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SELECTIVE TARGETING AND OCCLUSION OF TUMOURAL BLOOD VESSELS

OF TUMOURAL BLOOD VESSELS

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CONTENTS

1 SUMMARY

The formation of new blood vessels from pre-existing ones (angiogenesis) is a rare phenomenon in the adult, but a characteristic feature of many aggressive tumours and other relevant disorders. Molecules with high specific binding to new blood vessels, but not to mature vessels, could be used as selective vehicles and would open diagnostic and therapeutic opportunities. Furthermore, targeting of angiogenesis circumvents the need of different binders for different tumour types, as new-forming blood vessels appear to be a general feature of tumoural progression and invasion.

A number of cell surface and extracellular adhesion molecules are upregulated in newly formed blood vessels, demonstrating phenotypic differences between mature, quiescent vessels, and their newly formed (angiogenetic) counterparts. In particular, a fibronectin isoform with an extra domain B (ED-B) inserted by alternative splicing, is specifically present in the stroma of neoplastic tissues and around adult and neoplastic blood vessels during angiogenesis.

Using antibody phage display libraries and combinatorial mutagenesis, a high-affinity human antibody fragment, scFv(L19), directed against the ED-B domain of fibronectin, was isolated by our group. The high-affinity anti-ED-B scFv(L19) was demonstrated to target tumoural blood vessels in vivo with high specificity using fluorescent and radioactive techniques. Upon intravenous injection in tumour-bearing mice, scFv(L19) is rapidly eliminated from the blood through the kidneys and, unlike conventional monoclonal antibodies, does not accumulate in the liver or other organs. This antibody can be used for the selective delivery of a pro-coagulant factor to the tumoural vasculature and there induce thrombosis of the blood vessels, impairing transport of oxygen and metabolites to tumoural tissue resulting in subsequent tumoural cell death. A good choice among pro-coagulant factors is the human coagulation-inducing protein tissue factor (TF). Tissue factor is a cell surface glycoprotein and is a major initiator of

blood coagulation [Ruf et al., 1991]. At sites of injury, blood comes in contact with the membrane-bound TF which forms a complex with the serine protease FVIIa present in blood. The resulting complex activates factors IX and X, which leads to thrombin activation and ultimately to blood clotting. A truncated form of TF, tTF, consisting of only the extracellular soluble domain (residues 1-219), exhibits a five orders of magnitude lower ability to activate the clotting cascade in solution, but is fully active when re-targeted to a membrane surface [Huang et al., 1997]. An attractive feature of such a therapeutic approach is, that it involves endogenous components of the body in an amplification cascade, with the targeted tissue factor as a trigger in the very beginning of the cascade.

Three main issues were addressed in this thesis:

1 is it possible to target tTF to tumoural blood vessels by fusing it to an antibody specific for the angiogenesis related protein ED-B?

2 ED-B is a component of the modified extracellular matrix that surrounds tumour blood vessels. Can the delivery of a pro-coagulant factor at this *abluminal* site mediate the complete and selective *intraluminal* coagulation of tumour blood vessels?

3 is targeting of tTF to tumoural blood vessels of therapeutic benefit?

I show that a fusion protein, consisting of the L19 antibody fragment fused to tTF, mediates the complete and selective infarction of three different types of solid tumours (F9, C51 and FE8) in mice. Injection of 35 μg of fusion protein led to complete tumour eradication in approximately 30% of the treated animals in a dose-dependent fashion, while tTF-fusion proteins with binding specificity for an irrelevant antigen did not show any therapeutic effect. Furthermore, I demonstrate that targeting of tTF to ED-B potentiates chemotherapy with near 100% complete remissions in preliminary animal studies.

1 ZUSAMMENFASSUNG

Die Bildung neuer Blutgefässe ausgehend von alten, schon vorhandenen Blutgefässen (Angiogenese), kommt bei erwachsenen Menschen sehr selten vor, ist aber eine Eigenschaft vieler aggressiver Tumore. Moleküle, die an neugebildete Blutgefässe, aber nicht an schon vorhandene, mit sehr hoher Spezifität binden, können als Vehikel benutzt werden und bieten neue diagnostische und therapeutische Möglichkeiten.

Es gibt Proteine an der Zelloberfläche und in der extrazellulären Matrix, die hauptsächlich bei neu gebildeten Blutgefässen auftreten und bei alten Blutgefässen kaum vorhanden sind. Zum Beispiel, eine Fibronectin-Isoform, mit einer zusätzlichen Domäne B (ED-B), akkumuliert spezifisch um neugebildete Blutgefässe.

Ein menschlicher Antikörper, der ED-B mit sehr hoher Affinität und Spezifität bindet, scFv(L19), wurde in unserer Gruppe mit Phage-Display Technik isoliert. Es konnte in tumortragenden Mäusen gezeigt werden, dass sich der Antikörper nach intravenöser Injektion an tumoralen Blutgefässen anreichert.

Prinzipiell könnte die selektive Anreicherung eines Blutgerinnungsfaktors an tumoralen Blutgefässen dort lokal die Blutgerinnung auslösen und so die Sauerstoffzufuhr der Tumorzellen blockieren, und die Tumorzellen damit zum Absterben bringen. Ein wichtiger Blutgerinnungsfaktor ist der Gewebe Faktor, oder auch Tissue Factor (TF) genannt. TF ist ein glykosyliertes Zelloberflächenprotein und ist eins der wichtigsten Proteine zu Beginn der Blutgerinnungskaskade [Ruf et al., 1991]. Wo eine Wunde vorhanden ist, kommt Blut in Kontakt mit dem membrangebundenen TF in der Adventitia, es bildet sich ein Komplex mit der im Blut vorhandenen Serin-Protease Faktor VIIa, der dann die Faktoren IX und X zu IXa und Xa aktiviert. Faktor Xa aktiviert dann Thrombin, und zusammen mit Fibrinfasern und Blutplättchen bildet sich am Ende ein Blutgerinnsel. Eine verkürzte Form von TF (truncated TF, tTF), die nur aus der löslichen extrazellulären Domäne besteht, ist im Blut gelöst nicht aktiv, wird aber aktiviert, sobald es an eine Membran gebunden wird [Huang et al., 1997]. Eine solche Strategie ist sehr attraktiv, weil TF zu Beginn der Blutgerinnungskaskade wirkt,

und weil die schon im Blut vorhandenen Komponenten die Wirkung vervielfältigen; so kann ein einziges tTF-Molekül einen grossen Effekt ausüben.

In dieser Doktorarbeit wurden drei Themen untersucht:

1 Kann der scFv(L19)-Antikörper genutzt werden, um tTF zu dem tumoralen Blutgefässe zu transportieren?
2 Kann das scFv(L19)-tTF Fusionsprotein die Blutgerinnungskaskade immer noch aktivieren, wenn es über den Antikörper an das ED-B Protein gebunden ist?
3 Hat das scFv(L19)-tTF Fusionsprotein in tumourtragenden Mäusen einen therapeutischen Effekt?

Ich konnte in dieser Dissertation zeigen, dass ein prokoagulantes Fusionsprotein, bestehend aus dem Antikörperfragment scFv(L19) und dem verkürztem Tissue Factor tTF, die Blutgerinnung bei drei verschiedenen Tumortypen (F9, C51 und FE8) auslöst. Dieser Effekt war abhängig von der injizierten Dosis, bei der grössten Menge (35 µg Fusionsprotein) konnte 30% der Mäuse vom Tumor geheilt werden. Ich konnte bereits in Vorexperimenten mit Mäusen zeigen, dass scFv(L19)-tTF die Wirkung von Chemotherapie potenziert, mit nahezu hundertprozentiger Tumorheilung.

2 INTRODUCTION

2.1 Tumour targeting: concepts, targets and vehicles

Cancer chemotherapy today relies on the expectation that anti-cancer drugs will preferentially kill rapidly proliferating tumour cells rather than normal cells [Chari, 1998]. A large portion of the tumour cells has to be killed in order to obtain and maintain a complete remission. This often requires the use of large doses of drug, which cause significant toxicity towards proliferating non-malignant cells. The development of more selective anti-cancer drugs, with better discrimination between tumour and non-tumour cells and therefore better therapeutic ratios, is possibly the most important goal of modern anti-cancer research.

One way to improve the selectivity of therapeutic molecules would be to target them on the tumour site, thereby sparing normal tissues (Fig. 2.1). The concept of therapeutic "magic bullets", first formulated by Ehrlich at the end of the last century, gained momentum in the seventies, with the development of hybridoma technology for the production of monoclonal murine antibodies of defined specificity [Köhler and Milstein, 1975]. In principle, a large variety of molecules other than antibodies (e.g., globular proteins, peptides, small organic molecules, polymers, etc.) could be used as smart vehicles, if they have an affinity for the tumour cells, or if they localise at the tumour site by virtue of other mechanisms. "Tumour targeting" could therefore be defined in a broad sense as the area of scientific research that aims at achieving selective localisation of active compounds at tumour sites.

Fig. 2.1
Research in tumour targeting aims at achieving selective localisation of agents at the tumour site (typically administered by intravenous injection), for diagnostic or therapeutic applications.

The targeted delivery of molecules to tumours is attractive for a number of reasons:

1 The approach is a logical and intuitive one, which relies on experimental parameters that can be defined *a priori*. Given a tumour antigen which is specific, abundant and accessible, and sufficient amounts of a specific vehicle molecule with suitable affinity and pharmacokinetics, it should be possible to selectively deliver large doses of active molecules to the tumour.

2 The approach is modular, since tumour recognition and effector function are typically performed by different molecules linked together (e.g., an antibody and a radionuclide), or by different epitopes of the same molecule (e.g., different domains of a monoclonal antibody). In principle, the same targeting agent could be modified with several different bioactive molecules, thereby building a

strategy that is suitable for combination therapy or for the evaluation of the therapeutic potential of different targeted cytotoxic agents.

3 The level of selectivity of tumour targeting agents can be monitored by classical biodistribution analysis.

4 Selective targeting agents could be useful both for the diagnosis (e.g., by conjugation to radionuclides or fluorophores) and therapy (e.g., by conjugation to cytotoxic agents) of tumours. Even though the pharmacokinetic requirements for imaging and therapy may be different, the performance of a targeting agent in in vivo diagnosis may be predictive of the vehicle's potential for therapeutic applications.

5 Conjugation of some therapeutic agents to an antibody vehicle may render the agent biologically inactive until it reaches the target site. Once at the tumour site, the bioactive species may be liberated and display its full biological activity, sparing normal tissues.

Together with the positive features of tumour targeting strategies, there are critical issues that need to be considered. For example, the pharmacokinetics and immunogenicity of the vehicle-cytotoxic agent conjugates may be different from those of the vehicle and cytotoxic agent individually taken. The targeted delivery of an anticancer drug or another bioactive compound at the tumour site may not be sufficient for providing therapeutic benefit, as the appropriate subcellular localisation may have to be achieved.

It may be appropriate to briefly introduce a few definitions and concepts that are relevant for the next sections, before addressing the opportunities offered by phage display technology for the development of selective tumour targeting agents.

Let us consider as illustrative example a radiolabeled antibody Fab fragment directed against a tumour associated marker, which is injected intravenously in mice bearing an implanted tumour carrying the antigen of interest. Let us also assume that the radionuclide is stably associated with the antibody, so that the radioactivity in a tissue is directly related to the amount of antibody in the same tissue. It is then possible to monitor the tumour targeting performance of the recombinant antibody by biodistribution analysis (e.g., by weighing organs and counting radioactivity at various time intervals) and express the results in a number of ways, some of which are presented in Fig. 2.2.

Typically, in order to compare the antibody dose delivered to organs and fluids of different weight and size, targeting results are expressed as percent injected dose of antibody per gram of tissue or fluid (%ID/g). Fig. 2.2a shows a schematic profile of the antibody dose in tumour and blood at different time points. Blood levels contain information about antibody clearance rate, and are often predictive for antibody levels in irrelevant organs.

For some applications, knowledge of the tumour : blood and the tumour : organ ratio at a certain time point after injection is relevant (Fig. 2.2a, 2.2c). For example, in immunoscintigraphy it will be important to know whether at a certain time point these ratios are sufficiently large to permit the imaging of tumour lesions. For other applications (e.g., radioimmunotherapy of tumours with antibody-beta emitting radionuclide conjugates), the area under the curve (AUC, defined as the integral of a curve which describes the organ concentration vs. time for t=0 to t=∞) of tumour, blood and other organs will be relevant, since toxic radiation is released immediately after injection. For short lived nuclides, radioactive decay has to be taken into account, and profiles such as those of Fig. 2.2a and 2.2b have to be multiplied with a suitable exponential function.

Finally, in some cases it may be important to learn about the localisation of targeting agent at the tumour site. For example, heterogenous targeting of different areas of the tumour may be detected by microautoradiography or fluorescence microscopy (Fig. 2.2d). Furthermore, for the targeted delivery of toxic agents active intracellularly (e.g. toxins, drugs) it may not be sufficient to know that the antibody has been targeted to the tumour site. Knowledge of the successive steps of the antibody conjugate (dissociation, internalisation, metabolism etc.) may be required [Chari, 1998].

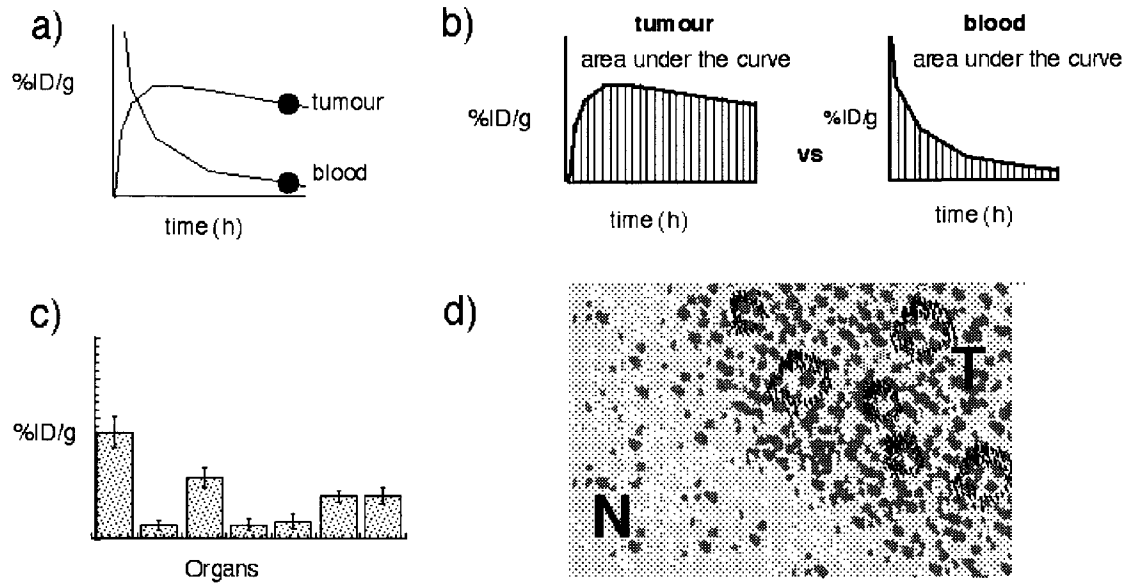


Fig.2.2

Schematic representation of different methods for the evaluation of tumour targeting efficiency.

1 The tumour targeting agent levels in tumour and blood, expressed as percent of the injected dose per gram of tissue or fluid, are obtained at different times, for example by biodistribution analysis in tumour bearing animals. The tumour:blood ratio at a particular time point may be a crucial parameter for imaging applications.

2 2 For therapeutic applications, in which a radionuclide is anchored on a targeting agent, radiation is delivered to the patient immediately after injection. It is therefore important to know the total dose of radioactivity delivered to tumour, blood and tissues, which is related to the area under the curve of the graph of Fig. 2.2a.

3 As in Fig. 2.2a, but the biodistributions at a certain time point are presented for a number of relevant organs.

4 Schematic microautoradiographic analysis (N=normal tissue; T=tumour), which reveals the potentially heterogenous localisation of the targeting agent within the tumour.

Traditionally, tumour targeting experiments have relied on the use of affinity reagents (typically monoclonal antibodies) specific for antigens preferentially expressed on the surface of tumour cells. Other classes of antigens could in principle be considered for targeting applications (Fig. 2.3).

The modified extracellular matrix of tumours is rich in tumour-associated antigens that are in general more abundant and possibly more stable than antigens on the cell membrane. Tumour targeting studies performed with monoclonal antibodies and antibody fragments directed against components of the modified extracellular matrix have shown promising results in animal models and in patients [Neri et al., 1998].

Angiogenesis, i.e. the sprouting of new capillaries from pre-existing blood vessels [Risau et al., 1997], is another characteristic feature of aggressive solid tumours [Folkman et al., 1995], which has been selected for tumour targeting applications [Huang et al., 1997; Pasqualini et al., 1997; Neri et al., 1997; Brekken et al., 1998]. With the exception of some ovary tissues and of the endometrium in the proliferative phase, angiogenesis is a rare phenomenon in the adult.

Rapid tumour cell proliferation is often accompanied by necrosis. For example, pseudopalisading around areas of necrosis may be a dominant feature of glioblastoma multiforme, a rapidly growing brain tumour [McKee et al., 1996]. It could therefore be conceivable to target necrosis areas in tumours for imaging and therapeutic purposes [Epstein et al., 1988; Chen et al., 1990; Frankfurt et al. 1996], or to exploit the selective presence of enzymes in necrotic areas [Bosslet et al., 1998].

Tumour targeting is usually associated with the concept of achieving a selective localisation by virtue of the specific binding of a tumour-associated marker. Selective localisation of a targeting agent could in principle be mediated by other processes, such as the extravasation of suitable targeting agents from fenestrated capillaries [Dvorak et al. 1990; Blumenthal et al., 1995; Riefke et al., 1996], or the trapping of particles in tumour capillaries [Håkansson et al. 1997]. However, the tumour targeting properties of

"non-specific" targeting agents, such as antibodies directed against irrelevant antigens, are typically very poor [Folli et al., 1994; Pimm et al., 1996; Syrigos et al., 1998]. For this reason, this introduction will focus on the development of tumour targeting agents with defined binding specificity towards a tumour-associated marker.

Relevant parameters for the evaluation of the performance of such vehicles depend ultimately on the effector function that they have to deliver. This will be the object of the next chapters.

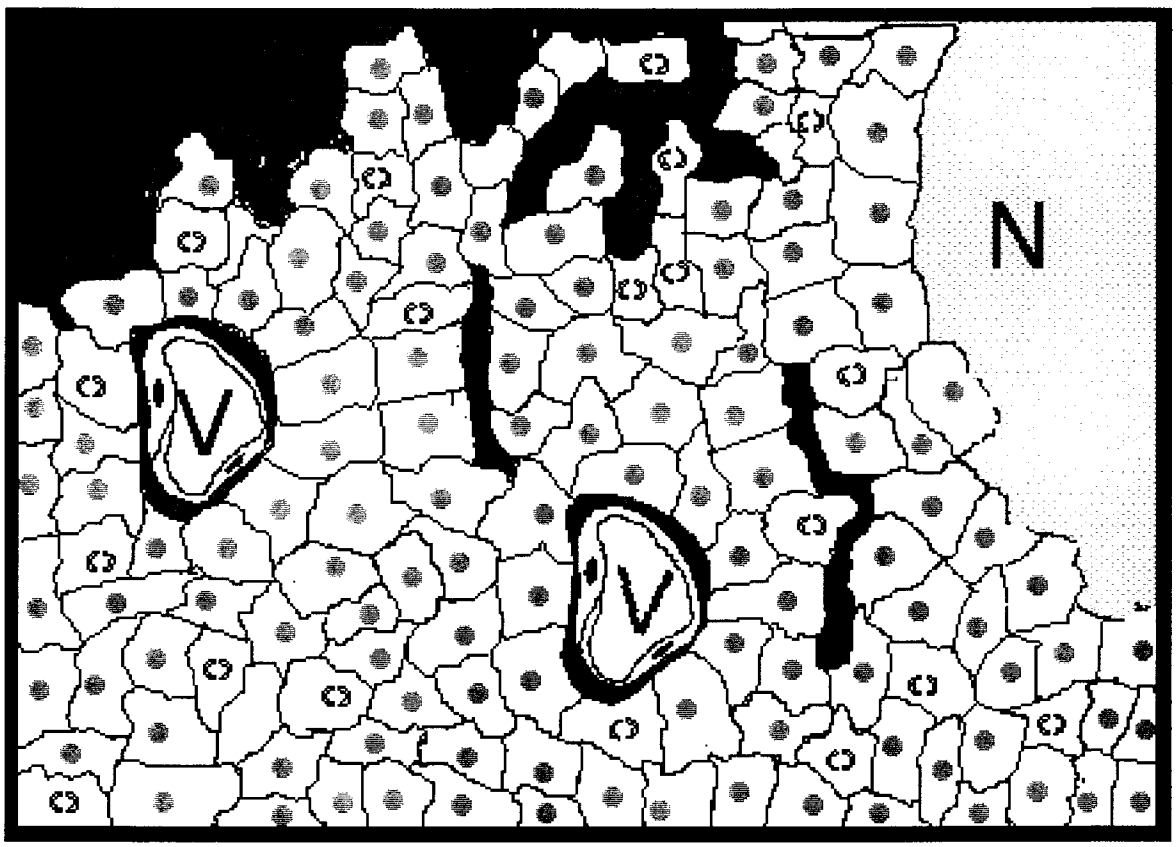


Fig. 2.3

Schematic representation of characteristic tumour features, that could be used for tumour targeting applications. In addition to the tumour cells, new forming blood vessels (V) could be considered for molecular intervention. Extracellular matrix compontents (ECM; dark gray) and necrosis areas (N; pale gray) may also represent useful targets for selective molecular localisation within the tumour site. Dividing tumour cells are represented with mitotic figures.

2.2 Antibodies as therapeutic agents

Antibodies, also called immunoglobulins, are important functional units of the immune system. They are B cell-produced glycoproteins which can trigger an immune response by directly interacting with the big variety of antigens that an organism encounters. It has been estimated that a human being makes at least 10^{15} different antibody molecules, each of them specific for a different antigen. Antibodies can be highly specific for their antigen, with affinities typically 10^5-10^7 M^{-1} for a primary and $10^8 M^{-1}$ or more for a secondary immune response [Winter et al., 1994]. Their affinities are improved by avidity, i.e. one antibody molecule has two binding domains which, when bound to the same target at the same time, increases the functional affinity of the antibody.
In 1975 the invention of hybridoma technology by Milstein and Köhler revolutionised the use of antibodies in cell biology, immunology, medicine. The technique allows the production of rodent monoclonal antibodies against a given antigen in virtually unlimited amounts.

In over two decades, with a myriad of variations in the experimental scheme, researchers have tried to exploit the binding specificity of murine antibodies towards tumour cells for the selective delivery of cytotoxic agents in animal models and in patients [Berkower et al., 1996]. This has led to some encouraging clinical results, particularly for the treatment of metastases, lymphomas and brain tumours [Riva et al., 1994; Maloney et al., 1994; Schneider-Gadicke et al., 1995; Press et al., 1995; Baselga et al., 1998]. However, rodent antibodies are immunogenic in humans. Furthermore, it is difficult to make rodent monoclonal antibodies against highly conserved antigens, or human monoclonal antibodies, especially against human self-antigens.

Phage antibody technology appears to offer a solution, as human antibody fragments can be isolated from repertoires of fragments displayed on filamentous bacteriophage [Winter et al., 1994]. The process does not require immunisation of humans, and

antibody fragments can be made against both foreign and human self-antigens. The fragments are secreted into the bacterial periplasm and culture medium [Skerra et al., 1988; Better et al., 1988] and can be produced in a large scale [Carter et al., 1992; Pack et al., 1993]. Indeed antibody fragments have already entered Phase I clinical trials for imaging of cancer [Casey et al., 1995; Begent et al., 1996]. The use of antibody fragments, rather than complete antibodies, may also have some advantages. The fragments would not be expected to be taken up into the liver, as occurs with complete antibodies [Abrams et al., 1993; Berkower et al., 1996]. They would also be expected to show improved penetration of tissues [Yokota et al., 1992; Yokota et al., 1993].

The next section addresses the engineering of tumour targeting agents isolated using phage display libraries. It mainly focuses on recombinant antibodies, since for this class of molecules a more extensive in vitro and in vivo characterisation has been reported. However, phage display represents a huge potential also for the engineering of peptides and other binding proteins for tumour targeting applications

2.3 Antibody phage technology

2.3.1 Phage display libraries for the isolation of specific ligands

Phage display was originally described in 1985 by Smith [Smith et al., 1985; Smith et al., 1991], who presented the use of the non-lytic filamentous bacteriophage fd for the display of specific binding peptides on the phage coat. The power of the methodology was further enhanced by the groups of Winter [McCafferty et al., 1990] and Wells [Lowman et al., 1991], who demonstrated the display of functional folded proteins on the phage surface. The technology is based on the fact that a polypeptide (capable of performing a function, typically the specific binding to a target of interest) can be displayed on the phage surface by inserting the gene coding for the polypeptide in the phage genome (Fig. 2.4). If one is able to purify a phage particle by virtue of the binding specificity displayed on its surface from a large repertoire of phage particles

(e.g., a phage library), one also isolates the genetic information coding for the binding protein, and can amplify the corresponding phage by bacterial infection (Fig. 2.5).

Several rounds of selections can be performed (typically 2-4 rounds of panning using antibody phage libraries; see below). As a consequence, even rare binding specificities present in large repertoires can be selected and amplified from a background of phages with irrelevant binding specificities.

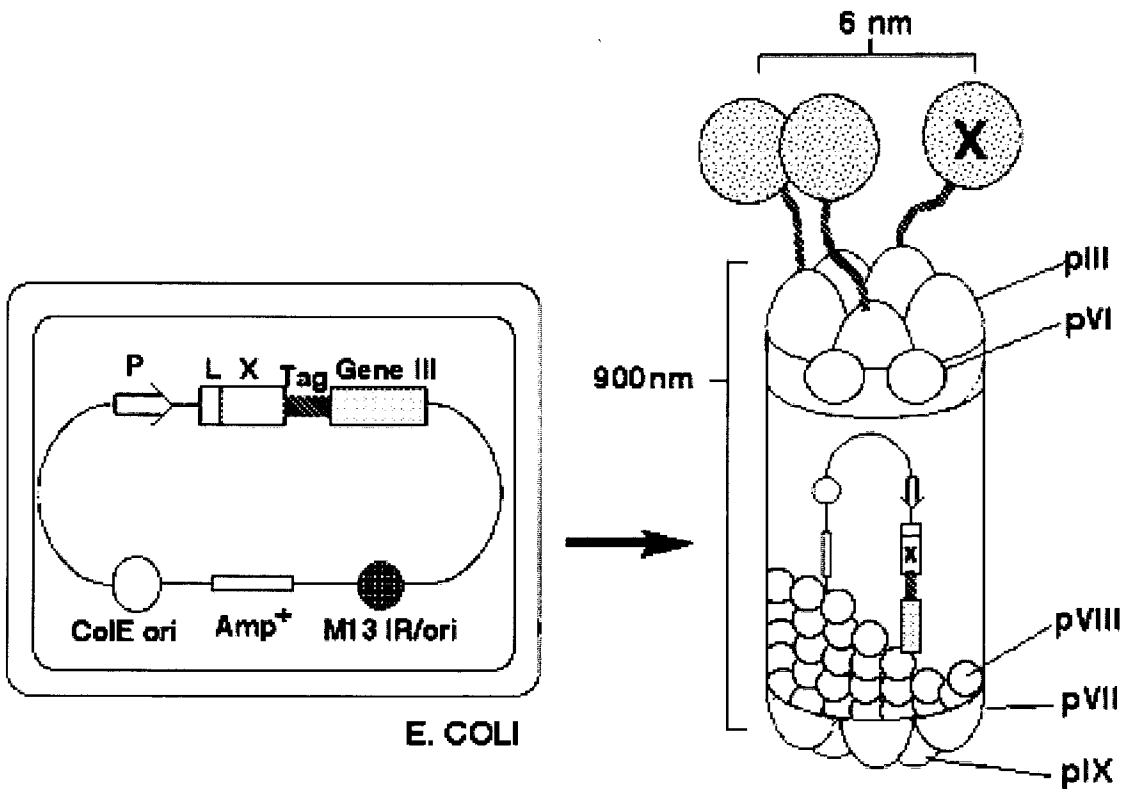


Fig. 2.4
Schematic representation of a phage or phagemid vector, carrying a promoter P, a leader peptide L, a gene X, a tag sequence, fused to filamentous phage gene III, which gives rise to a phage particle, displaying the protein "X" fused to the N-terminal extremity of the gene III product, at the tip of the phage. The peptidic tag is important for the efficient recognition of the product with an anti-tag antibody. Other relevant proteins and dimensions are also presented.

Filamentous phage infect strains of E.coli that harbour the F conjugative episome. Phage particles infect bacteria by attaching to the tip of the F pilus, and by translocating the phage genome (a circular single-stranded DNA molecule) into the bacterial cytoplasm. The genome is replicated involving both phage- and host-derived proteins, and packaged into elongated ("filamentous") viral particles of approximately 6 nm diameter and 900 nm length. For a review on phage biology, see Webster [Webster et al., 1996].

Filamentous phage particles are covered by approximately 3000 copies of a small major coat protein (pVIII). Few copies of the minor coat proteins pIII and pVI are displayed at one extremity of the phage particle, while pVII and pIX are present at the other extremity. The minor coat protein pIII, the product of gene III, is displayed in 3-5 copies and mediates the adsorption of the phage to the bacterial pilus. Peptides and/or proteins have been displayed on phage as fusions with the coat proteins pIII [Smith et al., 1985; Parmley et al., 1988] or pVIII [Greenwood et al., 1991]. Display of proteins encoded by a cDNA library as carboxy-terminal fusion with the minor coat protein pVI has also been reported [Jespers et al., 1995]. Fig. 2.6 depicts schematically some formats of binding molecules displayed on phage. A wide variety of molecules can in principle be expressed on the phage coat protein III including Fab fragments, scFv, VH domains, small globular proteins and peptides which may or may not be constrained by a disulfide bridge.

The first peptides and proteins were displayed on phage using phage vectors (essentially the phage genome with suitable cloning sites for pVIII or pIII fusions and an antibiotic resistance gene). Phage vectors carry all the genetic information necessary for phage life cycle. With pIII fusions in phage vectors, each pIII coat protein displayed on phage would be fused to the heterologous polypeptide. Using phage vectors, most peptides and folded proteins can be displayed as pIII fusions, while only short peptides containing no

cysteine and 6-7 residues give rise to functional phage when displayed as pVIII fusions [Iannolo et al., 1995]. Phagemids, a more popular vector for display, are plasmid vectors that carry gene III with appropriate cloning sites and a phage packaging signal [Bass et al., 1990; Hoogenboom et al., 1991; Garrad et al., 1991]. The phagemid encoding the polypeptide-pIII fusion is preferentially packaged into phage particles using a helper phage that contains a slightly defective origin of replication, such as M13K07 or VCS-M13, which supplies all the structural proteins. The resulting phage particles may incorporate either pIII derived from the helper phage or the polypeptide-pIII fusion, encoded by the phagemid. Depending on the type of phagemid, growth conditions used and the nature of the polypeptide fused to pIII, ratios of (polypeptide-pIII):pIII ranging between 1:9 and 1:1'000 have been reported [Bass et al., 1990; Kristensen et al., 1998; Sieber et al., 1998; Demartis et al., 1999]. Furthermore, the proteolytic cleavage of antibody-pIII fusions has been reported, contributing to further elevated levels of full-length wild type pIII [McCafferty et al., 1990; McCafferty et al., 1996]. Besides N-terminal fusions full-length pIII, fusions to sites downstream of gene III have been reported [Lowman et al., 1991; Barbas et al., 1991; Krebber et al., 1997].

Phage remains infective when treated with acids, bases, denaturants and even proteases. These properties allow a variety of selective elution protocols and have been used for applications other than selection for binding, such as the selection of proteins with altered thermal stability [Kristensen et al., 1998; Sieber et al., 1998] or the selection of catalytically active enzymes [Pedersen et al., 1998; Demartis et al., 1999].

The kind of polypeptides derived from phage display libraries which have been used most extensively in tumour targeting studies are recombinant antibodies and I will focus on these proteins.

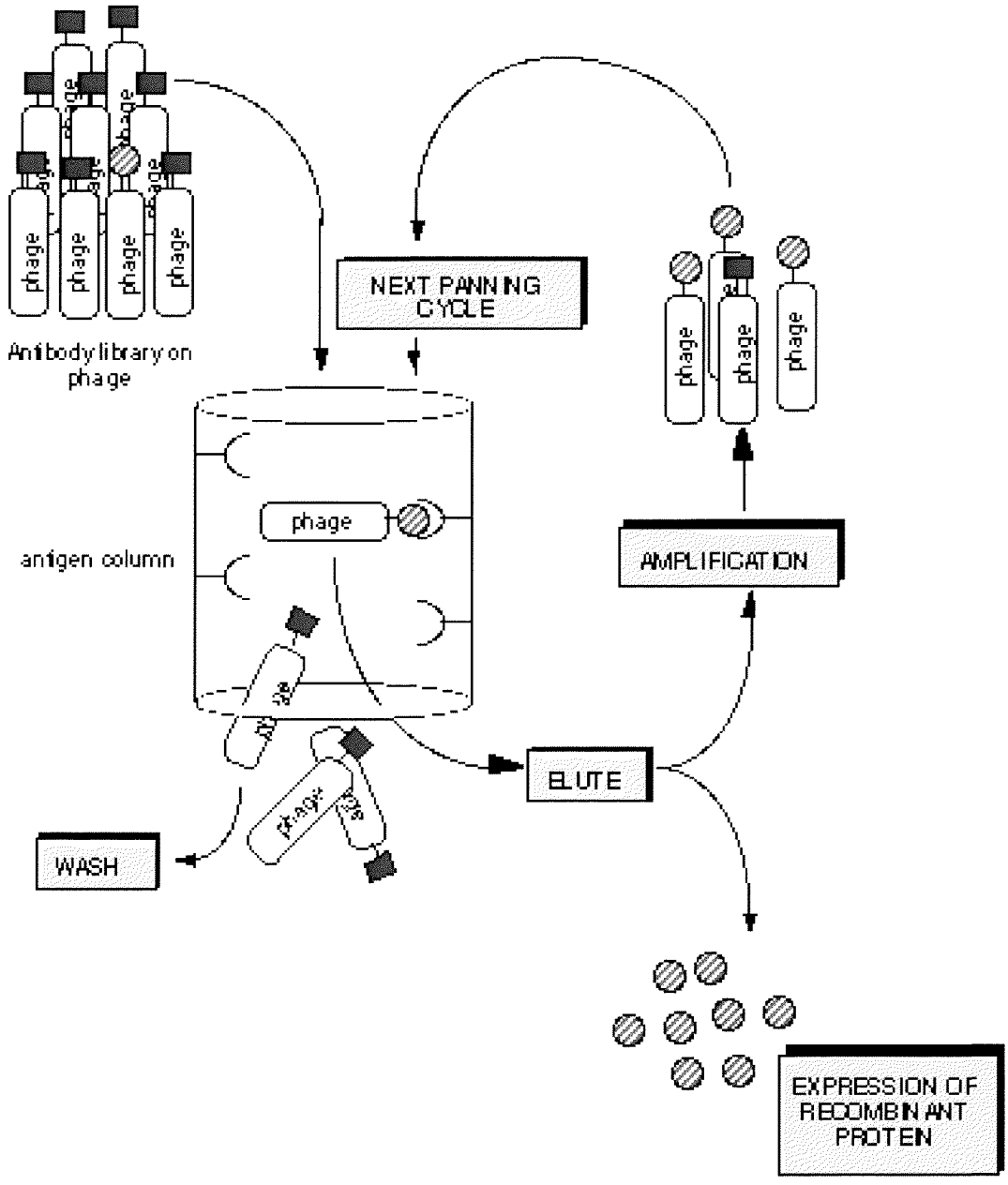
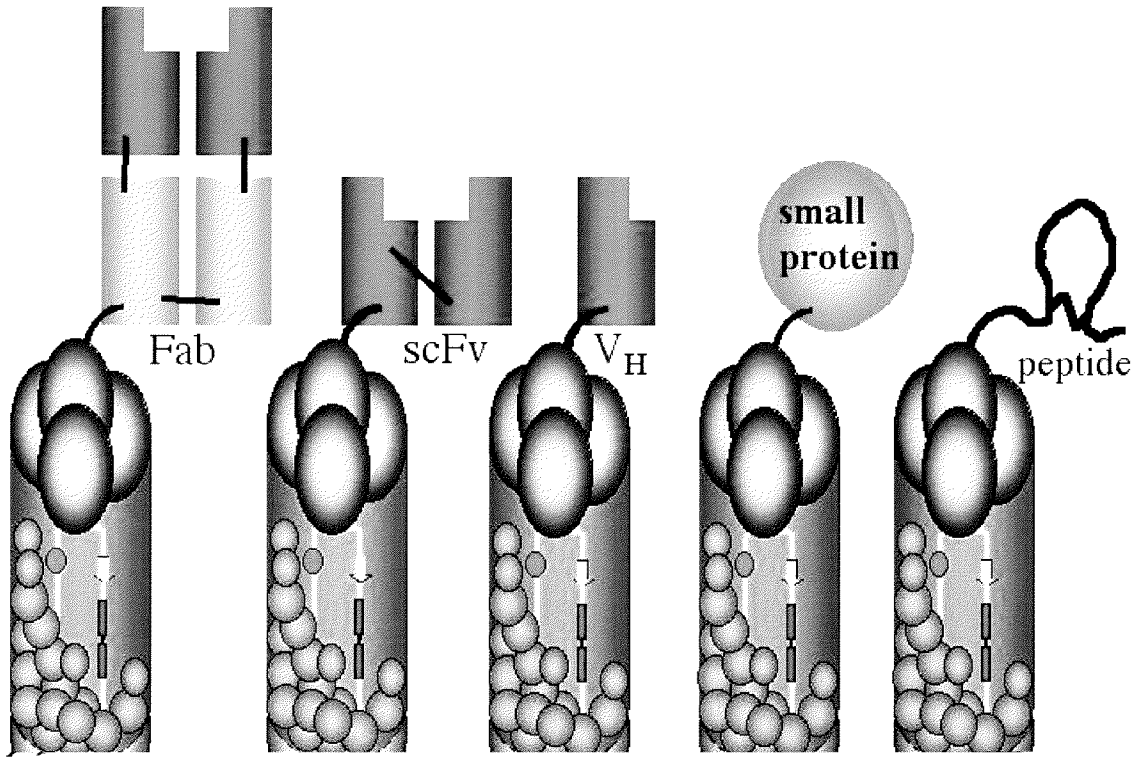


Fig. 2.5
Schematic representation of a round of panning. A phage display library is applied onto a solid support, coated with the antigen of interest. Only some binding specificities (circles) are retained on the affinity support and can selectively be eluted after washing. The eluted phage can be amplified by bacterial infection and used for a further round of panning. Alternatively, its genetic material can be used for the expression of the corresponding recombinant polypeptide in a suitable over-expression system.


