of the natural immunisation process, we used a subtractive panning strategy to select antibodies against discretely differing epitopes (8). This strategy is unique in its property to target complex cell membrane proteins, such as integrins, which cannot be immobilised without loss of function-determining conformation. The generated human scFvs are a promising basis for a new type of GPIIb/IIIa-blocking agents with several advantages: 1) Activation-specific inhibitors promise not to inhibit GPIIb/IIIa-mediated platelet adhesion to immobilised fibrinogen, which is for example exposed on injured vessel walls. Thus, the formation of a platelet monolayer at the site of vascular injury may still be possible. 2) Activation-specific scFvs do not bind and do not induce LIBS expression on resting platelets. Because thrombocytopenia is attributed mainly to an immune response directed against LIBS epitopes (63), thrombocytopenia is not expected to occur.

To demonstrate the advantages of activation-specific GPIIb/IIIa blockade *in vivo*, we chose a ferric chloride induced mouse carotid artery thrombosis model (Fig. 3). The antithrombotic effect of our activation-specific GPIIb/IIIa blocker was comparable to eptifibatid and tirofiban. In contrast to eptifibatid and tirofiban, the scFv did not prolong bleeding times. Moreover, the combination of an anticoagulant (enoxaparine) and a GPIIb/IIIa blocker, which is a typical combination used in the clinic, revealed an even stronger difference between eptifibatid and the activation-specific scFv (58).

In addition, we used the anti-LIBS scFv described above and genetically fused it to the potent, direct FXa inhibitor TAP (64). Antithrombotic efficiency was determined by Doppler-flow in a ferric chloride-induced carotid artery thrombosis model in mice. ScFv anti-LIBS-TAP prolonged occlusion time comparable to enoxaparine, recombinant TAP, and non-targeted mutant-scFv-TAP. ScFv anti-LIBS-TAP revealed antithrombotic effects at low doses at which the non-targeted mutant-scFv-TAP failed. In contrast to the other anticoagulants tested, bleeding times were not prolonged by scFv anti-LIBS-TAP.

Two scFvs that block the interaction between GPIb and von Willebrand factor (VWF) have been described as anti-platelet

agents. The human scFv Y1 binds specifically to sulfated epitopes in the platelet receptor GPIb, thereby competing with VWF for binding to human platelets and thus effectively inhibits platelet aggregation. This antibody recognises a sulphated epitope in GPIb receptor, efficiently inhibited platelet adhesion and aggregation, making it a candidate for a new anti-thrombotic agent (65). An anti-VWF scFv was generated by immunising mice with recombinant vWF domain A1 (VWF-A1) and screening the generated scFv for interaction with VWF-A1. The isolated scFv inhibited ristocetin-induced platelet aggregation but had no effect on thrombin-induced platelet aggregation (66).

ScFvs against intracellular targets: Intrabodies

Antibody applications are normally limited to the extracellular targets. However, advances in antibody engineering have allowed the generation of small fragments that can be stably expressed in cells. These entities are called intracellular antibodies or intrabodies and have been successfully applied, mainly in the scFv format, to inhibit the function of intracellular target proteins in specific cellular compartments in treatment of diseases such as cancer, AIDS or neuro-degenerative disorders (67, 68). Intrabody therapy combines the specificity of antibodies with a gene-therapeutic strategy to selectively affect an intracellular target protein. Most recently an intrabody against vascular adhesion molecule 1 (VCAM-1) has been described (69). VCAM-1 is involved in the recruitment of leukocytes to inflammatory sites. The study showed functional knockdown of VCAM-1 using an ER-retained antibody construct. Down-regulation of surface VCAM-1 might be a useful tool to reduce monocyte adhesion during the development of atherosclerotic disease.

Perspectives

Antibodies are highly promising in various clinical areas. Single-chain antibodies possess a number of major advantages: they are expected to be minimally immunogenic and can be produced on large scale at low cost in *E. coli*; scFvs are amendable to further optimisation by cloning and genetic engineering in bacteria; and they can be selected *in vitro* from human antibody libraries even against complex targets distinguishing between different conformations. This provides a unique opportunity to design antibodies that only target specific conformation and thereby only block specific functional states of the therapeutic target. Furthermore, the opportunity of constructing fusion molecules, imaging nanoparticles and immunosensors without loss of binding function guide the way to new applications. Highly selective antibodies carrying anticoagulants, fibrinolytics and contrast agents specifically to the target of choice can be generated in a single unique molecule.

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