Fab
scFv
V_H
small protein
peptide

For therapy of human tumours, the efficacy of murine antibodies appears to be hindered by additional hurdles, including the induction of a human anti-mouse antibody (HAMA) response [Frodin et al., 1992; Maher et al., 1992; Abrams et al., 1993; Berkower et al., 1996]. An avenue towards the solution of the HAMA problem is to use human antibodies. In principle, however, anti-idiotypic response could still develop upon repeated administration of human antibodies and has indeed been documented in patients [Isaacs et al., 1992; Schneider et al., 1993]. Human antibodies can be generated by humanisation of murine antibodies, for example by CDR grafting [Jones et al., 1986; Riechmann et al., 1988], by immunisation of transgenic mice expressing human antibodies [Taylor et al., 1992; Tuaillon et al., 1993; Taylor et al., 1994; Lonberg et al., 1994; Brüggemann et al., 1996; Jakobovits et al., 1998] or by using antibody phage

Table 2.1 Monoclonal antibodies approved in the US or EU

Product	Company	Therapeutic indication	Approved
Ortoclone OKT3 (Muromab CD3, murine Mab directed against the T-lymphocyte surface antigen CD3)	Ortho Biotech	Reversal of acute kidney transplant rejection	1986 (US)
OncoScint CR/OV (Satumomab Pendetide, murin Mab directed against TAG-72, a tumour-associated glycoprotein)	Cytogen	Detection/staging/follow-up of colorectal and ovarian cancers	1992 (US)
ReoPro (Abciximab, Fab fragments derived from a chimeric Mab, directed against the platelet surface receptor $GPII_b/III_a$)	Centocor	Prevention of blood clots	1994 (US)
Indimacis 125 (Igovomab, murine Mab fragment (Fab_2), directed against the tumour associated antigen CA 125	CIS Bio	Diagnosis of ovarian adenocarcinoma	1996 (EU)
CEA-scan (Arcitumomab, murine Mab, fragment (Fab), directed against human carcinoembryonic antigen, CEA)	Immunomedics	Detection of recurrent/ metastatic colorectal cancer	1996 (US) 1996 (EU)
MyoScint (Imiciromab-Pentetate, murine	Centocor	Myocardial infarction	1996 (US)

Table 2.1 continued:

Product	Company	Therapeutic indication	Approved
ProstaScint (Capromab Pentetate murine Mab directed against the tumour surface antigen PSMA)	Cytogen	Detection/staging/follow-up of prostrate adeno-carcinoma	1996 (US)
Tecnemab KI (murine Mab fragments (Fab/Fab_2 mix) directed against HMW-MAA	Sorin	Diagnosis of cutaneous melanoma lesions	1996 (EU)
Verluma (Nofetumomab murine Mab fragments (Fab) directed against carcinoma-associated antigen)	Boehringer Ingelheim/ NeoRx	Detection of small-cell lung cancer	1996 (US)
LeukoScan (Sulesomab, murine Mab fragment (Fab) directed against NCA 90, a surface granulocyte nonspecific crossreacting antigen)	Immunomedics	Diagnostic imaging for infection/inflammation in bone of patients with osteomyelitis	1997 (EU)
Rituxan (Rituximab chimeric murine Mab fragment (Fab) directed against CD20 antigen found on the surface of B-lymphocytes)	Genentech/ IDEC Pharmaceuticals	Non-Hodkin´s lymphoma	1997 (US)
Zenapax (Daclizumab, humanised Mab directed against the α-chain of the IL-2 receptor)	Hoffman La-Roche	Prevention of acute kidney transplant rejection	1997 (US) 1999 (EU)
Simulect (Basiliximab, chimeric Mab directed against the a-chain of the IL-2 receptor)	Novartis	Prophylaxis of acute organ rejection in allogeneic renal transplantation	1998 (EU)
Remicade (Infliximab, chimeric Mab directed against TNF-α	Centocor	Treatment of Crohn´s disease	1998 (US) 1999 (EU)
Synagis (Palivizumab, humanised Mab directed against an epitope on the surface of respiratory syncytical virus)	MedImmune (US) Abbott (EU)	Prophylaxis of lower respiratory tract disease caused by respiratory syncytical virus in pediatric patients	1998 (US) 1999 (EU)
Herceptin (Trastuzumab, humanised antibody directed against HER 2, i.e., human epidermal growth factor receptor 2)	Genentech	Treatment of metastatic breast cancer if tumour over-expresses HER2 protein	1998 (US)

Table 2.1 continued:

Product	Company	Therapeutic indication	Approved
Humaspect (Votumumab, human Mab directed against cytokeratin tumor-associated	Organon Teknika	Detection of carcinoma of the colon or rectum	1998 (EU)
directed against cytokeratin tumor-associated antigen)	Teknika	the colon or rectum	
Mabthera (Rituximab, chimeric murine Mab fragment (Fab) directed against CD20 antigen of B-lymphocytes)	Hoffman La-Roche	Non-Hodgkin´s lymphoma	1998 (EU)

Monoclonal antibodies approved in the US or EU (adapted from Nature Biotechnology (2000), 18, p831-833). Approvals as of May 2000. Several products have been approved for multiple indications, but only the first indication for which it was approved is listed. Products are listed by trade name. Sources: http://www.fda.gov, http://www.eudra.org/en_home.htm, http://www.phrma.org. Abbreviations: HMW-MAA, high-molecular-weight melanoma-associated antigen; Mab, monoclonal antibody; IL, interleukin; TNF, tumour necrosis factor.

When the desired binding specificities are rescued from an antibody phage library, one typically needs to express and purify recombinant antibody fragments from suitable over-expression systems. Since each immunoglobulin domain contains one disulfide bond, whose formation is generally important for the stability of the resulting antibody [Ohage et al., 1997], it is preferable to choose expression systems which secrete the

cloned into suitable expression vectors, for the production of the corresponding intact immunoglobulin in eukaryotic cells [Bradbury et al., 1995].

Fig.2.7 depicts schematically some of the different formats in which recombinant antibodies can be expressed. The smallest binding unit that retains the same affinity (yet not the same valence and avidity [Neri et al., 1996]) of the parental antibody is the Fv fragment. This heterodimeric recombinant antibody fragment consists of a variable heavy chain domain (VH) non-covalently associated with a variable light chain (VL). The stability of the Fv heterodimer can be in the micromolar range for some antibodies [Glockshuber et al., 1990]. At high dilutions and for in vivo applications, the two antigen and offering the hydrophobic interdomain interface for non-specific stickiness to unwanted targets. Single-chain Fv fragments (scFv), in which VH and VL are sequentially joined by an interdomain peptidic linker [Bird et al., 1988; Huston et al., 1988], may offer a solution to the problem. In fact, scFv fragments are possibly the most popular format for antibody fragments derived from phage display libraries. Furthermore, hybridomas can be used as a source of VH and VL genes for the construction of scFv fragments [Krebber et al., 1991; Clackson et al., 1991], for example using commercially available kits and protocols (e.g., the "Recombinant Phage Antibody System", Amersham Pharmacia).

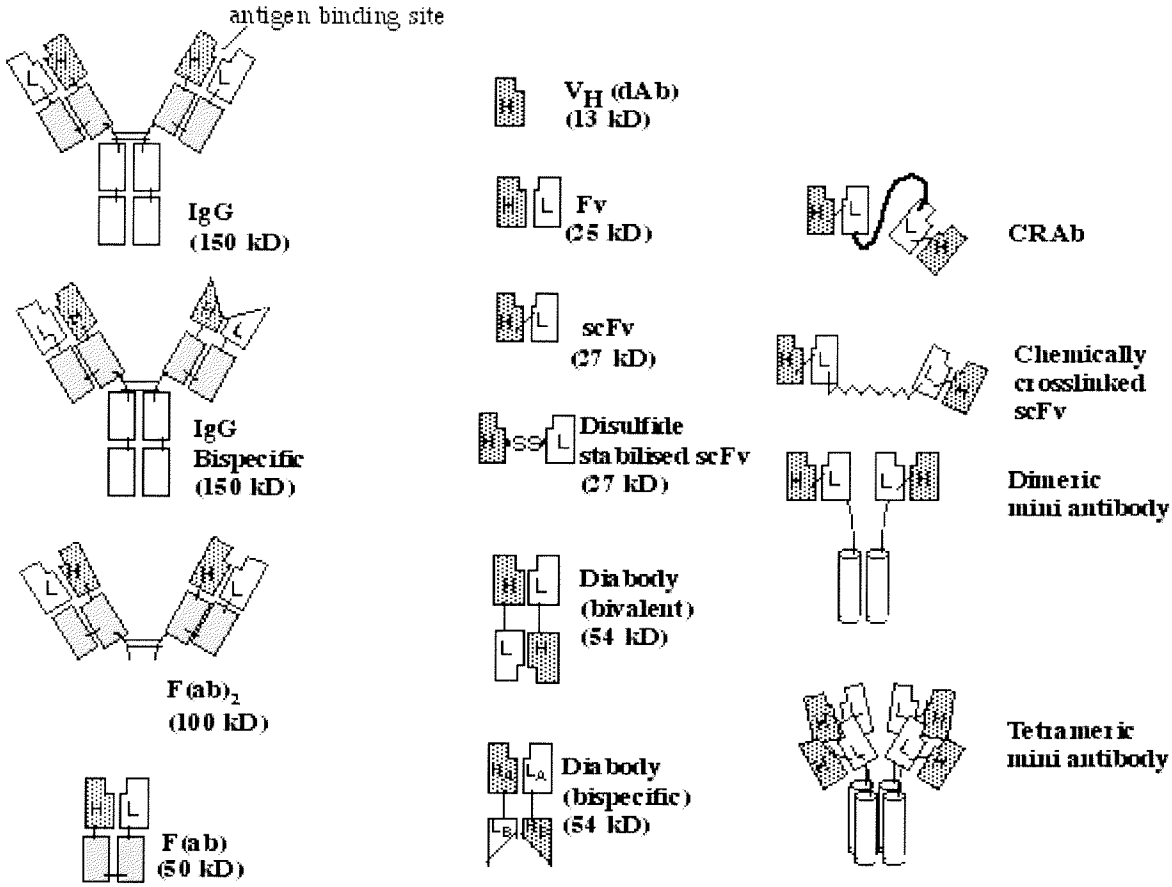


Fig. 2.7
Formats of antibodies and antibody fragments. H = variable heavy chain domain; L = variable light chain domain. Size is expressed in kiloDaltons (kD).

ScFv fragments may open the intramolecular VH-VL interface and dimerise [Marks et al., 1993; Griffiths et al., 1993; Nissim et al., 1994], trimerise [Iliades et al., 1997] and even form higher order oligomers [Neri et al., 1996] (Fig. 2.7). The tendency of scFv to oligomerise has been exploited by Holliger and Winter, who demonstrated that VH and VL scFv with a short polypeptide linker (0-10 aminoacid residues) cannot pair to form a monomeric antibody fragment, but rather homodimerise to form a small bivalent antibody fragment termed "diabody" [Holliger et al, 1993a; Holliger et al., 1993b]. Diabodies can be either homobivalent or bispecific (Fig. 2.7). Another way to assemble recombinant bispecific antibodies is either to chemically conjugate two antibody fragments of different specificity, or to sequentially fuse two scFv fragments (Fig. 2.7). Such scFv-scFv' constructs can also be directed against adjactent epitopes of the same

antigen to achieve high binding affinity and, in this case, are denominated CRAbs [Neri et al., 1995].

One way to stabilise Fv and diabodies, that may dissociate during tumour targeting applications [Fitzgerald et al., 1997], is to engineer interchain disulfide bonds, yielding disulfide-stabilised Fv fragments [Glockshuber et al., 1990; Brinkmann et al., 1993] and disulfide stabilised diabodies [Fitzgerald et al., 1997] (Fig. 2.7).

In order to gain binding avidity, it may be advantageous to oligomerise antibody fragments, creating "mini-antibodies" [Pack et al., 1992; Holliger et al., 1993; Pluckthun et al., 1997; Li et al., 1997] (Fig. 2.7). Several fusion proteins between an antibody fragment and an oligomerisation domain have been reported [Pack et al., 1992; Kipriyanov et al., 1996; Hu et al., 1996; Pluckthun et al., 1997]. It must be noticed, however, that oligomerisation units, coupled with the intrinsic tendency of scFv to dimerise, may result in the polymerisation of the corresponding fusion proteins and should therefore be used only with antibody fragments that are intrinsically monomeric.

The range of antibody formats that can be achieved by combining antibody fragments and other polypeptides is virtually unlimited. Some examples of less frequently occurring multimeric recombinant antibodies can be found in de Haard et al. [Haard et al., 1998] and Hoogenboom [Hoogenboom et al., 1997].

For some applications, for example in order to increase tissue penetration and accelerate antibody clearance from the blood [Yokota et al., 1992; Yokota et al. 1993], it may be advantageous to reduce the antibody size. After the original report by the Winter's group, showing that isolated VH domains (dAb [Ward et al., 1989], Fig. 2.7) may retain some of the binding affinity of the parental antibody towards the antigen, it was discovered that camel antibodies can be devoid of light chains and retain functionality [Hamers-Casterman et al., 1993]. Indeed, camel-derived VH domains [Arbabi Ghahroudi et al., 1997; Lauwereys et al., 1998] and "camelised" dAb antibodies [Davies et al., 1996] have been exploited, in combination with phage display methodologies, for the development of medium- to high-affinity monomeric antibody fragments of

molecular weight < 15'000 Daltons. Attempts to further reduce the size of antibody fragments capable of specific antigen binding, after initial promising results [Pessi et al., 1993], have so far yielded few additional binding specificities [Martin et al., 1994; Martin et al., 1996].

2.3.2.2 Antibody phage display libraries

This chapter and the next one on selection methodologies are to a large extent covered by the excellent recent reviews of Winter et al. [Winter et al., 1994], Winter [1998] and de Haard [Haard et al., 1998]. I therefore refer the reader who is interested in a comprehensive technical description to those articles. In this chapters I will therefore only briefly describe the basic concepts in antibody phage technology, the libraries more commonly used and the implications for tumour targeting applications.

Historically, demonstration of the principle that rare binding specificities displayed on filamentous phage could be selected and amplified from a pool of phage antibodies with irrelevant binding specificities was obtained by displaying on phage scFv fragments derived from a hybridoma [McCafferty et al., 1990]. After this "proof-of-principle" experiments, the first antibody libraries to be displayed on phage were "immune" libraries [Clackson et al., 1991; Burton et al., 1991], in which the source of variable immunoglobulin genes are B-cells from an immunised animal or an immune patient. The resulting libraries are enriched in antigen-specific immunoglobulin domains, some of which have already been matured by the immune system, and may therefore yield high-affinity antibodies even when the library size is not spectacular (e.g., 10^7 clones). For example, Chester [Chester et al., 1994] isolated from a murine immune library a well-behaved scFv fragment specific for the carcinoembryonic antigen (clone MFE-23) with affinity in the low nanomolar range. ScFv(MFE-23) has been shown to selectively target human tumours xenografted in nude mice, with some advantages over hybridoma-derived scFv fragments [Verhaar et al., 1995]. After the establishment of satisfactory procedures for the production and validation of recombinant antibodies for

Phase I clinical trials in patients with cancer [Begent et al., 1993; Casey et al., 1996], scFv(MFE-23) has been the first phage-derived antibody fragment to have been tested in the clinic for its tumour targeting performance in immunoscintigraphy [Begent et al., 1996]. ^{123}I-labeled scFv(MFE-23) enabled the scintigraphic detection of liver metastases of colorectal cancer in a patient, that had escaped detection by CT with contrast agents, suggesting that phage-derived antibodies can perform as well as those obtained using hybridoma technology [Acolla et al., 1980; Goldenberg et al., 1993]. Plenty of other examples (referenced in de Haard [Haard et al., 1998]) of monoclonal antibodies isolated from immune phage display libraries have been reported so far.

There are some disadvantages in isolating antibodies from immune repertoires. When the source of V genes is an immunised animal, the resulting antibodies are not human and therefore potentially immunogenic. Animal immunisation and library construction are necessary for each individual antigen, making the whole procedure long and somewhat labour intensive. In light of what is presented below for naive and synthetic repertoires, whenever a pure antigen is available, the use of immune libraries should be avoided. However, the isolation of human anti-tumour antibodies from phage repertoires of antibodies derived from cancer patients immunised with autologous tumour cells [Cai et al., 1995], or from their tumour-draining lymph nodes [Kettleborough et al., 1994] is a powerful strategy for the isolation of novel tumour-associated binding specificities. We envisage that immune libraries, obtained by immunisation against complex antigen mixtures and analysed using efficient selection schemes [Hoogenboom et al., 1997] and screening methodologies like high-throughput immuno-histochemistry (Leica immunostainer ST5050, [www.leica.com]; Life Span Biosciences [www.lsbio.com], Komiya et al. [Komiya et al., 1997]) in combination with tissue arrays on standard glass microscope slides, containing 600μm core tissue samples taken from normal human and mouse organs with up to 100 tissues spotted in duplicate [http://www.resgen.com], will be useful tools for the discovery of novel tumour markers.

Antibody phage technology is particularly powerful when "single-pot" libraries are used, i.e. libraries which contain virtually all the possible binding specificities, and which are not biased for a particular antigen. These single-pot libraries are cloned once, with the aim to reach a complexity > 10^8 clones and, if possible, > 10^9-10^{10} clones. The corresponding phages are stored frozen in aliquots [Neri et al., 1998] and can directly be used in panning experiments against a variety of different antigens. Typically, when using pure antigen preparations, specific monoclonal antibodies are almost always isolated in 2-4 rounds of panning (5-9 days of work). In general, both library design and library size contribute to the performance of the library, and to the quality of the antibodies isolated. Larger libraries have a higher probability of containing high affinity antibodies [Griffiths et al., 1994; Vaughan et al., 1996]. It is technically possible to make phage-display libraries of complexity >10^9 using brute force electroporation, and >10^{11} using combinatorial infection and cre-lox mediated recombination [Waterhouse et al., 1993; Griffiths et al., 1994; Fish et al., 1996; Sblattero et al., 2000]. However, the combinatorial diversity that can in practice be explored in panning experiments is limited by several factors, including the solubility of phage particles (typically ≤10^{13} transforming units/ml), the efficiency of antibody display on phage, and the phage recovery yields in biopanning experiments [Haard et al., 1998].

It is convenient to classify single-pot libraries into "naive" repertoires (in which V-genes are isolated from un-immunised animals or human donors, and are combinatorially assembled to create large arrays of antibodies) and "synthetic" repertoires (in which combinatorial diversity is introduced into a limited number of antibody scaffolds, for example by appending degenerate complementarity determining regions, CDRs, to a fixed antibody sequence).

Several naive human antibody phage libraries have been cloned so far. The first library of Marks et al. [Marks et al., 1991] has yielded several antibodies with different specificities, including scFv fragments directed against c*erb*B2, a tumour-associated

antigen over-expressed in approximately 30-40% of breast cancers, as well as in other solid tumours [Schier et al., 1995]. These antibodies, and their derivatives with improved affinity, have systematically been characterised in terms of their kinetic binding properties and tumour targeting performance (see below). A large naive human antibody library [Vaughan et al., 1996], containing 1.4 x 10^{10} independent clones, has so far yielded several dozens of different binding specificities [Vaughan et al., 1996; Merchant et al., 1998] with affinity in the low nanomolar or even in the subnanomolar range, including scFv fragments specific for CEA [Osbourn et al., 1996], VEGF (vascular endothelial growth factor) and HER3 (human epidermal growth factor receptor 3) [Merchant et al., 1998] and tenascin C [Carnemolla et al., 1999]. Recently, another large naive library has been reported to yield high-affinity antibodies against protein antigens [Sheets et al., 1998; Knappik et al., 2000].

While it is by now clear that high-affinity antibodies can easily be isolated from naive libraries if the corresponding pure antigen is available, potential disadvantages are (1) the lower affinity rescued when smaller repertoires are used; (2) the time and effort needed to construct these libraries; (3) the largely unknown and uncontrolled content of the library; (4) the need to sequence the isolated antibodies and to design custom primers for affinity maturation strategies based on combinatorial mutagenesis of CDRs. Furthermore, it remains to be seen how well naive libraries perform against conserved antigens (e.g., calmodulin [Eldik et al., 1987] or the ED-B domain of fibronectin [Zardi et al., 1987]), for which the immune system is tolerant.

Synthetic repertoires have the advantage that the content of the library (antibody structure, codon usage, knowledge of the antibody portions that are randomised and of those that are kept constant) is defined *a priori*. Furthermore, sequence diversity can be concentrated on those aminoacid residues which are supposed to be more important for antigen binding [Tomlinson et al., 1996]. Since antibody genes have not undergone any

immunological selection, the library is not biased against self antigens. Indeed, synthetic libraries have already yielded good-quality antibodies against conserved antigens, such as calmodulin [Griffiths et al., 1994], the ED-A [Borsi et al., 1998] and ED-B domain of fibronectin [Carnemolla et al., 1996; Neri, 1997; Pini, 1998], or against "difficult" antigens such as BiP [Nissim et al., 1994] and prion proteins [Leclerc et al., 2000].

Several synthetic antibody repertoires have been reported so far, and are described in de Haard [Haard et al., 1998]. As illustration of the principle, we here describe the design of the first synthetic repertoires, developed in Cambridge, UK. These libraries were based on a design in which a CDR3 loop, randomly mutated in aminoacid composition and length, was appended to the 49 human germline VH genes, while the light chain was kept constant [Hoogenboom et al., 1992; Nissim et al., 1994]. The "Nissim" library [Nissim et al., 1994], consisting of $5x10^8$ scFv fragments, with different VH domains and identical VL domain, has yielded binders against most antigens with which it was challenged, and has been distributed to over 200 academic laboratories worldwide. The antibodies isolated can be affinity matured by constructing mini-libraries in which the CDR3 of the light chain is randomised, followed by stringent selection [Neri et al., 1997; Pini et al., 1997]. Other synthetic or semi-synthetic repertoires have been reported:

- Barbas and colleagues used a single antibody gene sequence, in which they completely randomised a 16-aminoacid CDR3 loop of the heavy chain, resembling therefore a constrained antibody library implanted onto an antibody [Barbas et al., 1992].
- Griffiths and colleagues described a repertoire of 6.5×10^{10} Fab fragments, in which CDR3 loops, randomised in length and aminoacid composition, were appended to the ensamble of VH, Vκ and Vλ germline genes covering over 95% of all the unmutated human V-gene segment [Griffiths et al., 1994]. The authors reported the isolation of a large number of different antibodies, often with affinities in the low nanomolar range.

- de Kruif and colleagues used a defined set of VH-gene segments, fused to CDR3 loops which were 6-15 aminoacids long and partially randomised, and six light chain segments [De Kruif et al., 1995].
- Other authors have randomised the CDR3s in an undefined mix of human germline V-gene segments [Akamatsu et al., 1993], or have diversified all CDR3 loops in one gene segment [Garrad et al., 1991; Söderlind et al., 1995]
- Little and coworkers [Braunagel et al., 1997] described a synthetic library, in which they made use of trinucleotide chemistry in order to perform codon mutagenesis. The library was successfully tested on dinitrophenol, fluorescein isothiocyanate and 3-nitro-4-hydroxy-5-iodophenyl-acetic acid.
- Hughes-Jones and coworkers described the synthesis of Fv phage-antibodies using VH and VL germline genes [Hughes-Jones et al., 1999].
- Cai and Garen compared a VH fusion phage library, containing VH domains unassociated with VL, with a scFv fusion phage library [Cai et al., 1997].
- Knappik and colleagues has produced a large ($2x10^9$) synthetical commercial phage library, HuCAL, based on modular consensus framework [Knappik et al., 2000].

Recently, we have reported the construction and use of human scFv phage libraries, based on a single germline antibody scaffold, in which judiciously selected aminoacid positions were combinatorially mutated [Borsi et al., 1998; Pini et al., 1998; Kirkham et al., 1999]. From these libraries of intermediate size (approx. 3×10^8 individual clones), several antibodies against tumour-associated markers have been isolated with affinities in the 10^6-10^8 M^{-1} range. Some of these antibodies have subsequently been affinity matured by combinatorial mutagenesis of additional aminoacid residues followed by stringent selection and screening protocols (see below). Using similar principles, we have recently produced a novel synthetic library containing $> 5 \times 10^8$ individual clones (the "ETH-2 library", [Viti et al., 2000]), from which several dozens of different

binding specificities have already been isolated, and which has been distributed to over 90 academic laboratories.

2.3.2.3 Antibody selection with simple and complex samples

When good quality antibody phage display libraries are challenged with a pure antigen (typically a folded globular protein), monoclonal antibodies can in most cases be isolated with 1-2 weeks of work. Historically, affinity selections of specific phage antibodies were first performed with antigens immobilised on a resin. Soon afterwards, protocols were introduced, in which the antigen was coated on a plastic support [Marks et al., 1991]. Alternatively, selections could be performed using biotinylated antigens, followed by capture of the antigen-(phage-antibody) complex with streptavidin coated magnetic beads [Hawkins et al., 1992].

Several useful antibodies against tumour markers have been selected using antigens adsorbed on plastic (see for example Carnemolla et al., [Carnemolla et al., 1999]). However, when adsorbing proteins on a solid support (e.g., on immunotubes), the antigen may denature. The resulting antibodies may not recognise the native antigen and are typically of little utility for tumour targeting applications. Furthermore, small globular proteins (<15'000 daltons) do not coat well on plastic tubes. When raising phage antibodies against the ED-B domain of fibronectin, a marker of angiogenesis [Castellani et al., 1994], Carnemolla and colleagues had to covalently immobilise the ED-B on reactive plastic in order to perform selections, or to flank the ED-B domain with other protein domains in order to enhance adhesion to plastic [Carnemolla et al., 1996]. Immunohistochemistry and biodistribution experiments are possibly the most important assays to confirm that an antibody isolated using phage technology may be useful for tumour targeting applications [Chester et al., 1994; Carnemolla et al., 1996; Neri et al., 1997].

Selections with biotinylated antigens are attractive for a number of reasons. First, the antigen is likely to retain the native confirmation after biotinylation. Second, the

interaction between antigen and phage-antibody occurs in solution. Third, a number of experimental parameters can be controlled (antigen concentration, competition with non-biotinylated antigen), that directly dictate the stringency of the selection [Hawkins et al., 1992; Low et al., 1996]. Care must be taken, however, that the antigen is not over-biotinylated, since relevant epitopes may become unaccessible after chemical modification [Kirkham et al., 1999]. The use of biotinylated proteins in phage selections allows to reduce the amount of antigen needed in order to isolate antibodies from libraries. Indeed, we were able to isolate recombinant antibodies using 0.3 μg of biotinylated antigen isolated from two-dimensional gels [Pini et al., 1998].

More complex selection schemes, in which panning is performed using antigen mixtures, are reviewed in de Haard [Haard et al., 1998]. They include direct panning on cells, panning by cell sorting and selection on tissue sections. In principle, it would be desirable to use antibody phage libraries for the discovery of novel tumour markers by direct selection on cells. This approach has yielded interesting antibodies in a few cases (see for example work by Garen [Cai et al., 1995], Marks [Marks et al., 1993], Pereira [Pereira et al., 1997] and Logtenberg [Lekkerkerker et al., 1999]). In general, selections against tumour cell lines yield all expected binding specificities: pan-cell antibodies, antibodies specific for cells of a specific origin and antibodies against tumour markers [Roovers et al., 1998; Henderikx et al., 1998]. Selection schemes that feature rounds of panning on the target cells of interest, alternated with rounds of pre-adsorption on undesired cells or tissues, may in the future widen the scope of phage technology for the selection of antibodies against novel antigens anchored on the cell membrane [Siegel et al., 1994; Andersen et al., 1996; Stausbol-Gron et al., 1996; Van Ewijk et al., 1997; Ridgway et al., 1999; Hoogenboom et al., 1999; Mutuberria et al., 1999] or for internalising antibodies [Poul et al., 1999]. In practice, phage-antibody stickiness may limit the applicability of this technology for selections on cells and, more seriously, on tumour sections (Dario Neri, personal communication; de Haard [Haard et al., 1998]).

Potential solutions are offered by selection schemes in which the binding specificities of interest are isolated by cell sorting [de Kruif et al., 1995; Kipriyanov et al., 1996], or by in situ biotinylation strategies that enable selection for spatial proximity to a known molecule of interest [Osbourn et al., 1998]

In vivo selections, in which a phage library is injected into animals and binding specificities are isolated by elution from the tissue of interest after the animal has been sacrificed, have been reported for peptide libraries on phage [Pasqualini et al., 1996; Arap et al., 1998], but not yet for phage antibodies. It remains to be seen what will be the impact of this potentially attractive technique for the discovery of novel tissue- and pathology-specific antigens.

2.3.2.4 Relevance, characterisation and maturation of antibody affinity

Methods for the measurement of antibody-antigen affinity have recently been reviewed elsewhere [Neri et al., 1996; Huber et al., 1999]. I therefore address the interested reader to those articles, while only the basic information and concepts that are relevant for tumour targeting applications of recombinant antibodies will be mentioned here.

Let us consider the binding of a monomeric antibody fragment with an antigen in solution. When equilibrium is reached, the concentration of antibody and antigen, free and in complex, are linked to the dissociation constant K_d by the equation:

$$K_d = [Ab][Ag] / [Ab \bullet Ag] \qquad \text{[eq. 1]}$$

Let us consider the intuitive situation in which the antigen (e.g. a tumour marker) is present at low concentrations, much smaller than K_d. One mathematical implication of equation 1 is that at total concentrations of antibody greater than K_d, most of the antigen will be bound to the antibody. Conversely, at antibody concentrations lower than K_d, a large proportion of the antigen will be free [Neri et al., 1996]. In other words, the dissociation constant K_d (or its reciprocal, the affinity constant $K_a = 1/ K_d$) is a useful

parameter that tells us to which extent molecules will be associated in complex, at certain concentration conditions.

To a certain extent, equation 1 can guide the researcher when choosing suitable experimental conditions (e.g., amounts of antibody to be injected) for tumour targeting applications. In practice, however, the situation is complicated by a number of factors. First, a tumour targeting experiment can be described as a simple equilibrium process only to a first approximation, since clearance and tissue penetration continuously change the blood concentration of the targeting agent. Second, rebinding effects and multivalent binding to a tumour antigen on a tissue may significantly increase the "functional affinity" of a targeting agent, compared to the thermodynamically rigorous affinity obtained by solution measurements with monomeric molecules.

The dissociation constant K_d is also linked to the kinetic association and dissociation constants, k_{on} and k_{off}, through the relation:

$$K_d = k_{off} / k_{on} \qquad \text{[eq. 2]}$$

In conditions of irreversible dissociation of the antibody-antigen complex (e.g., when one of the binding partners is anchored on a solid support and the other one is washed away, as soon as the complex dissociates), the half life of the complex ($T_{1/2}^{off}$) is independent of concentration and is expressed by the equation [Neri et al., 1996]:

$$T_{1/2}^{off} = \ln(2) / k_{off} \approx 0.69 / k_{off} \qquad \text{[eq. 3]}$$

For example, a kinetic dissociation constant of 10^{-3} s^{-1} would correspond to a complex half-life of 690 s = 11.5 min; a kinetic dissociation constant of 10^{-5} s^{-1} would result in a half-life of 20 hours. One would be tempted to conclude that parameters such as residence time of a targeting agent at the tumour site could be directly predicted on the

basis of Eq. 3. Even though knowledge of the k_{off} constant is a precious information when planning tumour targeting experiments, in practice rebinding effects and multivalent binding can significantly increase residence time on tumour ([Milenic et al., 1991], see chapter 2.5.2).

Velocity of complex formation, in the initial rate approximation, is a function of ligand concentration and of the kinetic association constant, k_{on}. It could therefore be expected, that tumour targeting agents with high k_{on} values would target tumours more rapidly than agents with low k_{on}. However, tumour targeting is a multi-compartment pharmacokinetic problem and many parameters other than k_{on} are important. For example, the time it takes for a small antibody fragment like a scFv to reach the peripheral compartment after intravenous injection, is an important factor since the elimination from the central compartment is fast due to renal clearance. High k_{on} values may facilitate tumour targeting for binding agents that have a limited time to find and bind to their antigens, but it may very well be the distribution to the peripheral compartment (for example crossing of the endothelial cell layer) that is the rate limiting parameter. Thus, the influence of k_{on} values on tumour targeting, for different targeting agents, remains to be experimentally addressed. Fast association kinetics are likely to be important for one-compartment systems such as targeting of tumoural new-forming blood vessels (angiogenesis) [Otte et al., 1998]. High affinity constants (and possibly high kinetic association constants) are probably important determinants of the good tumour targeting performance of small tumour targeting agents such as radiobiotin (Paganelli [Paganelli et al., 1991]; Avicidin™, NeoRX Corporation, Seattle, U.S.A.) and octreotide derivatives [Otte et al., 1998].

A number of techniques can be used for the measurement of antibody affinity and kinetic constants, which are reviewed in [Neri et al., 1996; Huber et al., 1999] and will not be presented here. We will only mention that, in addition to standard techniques for

affinity measurements such as competition ELISA, fluorescence quench titration, isothermal titration calorimetry, recent advances in real time interaction analysis using biosensor technology [Jönsson et al., 1991] have revolutionised the field of antibody engineering. Although biosensor measurements are not devoid of potential limitations and artifacts, the amount of information that can be obtained on the antibody-antigen binding process is enormous [Huber et al., 1999].

Although the antibodies selected from primary phage libraries typically perform well in a number of applications such as ELISA, western blotting, immunohistochemistry and immunoprecipitation [Nissim et al., 1994; Pini et al., 1998], their affinity may not be sufficient for tumour targeting applications (see chapter 2.5.2), and may require to be matured (see chapter 2.5.3).

Recombinant antibodies, isolated from phage display libraries or derived from hybridomas, are affinity-matured using phage technology with variations of a single strategy , that can be summarised as follows. Typically, the sequence of one antibody clone (more rarely of a pool of clones) is combinatorially mutagenised, either randomly or at specific positions which are believed to be important for binding. The resulting repertoire of antibodies is displayed on phage, resulting in an "affinity-maturation" secondary library. Biopanning experiments in stringent conditions yield a number of candidate antibodies, which are screened for properties (mainly affinity and kinetic binding constants, but also expression yields, solubility, oligomeric state). If antibodies with the desired properties are not obtained at the end of this process, the procedure is repeated, possibly introducing diversity at different aminoacid positions.

Methods to introduce antibody sequence diversity in a non-targeted fashion include V-gene chain shuffling [Clackson et al., 1991], error-prone PCR [Hawkins et al., 1992], mutator strains [Low et al., 1996] and DNA shuffling [Crameri et al., 1996]. Targeted

introduction of sequence diversity is typically achieved by combinatorial mutagenesis of entire CDR loops [Yang et al., 1995; Thompson et al., 1996; Schier et al., 1996a; Schier et al., 1996b; Neri et al., 1997; Pini et al., 1997], or of few aminoacid positions [Pini et al., 1998; Chowdbury et al., 1999] which are predicted to be important for antigen binding on the basis of structural and evolutionary considerations [Tomlinson et al., 1996].

Biopanning experiments with biotinylated antigens can be performed in solution with protocols that favour the isolation of high-affinity binders [Hawkins et al., 1992; Chester et al., 1994; Yang et al., 1995; Low et al., 1996; Schier et al., 1996a; Schier et al., 1996b], or binders with reduced kinetic dissociation constant [Hawkins et al., 1992; Low et al., 1996]. In our experience, screening the affinity constants of several hundred clones isolated from the affinity maturation libraries is possibly an even more important factor for the success of an affinity maturation strategy [Neri et al., 1997; Pini et al., 1998; Carnemolla et al., 1999].

In general, whenever a purified protein antigen is available in sufficient quantity, antibody phage technology is so robust that binders with subnanomolar affinity can either be isolated directly from a large primary library, or can be affinity-matured in few weeks using one of the strategies outlined above. Synthetic antibody libraries are compatible with general affinity maturation strategies, in which the same degenerate oligonucleotides can be used for different antibodies without sequencing [Neri et al., 1997; Pini et al., 1997]. They may therefore represent one of the fastest avenues for the isolation of high-affinity antibodies, suitable for tumour-targeting applications [Neri et al., 1997, Viti et al., 1999].

2.4 Angiogenesis as a therapeutic target

2.4.1 The process of angiogenesis

Angiogenesis is the growth of new blood vessels from pre-existing ones. It occurs primarily during embryogenesis as an essential process for the development of the vascular network of arteries, veins, arterioles, venules and capillary blood vessels which nourish and protect the body's tissues [Bischoff et al., 1995].

Once the vascular network is in place in the adult, the endothelial cells (ECs) lining the blood vessels are quiescent and angiogenesis is normally triggered only locally and transiently during some processes such as the female reproductive cycle, hair growth, wound healing and inflammation [Bischoff et al., 1995]. In the exact location required, endothelial cells secrete proteases to degrade the extracellular matrix (ECM), then migrate into the perivascular space, proliferate and align themselves to form new vessels (see Fig. 2.8). When the process of angiogenesis is completed in a certain area, the endothelial cells become quiescent again and may regress if no longer needed. This transient triggering of angiogenesis relies on a balance between inhibitory controls and angiogenic inducers [Folkman et al., 1995a, b].

There are pathological conditions, however, in which this equilibrium is altered: blood vessels grow unabated and angiogenesis sustains the progression of the disease [Folkman et al., 1995a, b]. Angiogenesis is now recognised to take part in a wide range of human disorders including cancer, rheumatoid arthritis, diabetic retinopathy, age-related macular degeneration, psoriasis.

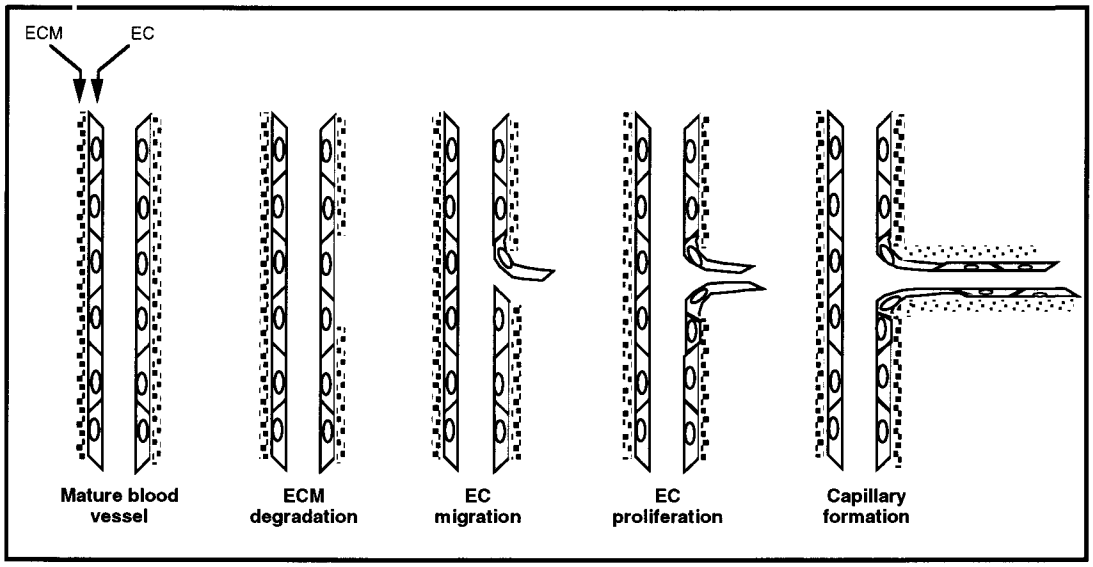


Fig. 2.8.
Model for the formation of new blood vessels from pre-existing ones.
The extracellular matrix (ECM) is degraded by proteases secreted by the endothelial cells (EC). Endothelial cells then migrate into the perivascular space, where they proliferate and align themselves to form a new vessel.

2.4.2 Angiogenesis in cancer

From studies performed on people who died for accidental causes (Folkman, oral communication, "Biological basis for Antiangiogenic Therapy", Milano, 1999) it is known that:

1 39% of 40-50 years old women have small in situ breast carcinoma
2 46% of 60-70 years old men have small prostate cancers
3 around 100% of 50-70 years old people have small thyroid cancer

However, only a small percentage of these lesions typically become pathologically relevant tumours and therefore only 1% of them would have been diagnosed.
Immunohistochemical analysis shows a common feature which accounts for the non-pathological behaviour of these in situ lesions: a low blood vessels density.
Many tumours in humans persist in situ for months or even years without neovascularisation, surviving as asymptomatic lesions rarely larger than 2 mm^3 [Folkman, 1971; Folkman, 1972a; Folkman, 1972b]. In these "prevascular" tumours,

the high rate of tumour proliferation is balanced by a high rate of tumour cell death, probably caused by the low level of blood perfusion (Fig. 2.9). Only when a group of tumour cells switches to the angiogenic phenotype the tumour mass expands and overtakes the rate of internal apoptosis by developing blood vessels. It is at this stage that the majority of tumours become clinically detectable and capable to invade surrounding tissues and metastatise [Blood et al., 1990; Folkman, 1995 a, b; Hanahan et al., 1998].

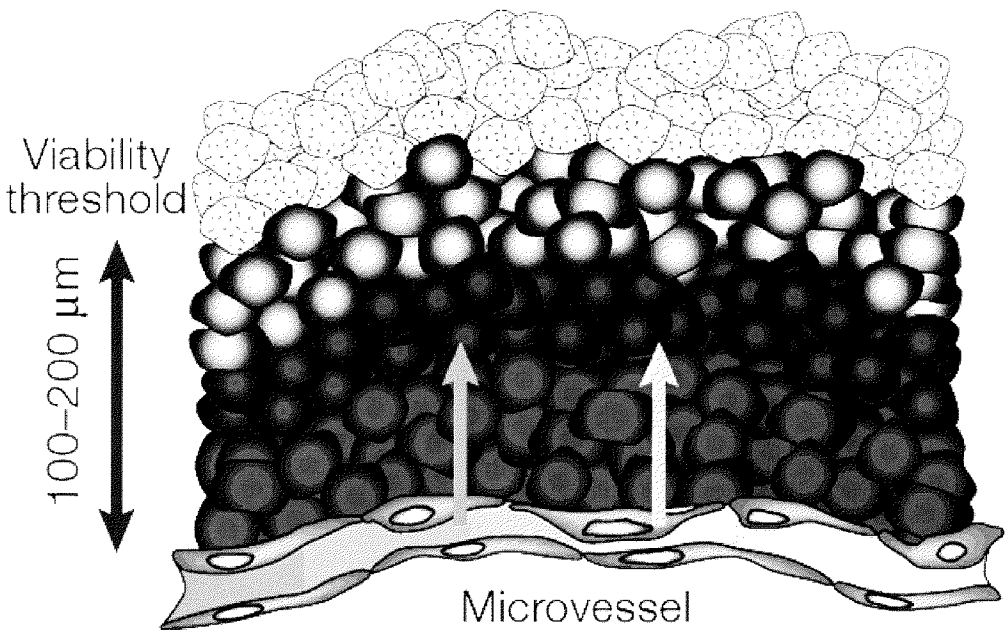


Fig. 2.9
Diffusion constraints govern tumour growth. Depicted is the constraint that tumour cells must be within 100–200 µm, the consumption-adjusted oxygen diffusion distance, from a vessel to remain viable. Tumour cells are shown in various stages of deprivation as a function of distance from the vasculature. The green arrow represents oxygen and nutrients from the vessel lumen. The yellow arrow represents the estimated 20 or so known endothelial derived mitogens and survival factors that influence tumour growth. (Picture from: Nature Reviews, Molecular Cell Biology 1; 76-79 (2000)).

The angiogenic switch in pathological conditions occurs when the balance between inhibitor and stimulators of angiogenesis, which normally accounts for the absence of angiogenesis in adult tissues, is upset. Table 2.2 lists some of the endogenous angiogenesis inhibitors and stimulators known at present.

In more detail [Brower et al., 1999] the transition from "dormant" to active vascularization is most likely triggered when hypoxia is present around the tumour cells. Recent discoveries have shown that hypoxia activates hypoxia-inducible transcription factors (HIFs), which function as master switches to induce expression of several angiogenic factors including VEGF, nitric oxide synthase (NOS), platelet-derived growth factor (PDGF), Ang2 and others (Fig. 2.10) [Semenza et al., 1998].

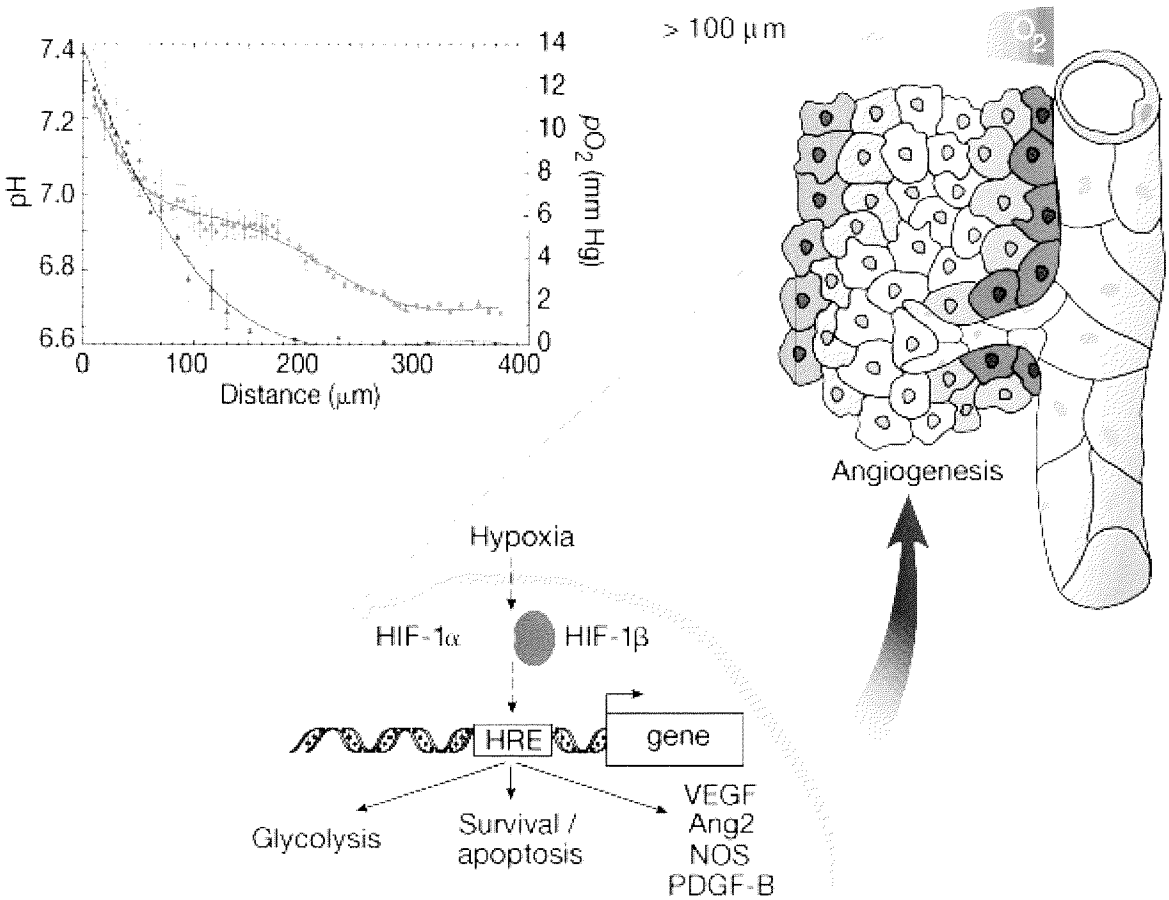


Fig. 2.10

Role of hypoxia in tumour angiogenesis. Because of the irregular pattern and organization of the tumour vasculature, some cells in tumours are located more than 100 μm (the diffusion limit for oxygen) away from blood vessels and become hypoxic (red-to-blue gradient indicates progressive hypoxia). Tumour cells survive fluctuations in oxygen tensions, in part because clones are selected in hypoxic tumours that switch to a proangiogenic phenotype. HIFs increase transcription of several angiogenic genes (for example, genes encoding VEGF, PDGF-BB and NOS). HIFs also affect cellular survival/apoptosis pathways. Inset: relationship between the distance of tumour cells from nearby vessels and their degree of hypoxia (blue symbols) and acidosis (red symbols) [Hemlinger et al., 1997]. (Picture from: Nature, 470, 249-257, (2000)).

As a response, ECs produce locally many other nonspecific angiogenic stimulators, including basic fibroblast growth factor (bFGF), acid fibroblast growth factor (aFGF), transforming growth factor β (TGF-β) and platelet derived EC growth factor, which also drive the invasive phenotype. In addition, tumour cells and ECs excrete proteolytic enzymes, such as matrix metalloproteinases (MMPs) and serine proteases (e.g. tissue plasminogen activator and urokinase plasminogen activator) which break down the ECM. Integrin adhesion molecules ($\alpha_v\beta_3$ and $\alpha_v\beta_5$) are expressed on the surface of ECs and mediate interactions with the ECM. Laminin, tenascin and type IV collagen are also produced to provide new basement membrane components. In addition, expression of EC growth factor receptor tyrosine kinase (e.g. VEGFR-1 and VEGFR-2) is upregulated, together with Tie-1 and Tie-2 receptors, which interact with angiopoietins to signal capillary organisation. The induction of cytokines and chemokines also recruits monocytes and leukocytes, producing local inflammatory reactions that aid in the process.

Up-regulation of angiogenic factors is however not sufficient in itself for a tumour cell to become angiogenic: negative regulators of vessel growth have to be down-regulated [Folkman, 1995b]. Thrombospondin [Rastinejad et al., 1989] was the first protein for which a downregulation during tumourigenesis was demonstrated: by the time tumour cells have become angiogenic, they are producing 4-6% of the thrombospondin originally generated by their normal precursor cells. Recently, a number of angiogenesis inhibitors have been identified as cryptic regions or fragments of intact molecules, which, in pure form, possess the capacity to block angiogenesis: angiostatin [O'Really et al., 1994], endostatin [O'Really et al., 1997], PEX [Brooks et al., 1998].

Also metastases are dependent on angiogenesis [Folkman, 1995a]. First, metastatic cells are not shed from a primary tumour until after it has become neovascularised (Fig. 2.11). Second, upon arrival at their target organ, metastatic cells must again undergo

neovascularization if a metastasis is to grow to a clinically detectable size. The clinical pattern of a cancer metastases (a metastasis can appear only after the removal of a primary tumours or it can be already present when the primary tumour is detected or it can even be detected with the primary tumour remaining occult) seems related to the intensity of angiogenesis in its vascular bed and to the balance between angiogenesis promoters and inhibitors [Folkman, 1995a]. Tumour dormancy exists where long-lasting angiogenic inhibitors, secreted by a primary tumour, are responsible for negatively modulating the growth of secondary tumours in distant sites [Folkman, 1995a]. For such a model, the removal of a primary tumour that secretes distant-acting angiogenic inhibitors, may cause a situation in which dormant micrometastases can escape from angiogenic inhibition and rapidly expand, resulting in death of the host. The quantitation of vascular density in a biopsy specimen may help in the diagnosis of tumours and, in the case that cancer is diagnosed, has proven to be helpful in the prediction of the risk of metastases or recurrance in different types of cancer [Folkman, 1994; Weidner et al., 1995].

2.4.3 Markers of angiogenesis

Angiogenic processes are regulated by a number of cell-surface and extracellular adhesion molecules. Molecules specifically expressed in tissues undergoing angiogenesis [Zetter et al., 1997], can be useful for the diagnosis and prognosis of angiogenesis-related diseases and also as targets for the selective delivery of therapeutic agents. For this reason, the identification of new angiogenesis markers is the goal of many studies at the moment. Only a few specific markers of angiogenesis have been discovered so far, including:

-Extra Domain B of Fibronectin	[Zardi et al., 1987]
-Endoglin	[Gougos et al., 1990]
-VEGF/VEGFR complex	[Brekken et al., 1998]
-Vascular Cell Adhesion Molecule-1 (VCAM-1)	[Ran et al., 1998]
-E-selectin	[Bevilacqua et al., 1993]
-Integrin $\alpha_v\beta_3$	[Brooks, 1994 et al.; Friedlander et al., 1995]
-Prostate Specific Membrane Antigen (PSMA)	[Chang et al., 1999]

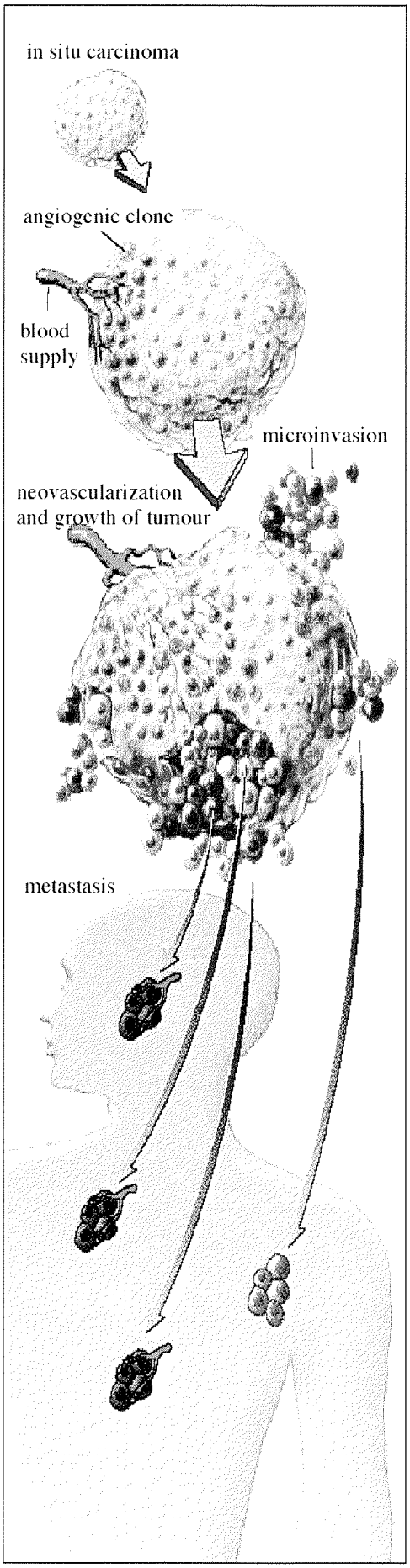


Fig. 2.11 The development of heterogeneity in the angiogenic phenotypes in a primary tumour and its relation to metastasis. (Adapted from Folkman J: "Clinical Application of Research on Angiogenesis", New Engl J Med 333, 1757-163, 1995). A neovascularized tumour may contain subgroups of cells that display the angiogenic phenotype (dark tumour cells) as well as cells that do not (light tumour cells). Tumour cells that do not express angiogenic phenotype may become dormant metastases. In contrast, angiogenic tumour cells may produce rapidly growing, clinically detectable metastases. Breakdown of the basement membrane and microinvasion (for example, around a breast duct containing carcinoma) have been observed with and without neovascularization (both shown here).

Table 2.2 Angiogenesis activators and inhibitors

Activators	Function
VEGF family members	Stimulate angio/vasculogenesis, permeability, leukocyte adhesion
VEGFR, NRP-1	Integrate angiogenic and survival signals
Ang 1 and Tie 2	Stabilise vessels, inhibit permeability
PDGF-BB and receptors	Recruit smooth muscle cells
TGF-β1, endoglin TGF-β receptors	Stimulate extracellular matrix production
FGF, HGF, MCP-1	Stimulate angio/arteriogenesis
Integrins $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_5\beta_1$	Receptors for matrix macromolecules and proteinases
VE-cadherin;PECAM	Endothelial junction molecules
Ephrins	Regulate arterial/venous specification
Plasminogen activators, MMPs	Remodel matrix, release and activate growth factors
PAI-1	Stabilise nascent vessels
NOS; COX-2	Stimulate angiogenesis and vasodilation
AC133	Regulate angioblast differentiation
Chemokines	Pleiotropic role in angiogenesis
Id1/Id3	Determine endothelial plasticity

Inhibitors	Function
VEGFR-1, NRP-1	Sink for VEGF, VEGF-B, PIGF
Ang 2	Antagonist of Ang 1
TSP-1,-2	Inhibit endothelial migration, growth, adhesion and survival
Angiostatin and related plasminogen kringles	Suppress tumour angiogenesis
Endostatin (collagen XVIII fragment)	Inhibit endothelial survival and migration
Vasostatin; calreticulin	Inhibit endothelial growth
Platelet factor-4	Inhibits binding of bFGF and VEGF
TIMPx; MMP inhibitors; PEX	Suppress pathological angiogenesis
Meth-1; Meth-2	Inhibitors containing MMP, TSP and disintegrin domains
IFN-α,–β,–γ, IP-10, IL-4, IL-12, IL-18	Inhibit endothelial migration; downregulate bFGF
Prothrombin kringle–2; antithrombin III fragment	Suppress endothelial growth

Table 2.2, continued

Inhibitors	Function
Prolactin (MW, 16K)	Inhibits bFGF/VEGF
VEGI	Modulate cell growth
Fragment of SPARC	Inhibit endothelial binding and activity of VEGF
Osteopontin fragment	Interfere with integrin signalling
Maspin	Protease inhibitor
Canstatin, proliferin-related proteins, restin	Mechanisms unknown

Angiogenesis activators and inhibitors (adapted from Carmeliet, 2000). List of selected examples, further information and references are available at http://steele.mgh.harvard.edu
Abbreviations: VEGF, vascular endothelial growth factor; VEGFR, VEGF receptors; Ang, Angiopoetin; MMP, matrix metalloproteinases, Id1/3, inhibitors of differentiation 1/3; NRP-1, neurophilin-1; TIMPs, tissue inhibitors of MMPs; PEX, proteolytic fragment of MMP2; IP-10, inducible protein-10; VEGI, member of the TNF family; SPARC, inhibits endothelial bining and activity of VEGF; PDGF,platelet-derived growth factor; NOS, nitric oxide synthase, HGF, hepatocyte growth factor, IGF-1, insulin-like growth factor; MCP-1, monocyte chemoattractant protein-1, TNF-α, tumour necrosis factor–α, TGF-β1, transforming growth factor β1.

2.4.4 The ED-B domain of Fibronectin

FNs are high-molecular mass adhesive glycoproteins present in the ECM and body fluids [for review see Hynes et al., 1990]. FN molecules are involved in diverse biological phenomena including the establishment and maintenance of normal cell morphology, cell adhesion, migration, hemostasis and thrombosis, wound healing and oncogenic transformation [Magnusson et al., 1998]. Mouse embryos homozygous for a loss of function mutation of the FN gene have been observed to die at about 8.5 days of gestation [George et al., 1993]. Embryonal death is associated with widespread defects in mesoderm-derived structures, including absence of somites and notochord, as well as developmental defects in the heart and vascular system.

FNs are dimers composed of two 250-280 kDa (≈ 2500 amino acids) monomers joined by two disulfide bonds at their carboxyl termini [Yamada et al., 1983; Hynes et al., 1985].

The FN modules are organised into functional domains that are resistant to proteolysis and contain binding sites for extracellular matrix proteins such as collagen and thrombospondin, cell-surface receptors such as integrins, circulating blood proteins such as fibrin and glycosaminoglycans such as heparin and chondroitin sulphate.

FNs are extended molecules, composed of serially repeating units, designated type I, II and III, which are indipendently folded modules arranged as "beads on a string" [Figure 2.12; Leahy et al., 1996].

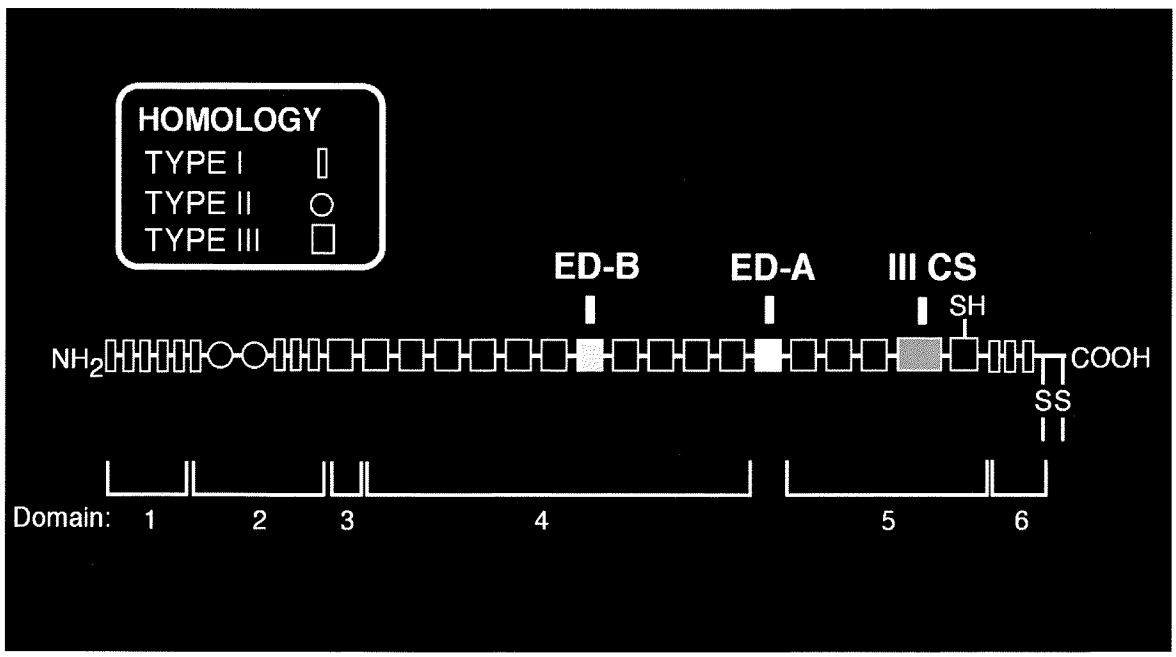


Fig. 2.12.
Model of the domain structure of a subunit of human Fibronectin. Fibronectin subunit is made up of a series of repeating units of three different types (type I, type II, type III). Two subunits are joined by two disulfide bonds at their carboxyl termini. Three repeats can be either inserted or omitted in the molecule by a mechanism of RNA alternative splicing: ED-B, ED-A, IIICS. Separate functional regions (in the figure named domains) have been identified that contain binding activities for other components of the ECM: domain 1 (can bind to heparin, DNA, fibrin), domain 2 (can bind to gelatin), domain 3 (can bind to heparin and DNA), domain 4 (can bind to cells, heparin, DNA), domain 5 (can bind to heparin and DNA), domain 6 (binds to fibrin).

Fibronectins are polymorphic proteins. Their polymorphism is due to alternative splicing patterns in three regions (IIICS, ED-A and ED-B) of the single FN primary transcript as well as to post-translational modifications. In transformed cells and in

malignancies, the splicing pattern of FN-pre-mRNA is altered [Castellani et al., 1986; Borsi et al., 1987; Vartio et al., 1987; Zardi et al., 1987; Carnemolla et al., 1989; Oyama et al., 1989, Oyama et al., 1990, Borsi et al., 1992; Kaczmarek et al., 1994; Castellani et al., 1994] leading to an increased expression of FN isoforms containing the IIICS, ED-A and ED-B sequences.

In particular, the FN isoform containing the ED-B sequence (B-FN) which, with some rare exceptions, is undetectable in normal adult tissues, is expressed in foetal and tumour tissues as well as during wound healing [Zardi et al., 1987; Carnemolla et al., 1989; Laitinen, 1991]. Furthermore, B-FN is accumulated around neovasculature during angiogenetic processes [Castellani et al., 1994] and thereby provides a marker of angiogenesis.

In B-FN, ED-B is inserted between the type III modules 7 and 8 (FN7 and FN8). It is a complete type III homology repeat composed of 91 aa (Fig. 2.13) and coded for by a single exon, which can be either completely included or completely omitted from the mature mRNA. ED-B is highly conserved among different species.

ED-B has been discovered during experiments of proteolytic cleavage of FN molecules obtained from different sources. Borsi [Borsi et al., 1985] reported that FN from tumour-derived or SV-40-transformed human cells contains, in the cell-binding domain 4 (see Fig. 2.12), a site sensitive to the proteolytic enzyme cathepsin-D, undetectable in FN from plasma and from normal human cells. This suggested a new splicing site and a transformed cell-specific splicing pattern leading to the expression of a new sequence in FN from transformed cells. In 1987, Zardi and co-workers [Zardi et al, 1987] demonstrated that a large percentage of FN molecules from transformed human cells contains, in the middle of the cell-binding domain 4, a new aminoacid sequence which introduces into the FN molecule a site sensitive to proteolytic enzymes: the ED-B domain of fibronectin. Since then, immunohistochemical studies with first a murine monoclonal antibody binding to FN only in the presence of ED-B [Carnemolla et al.,

1989], and then with recombinant antibody fragments binding to ED-B [Carnemolla et al., 1996], allowed the identification of ED-B as an angiogenesis-associated molecule.

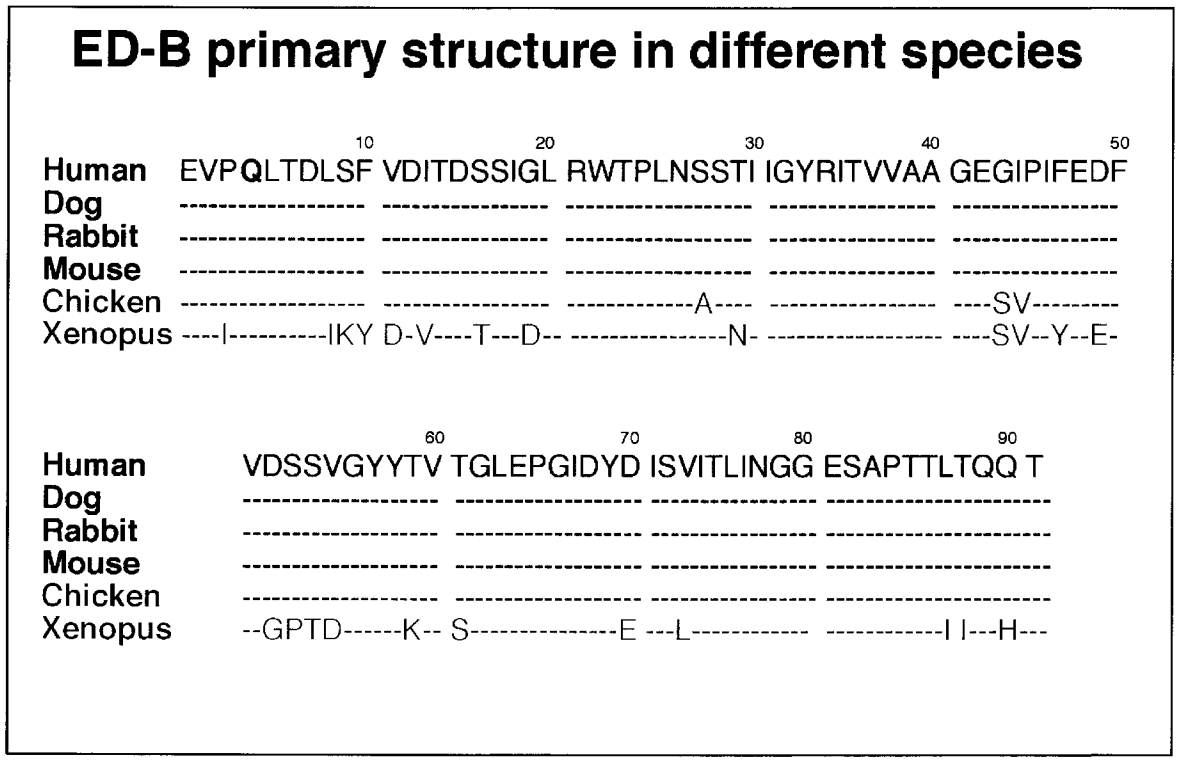


Fig. 2.13
Comparison of the aminoacid sequence of ED-B in different species. Human ED-B sequence is identical to that of dog, rabbit, rat, mouse. It has a homology of 96% with the chicken and of 80% with the Xenopus ED-B sequence.

2.4.5 Angiogenesis-based therapeutic strategies in neoplastic disease

The formation of new blood vessels is essential, at least to a large extent, for the progression of cancer and many other diseases. Therefore angiogenesis offers the possibility to attack these disorders at the level of the mechanism which sustains them. Extensive pre-clinical studies of angiogenesis-based therapies in animal models have performed impressively. As a consequence around 40 drug companies are now working on angiogenesis-related compounds and at least 27 antiangiogenic drugs are in clinical trials, see below Table 2.3.

There are two kinds of anti-angiogenesis therapeutic strategies. The first (and more exploited) one is based on the inhibition of angiogenesis. The second one consists in

targeting and delivering toxic molecules to new blood vessels, so called vascular targeting which will be discussed later. Moreover, antiangiogenic gene therapy is now emerging as a new possibility of angiogenesis-based therapy. For some disorders, an increase in angiogenesis is beneficial, however, I will concentrate on neoplastic pathologies related to a high level of angiogenesis.

In the treatment of solid tumours, there are many advantages of antiangiogenic therapies over chemo- and radio-therapies. Angiogenesis is a feature characterising almost all kinds of solid tumours. A unique angiogenesis-based therapeutic approach would therefore be suitable for many different kinds of tumours. Antiangiogenic therapy is generally less toxic than conventional therapies, since neovasculature is almost undetectable in normal adult tissue. Bone marrow suppression, gastrointestinal symptoms or hair loss normally occurring with conventional therapies usually do not take place. Angiogenesis-based therapies target ECs which, unlike cancer cells are stable and less prone to mutation [Kerbel, 1997]: the risk of developing resistance to the therapeutical agent is therefore low and thus angiogenesis-inhibiting therapies offer an alternative of tackling multi-drug-resistant tumours that have proven untreatable by conventional chemotherapy; since tumour cells depend on blood supply, local interruption of the tumour vasculature would produce an avalanche of tumour cell death; the targeted vascular endothelium -in direct contact with the blood- is easily accessible to drugs.

One of few drawbacks is that therapies targeting angiogenic endothelial cells are limited to the subfraction of tumour capillaries that expresses the immature angiogenic phenotype.

2.4.5.1 Inhibition of angiogenesis

The inhibition of tumour-associated angiogenic processes seems a powerful strategy to "starve" tumours and therefore block their growth and metastatic spreading.

Endogenous and synthetic angiogenesis inhibitors, inhibitors of the activity of the key regulators in the angiogenic cascade, signaling antagonists and proteolysis inhibitors represent good drug candidates and many of them are now being exploited in clinical trials.

In general, preclinical and clinical studies suggest two roles for inhibitors of angiogenesis in the treatment of patients with cancer. First, they could be used to potentiate conventional chemotherapy and radiotherapy, probably by inducing a decrease of tumoural blood pressure which correlates with an increase in the uptake of the toxic agent. Second they might be employed for the so called "dormancy therapy": after the completion of chemotherapy, radiation or surgery, antiangiogenic therapy may be continued for years, to prolong dormancy of microscopic metastases or to stabilise residual disease.

Table 2.3 (see below), lists some angiogenesis inhibitors in clinical trials. They can be devided into endogenous inhibitors, synthetic inhibitors, integrin inhibitors, signaling antagonists and proteolysis inhibitors.

Endogenous inhibitors

Many endogenous compounds with an antiangiogenetic effect have so far been discovered. They can usually inhibit (to a variable extent) the growth of tumours subcutaneously implanted in mice and the development of metastases in the same animal model (see above Table 2.2).

From extensive pre-clinical studies, general principles have emerged. It is therefore worth to briefly summarise the results obtained so far with two endogenous inhibitors of angiogenesis which are now being exploited in clinical trials: angiostatin and endostatin [O'Really et al., 1994; O'Really et al., 1997]. Both these molecules are proteolytic fragments of larger and abundant proteins (angiostatin of plasminogen and endostatin of collagen XVIII) and therefore ready to be mobilised from the storage as cryptic segments when the turning off of angiogenic processes is needed. Angiostatin has been

isolated from a subclone of Lewis lung carcinoma in which the primary tumour inhibited the growth of its metastases, endostatin from a murine hemangioendothelioma cell line. They both proved to be able to inhibit endothelial cell proliferation in vitro. They were further tested for their ability to inhibit angiogenesis in vivo, both using the chicken chorioallantoic membrane (CAM) assay and mice models in which they could inhibit growth of primary and metastatic tumours. Endostatin was also administered in repeated cycles to mice that harbored subcutaneous tumours. As soon as tumour nodules formed, the treatment was started, causing the tumours to shrink. Then, treatment was stopped, and the tumours regrew, at which point therapy was resumed until the tumour had regressed. This experiment showed that tumours did not develop resistance to endostatin, whereas when using cyclophosphamide (directly targeting cancer cells) tumours acquired drug resistance [Kerbel, 1997].

Recently [Mauceri et al., 1998], combined treatment with human angiostatin and ionising radiation therapy produced significant growth inhibition in comparison to radiation therapy alone in mice models, demonstrating the validity of a therapeutic approach that Folkman had already proposed several years ago: the use of angiogenesis inhibitors to augment the efficacy of chemotherapeutic drugs.

Synthetic inhibitors

Synthetic inhibitors of angiogenesis are more advanced in clinical trials than their biological counterparts. Among them are TNP-470 (a synthetic analog of the antibiotic fumagillin), squalimine (based on an extract of a product of the dogfish shark liver), IM-862 (a synthetic version of a natural peptide that upregulates IL-12 and inhibits VEGF and bFGF), combrestatin and thalidomide (see below Table 2.3).

Integrin inhibitors

Inhibition of the activity of any of the key regulators in the angiogenic cascade is another way of blocking tumour angiogenesis. Vascular cells undergoing angiogenesis

proliferate and migrate in an anchorage-dependent manner. Vascular cell adhesion molecules therefore contribute to the regulation of angiogenesis. Among them the integrins $\alpha_v\beta_3$ is preferentially expressed on the surface of proliferating ECs and it is crucial to angiogenic signaling and EC survival. Indeed, antibodies or peptides that block this receptor have been shown to result in rapid EC cell apoptosis and the shrinkage of neovasculature in animal models [Brooks et al., 1994; Brooks et al., 1995; Arap et al., 1998]. Companies are therefore now developing antibodies and peptides antagonising $\alpha_v\beta_3$.

Signaling antagonists

Another way of arresting EC growth and movement is to block growth factor function and signaling. An example is an antibody, currently in PhaseI/II trials, which binds the angiogenic stimulator VEGF [Kim et al., 1993], thereby disrupting activation of the cascade. Other examples are molecules which blocks the VEGF receptor VEGFR-2 (PTK787/ZK2284), [Drevs et al., 2000]; (SU6668), and antagonists for the PDGF receptor (SU6668) [NCI Database, www.cancertrials.nci.nih.gov].

Proteolysis inhibitors

The dependence of blood vessel and tumour growth on degradation of the ECM was one of the first processes recognised as a target for the development of anticancer therapies. As a result, several inhibitors of metalloproteinase (MMP) are in advanced clinical trials.

2.4.5.2 Antiangiogenic gene therapy

A new branch of gene therapy directed at tumour angiogenesis is now emerging and several studies are exploited to inhibit angiogenesis at the DNA and mRNA level.

Inhibition of growth of primary tumour and metastases has been obtained by injecting tumour bearing mice with an adenoviral vector to deliver a recombinant Tie2, which

blocks the activation of the Tie2 receptor in endothelial cells [Lin et al., 1998]. Another approach [Goldman et al., 1998], has been the use of an ex vivo gene transfer method to transfect stable human tumour cells with the cDNA of a truncated form of native soluble FLT-1, a receptor for VEGF. The s.c. growth of tumours from these cells was significantly inhibited as was the growth of lung metastases from i.v. injection of the transfected tumour cells. Moreover it has recently been reported [Blezinger et al., 1999] that direct injection of the endostatin gene into mouse muscle tissue resulted in sustained production of endostatin, regression of primary tumours and prevention of tumour spread. Finally a ribozyme that targets the mRNA of the VEGFR-1 has been developed, and early results of phase I clinical trials suggest that the drug has an acceptable toxicity profile (Table 2.3).

Table 2.3 Angiogenesis inhibitors in clinical trials

Drug	Sponsor	Description/Mechanism of Action- Stage.
Interference with proteolysis		
AG-3340	Agouron	Synthetic MMP-2/-9 inhibitor- Phase III,II
Bay-12-9566	Bayer	Synthetic MMP-2/-9 inhibitor- Phase III
BMS-275291	Bristol-Myers Squibb	Small-molecule MMP inhibitor- Phase I
CGS-27023A	Novartis	Synthetic MMP inhibitor- PhaseI/II
Marimastat	British Biotech	Synthetic MMP-2/-9 inhibitor- Phase III
Metastat	Collagenex	Synthetic tetracycline derivative- Phase I Inhibits MMP-2 and MMP-9
Neovastat	Aeterna	Shark cartilage extract- Phase I Inhibits MMP and VEGF
Blocking EC-ECM adhesion molecules		
EMD-121974	Merck KgcaA	Cyclic pentopeptide- Phase I/II Blocks $\alpha_v\beta_5$ on EC surface
Vitaxin	Ixsys	Mab against $\alpha_v\beta_5$ on EC surface- Phase II
Endogenous angiogenesis inhibitors		
Angiostatin	Entremed	Inhibits ATP synthase- Phase I
Troponin-I	Bostin Life	Inhibits EC growth- Phase I
Endostatin	Entremed	Solid tumours- Phase I

Table 2.3, continued.

Direct antagonists/inhibitors of angiogenesis Inducers		
Angiozyme	Ribozyme	Degrades VEGFR-mRNA- Phase I
Anti-VEGF Ab	Genentech	mAb against VEGF- Phase II/III
PTK-787/ ZK-225846	Novartis/Schering	Small molecule. Blocks VEGF Receptor signaling- Phase I
SU-101	Sugen	Blocks PDGF receptor signaling- Phase III
SU-5416	Sugen/Pharmacia	Blocks VEGFR-2 signaling- Phase I
SU-6668	Sugen	Blocks VEGF, FGF, EGF and PDGF receptor signaling and MMP formation- Phase I
Indirect angiogenesis inhibitors		
IM-862	Cytran	Upregulates IL-12 and inhibits VEGF and bFGF production- Phase III
IFN-α	Commercially available	Downregulation bFGF and VEGF production-Phase III
Pentosan	Georgetown University	Downregulates bFGF production- Phase I
Other approaches		
Carboxyamido triazole (CAI)	National Cancer Inst. Bethesda	Inhibitor of Ca influx. Suppressor of angiogenesis and metastasis- Phase II/III
CombrestatinA4	Oxigene	Binds to tubuline induces EC apoptosis- Phase I
Squalamine	Magainin Pharmaceuticals	Inhibits Na/H exchanger NHE3 interfering with anoxic response- PhaseI
TNP-470 (6-O-chloroacetyl-carbomyl) -fumagillol	TAP Pharmaceuticals	Synthetic analog of fumagillin – Phase II
Thalidomide	Celgene	Mechanism unknown- Phase II
ZD-0101	AstraZeneca	Endotoxin, binds to EC inducing local massive inflammatory response- Phase II

Adapted from Brower, 1999.

2.5 Antibody-mediated targeting of angiogenesis

2.5.1 Tumour targeting

To achieve selective tumour targeting, antibodies or other molecules must be directed towards abnormally expressed or over-expressed proteins or carbohydrates, called tumour-related antigens. However, specificity is only one of the major requirements for an antigen in tumour targeting. Besides, accessibility from the vasculature, abundance and possibly also stability are crucial parameters.

Since most research on tumour-specific molecules has been carried out using cultured neoplastic cells and immunological techniques, the majority of the markers described are on the surface of neoplastic cells. Carcinoembryonic antigen (CEA)[Chester et al., 1994], placental alkaline phosphatase (PLAP) [Kosmas et al., 1998], HER2/neu [Hung et al., 1999], MUC1 [Taylor-Papadimitriou et al., 1999] and EpCAM [Braun et al., 1999] are only a few examples. As a consequence, the majority of antibodies in clinical trials and those approved by the FDA for cancer therapy have, so far, been directed against cell surface proteins on malignant cells.

In a more general perspective, tumour-associated antigens can be grouped into three different categories, depending on their localisation in the tissue: antigens on the surface of malignant cells, antigens in the extracellular matrix and antigens in the neovasculature. Not all parts of a solid tumour become equally well vascularized, however, and in some areas tumour cell division may progress too fast for capillary growth to keep up. This often results in a tumour with a necrotic center of dead or dying cells surrounded by a layer of dormant cells and an outer "rim" of dividing cells. In theory, these necrotic areas can be considered as a further source of tumour associated antigens [Epstein et al., 1988; Frankfurt et al., 1997] (Fig. 2.14).

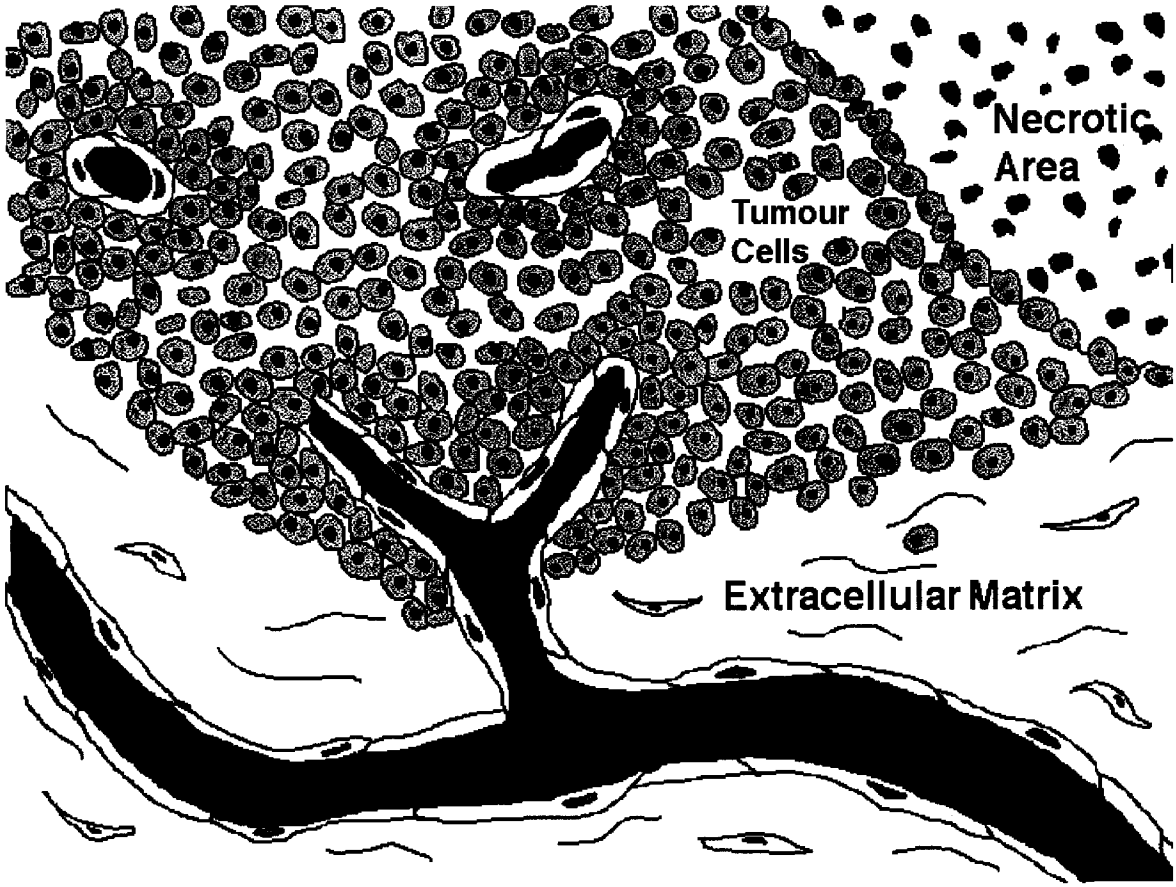


Fig. 2.14 Schematic representation of characteristic tumoural features, that could be used for tumour targeting applications. In addition to the tumour cells, new forming blood vessels (V) could be considered for molecular intervention. Extracellular matrix compontents and necrosis areas may also represent useful targets for selective molecular localisation within the tumour site.

2.5.1.1 Targeting tumour cells

Even though the scope of this chapter is targeting markers of angiogenesis, I briefly want to expand on targeting tumour cells, since important concepts, techniques and problems are common to both types of targeting. Moreover, most of our current expertise stems from studies targeting antigens on malignant cells.

Since tumour cells are normally separated from the blood by endothelial cells and extracellular matrix components surrounding the vasculature, the targeting process highly depends on the ability of the targeting agent (an antibody, for instance) to penetrate into the tumour tissue to encounter the cells. This process may be problematic

due to the overall architecture of the tumour vasculature, where high interstitial pressure, the absence of an anatomically well-defined functioning lymphatic network and low microvascular pressure may impede the extravasation process of targeting agents [Jain et al., 1987; Jain et al., 1988; Jain et al., 1998]. In addition, even upon extravasation, the antibody might still be separated from the most distant tumour cells by long diffusion distances [Denekamp et al., 1990]. It has also been observed, especially for high affinity antibodies, that agents targeting tumoural cells tend to become "trapped" by the antigens of tumour cells lining the vasculature, leaving only few unbound molecules to reach more distant sites [Kennel et al., 1991; Sung et al., 1993; Adams et al., 1998]. In fact, only a minute proportion of the total cell mass, predominantly the cells in close vicinity to the tumour blood vessels, is exposed to therapeutic immunoconjugates in the circulation. These peripheral targets form a so-called "binding site barrier" to the interior of the tumour [Juweid et al., 1992]. The relevance of this barrier may strongly depend on the kinetic stability of the antibody-antigen complex and on the relative stoichiometric abundance of the antibody over the antigen. In this context, several microscopic analyses and biodistribution experiments with different antibody formats have shown that antibodies clearing slowly from the blood might have a better targeting performance, in spite of the fact that in continuous infusion experiments, or in nephrectomised mice, small antibody fragments penetrate faster into the tumour [Yokota et al., 1992; Adams et al., 1998].

A further drawback from targeting malignant cells is the fact that tumour cells are genetically unstable and variants can arise which lack the target antigen [Engert et al., 1990; Burrows et al., 1994; Kerbel et al., 1997]. Nevertheless, if the antibody is able to reach the antigen, the tumour cell targeting approach may be rather effective since the malignant cells represent the target which immunotherapy ultimately seeks to destroy.

2.5.1.2 Targeting endothelial cells

Targeting tumoural endothelial cells features several advantages to targeting of tumour cells. Most importantly, vascular endothelial cells are freely accessible from the blood whereas tumour cells are, for the greater part, inaccessible. Unless the targeted antigen is localized on the abluminal side of the endothelial cells, no extravasation will be retarding or impeding the antigen binding process. Thus, accumulation of antibodies directed against markers on the endothelium will occur rapidly, efficiently and with a uniform distribution [Kennel et al., 1991]. Furthermore, if one considers that angiogenesis is a characteristic feature of almost all types of aggressive solid tumours, antibodies directed against common markers of the tumour endothelium might open up a general and widely applicable approach for targeting and therapeutic intervention, whereas antibodies against tumour cell markers usually only recognise a subset of tumours sharing the same antigen.

Endothelial cells, unlike cancer cells, have stable genomes and are less prone to mutate [Folkman, 1995; Kerbel, 1997]. Therefore, antigen loss or resistance to therapy is less likely to occur when the therapy is directed against the vasculature. Large numbers of tumour cells heavily depend on only few endothelial cells for their blood supply [Denekamp et al., 1990; Denekamp et al., 1993; Burrows et al., 1994]. Thus, damaging the tumour vasculature would have a vast amplification- or bystander-effect by causing an avalanche of tumour cell death. In fact, recent studies in preclinical models have demonstrated that directing therapy to antigens expressed on the tumour vasculature or stroma rather than to tumour cell markers can produce impressive results in the treatment of solid tumours [Borgström et al., 1996; Huang et al., 1997; Arap et al., 1998; Matsuno et al., 1999 and Nilsson et al., 2001].

Since angiogenesis and the concepts of anti-angiogenic therapy and neovascular targeting are rather young disciplines, some uncertainties still exist in respect to the relevance of angiogenesis in human tumours. The rate of tumour growth in human

cancers can differ substantially, with doubling times ranging from days, as for Burkitt's lymphoma and choriocarcinoma, to months as for several types of lung, breast and prostate cancers [Pratt et al., 1994]. However, most preclinical studies on tumour angiogenesis and anti-vascular therapy employ rapidly growing, subcutaneously grafted mouse tumours, or human tumour xenografts. These contain an extremely high proportion of newly formed blood vessels expressing angiogenic markers. As a consequence, it is likely that spontaneous, slow-growing tumours in humans have a much lower proportion of neovasculature, which could render therapies targeting markers of angiogenesis less effective. Further understanding on this issue is soon expected to emerge from immunoscintigraphic studies in patients using antibodies directed against the neovasculature.

2.5.1.3 Targeting the modified extracellular matrix

It has become increasingly apparent that the extracellular matrix (ECM) has an important role in the regulation of angiogenesis. A number of interactions between the ECM and tumour cells and endothelial cells, as well as the ECM's role in binding and releasing growth factors have been described. The degradation and remodelling of ECM components seem to be essential for tumour growth and create a new tumoural environment which appears to be more permissive and/or stimulating for further neovascularization and outgrowth of the tumour. In this process of modification, novel, characteristic markers of angiogenesis are generated which share many of the advantages for targeting with endothelial cells: components of the ECM are often abundantly expressed and may be common to many different types of solid tumours. The enhanced leakiness and fenestration of tumoural blood vessels favours the delivery of antibody to the ECM components in the perivascular space around the vessels, making them readily accessible for targeting. Tumour targeting studies performed with monoclonal antibodies and antibody fragments directed against components of the modified extracellular matrix have shown promising results in animal models [Neri et

al., 1998; Birchler et al., 1999; Tarli et al., 1999; Viti et al., 1999] and in patients [Mariani et al., 1997].

If the goal of targeting is to deliver therapy to the tumour, directing the antibodies to ECM components will have certain implications on the choice of effector function mediating cytotoxicity: as an example, drug- or toxin- conjugates, which require cellular uptake to exhibit their toxicity, will possibly be compatible with targeting of the extracellular matrix if the reagent can be released at the target site. On the other hand, effector mechanisms displaying a wider range of action, such as radioisotopes, may be succesfully employed even when remaining bound to the targeting agent. The therapeutic benefit from targeting immune stimulators, such as cytokines or chemokines, to the ECM still remains to be evaluated.

2.5.2 Antibody-mediated targeting of tumours

2.5.2.1 Quantification of tumour targeting

An important parameter in tumour targeting is the quantity of delivered antibody to the tumour and the non-targeted accumulation of antibody in irrelevant organs. A good method to monitor the tumour uptake over time, in total amounts and with respect to different organs, is the use of quantitative biodistribution studies with radiolabeled antibodies, plotting the percent injected dose per gram of tissue or, for immunoscintigraphic applications in patients, calculating the tumour area in spect-images. The first recombinant antibody isolated from a phage display library and used for tumour-targeting applications was scFv(MFE-23) [Chester et al., 1994]: a high-affinity antibody fragment specific for CEA isolated from an immune repertoire. This antibody fragment has distinctive tumour targeting advantages over one derived from a hybridoma [Verhaar et al., 1996] which binds CEA with lower affinity. Using intravenous injection of radioiodinated antibody in LS174 human tumour xenografts in nude mice, the tumour: blood ratio at 24 hours was 13:1 (but a tumour:kidney ratio of 2.7:1 was observed), with 3.2% injected dose per gram (%ID/g) of tumour at 24 h.

ScFv(MFE-23) could be expressed in high yields, is a stable monomer, and has enabled the development of production procedures compatible with Phase I clinical investigations in cancer patients [Casey et al., 1996], compatible with the British guidelines of the Cancer Research Campaign [Begent et al., 1993]. Indeed, Begent and colleagues demonstrated the usefulness of radioiodinated scFv(MFE-23) for the specific scintigraphic detection of liver metastases in patients with colorectal cancer [Begent et al., 1996]. Earlier, similar imaging results had been obtained by Mach and colleagues using radiolabeled anti-CEA Fab fragments [Bischof-Delaloye et al., 1989; Goldenberg,, 1997]. Now that several anti-CEA phage-derived recombinant antibodies are available [Osbourn et al., 1996], it will be interesting to learn how these molecules compare in terms of their tumour targeting properties. Meanwhile, scFv(MFE-23) fusions with carboxypeptidase G2 are being evaluated in pre-clinical models and in the clinic for the targeted prodrug activation at the tumour site [Martin et al., 1997].

2.5.2.2 Parameters to improve tumour targeting

Improving Ab-mediated tumour targeting: from IgGs to Ab fragments

The use of antibody fragments, rather than complete antibodies, has some advantages in the in vivo targeting of tumours. Fig. 2.15 illustrates schematically the antibody levels in the blood and on the tumour as they are typically observed for small scFvs (tumour values typically 2-4 % ID/g at maximum) or large IgGs (typically 30-40% ID/g) [Hu et al, 1996]. Even though IgGs do not penetrate well into the tissue, the concentration of antibody on the tumour may nevertheless be higher than in the case of scFv, since the slow blood clearance allows for more time to penetrate the tissue. Blood clearance values have been investigated for this two kinds of antibody formats using the CC49 antibody recognising the pancarcinoma antigen TAG-72 [Milenic et al, 1991]. For the CC49 scFv, a plasma half-life of 3.7 minutes in the distribution phase and a half-life of 1.5 hours in the elimination phase could be observed. Theese clearance values were much shorter than the ones observed for the full size CC49 IgG with a plasma half-life

of 39 minutes in the distribution phase and a half-life of 113 hours in the elimination phase.

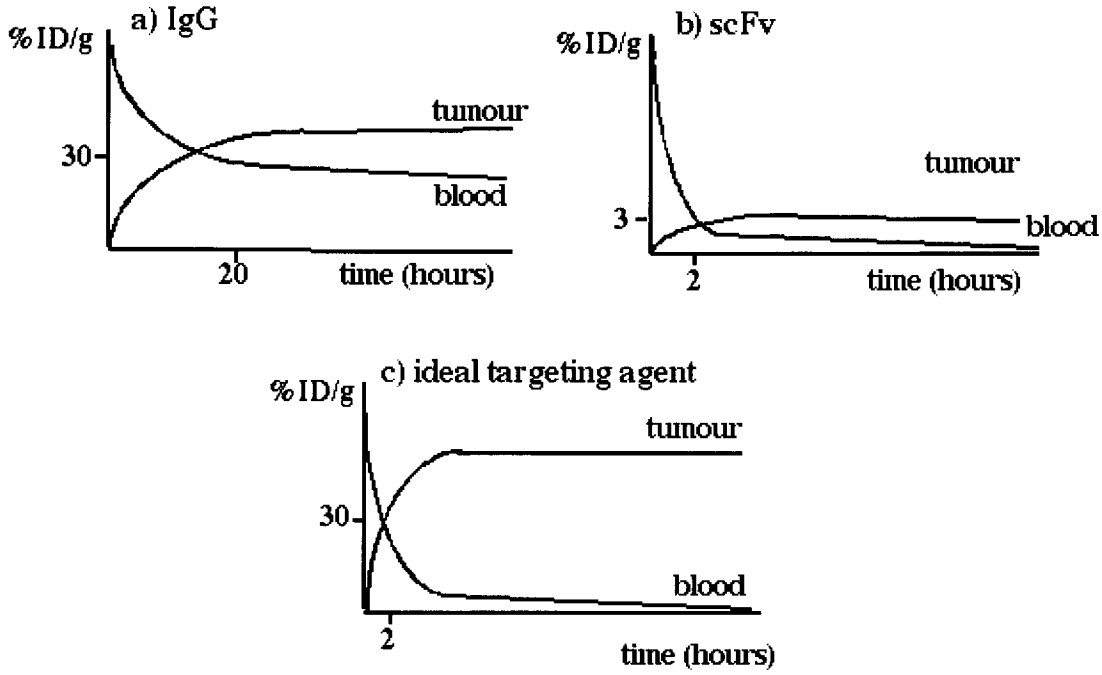


Fig. 2.15 Antibody localisation in tumour and blood with typical uptake time courses for intact antibodies and single-chain Fv [Wu and Yazaki, 1999]

a) Targeting performance (tumour and blood levels versus time) of an antibody in the IgG format (MW= 150 KDa)

b) Levels in tumour and blood of a scFv fragment specific for a tumour antigen.

c) Ideal targeting agent: it should localise at high levels in the tumour and have a fast blood clearance.

As can be seen schematically in fig. 2.15 and which follows from above, the blood clearance profiles of scFvs and of monoclonal antibodies differ considerably: scFvs or other small antibody fragments are rapidly cleared within hours, whereas whole antibodies may remain in the blood for days [Begent et al., 1996]. However, even with high doses delivered to the tumour, the ratio of tumour to blood levels of IgGs is typically rather low, as a consequence of the slow blood clearance. For immunoscintigraphic imaging, a high tumour to blood ratio is important and a short half life of the targeting agent might be favorable. For therapy, high concentrations of antibody on the tumour are required and therefore, a larger antibody fragment or even a

full size antibody may be the best choice. The fragments are of advantage since they are usually not taken up into the liver, as occurs with complete antibodie [Abrams, 1993; Berkower et al., 1996].

Improving Ab-mediated tumour targeting: the role of affinity

One of the attractive features of antibody phage technology is that several affinity mutants directed against the same epitope of a tumour marker can be isolated. In a collaboration between the University of California San Francisco and the Fox Chase Cancer Centre, Philadelphia, Adams and colleagues examined the tumour targeting properties of four monomeric scFv fragments directed against the same epitope of *cerb*B2, a tumour antigen overexpressed in certain forms of breast cancer [Adams et al., 1998], with K_d values ranging between $3x10^{-7}$ and $1x10^{-9}$ M. This is the first study in which tumour targeting agents with similar pharmacokinetic properties and directed against a single epitope of a tumour marker, but differing in affinity, were systematically investigated by accurate biodistribution studies in human tumour xenografted in mice. The affinity increase of the mutants was mainly due to lower kinetic dissociation constants, while the kinetic association constants remained unchanged. The authors found that increased antibody affinity resulted in improved targeting (0.19 %ID/g of tumour at 24h for a scFv with $K_d = 3x10^{-7}$ M, versus 1.42 %ID/g of tumour at 24h for a scFv with $K_d = 1x10^{-9}$ M). In the highest affinity antibody, tumour:kidney ratios at 24h were substantially better than those reported by Begent for MFE-23 (10:1 compared to 2.7:1). The authors also commented that antibodies with higher affinity ($K_d = 1.3 \times 10^{-11}$ M) did not show further improvements in tumour targeting performance. In this multi-compartment tumour targeting model, the rapid blood clearance of scFv fragments may indeed be the limiting step, with reduced antibody extravasation in perivascular spaces. Indeed, tumour targeting experiments with anephric mice (and consequently longer antibody half-life in the blood) showed

significantly higher levels of antibody accumulation in the tumour [Adams et al., 1998a; Adams et al., 1998b].

Our group, in collaboration with the groups of Greg Winter (Cambridge, UK) and Luciano Zardi (Genoa, Italy), reported the successful targeting of tumoural angiogenesis using scFv fragments (CGS-1 and CGS-2) directed against the ED-B domain of fibronectin, a marker of angiogenesis [Castellani et al., 1994, Neri et al., 1998]. In a first study with infrared photodetection [Folli et al., 1994], we found that these scFv fragments could efficiently target grafted tumours in nude mice if their antibody affinity was in the low nanomolar range, or if the antibody fragments were used in dimeric format [Neri et al., 1997]. The lesson learned from these preliminary experiments was confirmed by quantitative biodistribution analysis performed with anti-ED-B antibody fragments directed against an identical epitope, with K_d values of 4.1×10^{-8} M and 5.4×10^{-11} M respectively, assayed in both monomeric and dimeric antibody format [Viti et al., 1999]. The antibody with higher affinity (L19), targeted tumours significantly better than the lower affinity one. For comparison, it may be worth noting that L19, CGS-1 and CGS-2 recognise overlapping epitopes on the ED-B domain of fibronectin, suggesting that this portion of the ED-B surface is accessible in vivo [Fattorusso et al., 1999].

Although the antibodies selected from primary phage libraries typically perform well in a number of applications such as ELISA, western blotting, immunohistochemistry and immunoprecipitation [Nissim et al., 1994; Pini et al., 1997], their affinity may not be sufficient for tumour targeting applications and may need to be matured (see chapter 2.5.3). Recombinant antibodies, isolated from phage display libraries or derived from hybridomas, are affinity-matured using phage technology with variations of a single strategy that can be summarised as follows: Typically, the sequence representing the antigen-recognising moiety (often CDR-regions) of one antibody clone (more rarely of

a pool of clones) is combinatorially mutagenised. The resulting repertoire of antibodies is displayed on phage, resulting in an "affinity-maturation" secondary library. Biopanning experiments in stringent conditions yield a number of candidate antibodies, which are screened for different properties (mainly affinity and kinetic binding constants, but also expression yields, solubility, oligomeric state). If antibodies with the desired properties are not obtained at the end of this process, the procedure is repeated, introducing more diversity at different aminoacid positions.

Improving Ab-mediated tumour targeting: the role of size and valence

Results from several groups have suggested that fragments larger than scFv may be better suited for in vivo targeting applications. Greg Adams et al. [1993] evaluated the biodistribution properties of divalent forms of the anti-erbB2 scFv. In particular one of the forms which they analysed showed a two-fold improvement in tumour localisation. Moreover these scFv dimers showed better tumour uptake than the corresponding Fab (of the same molecular weight but monovalent), demonstrating the contribution of an "avidity" effect, although the tumour accumulation at 24 hours was still low (2% ID/g). The possibility of obtaining better tumour targeting with higher tumour/normal organs ratios by using dimeric scFvs, was explored by Anna Wu and colleagues [Wu et al., 1996]. They derived scFvs from T84.66, a monoclonal antibody with high affinity (K_d = 2.2 $x10^{-10}$ M) for CEA and performed biodistribution studies with both the monomeric and the dimeric form of this scFv. Both forms targeted xenografts efficiently, leading to high tumour:normal organ ratios (greater than 20:1 at 24 hours). However, the maximum tumour uptake of the monomeric form was 4-7% ID/g at 30-60 min post injection, whereas the value obtained with the dimer was of 10-15% ID/g of tumour at the same time points, showing the dimer as a prime candidate for a clinically useful agent [Wu et al., 1996].

Experiments in our group showed that an antibody with higher affinity (L19), targeted tumours significantly better than a lower affinity one both as a monomer and as a dimer [Viti et al., 2000]. The best targeting results were obtained with L19 dimer (12 %ID/g on tumour at 24 h, with tumour:organ ratios of 10:1 or better). Bearing in mind that both antibody constructs had a very high affinity, the fact that L19 dimer targeted the tumour better than L19 monomer suggests that a longer antibody half-life in the blood contributes to the efficient targeting of this marker of angiogenesis, located on the abluminal side of new-forming blood vessels.

Characterisation of antibody oligomeric state and ways to obtain multivalent recombinant antibodies.

A straightforward method to determine the size and oligomerization state of an antibody format is gel filtration analysis. Since the size of an antibody greatly influences its ability to penetrate into the tissue and also its half life in the blood, knowing this parameter may help to either predict or explain the performance of an antibody in vivo. Moreover, the oligomerization state of a recombinant antibody tells about its valency, a further characteristic which may be highly important for targeting: multivalent antibodies usually have apparently higher affinity constants than monovalent antibodies with the same specificity (Fv domains). This behavior is attributed to their higher avidity [Crothers et al., 1972]: bivalent antibodies, for example, can simultaneously bind to two antigens. In addition, if dissociation from the antigen at one or both binding sites occurs, they are more likely to rebind again to the antigen (Fig. 2.16).

ScFv fragments may open the intramolecular VH-VL interface and dimerise [Marks et al., 1993; Griffiths et al., 1993; Nissim et al., 1994] trimerise [Iliades et al., 1997] and even form higher order oligomers [Neri et al., 1996b] (Figure 2.17). The tendency of scFv to oligomerise has been exploited by Holliger and Winter, who demonstrated that VH and VL scFv with a short polypeptide linker (0-10 aminoacid residues) cannot pair

to form a monomeric antibody fragment, but rather homodimerise to form a small bivalent antibody fragment termed "diabody" [Holliger et al., 1993a; Holliger et al., 1993b]. By omitting the linker, a triabody can also be obtained [Iliades et al., 1997] (Fig. 2.17). Diabodies can be either homobivalent or bispecific. Another way to assemble recombinant bispecific antibodies is either to chemically conjugate two antibody fragments of different specificity, or to sequentially fuse two scFv fragments. Such scFv-scFv constructs can also be directed against adjacent epitopes of the same antigen to achieve high binding affinity and, in this case, are denominated CRAbs [Neri et al., 1995].

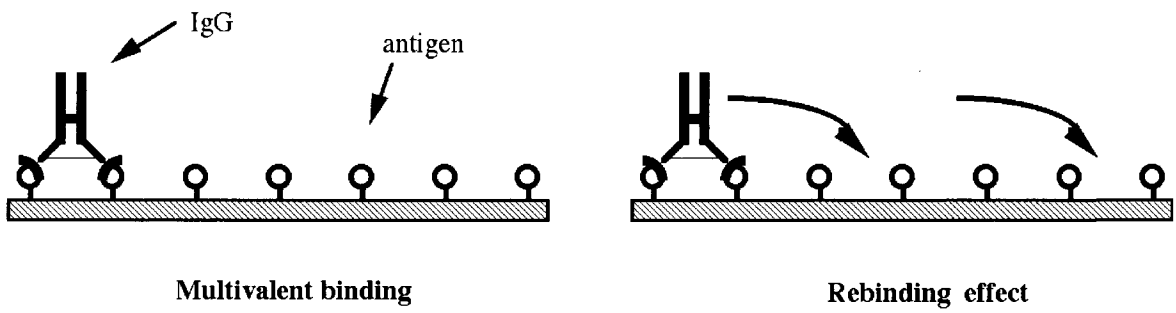


Fig. 2.16
The effect of avidity on antibody binding to the antigen. Rebinding effect: for a bivalent antibody, if dissociation from the antigen at one or both binding sites occurs, it is more likely to rebind again to the antigen compared to a monovalent antibody fragment.

One way to stabilise Fv and diabodies, that may dissociate during tumour targeting applications [Fitzgerald et al., 1997], is to engineer interchain disulfide bonds, yielding disulfide-stabilised Fv fragments [Glockshuber et al., 1990; Brinkmann et al., 1993] and disulfide stabilised diabodies [Fitzgerald et al., 1997].

In order to gain binding avidity, it may be advantageous to oligomerise antibody fragments, creating "mini-antibodies" [Pack et al., 1992; Holliger et al., 1993; Plückthun et al., 1997]. Several fusion proteins between an antibody fragment and an oligomerisation domain have been reported [Pack et al., 1992; Kipriyanov et al., 1996a;

Hu et al., 1996; Plückthun et al., 1997]. It must be noticed, however, that oligomerisation units, coupled with the intrinsic tendency of scFv to dimerise, may result in the polymerisation of the corresponding fusion proteins and are therefore of utility only with antibody fragments that are intrinsically monomeric. The range of antibody formats that can be achieved by combining antibody fragments and other polypeptides is virtually unlimited. Some examples of less frequently occurring multimeric recombinant antibodies can be found in Hoogenboom [1997a] and de Haard et al. [1998].

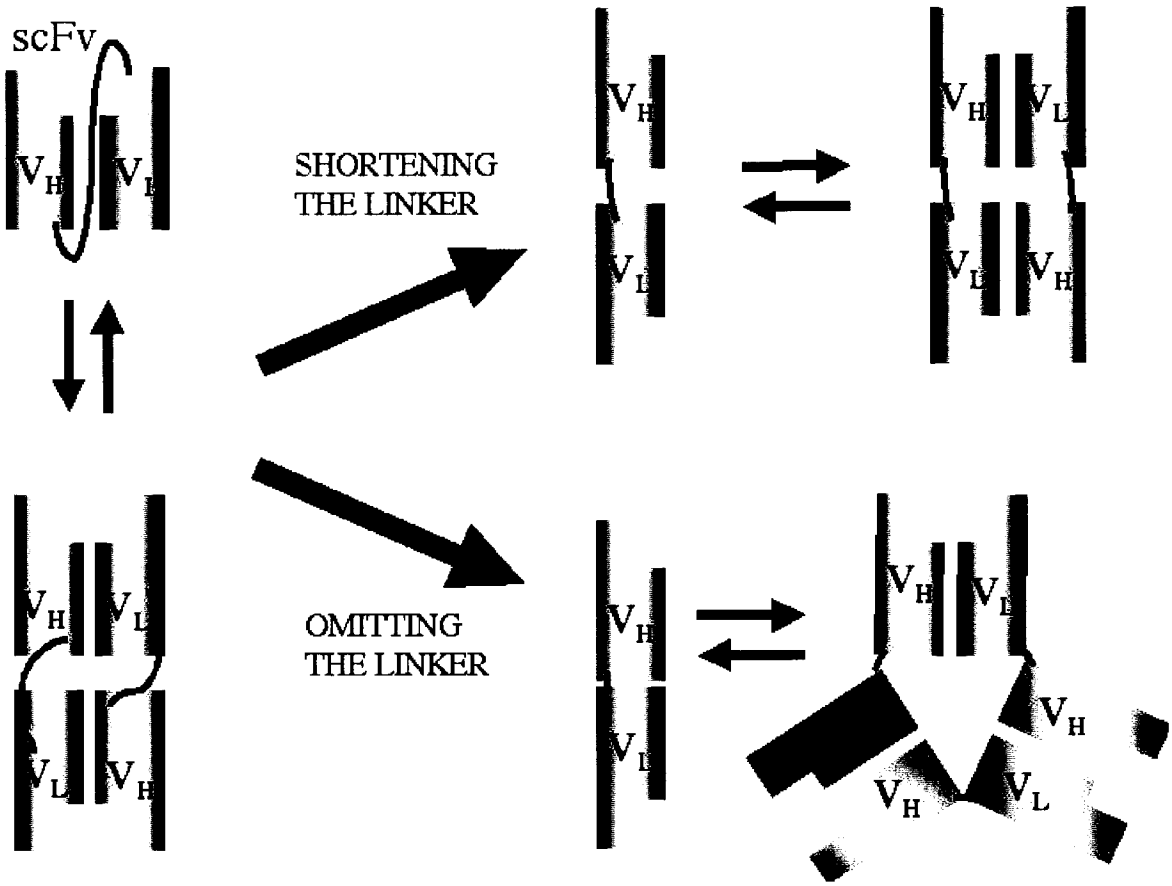


Fig. 2.17
Engineering "diabodies" and "triabodies" by shortening or omitting the linker between the light chain and the heavy chain domains of a scFv fragment.

For some applications, for example in order to increase tissue penetration and accelerate antibody clearance from the blood [Yokota et al., 1992; Yokota et al., 1993], it may be advantageous to reduce the antibody size. After the original report by the Winter's

group that isolated VH domains may retain some of the binding affinity of the parental antibody towards the antigen (dAb; Ward et al., 1989), it was discovered that camel antibodies can be devoid of light chains and retain functionality [Hamers-Casterman, 1993]. Indeed, camel-derived VH domains [Arbabi Ghahroud et al., 1997; Lauwereys et al., 1998] and "camelised" dAb antibodies [Davies et al., 1996] have been exploited, in combination with phage display methodologies, for the development of medium- to high-affinity monomeric antibody fragments of molecular weight < 15'000 Daltons. Attempts to further reduce the size of antibody fragments capable of specific antigen binding, after initial promising results [Pessi et al., 1993], have so far yielded few additional binding specificities [Martin et al., 1994; Martin et al., 1996].

2.5.3 The scFv (L19), an antibody fragment with high affinity for the ED-B domain of fibronectin

The extra domain B (ED-B) of fibronectin mentioned earlier in the introduction is a marker of angiogenesis, highly expressed in fibronectin molecules surrounding new blood vessels and undetectable, with some rare exceptions, in normal adult tissues (chapter 2.4.4). Since ED-B is highly conserved among different species, identical from mice to humans, it has not been possible to obtain monoclonal antibodies directly recognising ED-B by conventional hybridoma technology. However, phage antibody technology offers a solution, allowing the in vitro isolation of a human recombinant antibodies recognising also "difficult" antigens like haptens and antigens well conserved among species. Selected binders can be further mutagenised and subsequent phage display selection allows the isolation of recombinant antibodies with improved affinity affinity [Yang et al., 1995; Schier et al., 1996a, b; Neri et al., 1997; Pini et al., 1997].

A human recombinant antibody recognising the ED-B was isolated from the ETH-2 phage display library constructed in our laboratory [Viti et al., 2000] and the affinity was furtehr enhanced it as follows: in a first selection procedure, the antibody fragment

E1 with a moderate affinity in the 10^7-10^8 M^{-1} range was isolated which is typical for scFv fragments isolated from phage display libraries. The affinity of scFv(E1) was improved further with a limited number of mutations of CDR residues located at the periphery of the antigen binding site. The residues of the scFv E1 that were chosen for combinatorial mutagenesis by PCR using degenerate primers are shown in Fig. 2.18. The affinity maturation of E1 was performed in two steps (see Fig. 2.19).

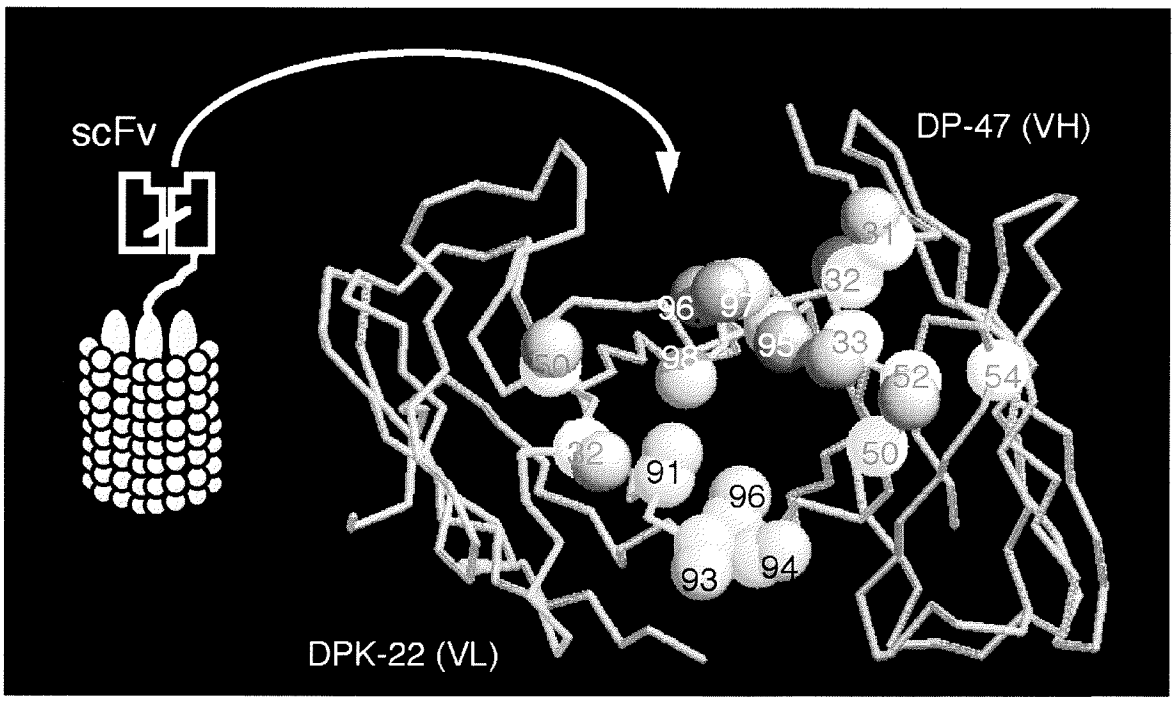


Fig. 2.18
Positions in the scFv scaffold subjected to combinatorial mutagenesis in the ETH-2 library and in the affinity maturation procedure. Antibody fragments are displayed on phage as pIII fusion proteins. In the antibody binding site, the VL CDRs are in yellow and the and CDR2 which can be mutated to improve antibody affinity are shown in gray spacefills.

Briefly, in a first step VH residues 31-33, 50, 52 and 54 were combinatorially mutated and the corresponding repertoire was displayed on filamentous phage. The resulting repertoire of 4 x 10^8 clones was selected for binding to the ED-B domain of fibronectin. After two rounds of panning, and screening of 96 individual clones, an antibody called H10 with 27-fold improved affinity was isolated. In a second step of affinity maturation, two residues located in the CDR 1 and CDR 2 of the variable light chain of the antibody

H10 were randomised. The resulting library, containing 400 clones, was displayed on phage and selected for antigen binding. From analysis of the dissociation profiles using real-time interaction analysis with a BIAcore instrument [Jönsson et al., 1991] and k_{off} measurements by competition experiments, a clone (L19) was identified with a dissociation constant to the ED-B domain of fibronectin of $K_d = 5.4 \times 10^{-11}$ M. This anti-ED-B scFv(L19) is one of the highest affinity antibodies isolated from phage display libraries.

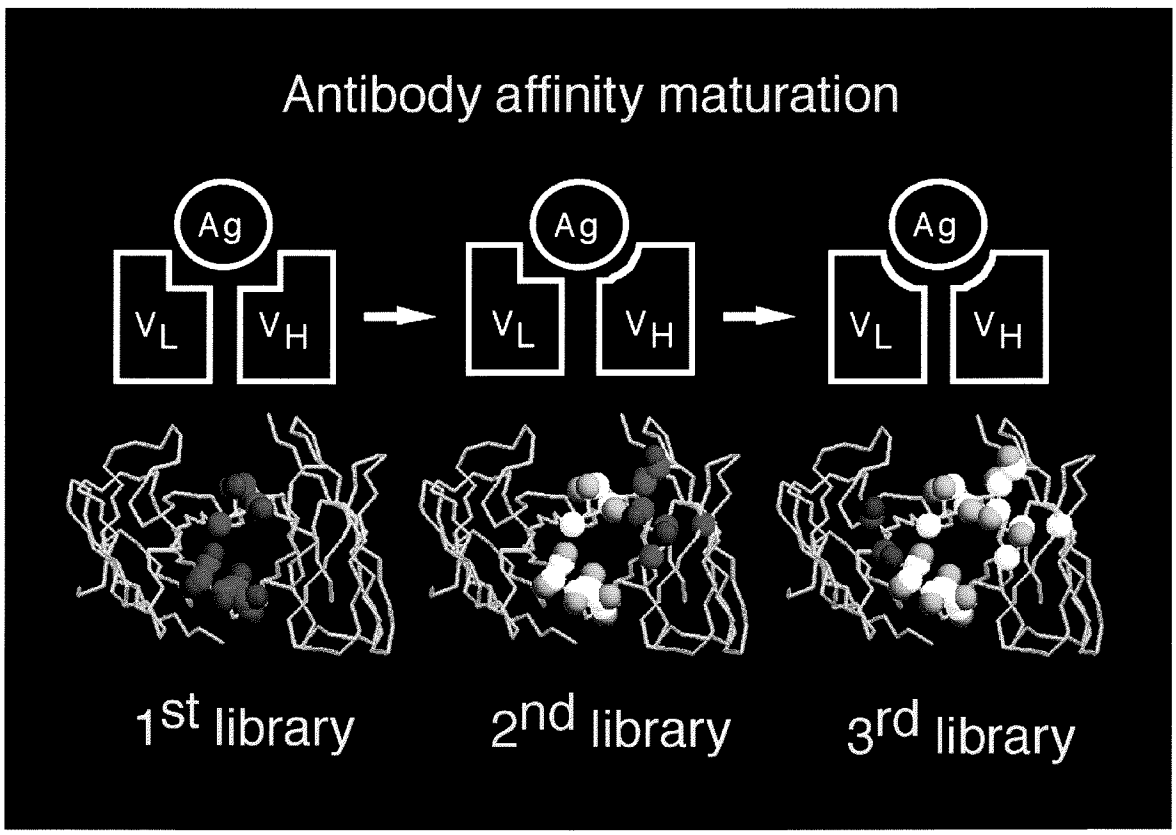


Fig. 2.19

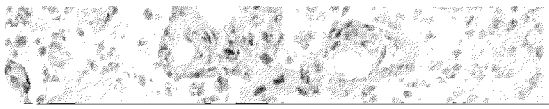
Strategy to improve antibody affinity. The affinity of an antibody selected from a primary phage display library (1st library), can be improved by selecting higher affinity binders from a second library, obtained by combinatorially mutating residues in the VH CDR1 and CDR2 of the antibody. The affinity of the antibody selected from the 2nd library can be further improved by building a third library where some more residues of the VL CDR1 and CDR2 are simultaneously mutated. From this 3rd library a binder with even better complementarity for the antigen can be selected. The residues subjected to random mutagenesis are shown in green; the residues kept constant (but already selected for binding to the antigen) as grey spacefills. In yellow are the VH and VL CDRs. Complementarity between antigen and antibody increases from the first to the third library.

To demonstrate its specificity for the ED-B domain present in new forming blood vessels, the L19 antibody fragment was tested in vitro as immunohistochemical reagent detecting growing blood vessels in sections of tissues undergoing angiogenesis (Fig. 2.20).

Moreover, ability to target angiogenesis in vivo was shown using the infrared photodetection methodology of Folli et al. (1994) in the implementation of Neri et al. (1997). Briefly, nude mice bearing the subcutaneously grafted F9 murine teratocarcinoma were injected with L19 antibody fragment labeled with the infrared fluorophore Cy7 [Neri et al., 1997]. As a control, identical nude mice were injected with a similarly labeled antibody fragment of irrelevant specificity, HyHEL-10. Fig. 2.21 shows infrared fluorescence images of tumour-bearing mice 24 hours after antibody injection. For L19, intense fluorescence was observed in the tumour, whereas after injection with the nonspecific control antibody HyHEL-10, no fluorescence localisation could be observed. Weak fluorescence outside the immediate environment of the tumour was observed only for the bladder and liver. Ex vivo microanalysis of the tumour confirmed that L19 localises around the new vessels.

These data suggests that the L19 antibody fragment may be a good agent for diagnostic imaging of tumoural lesions and that it also may be of use as a targeting agent in therapeutic settings.

A



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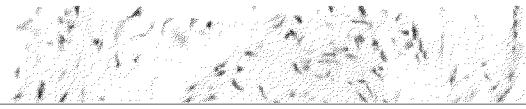


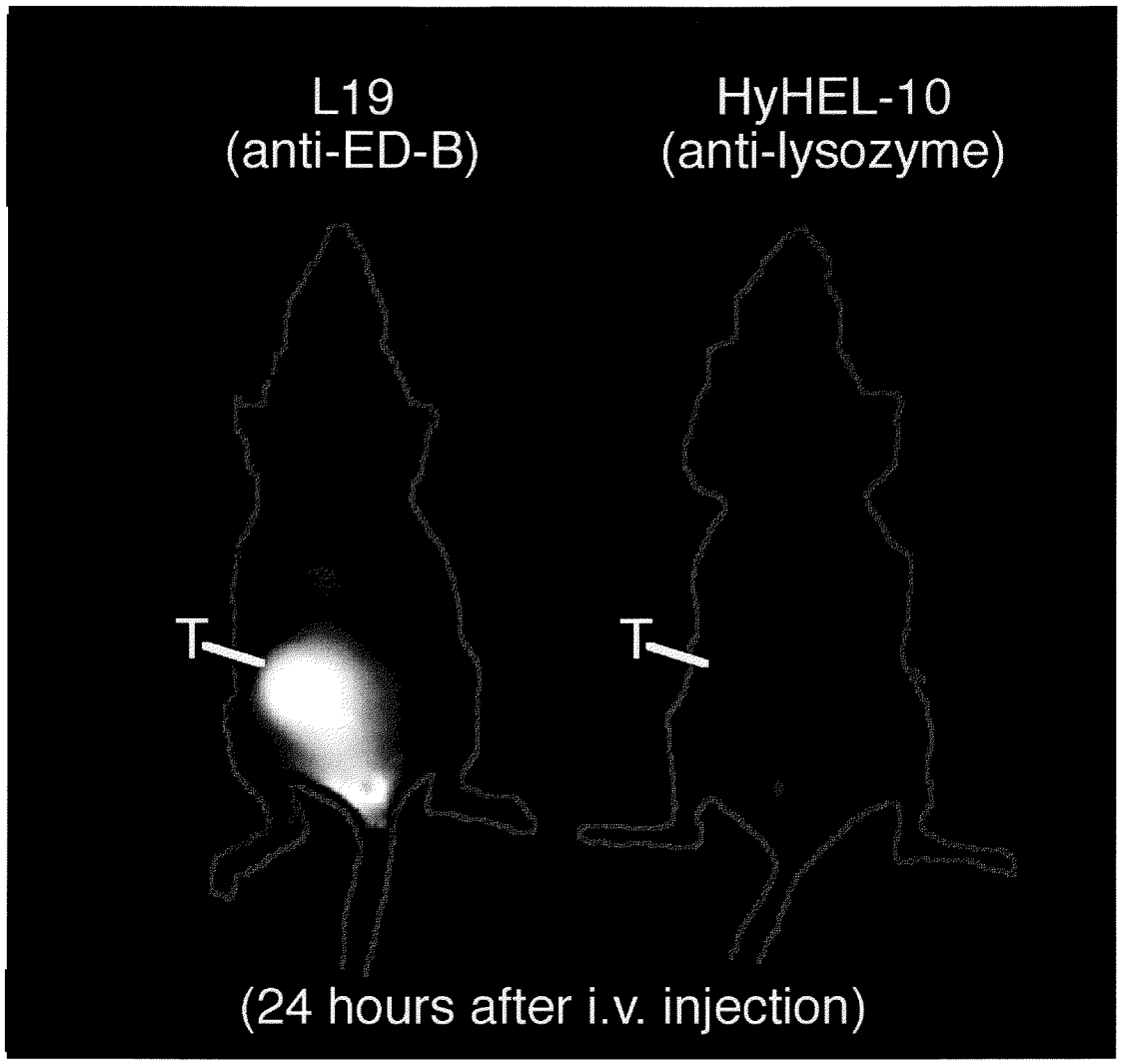


Fig. 2.21 Targeting of fluorescently labeled scFvs to F9 murine teratocarcinoma.
The data were obtained by infrared photodetection 24 h after injection of the labeled antibodies into nude mice bearing the tumour. The mice were injected with the specific anti-ED-B antibody L19 and with the irrelevant antilysozyme antibody HyHEL-10. For clarity, a line was drawn around the shape of the mice. The position of the tumour (T) is indicated.

2.5.4 Diagnostic applications

Recombinant antibodies derived from phage display libraries can have high affinity against tumour markers and can efficiently target tumours in vivo (see section above). In particular, they do not accumulate in the liver and are rapidly cleared through the kidneys, all parameters which are important for the successful development of a tumour imaging agent. It is therefore conceivable that in the future more and more imaging agents will be derived from antibody phage technology.

Recombinant antibodies can efficiently be radiolabelled with ^{99m}Tc, the radionuclide of choice for scintigraphic applications [Nicolini et al., 1994]. Several authors have described the tagging of recombinant antibodies with cysteine-containing C-terminal peptide tags, which stably bind technetium without impairing the antibody's ability to bind to the antigen [Sawyer et al., 1992; George et al., 1995; Liberatore et al., 1995; Verhaar et al., 1996].

Infrared fluorophores may be useful for the antibody-mediated detection of superficial tumoural lesions [Folli et al., 1994; Neri et al., 1997]. Indeed, infrared light at 800 nm exhibits approximately 10% transmittance through a variety of 1cm-thick tissues [Wan et al., 1981]. It should therefore be possible to combine infrared immunophotodetection with endoscopic fluorescence imaging modalities (for review, see Folli [1994]).

Finally, antibodies are being used as vehicles to deliver liposomes to tumour sites for both diagnostic and therapeutic applications. The antibody-mediated targeted delivery of gadolinium-loaded liposomes to tumoural vasculature for magnetic resonance imaging of tumour lesions in rabbits has recently been reported [Sipkins et al., 1998]. Future work will certainly shed more light on the applicability of this potentially interesting methodology in patients with cancer.

2.5.5 Therapeutic applications

Phage display offers great flexibility in the development of targeting agents, as exemplified in the successful isolation of binders against traditionally "difficult" targets such as markers of angiogenesis [Carnemolla et al., 1996; Pini et al., 1998]. The subsequent use of phage-antibodies as modular components of therapeutic agents in oncology will rely on the same armamentarium of bioactive molecules which are already being used in combination with hybridoma-derived antibodies.

The list of possible effector functions includes: therapeutic radionuclides [Liu et al., 1998], toxins [Kreitman et al., 1998], protease-activated pore-forming agents [Panchal et al., 1996], drugs [Chari, 1998], enzymes for pro-drug activation [Bosslet et al., 1998],

cytokines [Lode et al., 1998], bispecific antibodies [Featherstone et al., 1996], superantigens [Brodin et al., 1998], photosensitizers [Goff et al., 1996], liposomes [Morgan et al., 1992] and nanoparticles [Remsen et al., 1996].

Antibody fragments have also been engineered on the surface of cytotoxic T-lymphocytes [Fitzer-Attas et al., 1998] and retroviruses [Russel et al., 1993; Ager et al., 1996]. Furthermore, the variable regions of recombinant antibody fragments can be introduced in full immunoglobulin frameworks, in order to exploit the effector functions recruited by the constant portion of the antibody molecule [Maloney et al., 1994].

Some of the above mentioned therapeutic strategies, particularly the targeted delivery of drugs and toxins that act intracellularly, require either an efficient antibody internalisation and processing by the target cells, or the release of the toxic agent at the tumour site, which is then taken up by the target cells [Chari, 1998]. In this respect, it may be relevant to mention that selection for internalisation of ligands (antibodies or peptides) displayed on phage has recently been reported [Hart et al., 1994].

The use of phage antibodies for therapeutic applications in oncology is still in its infancy. Indeed, the antibody-based products approved by the American Food and Drug Administration (FDA) are based on technology which pre-dated the advent of antibody phage technology.

2.6 Vascular targeting

Selective induction of vascular damage within tumours represents an emerging approach to cancer treatment. Anti-tumour vascular targeting and angiogenesis inhibition are related cancer therapies that radically depart from conventional approaches to treating cancer. In contrast to traditional methods involving a direct attack on cancer cells, these new drugs target a tumour's life support system, the network of newly emerging blood vessels that forms as a result of angiogenesis. Preclinical studies have shown that the use of these therapies can cause a tumour to shrink and ultimately

disappear [O'Reilly et al., 1997; Huang et al., 1997; Chaplin et al., 1997; Chaplin et al., 1999; Nilsson et al., 2001], two examples will be high-lighted below.

According to the Cancer Research Campaign (http://www.crc.org.uk/), nearly 90 percent of all cancers -more than 200 types- are solid tumours and are therefore potential candidates for these new therapies. Despite advances in treatment with surgery, radiation and chemotherapy, serious problems with conventional treatments persist. Many solid tumours remain incurable, especially when the tumour has metastasised or is a large mass at the time of diagnosis. Also, and importantly, chemotherapy and radiation treatment damage healthy cells along with cancerous cells, resulting in serious side effects for patients and, in many instances, eventually induce drug resistance in the tumour. Treatments with a new mechanism of action are urgently needed, particularly ones that prove effective against growing, solid tumours and can potentially work in combination with chemotherapy or radiation.

While angiogenesis inhibitors and anti-tumour vascular targeting agents both target a tumour's blood vessels, they differ in their approach and in the end result. With angiogenesis inhibition, the aim is to prevent tumour growth by inhibiting the formation of tumour-specific blood vessels that feed and sustain the tumour. On the other hand, with anti-tumour vascular targeting the goal is to obliterate tumours by selectively attacking and destroying their existing blood vessels, creating a rapid and irreversible shutdown of these blood vessels. Such an effect is not observed with anti-angiogenesis drugs. Only antivascular targeting activity can destroy existing blood vessels supporting tumour growth leading to extensive tumour cell death as a result of metabolic depriviation.

There are a number of principal avenues in targeting and destroying existing tumoural blood vessels. A mechanical approach, similar to hepatic artery embolisation, is to inject

polymers in the artery supplying the tumour (or tissue area in which the tumour is located), with blood [Maurer et al., 2000]. Those polymers would then get trapped in the tumoural microvasculature and block the blood flow. Another mechanical approach could be to shut off the tumoural blood supply by physically blocking the tumour supplying arteries with ligatures. However, such mechanical therapies are only useful when the tumour is well localised, accessible and they do only make sense when surgery is not possible. Furthermore, the risk of systemic embolisation using large polymers must be considered, and treatments have to be carried out with great care in order to avoid lung and renal failure due to non-specific embolisation.

A more general therapeutic avenue is the systemic delivery of an antivascular targeting agent. In contrast to anti-angiogenic approaches, relatively small resources have been spent on drug discovery in this area. This is due, at least in part, to the absence of relevant in vitro evaluation procedures for predicting such effects. Vascular damaging properties have only been identified secondary to the drugs undergoing in vivo testing, based on promising cytotoxic or antiproliferative effects in vitro. There are, however, a few agents currently under investigation in pre-clinical and clinical studies. They can be grouped in small organic molecules and large polypeptides. The two main classes of small organic molecules that can induce vascular damage and subsequent haemorrhagic necrosis in tumours are the tubulin-binding agents [Hill et al., 1993, Chaplin et al., 1996] and drugs related to flavone acetic acid [Bibby et al., 1989; Zwi et al., 1989; Hill et al., 1995]. An example of the latter group, dimethylxanthenone-acetic acid (DMXAA), has proven its efficacy in pre-clinical experiments [Zwi et al., 1994; Lash et al., 1998] and is currently in Phase I clinical trials in the United Kingdom and New Zealand.

Tubulin-binding agents such as vincristine and vinblastine are potent anticancer drugs currently used in the clinic. These agents, applied close to the maximum tolerated doses, can also induce extensive vascular damage in animal tumours [Hill et al., 1993; Chaplin et al., 1996]. Colchicine was the first tubulin-binding agent discovered to have

antivascular effects, which resulted in hemorrhagic necrosis in human tumours [Seed et al., 1940]. Furthermore, it was noted that the endothelial cells of growing capillaries appeared sensitive to its toxic actions [Ludford et al., 1945]. The therapeutic window was, however, limited and its toxicity prevented any further clinical evaluation. More potent vascular-damaging agents with colchicine-like properties but with less side-effects are currently being sought. Combretastatin, which is a promising agent is discussed below.

One way to reduce side-effects is to increase the specificity of a drug, either by its mode of action or by targeting it to the relevant tissue. Small molecules and large polypeptides can be used in a highly specific fashion when fused to a targeting moiety. For example, high density accumulation of the Fc-portion of IgG antibodies in tumoural vasculature could induce the complement cascade, thereby stripping the blood vessels of their endothelial cells, a process that impairs the exchange of oxygen and metabolites and which promotes intraluminal coagulation. Conjugating a vascular targeting agent with a short range, high-energy alpha-emitting radioisotope like ^{211}astatine could result in the selective destruction of tumoural endothelial cells, preliminary studies show the selective tumour localisation of such a molecule in mice carrying subcutaneous tumours [Demartis et al., 2001]. The targeting of a pro-coagulant factor, inactive when soluble in the blood but regaining activity once bound to tumour represents another attractive avenue towards targeted vascular therapy. Such a molecule is the human tissue factor (TF) which is discussed below.

2.6.1 Combretastatin

Combretastatins are small organic molecules found in the bark of the African Bush Willow, the *Combretum Caffrum.* They were discovered and isolated a decade ago by Dr. Pettit and colleagues [Pettit et al., 1989]. One of them, CA-4, has a high affinity for tubulin at or near the colchicine binding site, causing the destabilisation of the tubulin polymers of the cytoskeleton [Pettit et al., 1989; Woods et al., 1995]. A more soluble

prodrug derivative of CA-4, CA-4-P, was synthesised [Pettit et al., 1995; Dorr et al., 1996]. The compound is cleaved to to active CA-4 by endogenous nonspecific phosphatases and was shown to cause vascular shut down in murine and human xenografted tumours at relatively nontoxic doses [Dark et al., 1997; Chaplin et al., 1999]. Studies in an isolated tumour perfusion system by Dark and colleagues indicated vascular shutdown within 20 minutes after drug administration [Dark et al., 1997]. Quantitative analysis of tumour sections showed a more than 90% reduction in functioning vascular volume at 6 hours following treatment and little recovery was seen after 18 hours. In vivo blood flow and histological studies confirmed the selective effect of the combretastatin A-4 prodrug. A rapid and prolonged blood flow shutdown was evident and both human and murine tumour models showed extensive necrosis within 24 hours. In spite of these impressive data though, there was a rather moderate tumour regrowth delay observed for CA-4-P administered as a single agent [Chaplin et al., 1999]. A wide spatial heterogeneity in tumour blood flow in different tumour microregions was observed and the drug affected peripheral tumour regions less than central regions, with a viable rim of tumour cells surviving to repopulate the tumour after CA-4-P treatment. Tozer and colleagues tried to increase the therapeutic efficacy of CA-4-P by combining it with a nitric oxide synthesis inhibitor, L-NAME [Tozer et al., 1999]. NO has vasodilatory properties , and it is possible that NO-production maintains some tumoural blood flow even in the face of endothelial cell damage induced by CA-4-P. Inhibition of NO synthesis has been found to enhance the tumour cytotoxicity induced by ischemia-reperfusion injury [Parkins et al., 1995, Parkins et al., 1997] and photodynamic therapy [Tozer et al., 1999] which are known to be vasculature-damaging strategies. The combination of L-NAME and CA-4-P significantly enhanced the vascular effect of CA-4-P. It remains to be seen how CA-4-P performs in clinical trials and how efficiently the anti-vascular effect can eradicate established tumours, alone or in combination with other compounds.

2.6.2 Tissue Factor (TF)

A novel approach to cancer therapy based on the antibody-directed targeting of the human coagulation-inducing protein tissue factor (TF) to tumour vasculature has recently been proposed by Thorpe and colleagues [Huang et al., 1997; Ran et al., 1998]. The approach is based on the concept that thrombosis of tumour vessels may stop the supply of nutrients and oxygen to tumour cells, and provides an alternative mode of action as compared to the small organic molecules described above. An attractive feature of such a therapeutic approach is, that it involves endogenous components of the body in an amplification cascade, with the targeted tissue factor as a trigger in the very beginning of the cascade.

TF is a cell surface glycoprotein and is a major initiator of blood coagulation [Ruf et al., 1991]. At sites of injury, blood comes in contact with the membrane-bound TF which forms a complex with the serine protease FVIIa present in blood. The resulting complex activates factors IX and X, which leads to thrombin activation and ultimately to blood clotting. A truncated form of TF, tTF, consisting of only the extracellular soluble domain (residues 1-219), exhibits a five orders of magnitude lower ability to activate the clotting cascade in solution, but is fully active when retargeted to a membrane surface [Huang et al., 1997] (Figure 2.22).

Thorpe and coworkers [Huang et al., 1997] used a bispecific antibody to target a truncated form of tissue factor (tTF) to an artificial marker of angiogenesis (MHC-II), experimentally induced on tumour vascular endothelium by grafting in mice neuroblastoma cells, which had been transfected with the interferon-γ gene. The investigators observed extensive intravascular thrombosis of the tumour, and complete regressions in 38% of the mice treated.

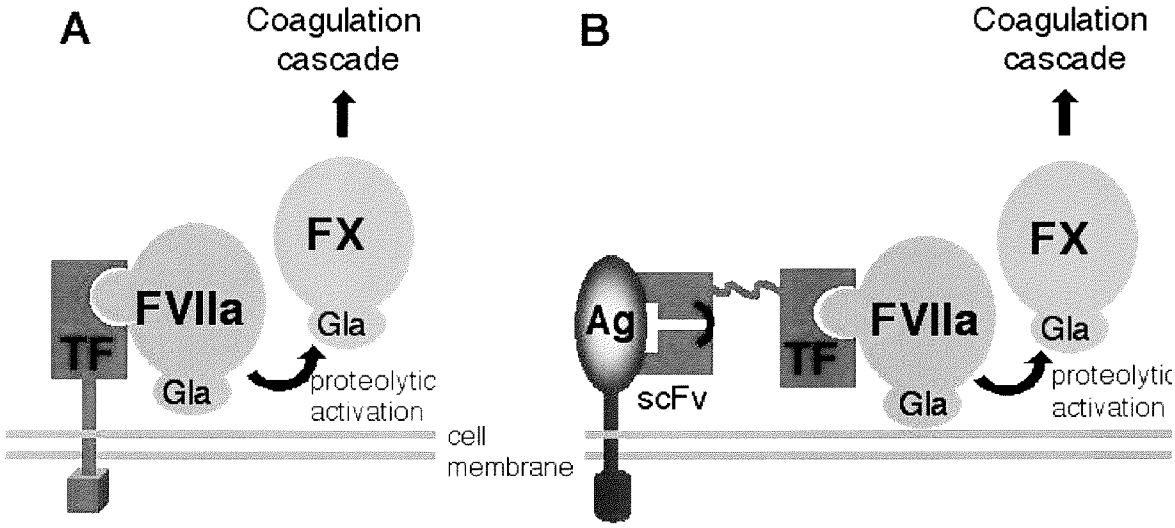


Fig. 2.22
(A) The membrane anchor of the wild-type human TF and the positively charged γ-carboxyglutamate domain (Gla) of the cofactor FVIIa guide the binary complex in an upright position relative to the cell membrane. In this way the proteolytic centre of the binary complex can interact with the cleavage sequence of FX, generating the activated form of FX, which will further activate the coagulation cascade [Davie et al., 1991]. (B) Antibody-mediated targeting and activation of FX by tTF using a scFv–tTF fusion protein recognising an angiogenesis-related antigen (Ag).

In a further step, immunoconjugates were used to target tTF to a naturally occurring marker of tumour vascular endothelium, vascular cell adhesion molecule-1 (VCAM-1) [Ran et al., 1998]. VCAM-1 is expressed by vascular endothelial cells in Hodgkin's lymphoma and in various solid tumours in mice and humans. It is also expressed in some vessels of thyroid, kidney and thymus (in humans), and in heart and lung (in mice) [Ran et al., 1998]. The authors observed selective localisation of tTF to VCAM-1-expressing vessels in the tumour, causing thrombosis of those vessels, a 50% reduction of tumour growth rate, but no complete remission. The immunoconjugate also localised to VCAM-1-expressing vessels in the lungs and heart, but did not induce thrombosis at these sites. An immunohistochemical evaluation of a monoclonal anti-phosphatidylserine (PS) antibody in mice showed that the VCAM-1-expressing vessels in the tumour also expressed PS, whereas VCAM-1-expressing vessels in the lungs and heart lacked PS. The authors concluded that PS expression on the luminal aspect of

blood vessels is needed to provide the procoagulant surface upon which coagulation complexes can assemble.

The targeted delivery of tTF would be of significant therapeutic relevance if it was directed against a naturally-occurring marker of angiogenesis, expressed in the majority of aggressive solid tumours, but undetectable in normal vessels and tissues, and if it mediated the selective thrombosis of tumour blood vessels. Attempts has been made to fuse the tTF to an antibody recognising a clinically relevant target molecule [Rippman et al., 2000] but so far, in vivo studies have not been reported with this immunoconjugate. A good-quality, clinically relevant marker for both tumoural and non-tumoural neovasculature is the extra-domain B (ED-B) of fibronectin described earlier in this work.

2.7 Goal of the thesis

I have investigated whether the selective antibody-mediated targeting of tTF to B-FN would result in thrombosis of tumour blood vessels and a therapeutic benefit for tumour bearing mice. The experimental part comprises the following issues:

1 design, construction and bacterial expression of a scFv-tTF fusion protein

2 in vitro and in vivo characterisation of the expressed scFv-tTF fusion protein

3 therapy studies with bacterially expressed scFv-tTF in tumour bearing mice

4 mammalian expression of a scFv-tTF fusion protein

5 in vitro and in vivo characterisation of scFv-tTF fusion protein expressed in mammalian cells

33 therapy studies with scFv-tTF fusion protein expressed in mammalian cells

34 combination of scFv-tTF fusion proteins with chemotherapy.

3. RESULTS

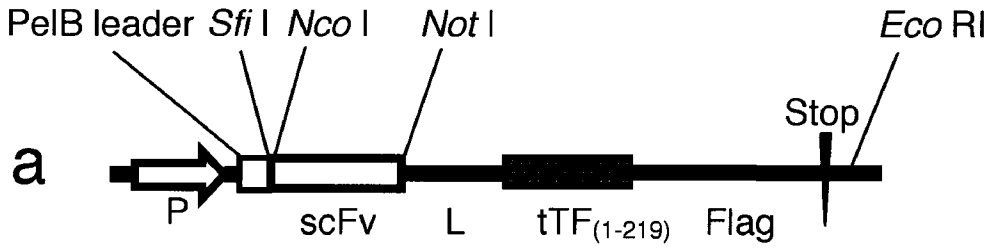
3.1 Bacterially expressed antibody-tissue factor fusion proteins

3.1.1 Cloning

The antibody fragment scFv(L19), which recognises the ED-B domain of fibronectin with affinity in the picomolar range [Pini et al., 1998] was used for the selective targeting of angiogenesis. ScFv(D1.3), which recognises hen egg lysozyme but not mouse lysozyme [Harper et al., 1987], was chosen as vehicle of irrelevant specificity for all the experiments performed with bacterially expressed protein described in this work. Both antibody fragments were fused to a truncated form of human tissue factor (residues 1-219) [Ruf et al., 1991] using a PCR assembly cloning strategy, for expression in *E. coli*. As illustrated in Fig. 3.1a, the tTF was appended at the C-terminus of the scFv antibody fragments, in order not to impair their immunoreactivity. Modelling studies based on the known three-dimensional structure of the tissue factor / Factor VII complex [Banner et al., 1996] suggested that modification of tissue factor at its N-terminal extremity does not interfere with complex formation and the consequent activation of the blot clotting cascade. A FLAG tag [Hopp et al., 1988] was appended at the C-terminal extremity of scFv-tTF fusions proteins, to facilitate immunodetection and to allow for affinity purification using the anti-FLAG M2 monoclonal antibody, immobilised on a resin.

3.1.2 Expression and purification

Similar to other antibody fragments [Winter et al., 1994; Neri et al., 1997; Tarli et al., 1999; Viti et al., 1999], scFv(L19)-tTF and scFv(D1.3)-tTF were expressed as secreted proteins in bacteria and purified to homogeneity using a multi-step procedure. This was based on affinity chromatography on antigen column, followed by purification on the M2 anti-FLAG resin, followed by dialysis, cation exchange chromatography and desalting by gel-filtration (Fig. 3.1b). Yields were typically in the range of 0.2 mg per liter bacterial supernatant.



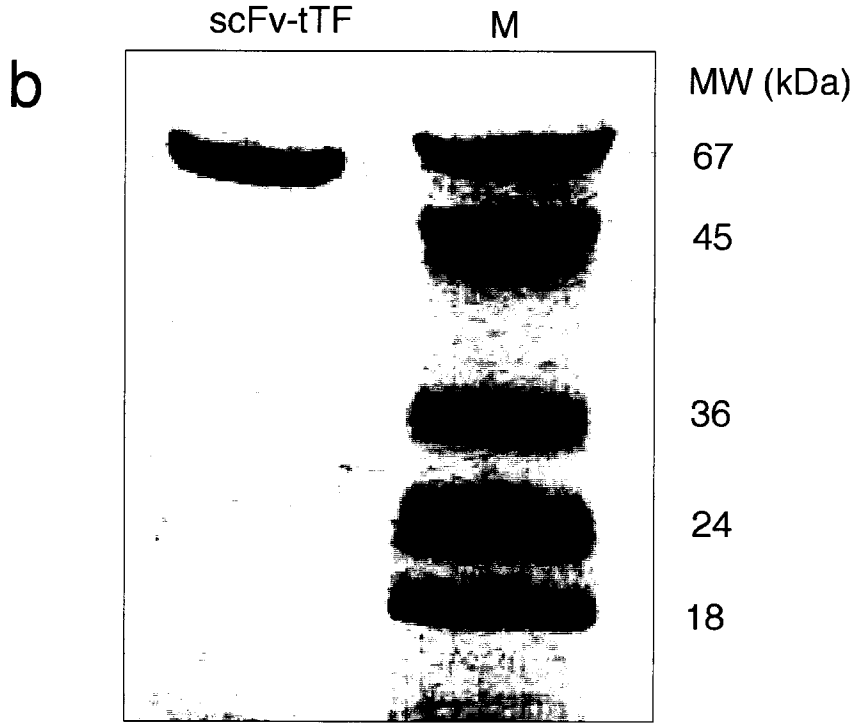


Fig. 3.1
a) Schematic representation of essential elements of vector pFN5, directing the soluble expression of scFv-tTF fusion proteins. The peptidic linker, L, consists of four glycine residues. P, lac promoter; relevant restriction sites are indicated; the FLAG-tag, DYKDDDDK, was used for detection of the protein; b) SDS-PAGE of a purified fraction of scFv(L19)-tTF. Molecular weight markers (M) are indicated.

3.1.3 In vitro characterisation

Both purified fusion proteins were monomeric, as assessed by a Superdex-75 gel filtration column. In order to check that both moieties of the fusion proteins were functional, immunoreactivity was measured both by affinity chromatography on antigen column [Tarli et al., 1999] and by BIAcore [Jönsson et al., 1991; Huber et al., 1999]. Tissue factor activity was measured as described [Ruf et al., 1991], detecting the cleavage of the FXa fluorogenic peptide mediated by the scFv-tTF/Factor VIIa

complex. An activity profile comparable with the one in fig. 3.14 was obtained, and a half-maximal activity was observed at 45nM protein concentration, in line with previously published values obtained with tTF [Ruf et al., 1991].

3.1.4 In vivo studies

The ability of scFv(L19)-tTF to selectively localise on tumour blood vessels was examined by quantitative biodistribution analysis in mice bearing a subcutaneously grafted F9 murine teratocarcinoma [Tarli et al., 1999], using a radioiodinated protein sample. Fig. 3.2 shows the results of this analysis, expressed in terms of percentage injected dose per gram of tissue (%ID/g), 24 hours after intravenous injection. At this time point, the %ID/g (tumour) was 16.8, with a tumour:blood ratio of 17:1, which is comparable to the ratio published for radiolabeled scFv (L19) [Tarli et al., 1999]. Tumour:blood ratios greater than 2 can be observed as early as 3 hours after injection. Considering that L19 localises to tumoural blood vessels, which represent only a small percent of the weight of F9 tumours [Tarli et al., 1999], the results of fig. 3.2 indicate that scFv(L19)-tTF is able to accumulate at high density around vascular structures.

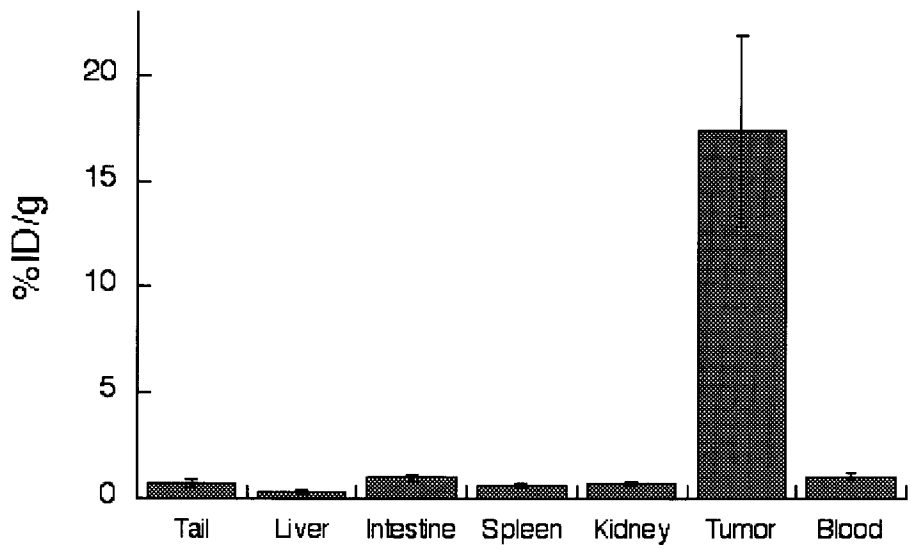


Fig. 3.2
Biodistribution results of mice bearing the F9 teratocarcinoma, 24 hours after injection with 1 μg of radioiodinated scFv(L19)-tTF. Antibody doses in the different organs are expressed as % injected dose per gram: 4 mice per group were used.

3.2 Targeted delivery of bacterially expressed antibody-tissue factor fusion proteins

3.2.1 Small tumours

In order to test whether the targeted delivery of tTF to B-FN, a component of the modified extracellular matrix which accumulates around vascular structures in aggressive tumours, was able to promote thrombosis of tumour vessels, we injected tumour bearing mice with scFv(L19)-tTF, scFv(D1.3)-tTF or saline. The tumours grafted subcutaneously in mice were the F9 murine teratocarcinoma [Bernstine et al., 1973], the C51 murine colon adenocarcinoma [Corbett et al., 1975] and the FE8 rat sarcoma [Schaefer et al., 1988].

Mice bearing small F9, C51 or FE8 tumours (< 500 mg) were injected at different time points with three doses of 14 μg scFv-tTF or saline, (Fig. 3.5). Few hours after the first injection, all tumours turned black with the L19 fusion protein (Fig. 3.3a), but not with saline or with scFv(D1.3)-tTF (Fig. 3.3b), suggesting that scFv(L19)-tTF did mediate a selective intraluminal blood coagulation in tumour blood vessels, as confirmed by histochemical analysis (fig 3.4).

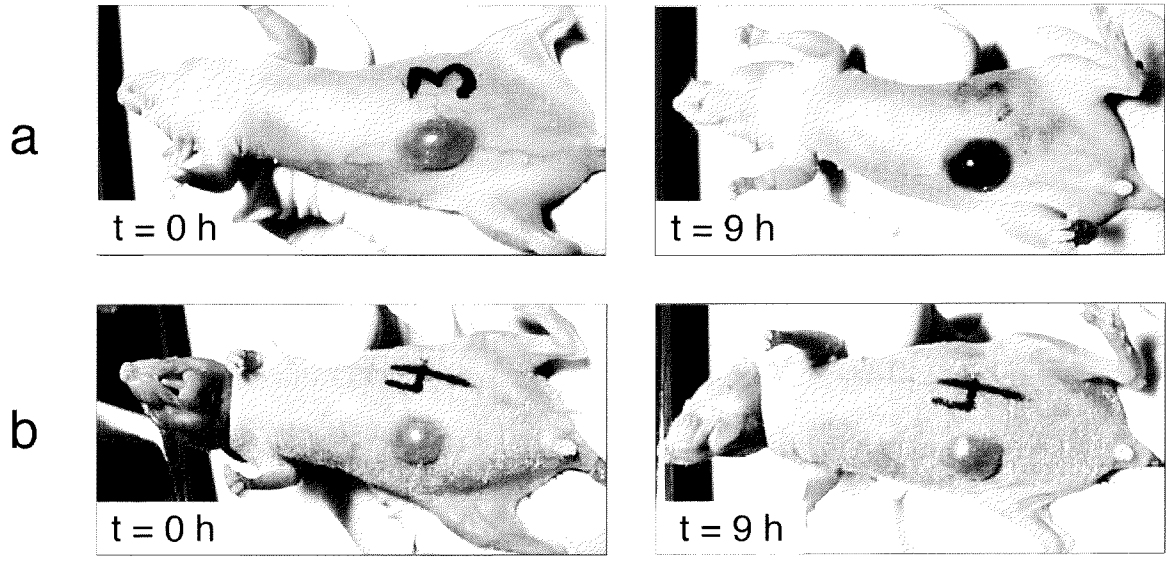


Fig. 3.3
Photographs illustrating that scFv(L19)-tTF (a), but not scFv(D1.3)-tTF (b), mediates the rapid infarction of tumoural blood vessels, after a single injection of 14 μg fusion protein.

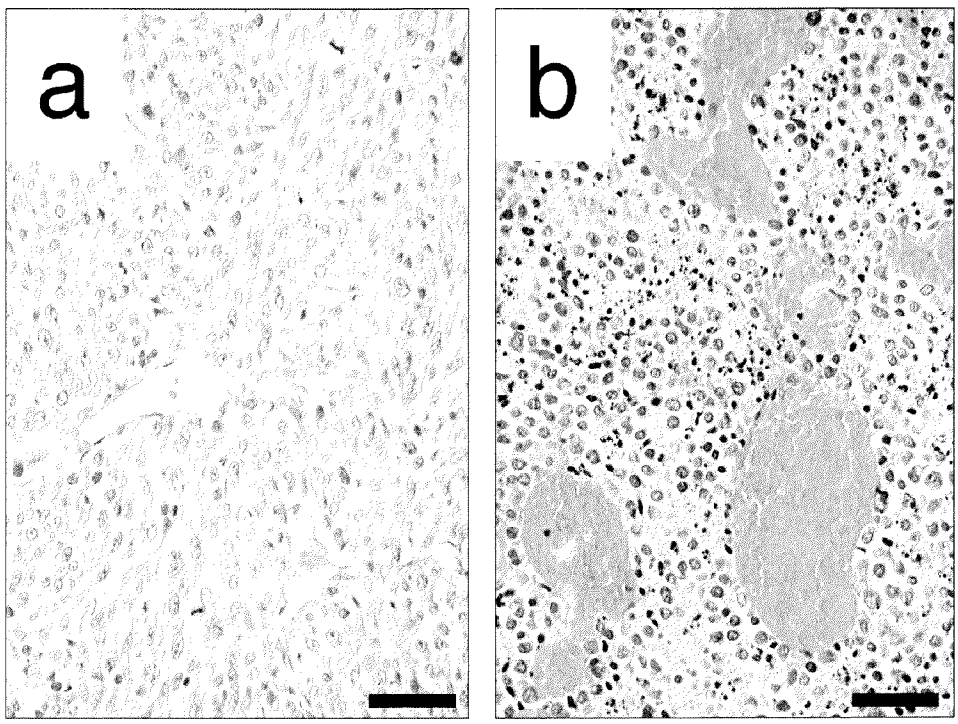


Fig.3.4
Haematoxylin and Eosin stained sections of FE8 tumours, one hour after injection of saline (a) or scFv(L19)-tTF (14 μg) (b), which mediates extensive thrombosis of blood vessels. Scale bars: 50 μm.

At the doses used, a statistically significant reduction in tumour growth rate was observed for all three tumour models, upon injection of scFv(L19)-tTF (Fig. 3.5).

However, animals were not cured. In the groups of negative control mice, there was no statistically significant difference between mice injected with saline or with the scFv(D1.3)-tTF fusion protein, confirming that the targeting of tTF to B-FN was necessary for the anti-tumour effect.

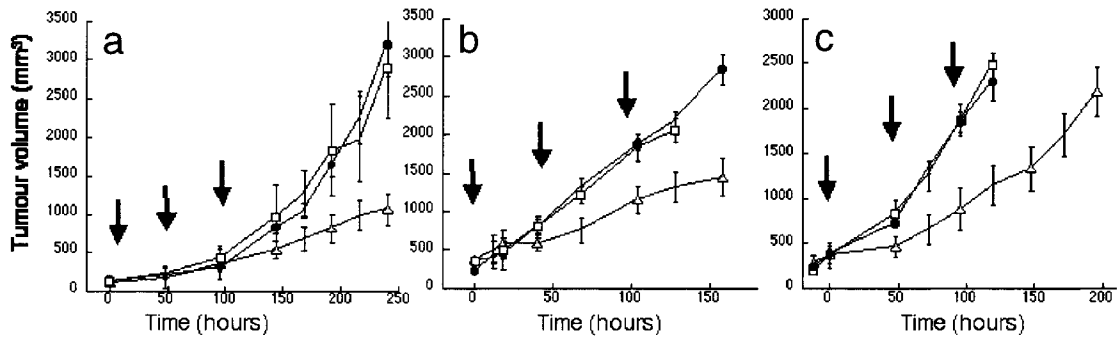


Fig. 3.5

Tumour growth retardation studies of three different tumour models [C51 (a), F9 (b) and FE8 (c) tumours)] in mice. In experiments a-c, cohorts of mice bearing small tumours were injected with three doses of 14 μg scFv-tTF or saline (arrows). Empty squares: scFv(D1.3)-tTF; filled circles: saline; empty triangles: scFv(L19)-tTF.

3.2.2 Large tumours

The effect of a single injection of 20 μg scFv(L19)-tTF in mice bearing larger tumours (> 1500 mg) was even more dramatic. Fig. 3.6 shows a plot of tumour volume versus time for the three different tumour models, after a single intravenous injection of D1.3-tTF or the L19-tTF fusion protein. Within 1-2 hours after injection of scFv(L19)-tTF (but not of scFv(D1.3)-tTF) tumours turned black. Two days after injection, most of the tumour had been converted into a crusty mass. However, a portion of the tumour typically survived at the border with the peritoneal cavity, which would eventually grow back and kill the animal (Fig. 3.7a). A histochemical analysis of tumours revealed that, at the dose used, a small fraction of vessels close to the peritoneal cavity were not occluded (Fig. 3.7b), in spite of the fact that all tumour vessels were ED-B positive. By contrast, blood vessels in other parts of the tumours were completely thrombosed.

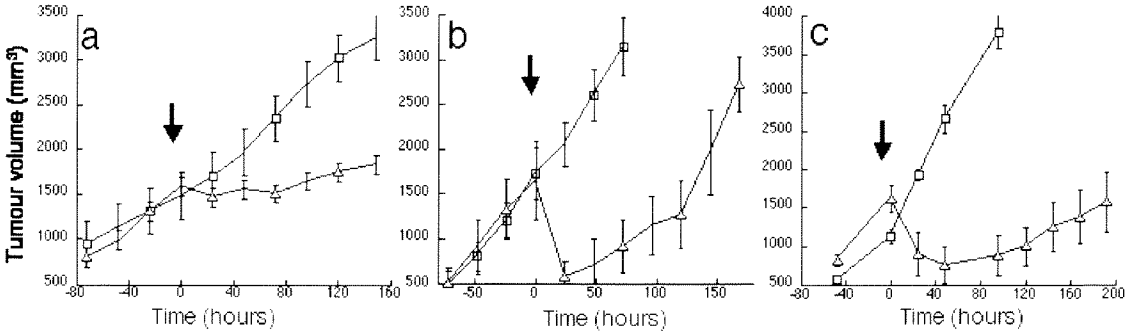


Fig. 3.6
Mice bearing large tumours (> 1 gram) were injected with a single dose of 20 μg scFv-tTF (arrow) in three different tumour models [C51 (a), F9 (b) and FE8 (c)] Tumour sizes were measured with a caliper; standard errors are indicated. Empty squares: scFv(D1.3)-tTF; empty triangles: scFv(L19)-tTF.

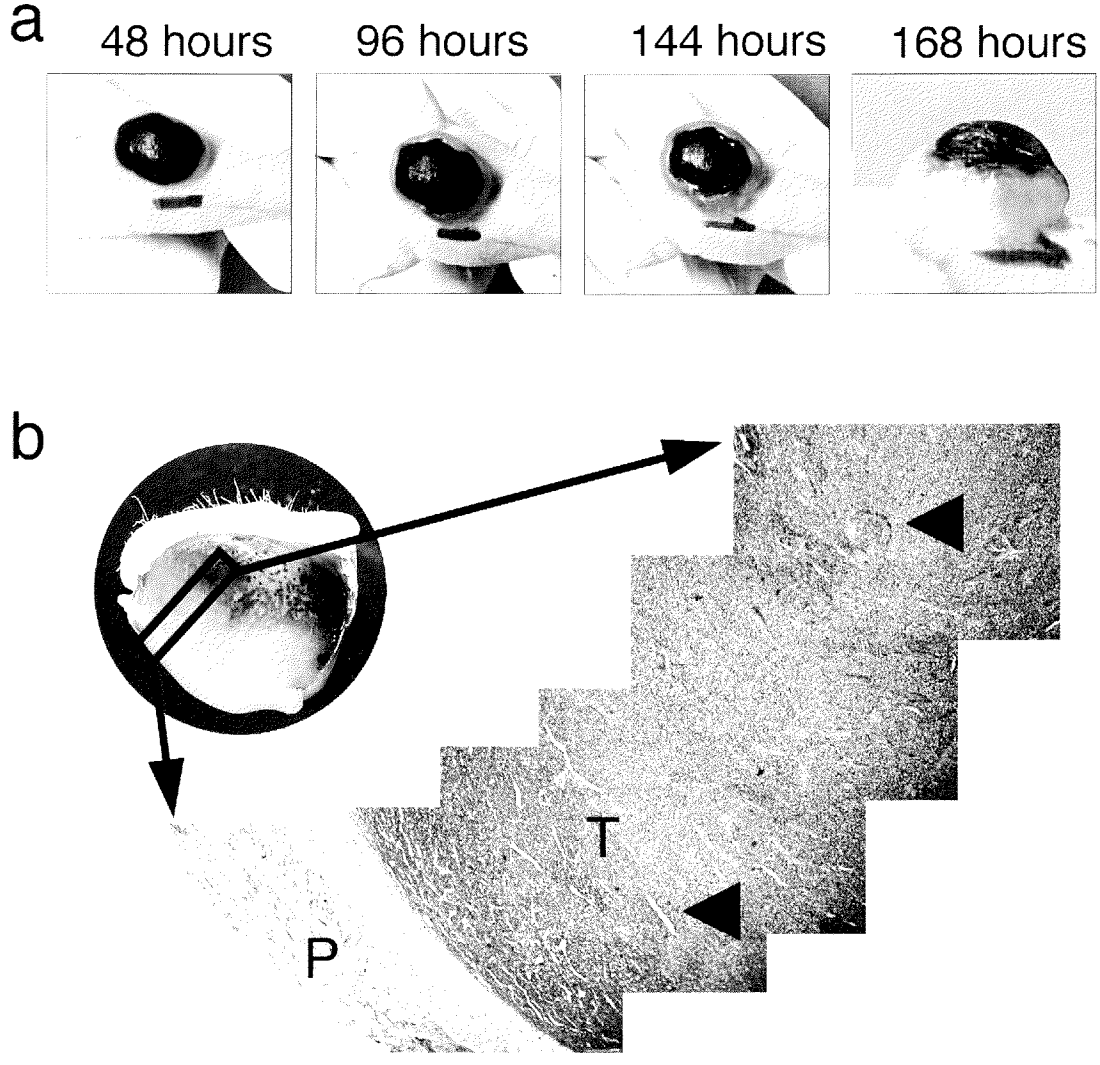


Fig. 3.7
ScFv(L19)-tTF mediates the rapid infarction of large tumours, after a single injection of 20 μg fusion protein. However, typically a rim of tumour cells, close to the peritoneum, survives and grows back (panel a). A microscopic study (panel b), reveals that tumoural vessels close to the

border of the peritoneum are less thrombosed. Open vessels close to the peritoneum (P), are indicated, as well as occluded vessels in the tumoural centre (T), depicted is a FE8 tumour.

3.2.3 A dose escalation study

In early experiments with intravenous administration of scFv(L19)-tTF to tumour-bearing mice, fusion protein at doses greater than 20 μg could not be administered since side effects were observed. In addition to loss of body weight, the tip of the mouse tail would become black and necrotic, and in some cases swelling of the hind limb was observed. These results were suggestive of aggregation of the fusion protein, being deposited at the extremities of animal's body. The antibody preparation did not show sign of aggregation when examined by gel filtration analysis, but a putative aggregate could in principle remain on the column and would therefore not be detected. Indeed, filtration of the fusion protein prior to intravenous injection abolished the side effects. This allowed us to perform a dose escalation study, with the aim to investigate whether, at high doses, complete remissions could be observed in mice.

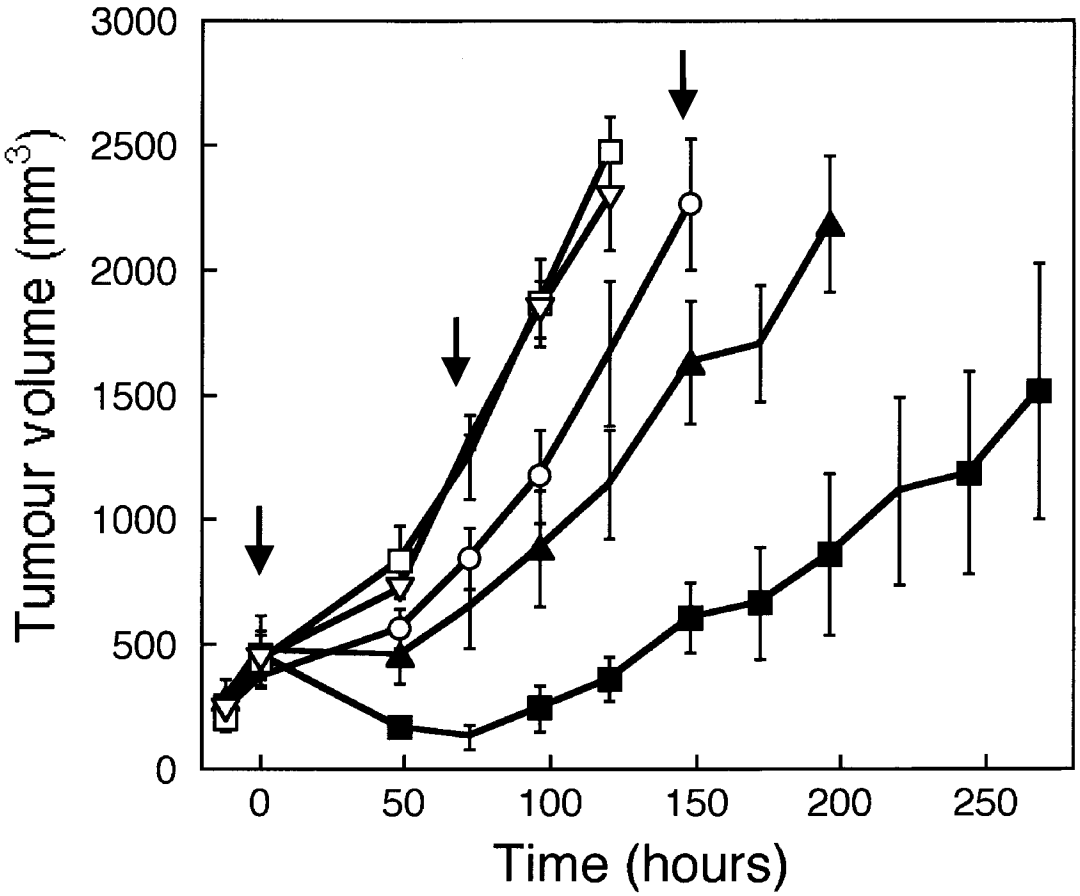


Fig. 3.8
Dose escalation study in mice bearing the FE8 sarcoma, after injection of three doses of 35 μg scFv(D1,3)-tTF, saline, or increasing dose of scFv(L19)-tTF. Standard errors are indicated. Empty squares: 35 μg scFv(D1.3)-tTF; empty triangles: saline; empty circles: 9 μg scFv(L19)-tTF; filled triangles: 15 μg scFv(L19)-tTF; filled squares: 35 μg scFv(L19)-tTF.

Fig. 3.8 shows FE8 tumour volumes, plotted versus time, of mice injected three times with saline, 35 μg of scFv(D1.3)-tTF, or escalating doses of scFv(L19)-tTF. Even at these high doses, scFv(D1.3)-tTF did not show any significant difference relative to the saline control. In contrast, scFv(L19)-tTF showed a therapeutic effect, which improved with an increase in the administered dose. At the highest dose tested (35 μg), approx. 30% of the mice exhibited a complete tumour regression. In these conditions, mice showed a transient loss of approx. 10% body weight, indicating the onset of some toxicity. Mice regained weight after the tumors were gone. Fig. 3.9 b and c shows representative pictures of the mice responding to treatment.

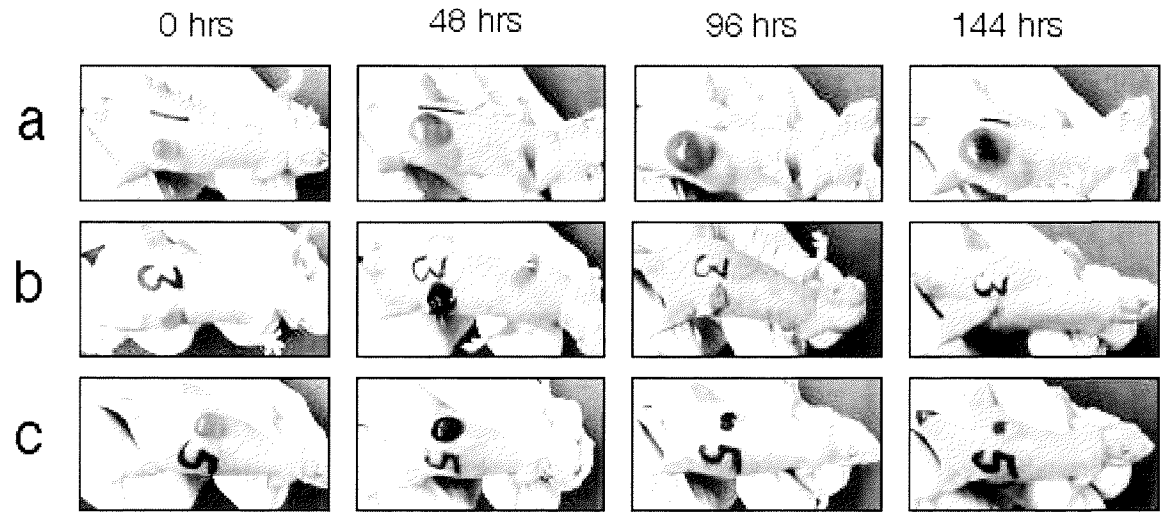


Fig. 3.9
Photographs of mice, bearing the FE8 sarcoma, after treatment with scFv(D1.3)-tTF (a) or scFv(L19)-tTF (b and c). Time after the first injection is indicated.

No apparent abnormality could be detected in organs of treated mice (lung, liver, spleen, intestine, kidney, heart, brain) at autopsy and by histological analysis 24 hours after injection (Fig. 3.10). Neither areas of haemorrhagia or necrosis nor thrombosis were observed. In contrast, approx. 50% of intratumoural blood vessels were completely occluded already 1 hour after injection. Four hours after injection, approx. 80% of the tumour vessels were thrombosed. The number of occluded intratumoural vessels did not change 24 hours after the injection, no additional thrombosis or thrombolysis could be observed.

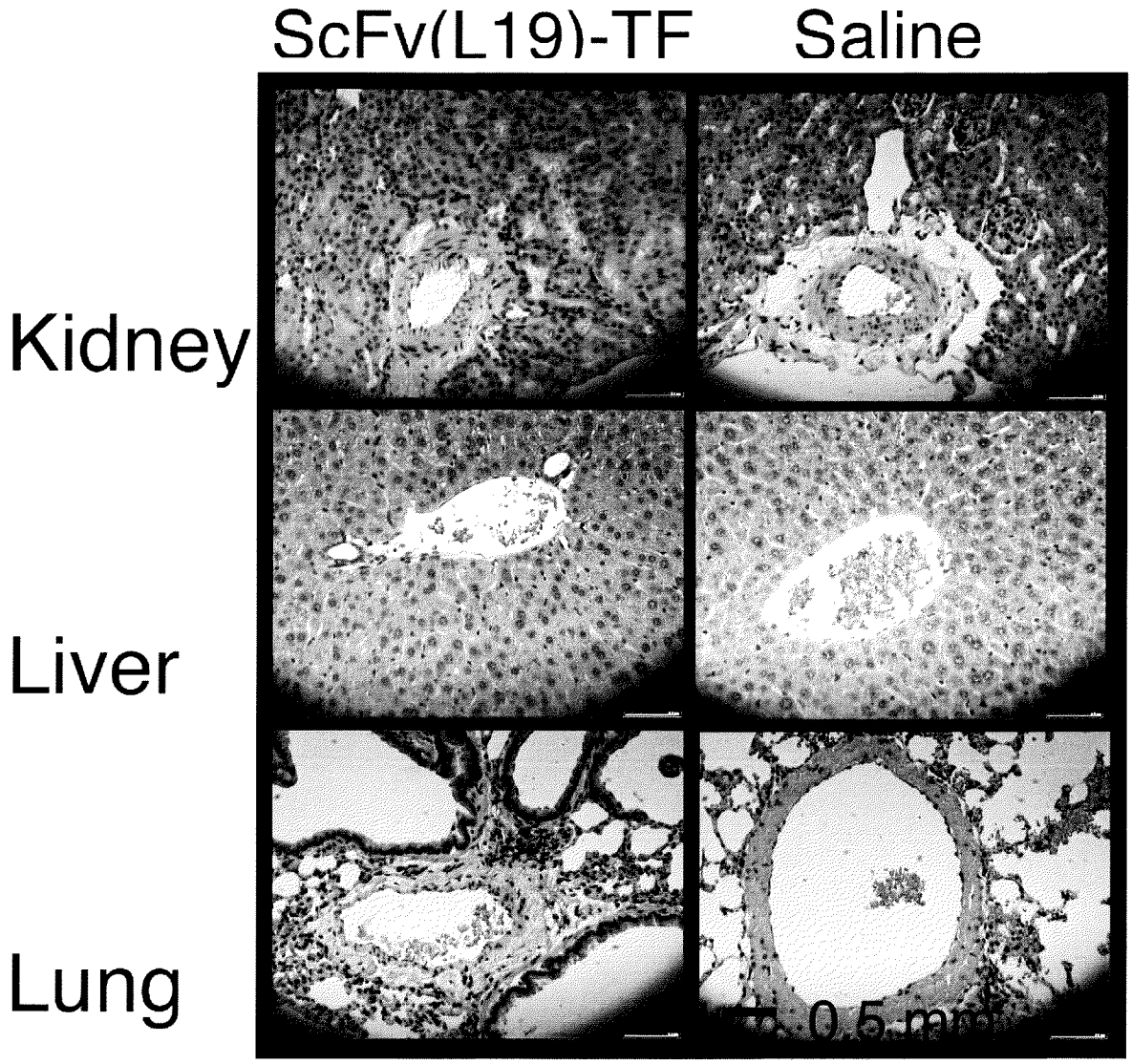


Fig. 3.10
Toxicity test. No thrombosis could be detected in a histochemical analysis of kidney, lung and liver, 24 hours after injection of a therapeutic dose (20µg) of the svFv(L19)-tTF.

3.3 Cloning and expression of antibody-tissue factor fusion proteins expressed in mammalian cells

3.3.1 Cloning

To increase the yield of expressed fusion protein, and to further enhance the in vivo solubility, the scFv-tTF fusion proteins where cloned in a mammalian expression system. Similar to the data presented in the previous chapters, the antibody fragment scFv(L19), which recognises the ED-B domain of fibronectin with affinity in the picomolar range [Pini et al., 1998] was used for the selective targeting of angiogenesis. The scFv(HyHEL-10) (abbreviated as HH10), which recognises hen egg lysozyme but not mouse lysozyme [Lavoie et al., 1992], was chosen as vehicle of irrelevant specificity for all the experiments with fusion proteins expressed in mammalian cells. Both antibody fragments were fused to a truncated form of human tissue factor (residues 1-219) [Ruf et al., 1991] using a PCR assembly cloning strategy, for expression in mammalian cells. As seen in Fig. 3.11, the resulting construct was a fusion gene almost identical to the one produced in bacteria, but adapted for mammalian expression with a mammalian peptide signal sequence for secretetion of the fusion proteins into the growth media. The only change was the introduction of a 5 aa peptide linker connecting the scFv to the tTF.

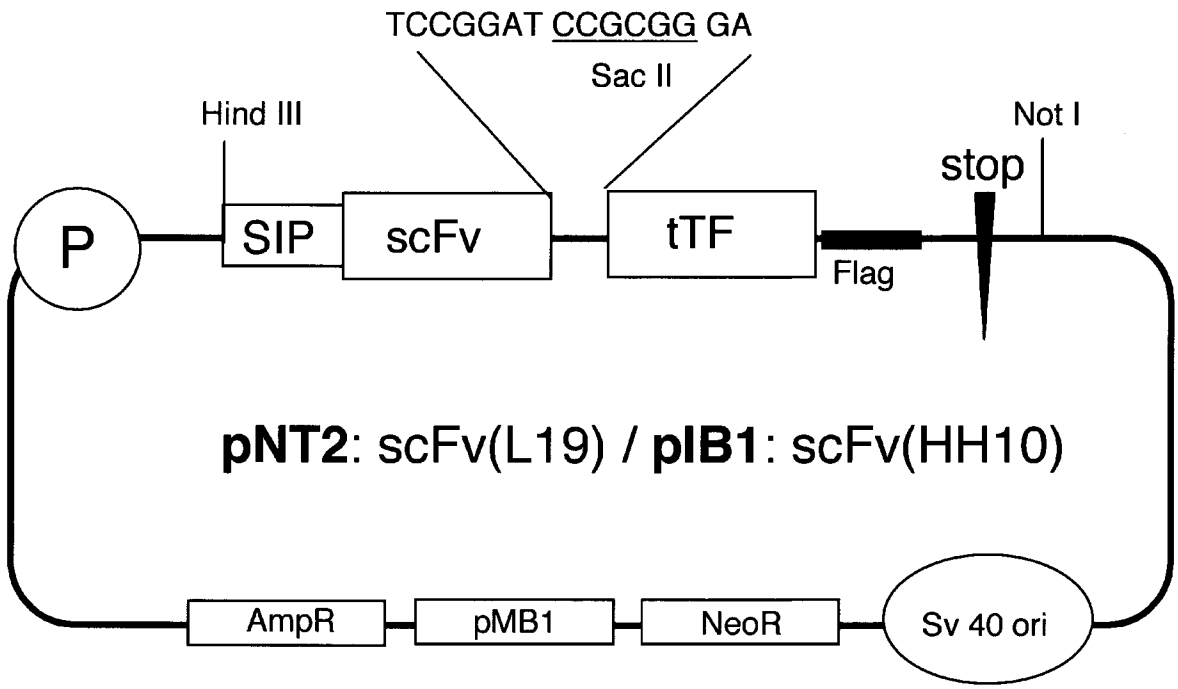


Fig. 3.11
Schematic representation of essential elements of vector pNT2 (the scFv(L19)-tTF fusion protein) and of vector pIB1 (the scFv(HH10)-tTF fusion protein) for the soluble expression of scFv-tTF fusion proteins. The peptidic linker consists of 5 amino acids, P, CMV-promoter, relevant restriction sites are indicated, the FLAG tag, DYKDDDDK, was used for detection of the protein.

3.3.2 Expression and purification

The fusion proteins were expressed as secreted proteins by mammalian HEK 293 cells growing in 150 cm^2 flasks. With harvest every third day, the optimal yield was 2 mg per liter. The supernatant was collected and stored frozen, then purified in a single step of affinity chromatography on antigen column, followed by dialysis. With this single step purification protocol, the fusion proteins were more than 98% pure with no need for further purification, as confirmed by gel analysis and gel filtration (Fig. 3.12 a and b).

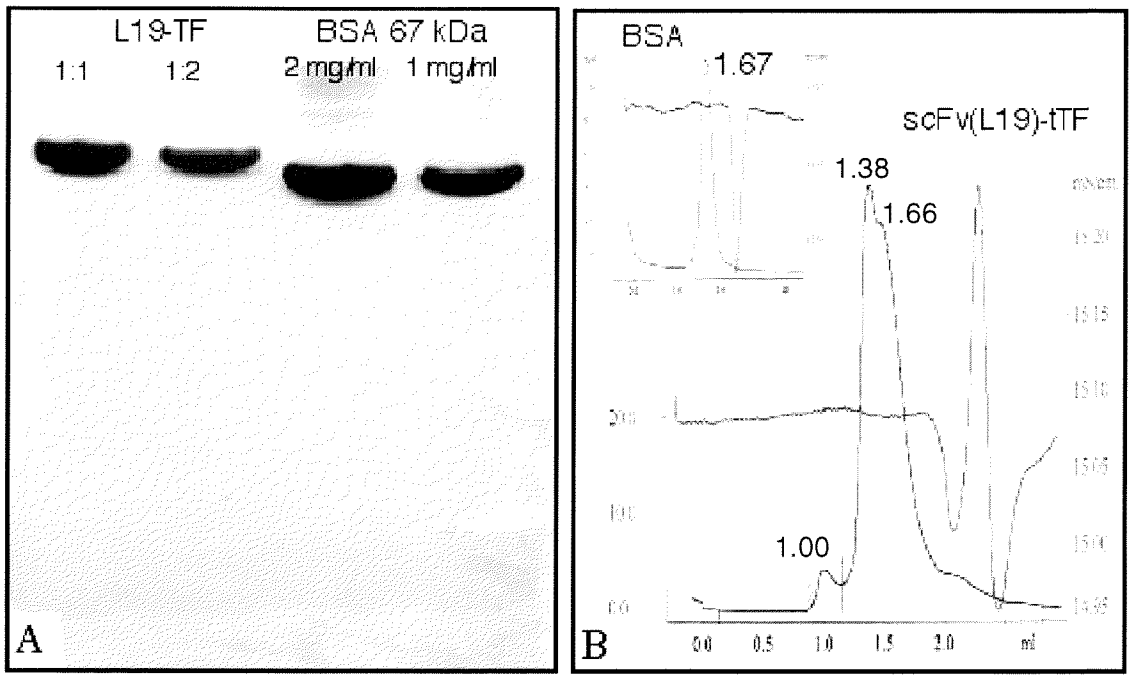


Fig. 3.12
Purified scFv-tTF. a) SDS-PAGE of a purified fraction of scFv(L19)-tTF compared with a BSA standard. b) Gel filtration of the native protein (scFv(L19)-tTF) on a Superdex-200 column. The fusion protein is a mixture of monomer and dimer, inset: BSA standard.

3.3.3 In vitro characterisation

Both purified fusion proteins appeared to be glycosylated as judged by an increase in molecular weight compared to the bacterially expressd fusion protein. Normally, the fusion proteins would have a size of 54 kDa, but when produced in mammalian cells, their apparent size was larger than 67 kDa. Gel filtration with a Superdex- 200 column showed that the purified scFv(L19)-tTF consists of a mixture of monomers and non-covalent homodimers. Freezing and thawing cycles did not result in precipitation, aggregation or any other change in oligomeric state (Fig. 3.12 b). In order to check that the targeting moiety of the fusion protein was funtional, immunoreactivity was measured by ELISA. The fusion protein was detected with two different secondary antibodies, either with one recognising the FLAG-tag on the C-terminal end of the tTF, or Protein A which binds to the folded VH-domain of the scFv (Fig. 3.13). Interestingly, the results were different. For the FLAG-tag detection, the ELISA profile of the fusion protein was identical to the bacterially produced control scFv. In ELISA experiments

with Protein A detection, the signal for the fusion protein was three times weaker than the control scFv produced in bacteria.

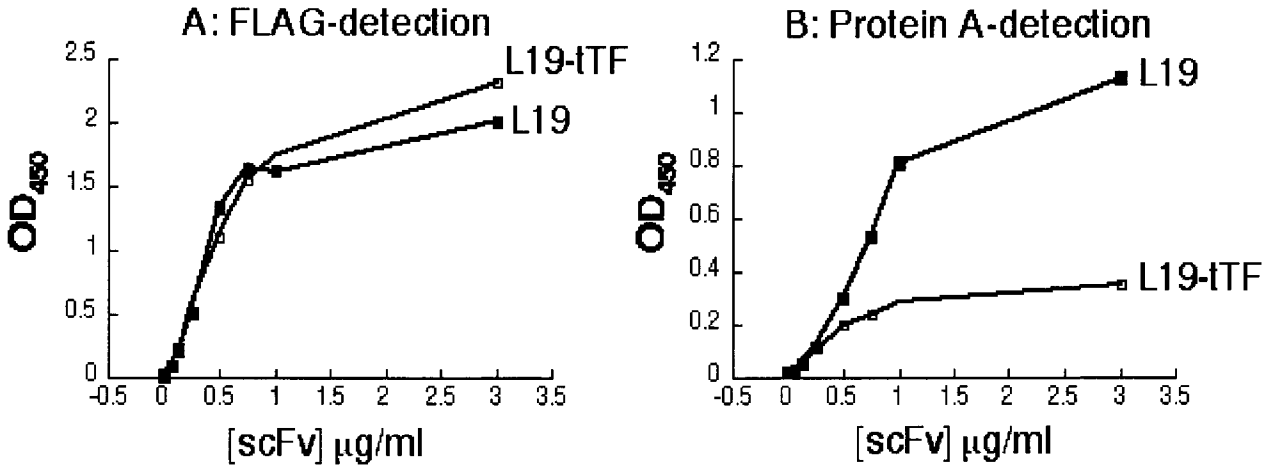


Fig. 3.13
Immunoreactivity of the fusion protein was identical when compared with the original bacterially produced scFv (L19) detecting the peptidic FLAG-tag in ELISA-assays (A). In contrast, detection by protein A gave a weaker signal for the fusion protein scFv(L19)-tTF (B). In the graph, absorbance at 450 nm is plotted versus standardised antibody concentration in µg/ml.

Tissue factor activity was measured as described [Ruf et al., 1991], detecting the cleavage of the FXa fluorogenic peptide mediated by the scFv-tTF/Factor VIIa complex (Fig.3.14). A half-maximal activity was observed at 45nM protein concentration, in line with the results from the bacterially produced protein and previously published values obtained with tTF [Ruf et al., 1991].

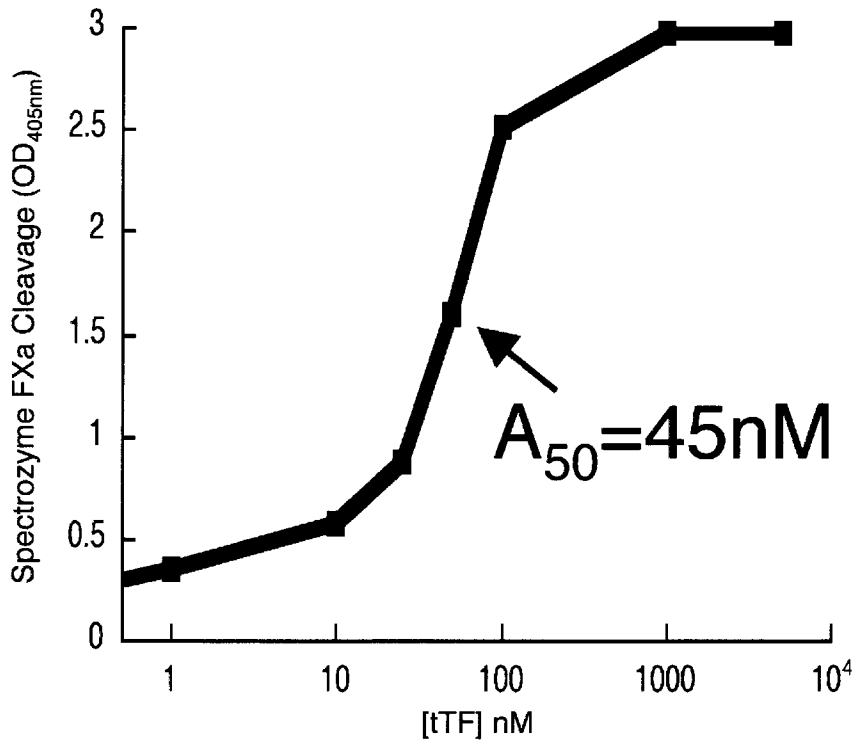


Fig. 3.14
Substrate cleavage by scFv-tTF/VIIa in solution. Hydrolysis of the peptidyl substrate Spectrozyme Fxa (1.25 mM) by 100 nM VIIa bound to increasing concentrations of scFv-tTF diluted in TBS. 5 mM $CaCl_2$ was present.

3.3.4 In vivo studies

The ability of HEK-produced scFv(L19)-tTF to selectively localise on tumour blood vessels proved to be comparable to the bacterially produced fusion protein, as examined by quantitative biodistribution analysis in mice bearing a subcutaneously grafted F9 murine teratocarcinoma [Bernstine et al., 1973], using a radioiodinated protein sample. Fig. 3.15 shows the results of this analysis, expressed in terms of percentage injected dose per gram of tissue (%ID/g), 24 hours after intravenous injection. At this time point, the %ID/g (tumour) was ~ 5% , with a tumour:blood ratio of 8:1, which is comparable to the ratio published for radiolabeled scFv (L19) [Tarli et al., 1999]. Tumour:blood ratios close to 2 can be observed as early as 4 hours after injection. The fusion protein did not show any signs of accumulation in the liver which is otherwise often observed with glycosylated proteins.

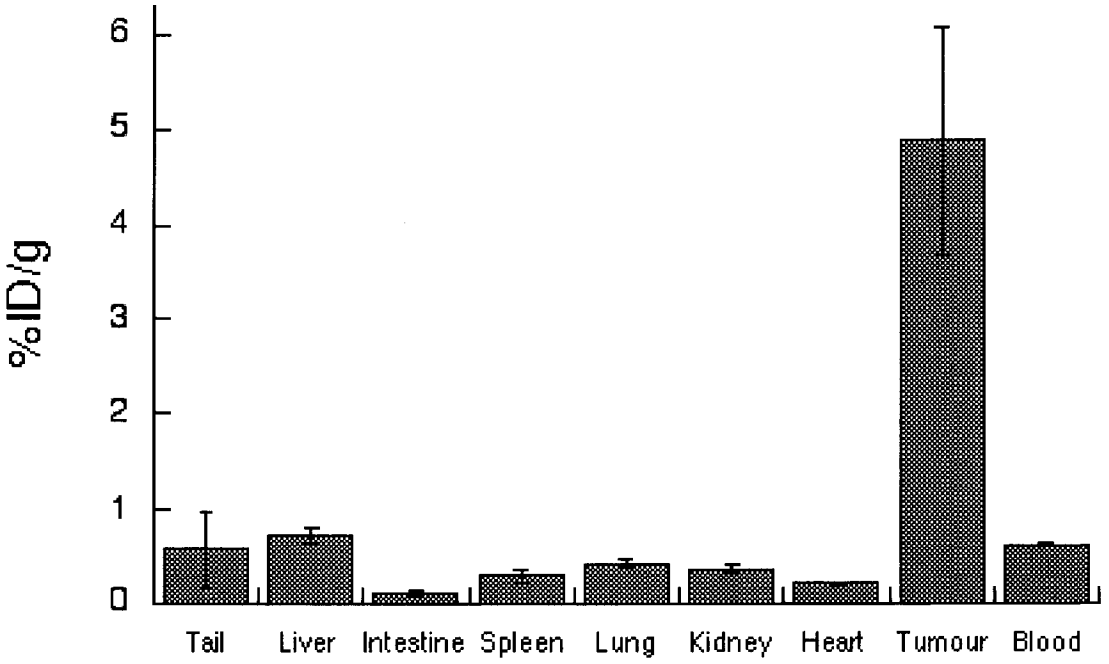


Fig. 3.15
Biodistribution of scFv(L19)-tTF in mice bearing the F9 teratocarcinoma, 24 hours after injection with 1 μg of radioiodinated fusion protein. Antibody doses in the different organs are expressed as % injected dose per gram; 4 mice per group were used.

3.4 Targeted delivery of antibody-tissue factor fusion proteins expressed in mammalian cells

In order to test the in vivo activity of the glycosylated fusion proteins, a small set of mice bearing large (1000-1500 mg) FE8 tumours was injected with 100 or 200 μg of the targeted tissue factor, scFv(L19)-tTF. The coagulative effect was monitored by optical examination (Fig. 3.16). The effect appeared to be dose-dependent with more extensive infarction of the tumours in mice injected with higher doses. No apparent side effects could be observed at these doses which were much higher than the maximum tolerated dose observed with the bacterially produced fusion proteins (35 μg fusion protein).

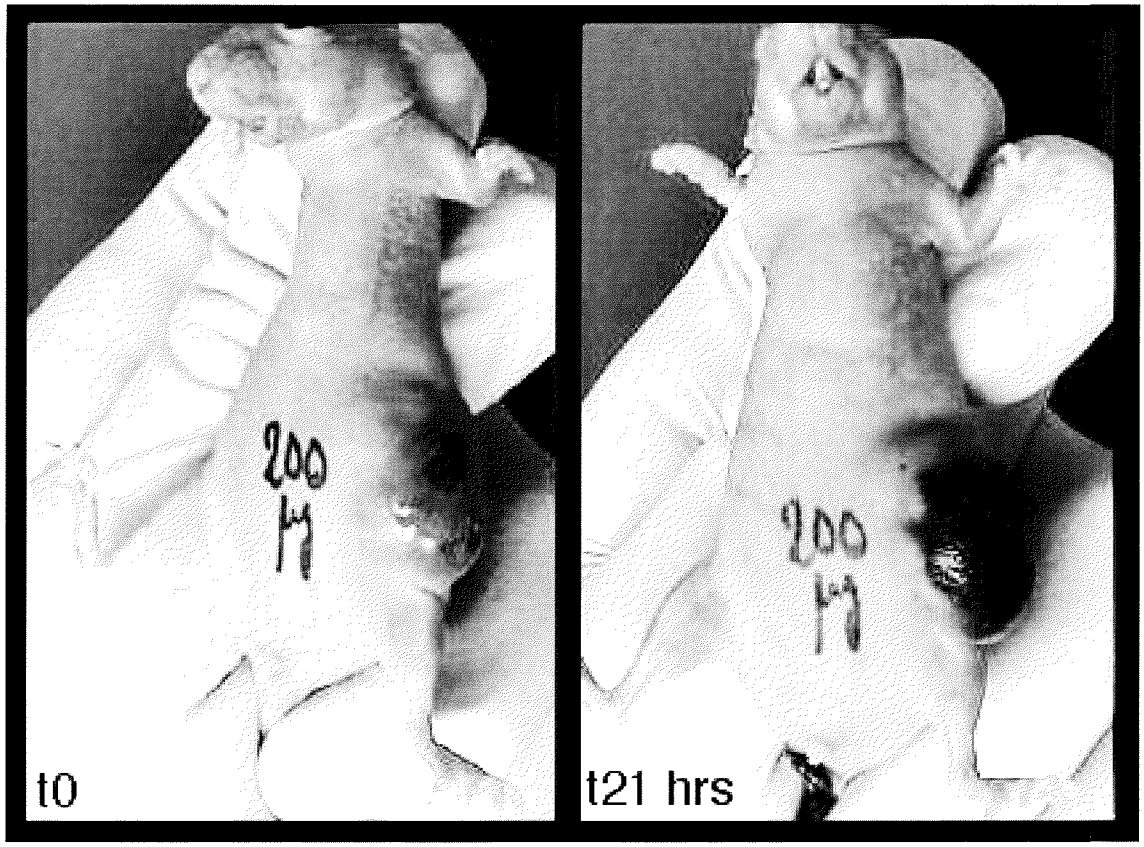


Fig. 3.16
Photographs illustrating that scFv(L19)-tTF produced in mammalian cells mediates the rapid infarction of tumoural blood vessels after a single injection of 200 µg fusion protein. No apparent side effects could be seen.

The next step was to perform a therapeutic study with the fusion proteins produced in mammalian cells. Mice grafted with FE8 sarcomas were divided into four cohorts with six mice in each. Grouping was necessary since for this study, the FE8 tumours developed large variations in size. Control groups were injected with saline or 100 µg of the negative control scFv(HH10)-tTF. In treated groups, mice received either 100 or 450 µg of ED-B specific scFv(L19)-tTF. Fig. 3.17 shows FE8 tumour volumes, plotted versus time, of mice injected three times with the doses indicated, at intervals of three days. In contrast to the results obtained with bacterially produced scFv-tTF fusion proteins, the saline control and the untargeted tissue factor differed in growth rate. When examined visually, the tumours of the control group injected with non-targeted tissue factor showed signs of thrombosis one day after injection, however, the tumours

continued to grow and the mice had to be euthanised six days after the first injection (Fig. 3.18). At this stage the mice in the treated groups had smaller tumours as compared to the control group, did not have significant weight loss and appeared to be healthy, allowing the study to be continued. Injection of targeted scFv(L19)-tTF produced in mammalian cells stabilised the tumour size for some time, then the tumour slowly continued to grow. No significant difference could be observed between the low and high dose of administered targeted tissue factor. Visual examination revealed extensive thrombosis after 24 hours. After 2 days, many tumours, but not all, were converted to crusty masses. For some tumours, only a portion of blood vessels was thrombosed and the non-affected part would continue to grow. This part could be thrombosed at a second injection three days later but in some cases, non-coagulated blood in parts of the tumour could be seen also at later time points (Fig. 3.19). At the highest dose used (450 µg), the onset of toxicity was registered as measured by body weight loss and rarely, as necrosis of the tail-tip.

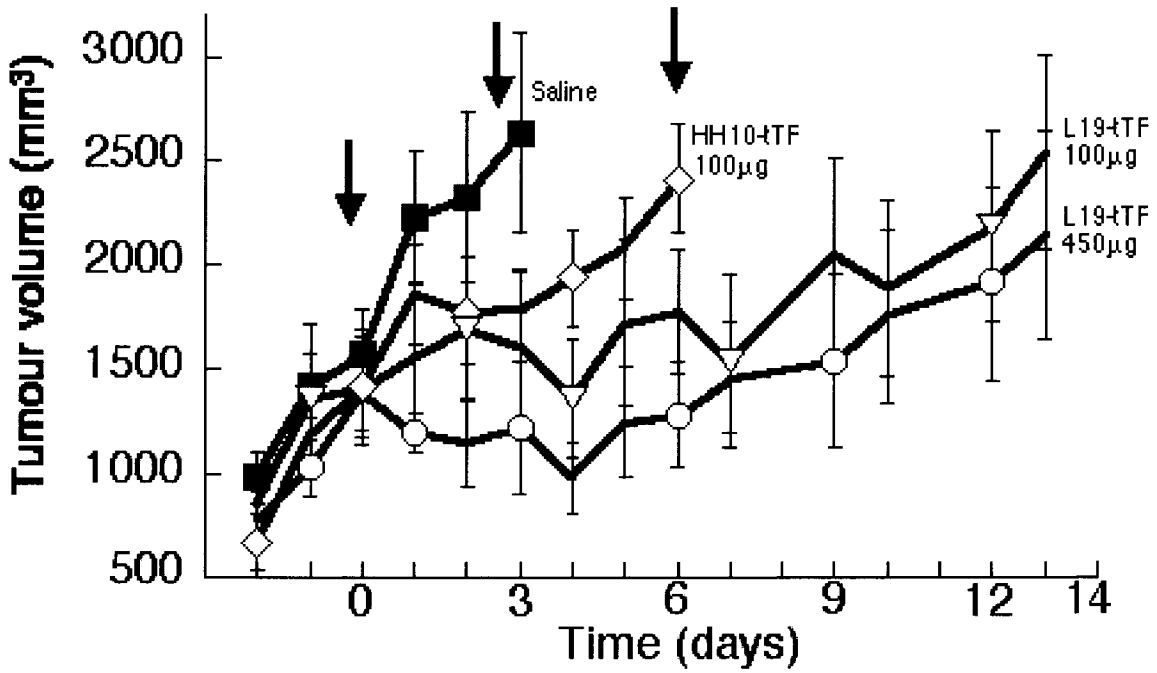


Fig. 3.17
Tumour growth retardation study in mice bearing the FE8 sarcoma after injection of three 100-µg doses of scFv(HH10)-tTF, saline, or two different doses of scFv(L19)-tTF. Means ±SE (n=4) are indicated. filled box, saline; empty diamond, 100 µg scFv(HH10)-tTF; empty triangle, 100 µg scFv(L19)-tTF,empty circle, 450 µg scFv(L19)-tTF. Injections are indicated by arrows.

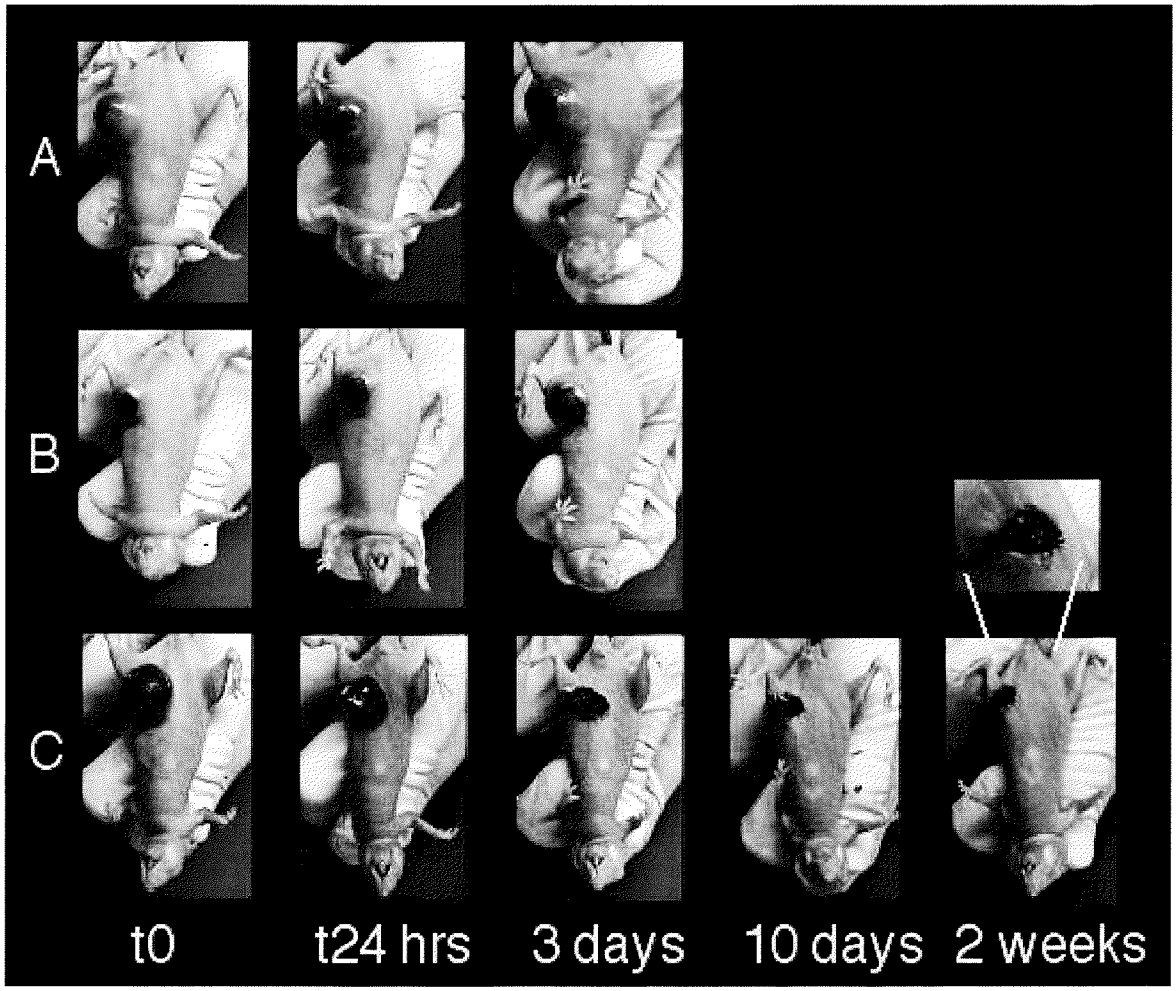


Fig. 3.18

Photographs of mice, bearing FE8 sarcoma after treatment with A: saline or B: scFv(HH10)-tTF, 100 µg or C; scFv(L19)-tTF, 100 µg. Time after first injection is indicated, injections were repeated on day three and six. Some coagulative activity of scFv(HH10)-tTF could be seen (B), but the effect after injection of scFv(L19)-tTF was much more pronounced with longer survival and even complete remissions (C).

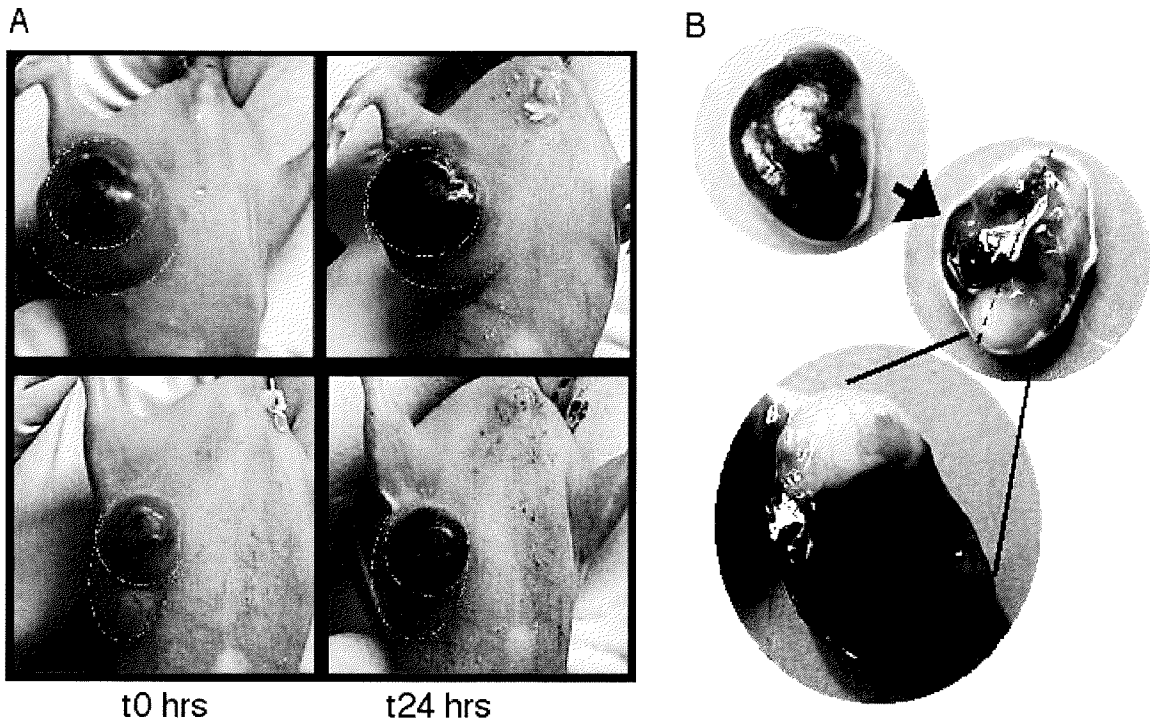


Fig. 3.19

Heterogenous response to scFv(L19)-tTF, 24 hours after injection of 100 µg. A) Blood vessels in some parts of the tumours are more readily thrombosed than others. B) Even tumours that appear to be well thrombosed when examined from outside (upper picture) may have non-infarcted parts at the tumoural base at the peritoneum as seen after excision (middle picture). Examination of a cross section (lower picture) reveals that there may be a distinct border between infarcted and non-infarcted parts of the tumour. Some blood vessels in the non-infarcted part, close to the border, are thrombosed but no damage to the tissue can be seen.

3.5 Combination of antibody-tissue factor fusion proteins with chemotherapy

In experiments with targeted tissue factor produced in either bacteria or mammalian cells, a small portion of the tumour typically survived at the peripheral parts, and eventually grew back. To investigate the possibility to eradicate these small portion of surviving tumour cells, a chemotherapy combination treatment modality with the widely used drug doxorubicin (Giuliani, 1981; Trail, 1993; Arap, 1998; Bosslet, 1998), was performed. First, the mode of administration and toxicity to two different tumour models was evaluated. Since the tissue factor fusion proteins are administered intravenously, it would be convenient to administer the doxorubicin intraperitoneally, thus avoiding two injections in the tail at the same time. Mice bearing either C51 carcinoma or FE8 sarcoma (300-400 mg) were injected intravenously with 50 μg doxorubicin or intraperitoneally with 150 μg doxorubicin. The tumour volume was monitored and in this setting, no difference could be seen for the different modes of administration. Interestingly, the tumours responded differently to the treatment. While the treated FE8 tumours continued to grow with the same rate as the saline control group, the C51 tumour model showed a two-fold reduction in growth rate as compared to saline controls (data not shown). The FE8 tumour model was selected for the combination study.

The next experiment was designed to monitor the effect of doxorubicin and targeted tissue factor in combination. Groups of mice bearing large FE8 tumours (>1500 mg) were injected at three different time points with either saline, doxorubicin alone or doxorubicin in combination with 450 μg scFv(L19)-tTF (Fig. 3.20). In contrast to the saline control group, or the animals treated with doxorubicin alone, there was a dramatic effect in the mice injected with the combination of doxorubicin and targeted tissue factor. After 24 hours, all tumours were thrombosed and two days after injection, most of the tumours had been converted into a crusty mass (Fig. 3.21). No signs of a

surviving tumoural mass could be seen. The dry crust eventually shrank and fell off. At the doses used, only a slight weight loss (< 5%) was observed and no cumulative toxicity of the doxorubicin occured.

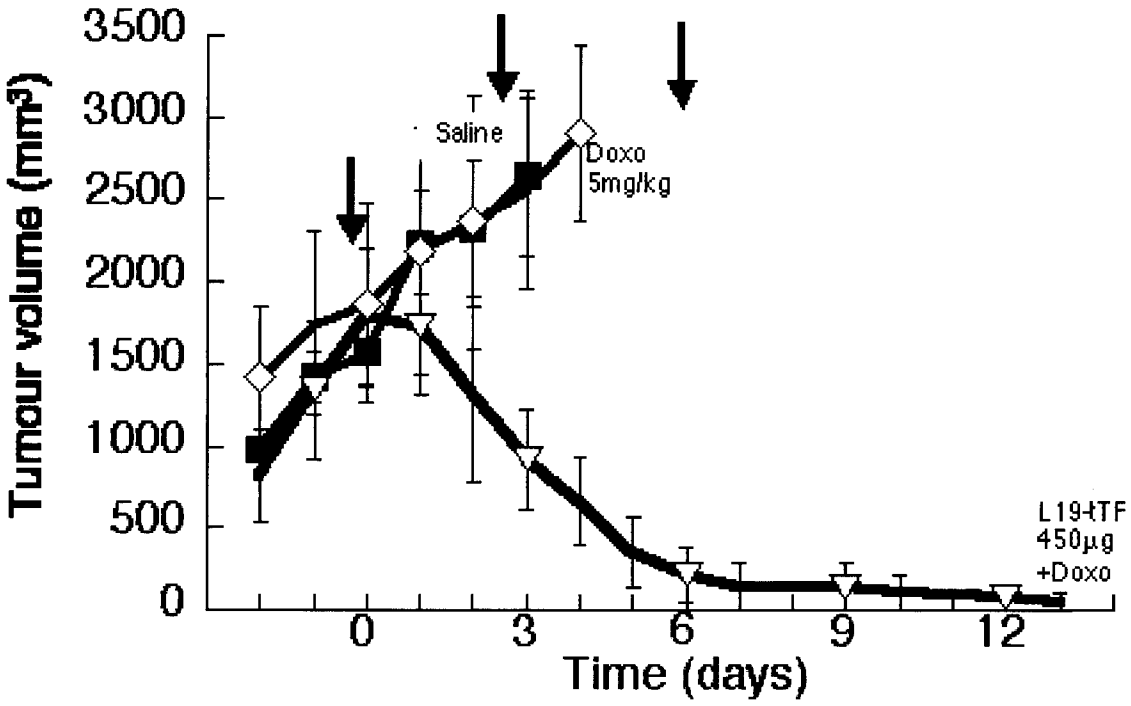


Fig. 3.20

Combination study in mice bearing FE8 sarcoma after three injections with saline (filled box), doxorubicin (5mg/kg) (empty diamond) or 450 µg scFv(L19)-tTF in combination with 5mg/kg doxorubicin (empty triangle).

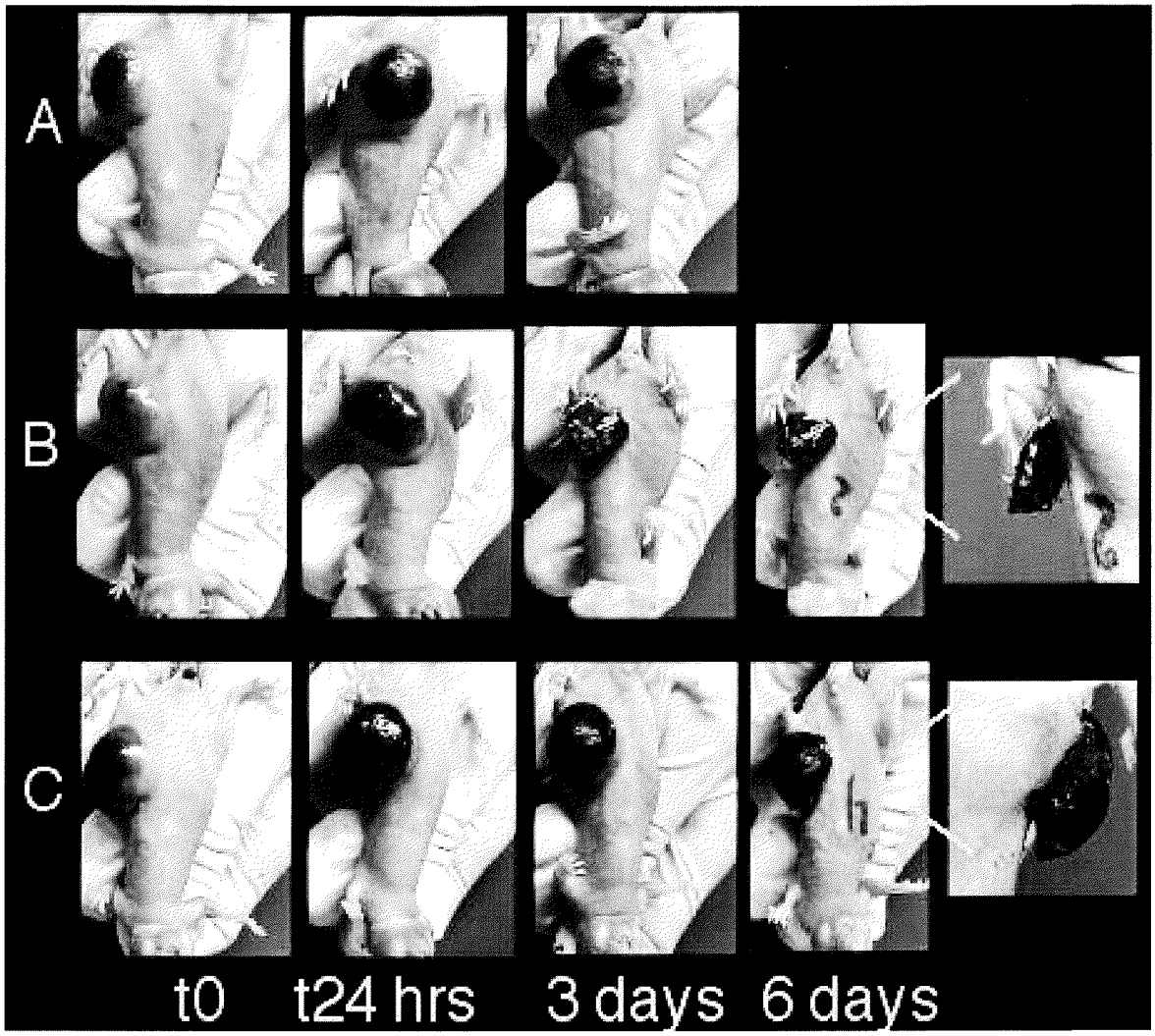


Fig. 3.21

Photographs of mice, bearing FE8 sarcoma after treatment with A: doxorubicin 5 mg/ kg or doxorubicin in combination with 450 μg scFv(L19)-tTF, B and C. Time after first injection is indicated, injections were repeated on day three and six. Mice in the control group were euthanised at day three due to their large tumours (A). At day six, the tumours of the treated mice were converted to a crusty mass as can be seen in side projections (B and C).

4 DISCUSSION

With the advent of phage technology some fifteen years ago, a new era started in protein engineering. The display of antibody fragments on the tip of phage made it possible to isolate high affinity antibodies in vitro. This procedure resulted in antibodies binding with high affinity also to "difficult" antigens (e.g., conserved antigens), against which it is difficult to raise antibodies using conventional hybridoma technology. Phage antibodies can be affinity matured, resulting in high affinity binding molecules.

A number of human diseases rely on the formation of new blood vessels from pre-existing ones in a process called angiogenesis. The ED-B domain of fibronectin is a highly conserved marker of angiogenesis, identical from mice and dog to human. Using a phage display library constructed in our laboratory, our group have recently isolated an antibody fragment, scFv(L19), with high affinity and specificity for ED-B. The L19 antibody has proven to bind to new forming blood vessels in tumoural tissues in in vivo biodistribution experiments.

In this dissertation, I show that targeting of a pro-coagulant factor to tumour vasculature by means of scFv(L19), thereby inducing occlusion of tumour blood vessels with the subsequent starvation and death of tumour cells, represents an attractive avenue towards tumour therapy. This concept was first proven by the group of prof. Thorpe some years ago. The first report by Huang and colleagues [Huang et al., 1997] on the targeted induction of intraluminal blood coagulation in tumoural blood vessels using an artificial marker of angiogenesis, generated a great interest to learn whether the same strategy would work in tumour models carrying natural markers of angiogenesis. The second report on this strategy, featuring the targeting of the vascular cell adhesion molecule-1 (VCAM-1), was less impressive, as only a 50% reduction in tumour growth rate was observed. The authors also postulated that the simultaneous display of target antigen and phosphatidylserine (PS) on the luminal surface of blood vessels was an essential requirement for starting the blood coagulation cascade.

At this stage, it was not known whether targeting of a truncated soluble version of the tissue factor (tTF) to the ED-B domain of fibronectin (B-FN) would mediate a coagulative effect in tumour blood vessels since B-FN is a component of the modified extracellular matrix which accumulates at the abluminal side of tumour blood vessels. A rational for targeting tTF to B-FN would be that the fenestration and leakiness of tumour blood vessels would allow the extravasation of Factor VIIa, which could then

bind to the tTF anchored at high density on B-FN via the fusion protein. Conversion of Factor X into Factor Xa in the perivascular space immediately around the blood vessels would facilitate the diffusion of Factor Xa into the blood stream, with consequent continuation of the blood clotting cascade.

To investigate the targeting of tissue factor to B-FN, a fusion protein consisting of the scFv(L19) and the tTF was cloned and expressed in bacteria. The production was difficult, for a number of reasons. The yields were low, as could be expected for bacterial expression of a protein containing four disulfide bridges. Furthermore, the protein was sensitive to proteolysis even after an initial affinity purification. Thus, a number of purification steps had to be carried out, and the material had to be kept at 4° C all the time. Finally, the resulting pure fusion protein was prone to aggregation, especially at high concentrations and after being frozen. As I later found out, these aggregates could not be detected in gel filtration, as they remained on the column. However, they could be removed by filtering the sample using 0.22 μm cut off filter. The fusion protein was shown to be functional in vitro in immunoassays and in enzymatic assays; both moieties proved to have activity similar to the one of the individual components. In vivo, scFv(L19)-tTF displayed a high accumulation on neovasculature few hours after injection, as measured by quantitative biodistribution analysis using radioiodinated fusion proteins.

In a first set of experiments, the therapeutic effect of scFv(L19)-tTF was examined in three different tumour models, either in small tumours and several injections per mouse, or in large tumours and a single injection. In the first part of the study, at the low dose of 14 μg used, only a retardation in tumour growth could be seen, along with some signs of thrombosis. The thrombosis was much more prominent for a single injection of 20 μg, suggesting that an increase in dose would be of therapeutic benefit. At this dose, however, toxicity could be observed. The mice lost body weight, the tip of the mouse tail would become black and necrotic and in some cases swelling of limbs could be observed. These results were suggestive of aggregates of the fusion protein, undetectable in gel filtration analysis, which would deposit at the extremities of animal's body. Filtration of the sample immediately before i.v. injection abolished the side effects and allowed me to perform a dose escalation study.

The therapeutic efficacy of the scFv(L19)-tTF was clearly dose-dependent, with a decrease in tumour growth rate corresponding to the doses administered. At higher doses, some of the tumours would not only turn black and crusty after two days, but would also completely disappear, leaving the mice cured. At the highest dose (35µg), complete remissions were observed in 30% of the mice treated. This was comparable to published data by Huang and colleagues using an artificial tumour marker and better than the 50% growth inhibition of Ran et al. [1998] using the VCAM-1 as a target antigen. All tumours showed extensive thrombosis of blood vessels, but in some cases residual tumour cells grew back. Typically, these cells would be located in tumour areas in which blood vessel occlusion was not complete. The clear dose dependence of therapeutic benefit obtained with bacterially produced scFv(L19)-tTF suggests that the efficacy of this therapeutic fusion protein would further improve using higher doses. At that stage, it was hard to increase the dose since the onset of toxicity could be seen.

In principle, a better formulation of the fusion protein and combination with other therapeutic modalities (e.g., chemotherapy) are avenues towards increasing the dose administered and/or the therapeutic index of scFv(L19)-tTF. To increase the solubility of the protein and aiming at a more stable production system, scFv(L19)-tTF was produced in stably transfected human embryonic kidney cells. A glycosylated fusion protein with apparent biological activity similar to its counterpart produced in E. coli, but less prone to aggregation, as evidentiated by freeze-thaw cycles and chromatographic analysis, was obtained. The yield of 2 mg/ml was ten times higher than the yield of the bacterially produced fusion protein and no signs of proteolysis could be seen. Interestingly, when monitoring the immunoreactivity of the fusion protein produced in HEK cells using an ELISA assay, a significant difference in signal depending on the detecting secondary agent was observed. ELISA signals were similar to the positive control scFv(L19) produced in bacteria when detecting the C-terminal FLAG-tag peptide. However, when detecting the scFv using Protein A, the signal was a factor three lower for the fusion protein as compared to the bacterially produced scFv. This may indicate glycosylation of the Protein A recognising an epitope on the scFv, which impaires Protein A binding. Further studies with de-glycosylated fusion protein will prove if this is the case. No changes in activity could be seen when comparing the in vitro enzymatic activity of the fusion protein with its bacterially produced counterpart, in line with published data for tTF [Ruf et al., 1991]. It should be noted that the enzymatic assay only measures the activity of the tTF:FVIIa complex cleaving a

chromogenic peptide and not the catalytic activity converting factor X to Xa. The ability of the fusion protein produced in HEK cells to localise on tumour blood vessels was comparable to the one of bacterially produced fusion protein as monitored in quantitative in vivo biodistribution studies. No accumulation in the liver was seen, which is otherwise often observed with glycosylated proteins.

The in vivo activity of the glycosylated fusion proteins was first evaluated for the targeted tissue factor in a small experiment with mice bearing large FE8 tumours. The high doses used, 100 and 200 µg, were not toxic and extensive shutdown of the tumoural vasculature could be seen macroscopically, as the tumours turned into black masses. After this pilot study, a larger study was performed. The aim was to monitor the increase in therapeutic efficacy for targeted versus non-targeted tissue factor and to see if the respons was dose-dependent. In contrast to the previous experiments performed with bacterially produced fusion proteins, at this high doses, the untargeted tissue factor showed some pro-coagulant activity. It is possible that the resident thrombogenic activity of tumour vasculature [Zacharski et al., 1993; Murray et al., 1991] renders these vessels more susceptible to thrombosis even by untargeted tTF. The effect seemed to be specific for the tumour since other organs were unaffected. The therapeutic effect in animals treated with targeted tTF was however much more pronounced and with longer survival of the mice. At 100 µg administered fusion protein no toxicity was seen but at the highest dose used, 450 µg, weight loss and necrosis of the tail-tip could be seen in some of the mice, thus this dose was considered close to the maximum tolerated dose (MTD). There was no significant quantitative increase in therapeutic efficacy for the higher dose used. Data for 100 µg show a similar effect to 450 µg after some days, indicating that the dose used is either saturating the antigen with the targeting antibody or close to the maximal activity for tissue factor. Considering the doses used, the therapeutic efficacy was surprisingly low. Whereas the bacterially produced fusion protein was active in low doses, no striking increase in therapeutic efficacy could be observed when administering a dose three times higher. The fusion proteins expressed in mammalian cells seemed to be less active in vivo than the bacterially produced scFv-

deglycosylated fusion proteins remains to be performed; in vitro ELISA assay with protein A detection, and in vivo studies, examining the thrombogenic effect.

Despite the extensive shutdown in vascular function and the associated tumour cell death, targeted tissue factor results in complete remissions for only a part of the mice treated. Typically, the majority of the tumours are thrombosed within a day, they later turn necrotic with a dramatic decrease of viable tumour, but many mice retain a small surviving fraction of tumour cells. This fraction may be located as a larger lobe in the tumour mass, but is most commonly seen in the perifery of the tumour, close to the skin and peritoneum for subcutaneously grafted tumours. Increased blood flow in the adjacent normal tissue, together with probable upregulation of angiogenic factors such as VEGF, as a result of ischaemic insult, facilitates rapid growth and expansion of the few surviving tumour cells. These tumour cells observed following vascular targeting

[Pedley et al., 1996; Pedley et al., 1999; Chaplin et al., 1999]. To address this possibility, the addition of targeted tissue factor to a doxorubicin treatment was investigated. The results show a dramatic enhancement of the therapeutic efficacy of doxorubicin. The FE8 tumour model was chosen since large established tumours were non-sensitive to doxorubicin treatment as studied in a pilot experiment. When treating tumour bearing mice with doxorubicin alone at half-maximal dose, the grafted tumours grew as fast as the saline injected mice. In contrast, in mice injected with the combination of doxorubicin and tTF, the blood vessels became occluded and the tumours necrotic due to the initial effects of the tTF. Furthermore, no surviving tumour cells could be seen, either after 24 hours or at later time points. Similar to data obtained with other vascular targeting agents [Chaplin et al., 1999; Pedley et al., 1999], tTF was administered some time after injection of doxorubicin, allowing the drug to spread throughout the body and then to become captured within the occluded blood vessels in

It is interesting to compare combination studies of scFv(L19)tTF fusion protein to another vascular targeting agent, the Combretastatin. The swedish company Oxigene has licensed Combretastatin to Bristol-Meyers Squibb in a $70 million deal, and the animal data in combination studies are far from convincing. In a combination trial with cis-platin, a 25% tumour growth retardation at 15 days after treatment was shown [Chaplin et al., 1999]. The results of Combretastatin in combination with radiation were more promising with a 50% tumour growth inhibition after 90 days, still, the combination studies were not curative in animals. Since the scFv(L19)-tTF and Combretastatin have similar modes of action -disruption of the tumour vasculature- it would be interesting to compare them side by side.

Due to its mode of action, targeted tissue factor is an ideal partner for virtually any combination therapy with conventional treatments including surgery, radiation therapy, systemic cytotoxic or cytostatic treatments. Targeted tissue factor should also potentiate the activity of agents such as anti-angiogenesis compounds, matrix metalloproteinase inhibitors and immunomodulatory proteins (e.g. cytokines and chemokines). Furthermore, one could imagine that tissue factor-mediated thrombosis of tumour blood vessels could help trapping cytotoxic agents in the tumour environment. In principle, tissue factor-induced tumour ischemia could be facilitated by NO-synthase inhibitors.

The sequence of the ED-B domain is identical from mice to humans, allowing the use of scFv(L19)-tTF in a number of different syngeneic animal models. Whereas the results described in this work suggest that scFv(L19)-tTF may offer a benefit to cancer patients in a therapeutic setting, experiments with slow growing tumours and studies in larger animals (preferentially in animal patients with spontaneous disseminated tumours) are required before considering clinical trials. It should be noted that the therapeutic responses seen in this dissertation differed depending on the tumour model used. Although all tumour models were extremely aggressive, the slower growing and therefore less angiogenic ones showed a lower response. Considering that these experimental tumours have a doubling time of at least 2-3 days and that many human tumours are growing much slower, it remains to be seen what the impact of anti-angiogenesis or anti-vascular treatments will be in the clinic. For example, the ability of drugs to produce a cure in the clinic is quite good for rapidly growing malignancies like Burkitt´s lymphoma, Hodgkins disease or Testicular embryonal carcinoma which all

have doubling times within days. The prognosis for slower growing, and unfortunately also more frequently occurring tumours such as colon, breast and lung cancer, with doubling times close to 100 days is worse.

5 MATERIAL AND METHODS

5.1 Cell lines, genes and antibodies

The tumoural cell lines used were F9 murine teratocarcinoma [Bernstine et al.,1973], C51 murine colon adenocarcinoma [Corbett et al., 1975] and FE8 rat fibroblast sarcoma [Schaefer et al., 1988]. Bacterial protein expression was performed in the *Escherichia coli* strain TG1 (K12, D(*lac-pro*), *supE*, *thi*, *hsdD5/F'traD36*, $proA^{+}B^{+}$, $laqI^{q}$, *lacZDM15*), for mammalian protein expression, HEK 293 cells were used (Invitrogen, Groningen, The Netherlands). The scFv(L19), scFv (D1.3) scFv and (HyHEL-10) have been described elsewhere [Tarli et al., 1999, Harper et al., 1987]. The cDNA for the TF was obtained from American Type Culture Collection (ATCC; Rockville, MD).

5.2 Cloning, expression and purification of scFv-tTF fusion proteins

5.2.1 Bacterially expressed scFv-tTF fusion protein

The scFv (L19)-tTF expression vector (pFN5) was constructed by cloning of a synthetic DNA sequence, coding for the human tTF, at the 3´end of the DNA sequence encoding the human scFv (L19), using the Not1/EcoR1 sites of a derivative of vector pDN5 [Neri et al., 1996], in which the scFv (D1.3) gene had been replaced by the scFv (L19) gene. The human tTF DNA sequence was modified by PCR as follows:

1 the primer TF-banot (5'-T GAG TCA TTC GCG GCC GCA GGT GGC GGT GGC TCT GGC ACT ACA AAT ACT GTG GCA-3´) introduced at the 5´end of the tTF DNA sequence a restriction site for the endonuclease Not 1. It also introduced a short linker C-terminally of the restriction site consistent of four glycins (GGGG);

2 the primer TF-fostueco1 (5'-GTC CTT GTA GTC AGG CCT TTC ACG GAA CTC ACC TTT CTC CTG GCC CAT ACA-3') introduced at the 3´end of the tTF DNA sequence a Stu 1 endonuclease restriction site and the first four residues of the FLAG-tag. It also removed a EcoRI restriction site in the codon for the aminoacid 216 in the tTF DNA sequence by a silent mutation.

3 the primer TF-fostueco2 (5'-AGA GAA TTC TTA TTA CTT ATC GTC ATC GTC CTT GTA GTC AGG CCT TTC ACG-3') introduced at the 3'end of the product of TF-banot and TF-fostueco1 the rest of the FLAG-tag (DYKDDDDK), two stop codons and a EcoRI restriction site.

The scFv (D1.3)-tTF expression vector was constructed in a similar fashion as described above for scFv (L19)-tTF. In short, the tTF gene was cloned in the Not1/EcoR1 sites of vector pDN5 [Neri et al., 1996] which already contains the scFv (D1.3) gene.

The vectors were introduced by electroporation in TG1 *Escherichia coli* cells. Protein expression and the first affinity purification step on antigen column were performed as described [Tarli et al., 1999; Neri et al., 1999]. In the case of scFv(L19)-tTF, eluted fractions were neutralised with 1 M Tris/HCl pH 7.4 and loaded on a Sigma M2-resin for affinity purification using the FLAG-tag (Sigma, MI USA). After serial washes, the protein was eluted with 0.1 M Glycine/HCl pH 3.5 and put directly onto a Resource S cation exchange column (Amersham Pharmacia Biotech, Uppsala, Sweden). The monomeric peak fraction was collected and desalted with a preparative gelfiltration column PD-10 (Amersham Pharmacia Biotech, Uppsala, Sweden). Typical yields of tri-purified therapeutic fusion protein were approximately 0.2 mg/l culture, mainly due to the low capacity and limited life of the α-Flag M2 resin. Protein yields after the first affinity chromatography step were approximately 1-2mg/l.

In the case of scFv(D1.3)-tTF, fractions eluted from a lysozyme column were neutralised with 1 M Tris/HCl pH 7.4 and loaded on a Sigma M2-resin for affinity purification using the FLAG-tag (Sigma, Mi, USA). After serial washes, the protein was eluted with 0.1 M Glycine/HCl pH 3.5 and pH adjusted to 8.0. The sample was loaded onto a Resource Q anion exchange column (Amersham Pharmacia Biotech, Uppsala,

Sweden). The monomeric peak fraction was collected and desalted with a preparative gelfiltration column PD-10.

Fusion proteins were analysed under denaturing conditions on SDS-PAGE and in native conditions by FPLC gel filtration on a Superdex S-75 (Amersham Pharmacia Biotech, Uppsala, Sweden).

5.2.2 ScFv-tTF fusion protein expressed in mammalian cells

The scFv(L19)-tTF expression vector (pNT2) (fig. 3.11) was constructed by cloning of a synthetic DNA sequence, coding for the human tTF, at the 3´end of the DNA sequence encoding for the human scFv(L19) using the Sac II/ Not I sites of a derivative of the pcDNA3 vector (Invitrogen, Groningen, The Netherlands) into which an IgG secretion signal had been engineered followed by the sequence for the scFv(L19) [Li et al., 1997]. The human tTF DNA sequence was modified by PCR as follows:

1 1 the primer TFbaSacII (5'- TCT TCC CCG CGG TTA TTA CTT ATC GTC ATC GTC CTT GTA-3') introduced at the 5´end of the tTF DNA sequence a restriction site for the endonuclease Sac II;

2 the primer TF-fostueco1 (5'-GTC CTT GTA GTC AGG CCT TTC ACG GAA CTC ACC TTT CTC CTG GCC CAT ACA-3') introduced at the 3´end of the tTF DNA sequence a Stu 1 endonuclease restriction site and the first four residues of the FLAG-tag. It also removed a EcoRI restriction site in the codon for the aminoacid 216 in the tTF DNA sequence by a silent mutation.

3 the primer flagfoNotI (5'-ACT CAG TAA GGC GGC CGC CTA TTA CTT ATC GTC ATC GTC CTT GTA GTC-3'), introduced at the 3´end of the tTF-FLAG DNA sequence the rest of the FLAG tag (DYKDDDDK), two stop codons. and a Not I restriction site.

The scFv(HH10)-tTF expression vector (pIB1) (fig. 3.11) was constructed by replacing the scFv(L19) gene of the scFv(L19)-tTF expression vector (pNT2) with the scFv(HyHEL-10) gene using the Hind III restriction site at the 5´end of the SIP secretion sequence and the Sac II restriction site in the linker between the scFv and the tTF (Fig. 3.11). The scFv(HH10) DNA was modified as follows:

1 1 the scFv(HH10) was amplified by PCR with the primer HH10ba (5´-GAG GTG AAG CTG CAG CAG TCA GGA -3´) and the primer SacIIHELfo (5´-TCC CGC GGA TCC GGA TTT TAT TTC CAG CTT GGT CCC-3´) introducing a restriction site for the endonuclease Sac II at the 3´end of the scFv(HH10) DNA sequence.

2 the SIP secretion sequence was amplified by PCR using the primer T7, annealing on the T7 promoter binding site (5´-AAT ACG ACT CAC TAT AG-3´) and the primer HH10SIPfo (5´- TCC TGA CTG CTG CAG CTT CAC CTC CGA GTG CAC ACC TGT GGA GAG AAA -3´) introducing a 3´ sequence complementary with the start of the scFv(HH10) gene.

3 The thus resulting inserts (1+2) were assembled in a PCR assembly reaction, digested Hind III and Sac II and ligated into the empty, Hind III and Sac II digested pNT2 vector.

The vectors were introduced by electroporation in TG1 *Escherichia coli* cells and grown over night. Maxipreps were made (Qiagen), the DNA linearised with the endonuclease Bgl II to facilitate stable integration, and then transfected into HEK 293 cells using a Fugene method according to manufacturer instructions (Roche Biochemicals). 8 hours before transfection, 10 dishes of 6 cm diameter were filled with 4 ml culture medium. The cells from one confluent flask were trypsinised, 8 ml medium was added to the cell solution and 1 ml of the cells (one tenth) was then distributed to each dish. At transfection time, 6 µl Fugene (Roche Biochemicals) were added to 92 µl Eagle modified Dulbecco medium (Gibco), afterwards 2 µg of plasmid solution was pipetted in the same tube. Incubation at room temperature for 1h followed. The solution was then

pipetted dropwise to the cells. One day after transfection the medium was exchanged to medium supplemented with 0.5 ml/L neomycin (Sigma). The cells were kept in culture for 10 days, changing the medium every 24 hours in order to keep the selection pressure constant and wash away dead cells. Once all negative control cells (nontransfected) were detached , the cloning procedure was started.

The transfected cells with a Neomycin resistance were trypsinised and counted. They were diluted to have 3 cells per 20 µl of dilution (150 cells/ml). 20 µl of this cell suspension was added to each well of four 96-well plates that were previously filled with 80 µl neomycin (0.5 mg/L) supplemented medium. After one week of incubation, the neomycin medium was changed in order to re-establish the neomycin concentration. After one more week, wells with only one clone were selected. Supernatant from monoclonal wells was checked for expression by ELISA.

The 24 best clones were chosen and transfered to a 24-well plate: After trypsinisation of the culture with 40 µl trypsine, the cells were resuspended in 80 µl medium (containing neomycin) and diluted in 400 µl more medium in the 24-well plate. The cells grew for a few days in the 24-well plates, then checked for expression by ELISA. Several candidates were then grown in 75 cm^2 tissue culture flasks (TPP) in order select the best expressing clone. Cryotubes were made of the best expressing clone for each construct and cells were cultured in 150 cm^2 flasks for protein production. Medium was exchanged every third day and stored at –20°C.

ScFv-tTF fusion proteins were purified in a single step of affinity chromatography on antigen column, followed by dialysis in PBS, as described [Tarli et al., 1999; Neri et al., 1996]. In the case of scFv(L19)-tTF, an ED-B column was used, in the case of scFv(HH10)-tTF, a hen egg lysozyme column was used. Typical yields of purified and dialysed therapeutic fusion protein were approximately 2-4 mg/l culture. Fusion proteins were analysed under denaturing conditions on SDS-PAGE and in native

conditions by FPLC gel filtration on a Superdex S-75 (Amersham Pharmacia Biotech, Uppsala, Sweden).

5.3 Measurement of immunoreactivity and tissue factor activity

The immunoreactivity of the scFv-tTF fusion protein was analysed by BIAcore and by affinity chromatography on antigen column as described [Neri et al., 1997; Tarli et al., 1999; Viti et al., 1999] and by ELISA immunoassay: 100µl of biotinylated antigen (10^{-7}M) in PBS was added to each well of a streptavidin-coated plate (Roche). The plate was incubated at 37^{0}C for 15 minutes. Afterwards, the wells were washed once with PBS, the plate was blocked with a solution of 4% of milk powder in PBS (MPBS) at room temperature for 30 minutes and then washed again with PBS. 100µl of protein sample in 2% MPBS was applied to the wells. After incubation 45 min at room temperature, horseradish peroxidase labeled Protein A (Sigma, working dilution 1:6000) or horseradish peroxidase labeled M2 anti-FLAG antibody (Sigma, working dilution 1 µg/ml) was added as detection reagents. Subsequently, the wells were washed three times with PBS containing 0.1% Tween and then three times with PBS. 100µl BM BluePod (Roche Biochemicals) was added to each well. When the solution turned bluish, the reaction was quenched with 60µl of 1M H_2SO_4 (Fluka) and the plate read with a VERSAmax microplate reader (Molecular Devices) at 450nm. The 650nm value was subtracted.

The enzymatic activity of the scFv-tTF fusion protein was analysed using the Spectrozyme FXa assay (American Diagnostica, Pfungstadt, Germany) as described by Ruf et al. [Ruf et al., 1991]. The catalytic activity of VIIa in complex with soluble scFv-tTF was analysed by hydrolysis of Spectrozyme Fxa. 1, 10, 25, 50, 100, 1000 or 5000 nM of non-phospholipid-associated scFv-tTF was incubated with 100 nM VIIa (Novo Industri, Bagsvaerd, Denmark) in 0.2 % BSA, 5 mM $CaCl_2$ and 1.25 mM Spectrozyme FXa (American Diagnostica). The rate of the product formation was monitored at 405

nm. The absorbance increase was determined in a VERSAmax microplate reader reader and plotted versus tTF concentration.

5.4 Tumour mouse models

Tumour bearing mice were obtained by subcutaneous injection of 10^6 tumour cells of C51 murine colon adenocarcinoma, FE8 *ras*-transformed rat fibroblasts or F9 murine teratocarcinoma in female balb-C nude mice (Labortierkunde der Universität Zürich, Switzerland). Mice were monitored daily, and tumour volume was measured with a caliper, using the formula volume = length x width^2 x $\pi/6$. Experiments were performed in agreement with Swiss regulations, and under a Project Licence "Tumour Targeting" issued to professor Dario Neri by the Kantonales Veterinäramt des Kantons Zürich (Bewillgung 53/97). In addition to the experiments with nude mice, a set of experiments with F9 and C51 tumours was also performed with immunocompetent mice in a syngenic setting, yielding results comparable to the ones obtained with nude mice (data not shown). According to our Project Licence, mice had to be euthanised when tumours became too large, if animals lost more than 20% of body weight or showed sign of pain during the therapeutic experiments.

5.5 Biodistribution experiments

The in vivo targeting performance was evaluated by biodistribution analysis as described by Tarli et al. [Tarli et al., 1999]. Briefly, purified scFv (L19)-tTF fusion protein was radioiodinated and injected into nude mice with subcutaneously implanted F9 murine teratocarcinoma. Mice were sacrificed at 24 hours after injection. The organs were weighed and the radioactivity counted. Targeting results of representative organs are expressed as percent of the injected dose per gram of tissue (%ID/g).

5.6 Treatment of tumour bearing mice with scFv-tTF fusion proteins

5.6.1 Bacterially expressed scFv-tTF fusion protein

Cohorts of mice with tumours of volume ca 200-300mm^3 ($n \geq 4$) were injected with 14μg scFv-tTF fusion protein in 200 ul saline or injected with saline only. The injection was repeated after 72 and 144 hours. Mice with tumours of volume ca 1500mm^3 were injected with a single dose of 20μg scFv-tTF fusion protein in 200ul saline.

In a dose escalation investigation, mice with FE8 tumours of volume ca 300-500mm^3 were injected with 9,15 or 35 μg scFv-tTF fusion protein in 200 ul saline or injected with saline only. The injection was repeated after 72 and 144 hours. In all experiments, mice were monitored by tumour volume, weight and photographic documentation.

5.6.2 ScFv-tTF fusion protein expressed in mammalian cells

A small set of mice (n=6) with tumours of volume ca 1000-1500 mm^3 were injected intravenously with either 100 or 200 μg scFv(L19)-tTF fusion protein.

In a dose finding investigation, cohorts of mice with tumours of volume ca 1300-1500 mm^3 (n =6) were injected intravenously with 100 or 450 μg scFv(L19)-tTF fusion protein in 200 ul saline, 100 μg scFv(HH10)-tTF fusion protein in 200 ul saline or injected with saline only. The injection was repeated after 72 and 144 hours.

In a combination therapy experiment, groups of mice (n=4) were injected intravenously with a combination of 450 μg scFv(L19)-tTF fusion protein and 5 mg/kg doxorubicin (110 μg doxorubicin per mouse) or 5 mg/kg doxorubicin alone or saline only. The injection was repeated after 72 and 144 hours. In all experiments, mice were monitored by tumour volume, weight and photographic documentation.

5.7 Histology

In order to assess the toxicity of treatment by histological analysis, organs (lung, liver, spleen, intestine, kidney, heart, brain) were collected 24 hrs after injection of 20μg scFv-TF or saline, fixed in 4% buffered paraformaldehyde and embedded in paraffin. 4μm sections were cut, stained with haematoxylin and eosin and analysed. At least one slide was available per 0.5 cm diameter of the sample.

Mice were sacrificed at different time points after injection of 20 μg scFv (L19)-TF (1 hr, 4 hr, 6 hr, 12 hr, 24 hr). The tumours were excised, fixed in 4% buffered paraform-aldehyde and embedded in paraffin. Sections were cut, stained with haematoxylin and eosin. At least one slide was available per 0.5 cm tumour diameter. Thrombosis of intratumoural vessels was defined according the following criteria: total or incomplete occlusion by closely packed erythrocytes with blurred outline.

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8 CURRICULUM VITAE

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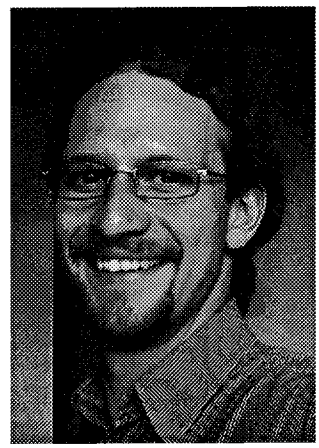

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